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Title	From youth to old age: therapeutic potential of microbiota interventions in the gut-brain-axis
Authors	Minuto, Chiara
Publication date	2022-08-17
Original Citation	Minuto, C. 2022 From youth to old age: therapeutic potential of microbiota interventions in the gut-brain-axis. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
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Download date	2026-11-06 18:38:39
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From Youth to Old Age: Therapeutic Potential of Microbiota Interventions in The Gut-Brain-Axis

Thesis presented by

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under the supervision of

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for the degree of Doctor of Philosophy

2022

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Declaration

This thesis submitted is my own work and has not been submitted for any other degree, either at University College Cork or elsewhere.

Author Contributions

All of the work conducted in this thesis was performed independently by the author with the following exceptions:

Chapter 2: Dr. Karen Scott assisted with behavioural experiments. Dr. Anna Golubeva helped with the Ussing chambers experiment. Veronica Peterson carried out the bioinformatic analysis. Marcel van de Wouw performed the Flow Cytometry analysis. Dr. Karen Scott helped with statistic analysis. Timothy G. Dinan & John F. Cryan discussed the data and assisted in writing the manuscript.

Chapter 3: Dr. Marcus Boehme assisted with animal care. Dr. Anna Golubeva trained me with statistical analysis. Timothy G. Dinan & John F. Cryan discussed the data and assisted in writing the manuscript.

Chapter 4: Dr. Lis London and Dr. Catherine Stanton provided prebiotics and probiotics. Dr. Alan E. Hoban and assisted with brain dissection. Timothy G. Dinan & John F. Cryan discussed the data and assisted in writing the manuscript.

Acknowledgments

Firstly, I would like to sincerely thank my supervisors Prof. Ted Dinan, Prof. John Cryan and Dr Gerard Clark for their support, encouragement and guidance throughout my PhD.

I owe gratitude to Dr. Anna Golubeva, Dr. Gerard Moloney, Pat Fitzgerald, Dr. Kieran Rea and Dr. Ken O'Riordan, for their scientific and professional advice during my PhD. I would also like to express my gratitude to my colleagues at the Laboratory of Neurogastroenterology and Department of Anatomy and Neuroscience in general, which provided a continuous source of help and support and where I, most importantly, made some great friends.

In particularly, I would like to sincerely thank my mentor Prof. Massimo Cocchi for his help and support. For me it was an honor to collaborate with him and learn so much from his genius. Thanks to my family for the support and to my brother Andrea to help me in all difficult moments.

Publications and presentations

John R.Kelly, **ChiaraMinuto**, John F.Cryan, GerardClarke, Timothy G.Dinan (April 2020)
The role of the gut microbiome in the development of schizophrenia. 'Schizophrenia Research 234(Part B). DOI: 10.1016/j.schres.2020.02.010

Boehme, M. Boehme, Marcus, van de Wouw, Marcel, Bastiaanssen, Thomaz F.S., Olavarría-Ramírez, Loreto, Lyons, Katriona, Fouhy, Fiona, Golubeva, Anna V., Moloney, Gerard M., **Minuto, Chiara**, Sandhu, Kiran V., Scott, Karen A.,Clarke, Gerard, Stanton, Catherine, Dinan, Timothy G., Schellekens, Harriët, Cryan, John F. (2020) 'Mid-life microbiota crises: middle age is associated with pervasive neuroimmune alterations that are reversed by targeting the gut microbiome', *Molecular Psychiatry*. Springer US. doi: 10.1038/s41380-019-0425-1.

Chiara Minuto, Massimo Cocchi (June,2020). Cholesterol and Linoleic Acid: a forgotten cooperation? Progress in Nutrition 2020; Vol. 22, N. 2: 357-360 DOI: 10.23751/pn.v22i2.9560.

John R Kelly, **Chiara Minuto**, John F Cryan, Gerard Clarke, Timothy G Dinan
(2017) 'Cross talk: The microbiota and neurodevelopmental
disorders', *Frontiers in Neuroscience*, 11(SEP). doi: 10.3389/fnins.2017.00490.

Massimo Cocchi, **Chiara Minuto**, Lucio Tonello, Fabio Gabrielli, Gustav Bernroider, Jack A Tuszynski, Francesco Cappello, Mark Rasenick (2017). 'Linoleic acid: Is this the key that unlocks the quantum brain? Insights linking broken symmetries in molecular biology, mood disorders and personalistic emergentism', *BMC Neuroscience*, 18(1). doi: 10.1186/s12868-017-0356-1.

Conference posters and oral presentations

Chiara Minuto, Lis London, Catherine Stanton, John F. Cryan & Timothy G. Dinan. 'Effects of Anti-hypercholesterolemic Diets on the Brain in a mouse model of Cardiovascular Disease'. Poster presentation, NeuroGASTRO meeting, University College Cork, Cork, Ireland, August 2017.

Chiara Minuto. "Membrane, Cholesterol and Linoleic Acid: A Milestone in Brain Evolution?" June 2017, The 5th qpp Meeting, Quantum Paradigms of Psychopathology, Bologna university, Italy. Invited speaker

Chiara Minuto, Ted Dinan. “Brain-gut-microbiota axis:implications for psychopathology”. September 2019. The views from Philosophy, Physic, Mathematics, Biochemistry, Neuroscience, Psychiatry and Psychology Perspectives. The human brain seen from multiple perspectives. Torino University. Torino. Invited speaker.

Abbreviations

bdnf	Brain-derived neurotrophic factor
CRP	C-reactive protein
CVDs	Cardiovascular diseases
EPS	exopolysaccharide
fgf21	Fibroblast growth factor 21
G6PC	Glucose-6-phosphatase, catalytic subunit
HFD	High fat diet
HPA	hypothalamic–pituitary– adrenal
IBS	Irritable bowel disease
L.	Lactobacillus
LK	Lactobacillus mucosae heat-Killed
LR	Lactobacillus reuteri
MRS	Man Rugosa Sharp
nmda	N-methyl-D-aspartate
O	Oat beta glucans
P	Plant sterol esters
PBS	Phosphate buffered saline
PC	Pyruvate carboxylase
pck1	Phosphoenolpyruvate carboxykinase 1
PparGC1A1	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PparS	peroxisome proliferator-activated
rxrs	retinoid X receptors; fgf21= Fibroblast growth factor 21
SIRT1	sirtuin (silent mating type information regulation 2 homolog)1
STAT	Statin

Abstract

There is a growing recognition of the involvement of the gut microbiota in the regulation of certain physiological and metabolic parameters across the life cycle of animals. A greater understanding of the microbiota changes throughout the life cycle may provide a novel therapeutic target for treating various age-related disorders from autism in childhood to dementia in old age. The increase in the elderly population with a rapid rise in dementia, cardiovascular and metabolic disease, leads to the growing demand for new interventions to decelerate the senescent decline. However, if the microbiota is to become a therapeutic target a far greater understanding of the structural changes which take place throughout the lifespan is required.

In this thesis, we focus attention on microbiota-targeted interventions in young, middle age and old animals. The studies examine behaviour, physiology, and metabolism, exploring effects both in the periphery and in the brain, across a range of treatments from prebiotics to polyphenols. We highlight the physiological differences between the various age groups. Intestinal permeability and neuronal plasticity were the only modifiable parameters in the elderly. In younger animal prebiotics impact metabolism and inflammation.

Polyphenols rich diets (grape pomace and rafuma) significantly alter intestinal permeability reversing the damage to the intestinal barrier due to ageing. The effect was not directly linked to changes in microbial diversity of the gut and did not significantly ameliorate the impaired immune system of the aged animals' intestine or hippocampus. Only a significant increase in the number of dendritic cells emerged in conjunction with a reduced level of TNF-α in the blood. Neither did the polyphenols reverse some of the behavioural deficits associated with ageing. Cumulatively, these data show the specific effect of polyphenols in the elderly.

We next investigated whether inulin, the most widely studied prebiotics, could improve host metabolism in middle-aged mice. We tested 52 metabolic markers involved in hypothalamic-pituitary-adrenal (HPA) axis regulation, cholesterol and glucose homeostasis and immune regulation. Interestingly, the liver, the brain and the mesenteric fat did not show any modification in middle-age animals. Inulin had a

greater impact in young animals and stimulated the lipid and glucose metabolism, increasing the level of Sirtuin1, a fundamental metabolic sensor, without impacting transcriptional factor changes in the liver. The study also uncovered a range of changes in the HPA. In fact, inulin decreased the level of the glucocorticoids receptors (Nr3c1) and corticotropin-releasing hormone (Crhr1) receptors in the amygdala of young mice.

In an attempt to understand the mechanisms through which microbiota-targeted interventions exert the anti-aging effects, we used a model of premature ageing (ApoE deficient mice). The effect of two different strains of lactobacillus *(L.reuteri* and *L.mucosae*) and two prebiotics derived from plants (plant sterol esters and oat β-glucan) were examined. Neural plasticity was restored in the Prefrontal cortex after 24 weeks of prebiotic administration and the level of claudin 5 increased in the hippocampus following consumption of *L. mucosae*.

Overall, these results provide novel insights regarding the impact of a portfolio of microbiota-targeted interventions on age-related alterations in physiology and brain function and suggest the importance of using target-specific therapeutic options during particular time windows for maximum gain.

Chapter 1

Introduction

1.1 Gut microbiota

Billions of organisms, especially bacteria, populate our intestines; a recent estimation of bacterial cells in the body in relation to human cells was calculated at ratio of at least 1:1(Sender, Fuchs and Milo, 2016). This ratio highlights the important impact that the microbiota potentially has on the physiology and metabolism of the host where the 100 trillion microbes including bacteria, virus, fungi and archaea, can modulate the health and disease state of the host or *vice versa.*

The microbiota may be significant rapidly altered by external factors such as diet, mode of delivery, diet, drugs, physical exercise or the type of microorganisms present in the daily environment. During the lifespan, the microbiota composition mutates and the diversity of the bacterial phyla changes in conjunction with the different evolutionary steps of life (Ottman *et al.*, 2012). Infants, during the first three years of life, present a low diversity in microbiota phyla with a dominance of *Proteobacteria* and *Actinobacteria* and then the microbiome assume the diversity of the adult gut that is stable for many years and is generally characterized by four main phyla: Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Yang *et al.*, 2016). From a state of equilibrium called “eubiosis”, it is therefore possible to experience the opposite condition of “dysbiosis”. It is to the latter that we owe the increased incidence of metabolic, cardiovascular, inflammatory, neurological and oncological pathologies.

1.2 Microbiota gut brain axis

The gut microbiota interfaces with the gut environment and through different mechanisms it communicates with the host system involving various signalling networks and their mediators such as hormones, neurons or cytokines. The brain-gut-axis connects the gastro-intestinal system with the central nervous system and this bidirectional communication takes place through different pathways (**Figure 1.0.1**).

A gut dysbiosis generates a cascade of signalling molecules that ultimately communicate with the brain through neural systems (vagal nerve), endocrine signalling (including the hypothalamus-pituitary-adrenal (HPA) axis) and the immune system (cytokines), modulating brain function, behaviour and, most remarkably, cognition (Quigley, 2017).

It has already been demonstrated that pathophysiology of numerous mental and neurological diseases is associated with intestinal dysbiosis; an imbalance of the microbiota compromises the diversity of the microbial families that colonize the intestine (Dinan et al., 2013). Generally a diversified microbiota is associated with improved learning/memory with consequences on the behaviour and neural plasticity; conversely, low microbial diversity is connected with impairment of cognitive abilities (Davidson *et al.*, 2018), which influences various mental processes and is involved in the pathophysiology of numerous mental and neurological diseases (Quigley, 2017).

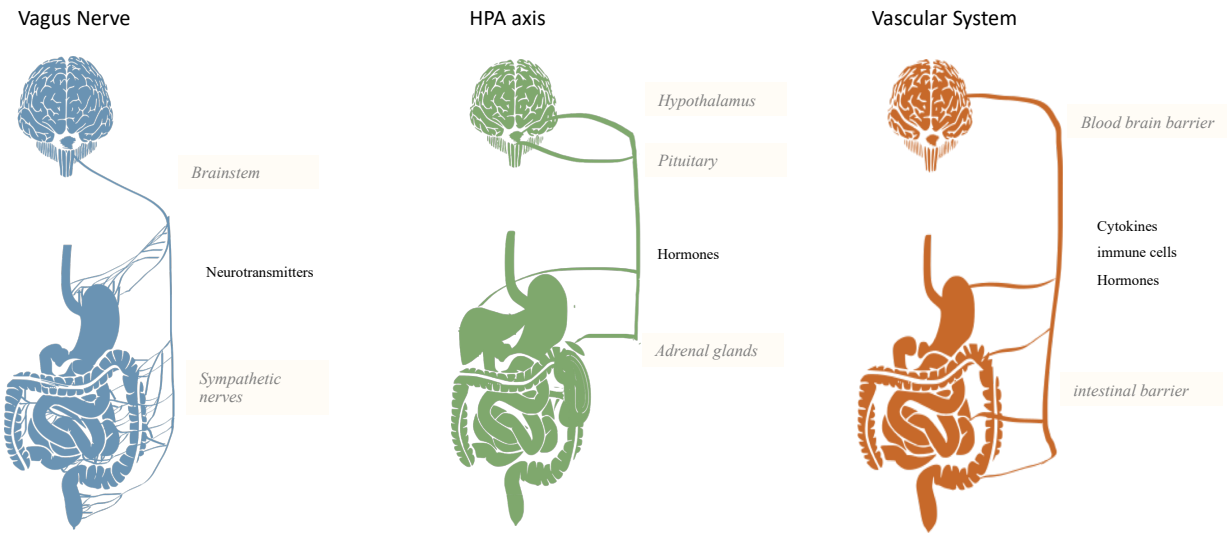


Figure 1.0.1

Pathway of comunication between the gut and brain.

The first blue gut-brain axis represents the *via vagus nerve* where the brainstem of the central nerveus system intefaces with the sympathetic efferent nerves that controls the lungs, heart, and the digestive tract though the neurotrasmitters. The second green gut-brain axis shows the hypothalamic–pituitary–adrenal axis (HPA axis) in relation to the gut microbiota where the interaction among the hypothalamus, the pituitary gland and the adrenal glands is moderated by direct hormonal signals and feedback in situations related to stress. The third red gut-brain axis represents the communication between the brain and the intestinal bacteria through the vascular system. The immune cells, cytokines, hormones and also SCFAs can act on the brain and on the microbiota thorough the alteration of blood brain barrier and the blood gut barrier.

1.2.1 Extrinsic factors modulating the gut microbiota

Throughout the lifespan, the microbiota can be altered by a number of different internal and external factors (**Figure 1.0.2**). Intrinsic factors are key for microbiota diversity, these include genetics, ageing and mode of delivery. Instead, diet, use of probiotics, lifestyle, stress and use of antibiotics represents extrinsic factors that can be modified daily (Wen and Duffy, 2017); in fact, they may be a key point of interest for many diseases that are now known to be associated with the microbiome.

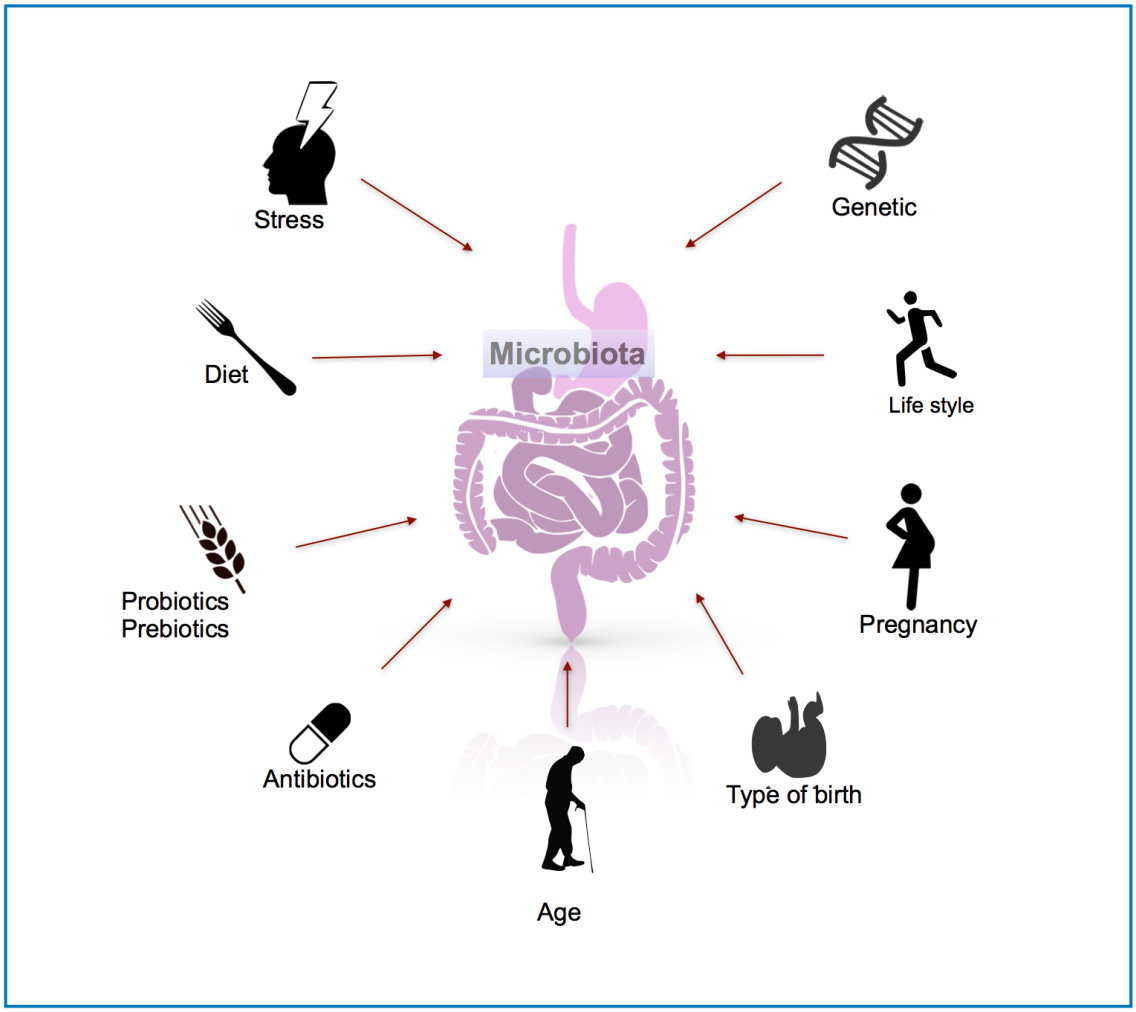


Figure 1.0.2

Intrinsic and extrinsic factors manipulate the gut microbiome during the human life.

Different factors influence the gut microbiota throughout life. Microbiota can be influenced by daily occurrences such as diet, lifestyle, ingestion of prebiotics or probiotics, use of antibiotic and psychological stress. But also, intrinsic factors that are outside of our control, such as genetics, ageing and type of birth.

1.2.2 Diet and microbiota

Of all the extrinsic factors affecting the gut microbiota, diet appears to have the largest effect. Recent research on the effects of dietary interventions has shown that the gut microbiota can change dramatically with diet (Lawrence A David *et al.*, 2014). Even

if each individual has a unique heritable microbiota determined by his own genetics (Goodrich *et al.*, 2017), human studies demonstrated that diet can rapidly alter the abundance of microbial families (Faith *et al.*, 2011). For example, a diet rich in fibre assists the maintenance of a healthy microbiota associated with increased diversity. The high fibre intake stimulates mucus production and secretion from the epithelium and, it contributes to increase the expression of tight junction proteins (Makki *et al.*, 2018). On the contrary, the negative impact of the high-fat diet (60% fat) was demonstrated in humans and rodents (Hussain *et al.*, 2016; Rohr *et al.*, 2019). Specifically, a high-fat diet (HFD) seems to decreases the diversity in the gut microbiota, and the composition of the bacteria between mice given a HFD compared to those given a regular diet (Wen and Duffy, 2017). The consumption of high fat diet is one of the main factors contributing to the development of obesity (Showalter *et al.*, 2018); it has been reported in animal and clinical studies that both obesity and HFD are associated with changes in gut microbiota compromising immunological and metabolic functions of the host (Morales *et al.*, 2016). In fact, HFD affects the microbial community reducing the ratio of Gram-positive and Gram-negative bacteria and increases lipopolysaccharide (LPS) concentrations in intestinal epithelia (Serino *et al.*, 2009). This latter event was found to be associated with impairment of barrier function, increasing the intestinal permeability and the production of pro-inflammatory cytokines secretion with subsequent development of systemic inflammation, insulin resistance, diabetes and obesity (Martinez-Medina *et al.*, 2014; Morales *et al.*, 2016).

The diet is able to affect different species of bacteria and this has a strong impact on the individual health (Kolodziejczyk, Zheng and Elinav, 2019). The HFD with a low-fibre content results in a decrease in Bacteroidetes and an increase in Proteobacteria, Firmicutes and Cyanobacteria in mice (Zhang *et al.*, 2012). More specifically, the body weight increased after administration of HFD was positively associated with *Allobaculum* and *Lactococcus*, but was negatively associated with *Akkermansia* (Parks *et al.*, 2013). *Akkermansia* is a mucin-degrading bacteria that damages the mucus barrier after a prolonged lack of dietary fibres (Desai *et al.*, 2016).

The impact of diet and how it causes an imbalance of the microbiota can have a consequence on mood and behaviour through the gut-brain axis (**Figure 1. 0.3**) In fact, it was demonstrated in mice that HFD led to a depression-like phenotype as revealed by reduced sociability in the three chamber test and sucrose preference (Hassan *et al.*,

2018). More precisely in the brain, HFD can lead to modification of the *bdnf* levels a neuroendocrine effector involved in the response to stress and in the neurobehavioral changes associated with depression (Berry *et al.*, 2012). During a stressful situation HFD has a stronger negative effect on the *bdnf* but also on *nmda* where the reduction of the *nmda* level is characterized by neuronal plasticity (Rothman and Mattson, 2013),(Nam *et al.*, 2017),(Valladolid-Acebes *et al.*, 2012). It is interesting to note that all of these consequences have in common a systemic inflammation. In fact it is accepted that inflammation generated by HFD increases circulating proinflammatory cytokines with macrophage infiltration in the intestine and in the adipose tissue (Kim *et al.*, 2012). Studies in rodents demonstrated that animals fed a diet high in saturated fatty acids also show increased brain inflammatory markers (Diane E. Handy, Rita Castro, 2011). Furthermore, a HFD is able to modify the performance of an episodic memory task following only one day of treatment which rapidly reverses when switching mice to a low-fat diet (McLean *et al.*, 2018).

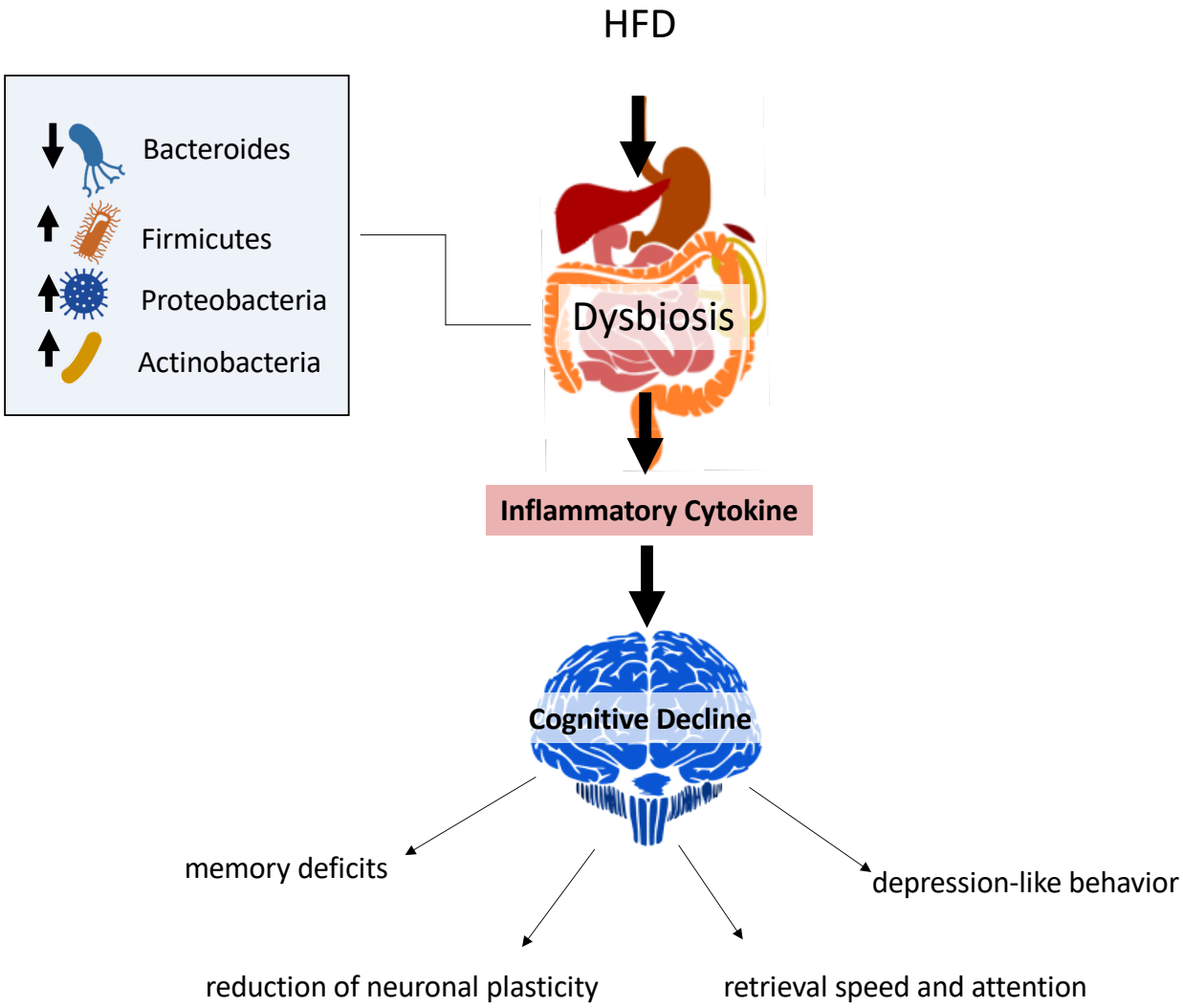


Figure 1. 0.3

The effect of a high-fat diet (HFD) on the microbiota with negative consequence on cognitive function. Consumption of HFD induces intestinal dysbiosis changing the microbiota composition. Bacteroides phylum increased, instead Firmicutes, Proteobacteria and Actinobacteria phylum tends to decrease. The HFD triggers a systemic inflammation with the release of pro-inflammatory cytokines. The dysbiosis connected to the inflammation can lead to neurodegenerative disease and brain dysfunction. A cognitive decline is manifested by mood alteration as depression and deficit on memory and attention.

1.2.3 Prebiotics and microbiota

The definition of the term "prebiotic" has undergone numerous changes in the course of the last two decades. Gibson and Roberfroid in 1995 were among the first to

introduce the concept of prebiotics as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon and thus attempt to improve host health" (Gibson and Roberfroid, 1995); this definition was limited exclusively to non-digestible carbohydrates with direct action to the intestine for the health of the host. However, over the years, the increase of microbiota research, led to the term "prebiotic" becoming more and more complex; after 15 years, it was redefined from Robertfroid as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" (Roberfroid, 2007). Within this context the microbiota is viewed as a rather plastic virtual "organ". Over the last decade, emerging research suggests that earlier definitions of prebiotics are too narrow: the concept is now extended to any element that is able to feed the intestinal microbiota, select beneficial bacteria with consequent production of short-chain fatty acids which when released into the blood circulation can have beneficial effects in other distant organs (Davani-Davari *et al.*, 2019).

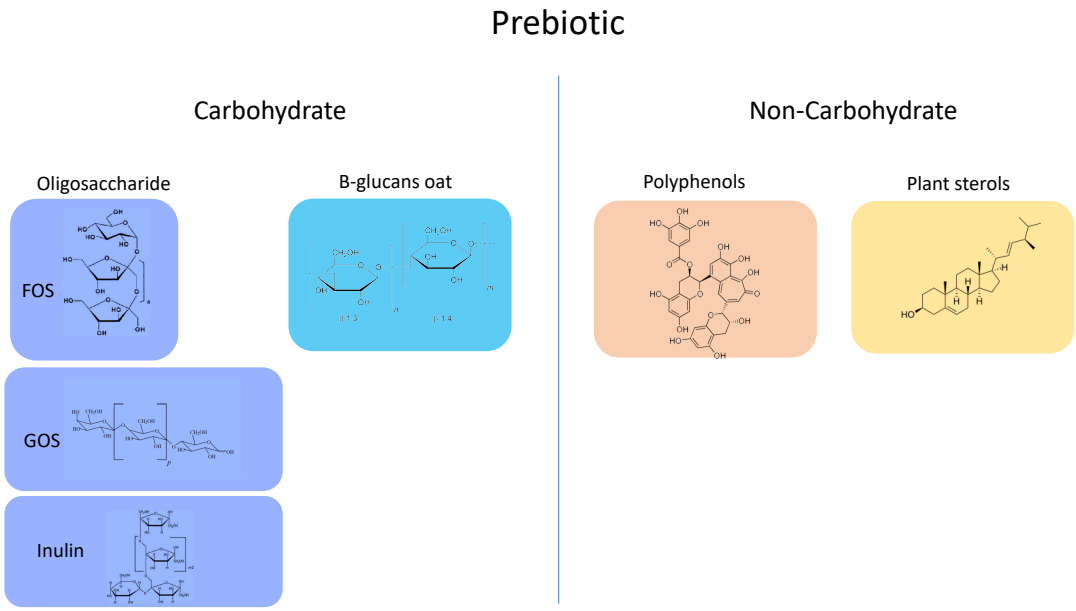


Figure1 0.4 Classification of the most common prebiotics and their chemical structure.
It is possible to classify prebiotic in Carbohydrate and non-Carbohydrate. Oligosaccharides and beta-glucans are a class of carbohydrates possessing 2–10 monosaccharide units. fructooligosaccharides (FOS), galactooligosaccharides (GOS) and inulin are the most important oligosaccharides used as prebiotic commercially available. Non- carbohydrate prebiotic are polyphenols and plant sterols.

Prebiotics can be classified into many different types (**Figure1 0.4**). The most widely studied are a subset of carbohydrate groups and are mostly oligosaccharide carbohydrates. They are a host-indigestible dietary fibre, that passing unaltered though the small intestine, are fermented by the microbiota with beneficial consequence for the host. Fructooligosaccharides (FOS) together with inulin and galactooligosaccharides (GOS) have been studied for their positive effect on gastro-intestinal transit and on immunity and allergies (Gibson *et al.*, 2017). Cereal ß glucans are also another non-digestible carbohydrate considered as a prebiotic that pass undigested through the gastro-intestinal tract, are fermented by the microbiota increasing the number of beneficial bacteria (Bigliardi and Galati, 2013). The non-carbohydrate prebiotics have been considered as probiotics only recently. Substances like polyphenols or plant sterols have been shown to have a positive impact on the intestinal microbiota inhibiting the growth of pathogens and consequently on the host health (Filosa, Di Meo and Crispi, 2018).

1.2.3.1 FOS and GOS positive and negative aspects

Fructooligosaccharides (FOS) are oligosaccharides that are naturally present in vegetables such as chicory, garlic, asparagus, onion, tomato and many others and are obtained by hydrolysis of inulin. Galacto-oligosaccharides (GOS) instead occurs more often in beans and legumes (Martins *et al.*, 2019).

FOS are composed of linear chains of fructose units that are not hydrolysed by small intestinal glycosidases; they reach the cecum structurally unchanged. Here, the gut microbiota metabolizes FOS into short fatty acid such as acetic, propionic and *n*-butyric acids, carbon dioxide, hydrogen, methane, amino acid and vitamins (Rowland *et al.*, 2018) and used by the host as an energy source (Tokunaga, Oku and Hosoya, 1989; Hidaka and Hirayama, 1991; Tuncil et al., 2018). Much evidence has been reported that the environment in the gastrointestinal tract is improved by ingestion of FOS and the benefit such as facilitating defecation, repressing pathogenic bacteria, reducing hepatic responsibility for detoxification, decreasing decomposing matter and improving stool condition in the intestinal tract are well describes (Sabater-Molina *et al.*, 2009; Slavin, 2013).

The effects of FOS are not limited to the gut. The capacity of this prebiotic to influence the lipid and glucose metabolism was demonstrated, for example, in a rat study where

type 2 diabetes mellitus was induced, the significant reduction of plasma triacylglycerol and very low-density lipoprotein level with decrease in severe glucosuria, proteinuria, blood creatinine, urea and advanced glycation end products was observed in rat treated with FOS (Bharti *et al.*, 2013). In another clinical study, it was demonstrated that the treatment with 2g of FOS daily, raised HDL cholesterol on the serum but the total cholesterol and triglycerides level was unchanged (Moroti *et al.*, 2012). Furthermore, rats fed with inulin showed higher excretions of faecal lipid and cholesterol, mainly attributed to reduced cholesterol absorption (Ooi and Liong, 2010). Another study showed that treatment with 10g of FOS daily presented no statistically significant reduction of cholesterol in the serum (Giacco *et al.*, 2004). All of these data suggest that FOS seems to work only in certain conditions; for example happened in another clinical study where FOS supplements reduced the LDL and steatosis but only in a group of patients who, in addition to FOS supplementation had a change of lifestyle, with physical exercise practice and a balanced diet (Malaguarnera *et al.*, 2012). Similarly, the reduction of body weight and total cholesterol in beagle dogs was brought about by FOS but only in conditions of calorie-restricted diet (Ricci et al., 2011). Costa *et al.*, speculate that FOS intake have a beneficial effect on cholesterol metabolism only in subjects that change their lifestyle (Costa *et al.*, 2015). In fact, the microbiota can be quickly and profoundly altered by common human actions and experiences (Lawrence A. David *et al.*, 2014) and this underlines the close relationship between the host and microbiota where evidently the positive effect of FOS is not appreciable without other interventions. Interesting, through the gut brain axis is the indirect effect of FOS on the brain. For example, FOS from the plant Morinda officinalis is used to improve the memory ability on Alzheimer- like disease animals regulating the synthesis of neurotransmitter as norepinephrine and 5-hydroxytryptamine (D. Chen *et al.*, 2017).

FOS in addition to GOS exhibits both antidepressant and anxiolytic effects. The administration of GOS and the FOS+GOS combination reduced stress-induced corticosterone release. FOS in particularly, modified specific gene expression in the hippocampus and hypothalamus such as *bdnf* but GOS was more effective on *nmda* signalling than FOS (Burokas *et al.*, 2017) and, may reflect the potency of GOS and FOS on microbiota thorough the gut brain axis (Savignac *et al.*, 2013).

In fact, FOS and GOS are a well-known to be able to selectively stimulate the growth of *Bifidobacteria* in the gut (Gibson *et al.*, 2004; Ben *et al.*, 2008) and in some cases

Lactobacilli (Roberfroid, 2007). The growth of *Bifidobacterium* was observed both for FOS and GOS, while the butyrate-producing bacteria like *Phascolarctobacterium* was stimulate only by FOS while *Ruminococcus* seems to be reduced by GOS (Liu *et al.*, 2017).

An ob\ob mice model study revealed that FOS administration increases *Bacteroidetes* and decreases *Firmicutes*, and changed more than 100 taxa of bacteria (Everard *et al.*, 2011).Another intervention study in obese subjects with FOS resulted in the increase of *Faecalibacterium prausnitzii* and *Bifidobacterium* (Dewulf *et al.*, 2013). However, FOS has the capacity not only to stimulate the growth of beneficial bacteria but also some pathogenic strains such as *E. coli* (Schouler *et al.*, 2009). *Salmonella spp* was found increased in caecal contents in rats, after a diet enriched with inulin and FOS despite the proliferation of intestinal *Lactobacilli* and *Bifidobacterial* (Ten Bruggencate *et al.*, 2004).This peculiarity of a bacteria to be a substrate for a pathogenic bacteria might explain the side effect that FOS can have in particular situation. For example, in a mouse surgical study, after a ileocecal resection, the administration of FOS increased the inflammation accompanied by a reduction in intestinal barrier function with a decline of bacterial diversity (Laffin *et al.*, 2017) and it was hypothesized that the massive stimulation of *Bifidobacteria* consequently inhibited the growth of butyrate-producing anaerobic organisms necessary for gut homeostasis (Laffin *et al.*, 2018). The increase of inflammation by FOS was observed in a previous study where inulin was used as prebiotic in a mice model of atherosclerosis accelerating the process (Hoving *et al.*, 2018). The pro-inflammatory cytokine tumour necrosis factor α (TNFα) level increased in monocytes in *vitro* anther inulin administration (Capitán-Cañadas *et al.*, 2014). Recently, a study highlighted the possible side effect of a massive increase in *bifidobacterium* and Lactobacillus in active phase of inflammatory bowel disease due to the reduction of bacteria that produce butyrate (Wang *et al.*, 2014). In fact, particular strains such as *B. longum* were able to stimulate cytokine interleukin (IL)-10 and proinflammatory cytokine TNFα production in vitro (Medina *et al.*, 2007). In particularly in another *in vitro* study, *Bifidobacterium adolescentis* and *B. longum*, induced pro-inflammatory cytokine secretion, IL-12 and TNF-alpha (He *et al.*, 2002). In contrast, another study demonstrated that *Bifidobacterium* strains isolated from faeces of healthy infants inhibited inflammatory responses (Khokhlova *et al.*, 2012). In conclusion, it is evident

the positive effect of FOS and GOS on the microbiota and on the brain belong to the gut brain axis; however, more studies are needed to elucidate the impact of these prebiotics on the body and in different conditions.

1.2.3.2 Beta glucans

β-glucans, a type of soluble fibre, are non-starch polysaccharide that are composed of six-sided D-glucose rings connected at varying carbon positions and they are naturally present on the cell walls of bacteria, fungi and cereals (Wood, 2011). The β-glucans of the cereal are completely linear, consisting of consecutively linked of cellulosic oligomers and water soluble especially from oat and barley (Zielke *et al.*, 2017). It is well proven that β-glucans possess several properties such as hypoglycaemic (Kim *et al.*, 2005), anti-inflammatory actions (Du *et al.*, 2015), immune modulation (Stier, Ebbeskotte and Gruenwald, 2014) and anticancer potential (Choromanska *et al.*, 2015). The type of linkage, branching and degree of solubility gives different characteristics to β-glucans and soluble glucans can induce inflammatory cytokine production strongly compared to least soluble ones (Soltanian *et al.*, 2009). In fact, the β-glucans with β (1,3)-linkages associated to β (1,6) branches have a strong immune action by stimulating proinflammatory cytokines such as IL-1β, IL-6, and TNF-α (Vannucci *et al.*, 2013).

In oat, glucose monomers are linked via β-(1→4) and β-(1→3) glycosidic bonds and as a result are completely insoluble in water (Bohn Dz and Bemiller, 1995).Water-solubility and molecular weight of β-glucan is considered to control its hypocholesterolemic effect (Bae *et al.*, 2009). In particular, the cholesterol-lowering properties of oat β-glucans are well known from 1997 where the Panel on Dietetic Products, Nutrition and Allergies in UK recognized the capacity of β-glucans to reduce the risk of coronary heart disease by the effect of reduction LDL in the blood (EFSA, 2010). The ability of oat β-glucans to intervene on human and mice health seems to be multifactorial. The mechanism involved in the lowering of the cholesterol is still not totally clear because the β-glucans intervention seems to act directly on the cholesterol and lipid digestion but also act as a substrate for microbial fermentation (Gibson, 2004). The cholesterol metabolism has a complex pathway and it involves the bile salts homeostasis with a feedback mechanism of recycling intestinal bile regulate the cholesterol absorption and excretion (see 1.3.1 paragraph) (Mishra, Somvanshi and Venkatesh, 2014). Oat- β-glucans act *via* the bile salts metabolism

recycling them by dynamic interactions (Gunness *et al.*, 2016) and consequently an impact on the cholesterol metabolism (Grundy *et al.*, 2017). This may be explained by the natural characteristic of a water-soluble fibre of β-glucans that interfere directly with lipid emulsification (Pasquier *et al.*, 1996) and lipase activity (Wilcox *et al.*, 2014) in the intestinal tract.

However, β-glucans interact with microbiota homeostasis and a recent study performed an *in vitro* using an anaerobic batch-culture showed a that the phylum *Bacteroidetes* is increased after 24 hours of β-glucan treatment (Kristek *et al.*, 2019). The tendency to increase *Bacteroidetes* at the expense of *Firmicutes* by *β*-glucan effect is reported in a recent clinical study where the genus of *Prevotella* raised and *Dorea* reduced the abundance in conjunction with the attenuation of cardiovascular risk as well as blood pressure, triglyceride levels and body mass (Wang *et al.*, 2016).

1.2.3.3 Polyphenols

Polyphenols are an abundant biological molecule characterized by the presence of large multiples of phenol structural units. They are a vast family of molecules produced by plants that play a protective role as a secondary metabolites against exogenous stresses(Lattanzio, 2013). Specifically, polyphenols protect plants against ultraviolet radiation, reactive oxygen species, pathogens, parasites and plant predators (Beckman, 2000). They are classified into two different classes according to the shared structural characteristics of their carbon skeletons: Simple and complex structure (Table 1.0.1). The simple structure classes include phenolic acids derivatives, where in particularly, the hydroxybenzoic acid content of edible plants is for example, generally low, with the exception of certain red fruits and onions, which hydroxybenzoic acid can be very high (Concenço *et al.*, 2014); and the hydroxycinnamic acids that consist chiefly of *p*-coumaric, caffeic, ferulic and sinapic acids (Pandey and Rizvi, 2009); flavonoids are part of the complex structure of polyphenols as flavanols, flavanols, flavones, flavanones, isoflavones, chalcones, anthocyanins, but also tannins, stilbenes, lignans, coumarins and lignins are an important fractions of this class (Naczk and Shahidi, 2006).

Table 1.0.1 Classification of the most representative natural polyphenols divided into simple and complex structures. Table adapted from Freyssin et al., 2018

simple	Phenolic acid derivatives	Hydroxybenzoic acid: Gallic acid
		Hydroxycinnamic acid: Curcumin Ferulic acis Rosmarinio acid Verbascoside
Complex	Flavonoids	Flavanols: Catechin Epicatechin Epigallocatechin galate
		Flavonols: Kaempferol Morin Myricetin Quercetin
		Flavones: Apigenin Baicalein
		Anthocyanidins: Delphinidin Proanthocyanidins
	Tannins	Tannic acid
	Other polyphenols	Stilbens: Piceatannol Resveratrol Viniferin
		Lignans: Nordihydroguaiaretic acid

A protective effect like that seen in plants has been amply demonstrated to be exercised on animals and humans through the consumption of vegetable and fruit rich in polyphenols. Epidemiological studies and associated meta-analyses strongly

suggested that the consumption of diets rich in plant polyphenols offered some protection against diabetes, cardiovascular diseases, development of cancers, and neurodegenerative diseases (Pandey and Rizvi, 2009). In fact, the positive effect of a polyphenol diet to reduce hyperglycaemia and improve insulin secretion and sensitivity was widely demonstrated in studies on diabetes (Aryaeian, Sedehi and Arablou, 2017). Moreover, in a recent cardiovascular disease clinical study, polyphenols such as flavonoid from apples and nitrate from spinach were shown to enhance endothelial function and lower blood pressure (Bondonno *et al.*, 2012). Polyphenols and its particular phenol structure seems to have the ability to interfere directly with proteins, lipids and polynucleotides through electrostatic, hydrophobic, and even covalent binding (Hendrich, 2006) but also with membrane and intracellular receptors of the cells. In fact, for example different receptors of immune cells such as Toll-like receptors, T-cell receptors αβ, and IgM-B-cell receptor recognize and allow cellular uptake of polyphenols, which subsequently activate signalling pathways to initiate immune responses (Ding, Jiang and Fang, 2018). Such interactions also include modulation of signalling cascades, interaction with the basic enzymes involved in tumour promotion and metastasis, interaction with oncogenes and oncoproteins, and, finally, direct or indirect interactions with nucleic acids and nucleoproteins (Kampa *et al.*, 2007). In fact, several animal models and a limited number of human studies have revealed the potential polyphenol mechanisms included antioxidant, anti-inflammation as well as the modulation of multiple molecular events were involved in carcinogenesis (Zhou *et al.*, 2016).

Polyphenols also regulate the intestinal mucosal immune response. It is demonstrated that polyphenols prevent gastrointestinal tract tumours *via* decreasing the number of abnormal crypts (Xu *et al.*, 2010). *In vivo* experiments shows that polyphenols enhance intestinal mucosal immunity by increasing the populations of intraepithelial T-cells and mucosal eosinophils in the colons of pigs infected with *Ascaris Suum* (Williams *et al.*, 2017). The influence of polyphenols on the gut health is underlined also by the relationship with the microbiota. Microbiota transforms polyphenols into metabolites improving their bioavailability and the positive health effects are observable in the modulation of the microbiota community (Cardona *et al.*, 2013). The prebiotic activity of polyphenols is expressed by their capacity to inhibit and increase the growth of specific gut bacteria, changing the ratio of *bacterioides* and *firmicutes* in favour of

bacterioides (Rastmanesh, 2011). More specifically, different polyphenols can increase beneficial strains such as *bifidobacteria* and *lactobacilli*, reducing the number of pathogens such as *Clostridium perfringens* and *Clostridium histolyticum* (Dueñas *et al.*, 2015). The grape pomace, a huge important pool of different polyphenols, demonstrated the beneficial anti-inflammatory property to stimulate the growth of *Bifidobacterium* following long-term intake (Chacar *et al.*, 2018). Moreover, the typical microbiota associated to HFD such as *desulfovibrio* and *lactococcus* decreased after intake of grape pomace in mice and *allobaculum* and *roseburiawere* instead increased highlighting the positive effect of grape pomace on the microbiota composition (Van Hul *et al.*, 2018).

So the capacity of polyphenols to perturb the microbiota homeostasis can also have effects on brain function through the gut-brain axis (Marizzoni *et al.*, 2017). These natural bioactive molecules derived by plants, could be used as therapeutic agents directed towards brain functions that characterize neurodegenerative diseases. For example, the capacity of flavonoids like quercetin, rutin, luteolin and daidzein is well known to have neuroprotective capacity by controlling and reducing neuroinflammation (Spencer *et al.*, 2012). Their direct mechanisms on counteracting neuroinflammation and oxidative stress (Kennedy, 2014) and improving memory and cognitive function (Spencer, 2009) reflected the effect on chronical neurologic disease. In recent years, studies on the benefits of dietary polyphenols in dealing with neurological disorders, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis and ASD was investigated. In a pre-clinical study the positive effect of resveratrol for the treatment in Alzheimer’s disorder was demonstrated (Moussa *et al.*, 2017). Resveratrol was also used to improves memory performance in 50-80 year old adults activating the hippocampal function (Veronica Witte *et al.*, 2014). Another clinical study demonstrated that a treatment with a blueberry beverage rich in flavonoids influenced positively the cognitive abilities of healthy old adults amending cerebral blood flow in the occipital cortex and frontal and parietal lobes of the brain (Rendeiro, Rhodes and Spencer, 2015). Polyphenols derive from grape are widely used as adjunctive treatment for the prevention of neuropathology associated with ageing as Alzheimer’s disease, Parkinson’s disease and Chronic Traumatic Encephalopathy by simultaneously ameliorating inflammation and oxidative stress that influence those pathologies (Herman *et al.*, 2018).

1.2.3.4 Plant sterol

Plant sterol are a group of substances made in plants and are found in the highest amounts in foods like nuts, seeds and vegetable oils. Plant sterols are bioactive compounds that are structurally similar to mammalian cholesterol and can be also defined asphytosterols. Cholesterol and plant sterols have similar structures derived from squalene, comprising a tetracyclic cyclopenta[α]-phenanthrene structure and the hydrophobic region with a polar region organized in hydroxyl group at C-3, however plant sterols have an extra hydrophobic carbon chain (methyl or ethyl group) at the C-24 position (Figure 1.0.5) (Jayaprakasha G. K., 2015).

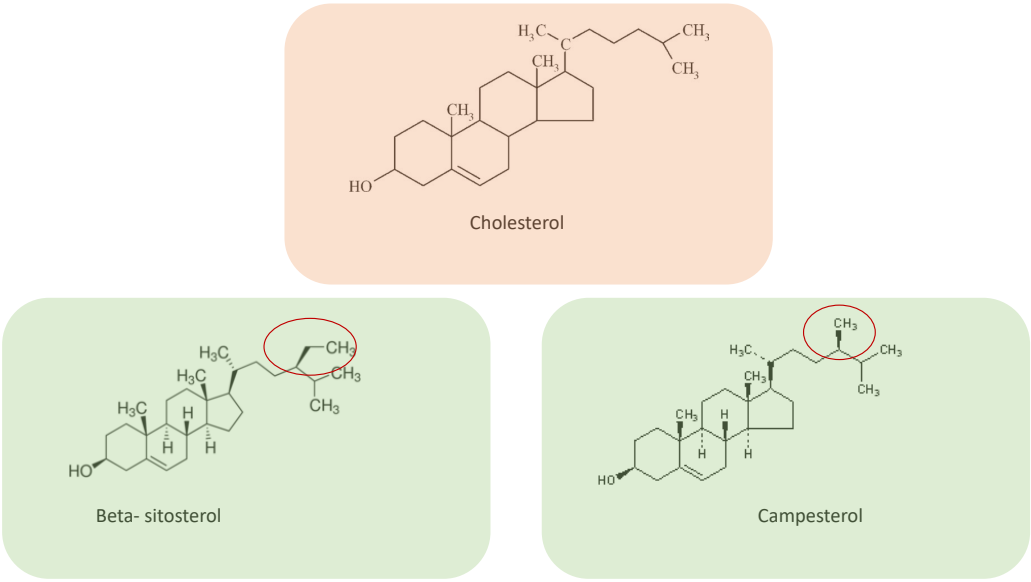


Figure 1.0.5 **the structure of cholesterol compared to plant sterols**. The key differences in structure are highlighted in the red circles. Cholesterol is structurally similar to plant sterols. The difference being that plant sterols have a one- or two carbon substituents with variable stereochemistry in the side chain at C-24.

Many animal and human studies report the capacity of plant sterols in promoting body health and in particularly as enriched functional food with the potential to reduce atherosclerotic risk (Köhler *et al.*, 2017). More specifically, plant sterols reduce the serum LDL cholesterol and lower atherosclerotic lesion development (de Jong, Plat and Mensink, 2003). Ryan *et al.*, 2017 show the capacity of plant sterol ester to allow the regression of atherosclerotic plaque in the aorta artery. It seems that the major activity of plant sterols is to reduce the inflammation and oxidative stress during the

atherosclerosis (Scolaro, Soo Jin Kim and de Castro, 2018). However, over many years the mechanism of action was not totally clarified. The most commonly used hypothesis is based on the concept that plant sterols compete with intestinal cholesterol for incorporation into mixed micelles or directly transports in the membranes of enterocytes (Smet, Mensink and Plat, 2012). It is interesting to note that in the mammal's intestine cholesterol is absorbed in a proportion of between 30-60%, while the absorption for plant sterols is lower around 2-3% (García-Llatas and Rodríguez-Estrada, 2011) and this was in contrast with the high capacity to reduce the cholesterol level on the serum. In the last years the predominant role of microbiota emerged. In fact, it was demonstrated that the unabsorbed sterols reach the colon where they are bio-modified by the microbiota and become a fermentable substrate for the microbial population and for energy supply to the host through anaerobic fermentative processes (Cuevas-Tena *et al.*, 2018).

During fermentation plant sterols decrease, and metabolites increase in conjunction with the global changes of the microbiota where the proportion of Erysipelotrichaceae species decrease but the abundance of phylotypes associated with *Eubacterium hallii* increased. In fact, most of the members of the genus *Eubacterium* isolated are cholesterol-reducing bacteria (Gérard *et al.*, 2007). *Eubacterium hallii* is an anaerobic bacterium belonging to the butyrate-producing *Lachnospiraceae* family and it was demonstrated as probiotic to increase faecal butyrate concentrations, to modify bile acid metabolism and, to improve insulin sensitivity (Udayappan *et al.*, 2016).

Plant sterols with a simple side chains structure as sitosterol and stigmasterol, easily are transported across the blood–brain barrier, and can be accumulated in the brain after a prolongate diet with plant sterols (Vanmierlo *et al.*, 2012a; Saeed *et al.*, 2014). Although, there are very few studies that examine the influence of plant sterols on the brain. A study in phytosterolaemia investigated the effect of plant sterols in the brain showing their influence on cholesterol homeostasis but no phenotypic effect in memory or anxiety related behaviour in the hippocampus and cortex (Vanmierlo *et al.*, 2011). Another study focusing in ageing and Alzheimer disease show how plant sterol diet prevents long-term potentiation impairment and cognitive deficits in SAMP8 mice without altering cholesterol levels but reducing the abnormally high amyloid peptide levels in the brain (Pérez-Cañamás *et al.*, 2016). The β-sitosterol extracted from a brown seaweed *Sargassum horneri* demonstrated the capacity to increased 5-ht on the brain underlining the antidepressant-like potentiality used as treatment (Zhao *et al.*,

2016). However, the real mechanism of plant sterols on the body is not totally investigated and the mechanism may involve the microbiota must be investigated.

1.2.4 Probiotics and microbiota

In 1965, Lilly and Stillwell described for the first time probiotics as "microorganisms stimulating the growth of other microorganisms" (Lilly and Stillwell, 1965). The definition of probiotics has been changed many times in the last fifty years. Fuller in 1989 declared that probiotics must be viable microorganisms and must exert a beneficial effect on their host (Fuller, 1989). The definitive version which is still in force currently was formulated by WHO (World Health Organization) and FAO (Food and Agriculture Organization of the United Nations) and describes probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host"(FAO, 2002). Probiotic act in in prophylactic manner in the treatment of infections and in the maintenance of balance between pathogens and the bacteria that are necessary for a normal function of the organism (Oelschlaeger, 2010). Probiotics are effectively able to inhibit pathogenic bacteria as was demonstrated by *Clostridium perfingens* (Schoster *et al.*, 2013)*, Salmonellla Enteritidis* (Carter *et al.*, 2017)*, Yersinia* (De Montijo-Prieto *et al.*, 2015) or various species of *Shigella* (Hussain *et al.*, 2017). More, probiotics have a positive effect on the treatment of candidoses (Kumar *et al.*, 2013) and other digestion processes as treatment of food allergies (Castellazzi *et al.*, 2013). However, the benefit of probiotics was demonstrated not only on gastrointestinal area (Sebastián Domingo, 2017) but also in the entire host. Many clinical studies have proven the positive effect of probiotics on systemic disease such as obesity, insulin resistance syndrome, type 2 diabetes, and non-alcoholic fatty liver disease (Koutnikova *et al.*, 2019). An important aspect is the effectiveness of probiotics on the immune system, human and animal health have been demonstrated by increasing the body's immunity (immunomodulation)(Azad, Sarker and Wan, 2018) and interfere with the body metabolism as adiposity and energy balance (Le Chatelier *et al.*, 2013). Reports also show the benefits of administration of probiotic strains may exert some prophylactic and therapeutic effects in a wide range of neurologic conditions (Umbrello and Esposito, 2016). In fact, live bacteria recently called "psychobiotics" (Dinan *et al.*, 2013) have been demonstrated to have mental health benefits. Several mechanisms for psychobiotic activity have been proposed

including an impact on the core stress system, namely, the hypothalamic–pituitary–adrenal (HPA) axis (Farzi, Fröhlich and Holzer, 2018a).

1.2.4.1 Mental disease and Probiotics

Many psychiatric disorders have been associated with gut microbiota changes (Rogers *et al.*, 2016). It has been demonstrated that some probiotic treatments (psychobiotics) play important roles in controlling the neural excitatory-inhibitory balance, thus influencing mood, cognitive functions, learning and memory processes (Bermúdez-Humarán *et al.*, 2019) though the gut microbiota brain axis. Indeed, the psychobiotics may intervene on different mental disorders (Sarkar *et al.*, 2016) and lead the regulation of neurotransmitters and proteins such as gamma-aminobutyric acid (GABA), serotonin, *nmda* and *bdnf.* The gene expression of *bdnf* and *nmda* regulate neurogenesis and synaptic plasticity in different brain area and a low level was observed in patients with major depression and in suicide (Dunham *et al.*, 2009; Guilloux *et al.*, 2012). In particular, *bdnf* was associated with increased susceptibility to neuropsychiatric disorders including depression, anxiety-related dysfunction and bipolar disorder (Lang *et al.*, 2005; Monteggia *et al.*, 2007; Post, 2007). The application of probiotics in psychiatry to improve synaptic functions and intervene in depression has been widely investigated in rodents and in animal study, while less evidence exists for probiotic actions on human population. In rats, for example, intake of *Bifidus infantis* was shown to change depression related biomarkers (Desbonnet *et al.*, 2008). In another study by Desbonnet et al., it is demonstrated that chronic-stressed mice no longer displayed depressive-like behaviours after *B. infantis* intake (Desbonnet *et al.*, 2010). Most psychobiotics belonging to the *Lactobacillaceae* family appear to have in preclinical studies, the most valuable benefits over anxiety- and depression-like behaviour though the modulation of *bdnf* and the central 5-HT system. *Lactobacillus helveticus* (Liang *et al.*, 2015) and *L. reuteri* show positive effect on the intestine as alleviated colitis and in the same time are able to increase the hippocampal *bdnf* in stressed mice (Jang, Lee and Kim, 2019). *L. helveticus* may has positive influence on the cognition increasing the *bdnf* expression and the central 5-HT system (Liang *et al.*, 2015). While *L. fermentum* seems to be able to reverse the antibiotic effect acting on *nmda* expression on the brain (Wang *et al.*, 2015).

In human studies, probiotics seem to have similar anxiolytic and antidepressant effects as those seen in preclinical studies, however clinical studies are few and inconsistent.

B. longum is able to reduce depressive-like symptoms in major depression patients (Kazemi *et al.*, 2019) and reduces anxiety-like behaviour alleviating psychological distress in healthy men and women (Messaoudi *et al.*, 2011). Depression is accompanied by immune system dysregulation with high levels of cytokines (Huang *et al.*, 2018) and the administration of Psychobiotics also play an important role in inflammatory modulation. *Lactobacillus rhamnosus and Lactobacillus brevis* have shown to elevate the production of *gaba* via glutamate decarboxylase in substantial amounts (Barrett *et al.*, 2012; Yunes *et al.*, 2016). *L.helveticus* acts in the immune system increasing anti-inflammatory cytokines such as IL-10 in the serum in conjunction with improved memory and cognitive performance (Liang *et al.*, 2015).
L. paracasei was able also to interfere on the concentration of *tnf-α* and MCP-1 in senescence accelerated mouse prone 8 (Huang *et al.*, 2018). Patients with inflammatory conditions (Groeger *et al.*, 2013) reduce plasma IL-6 levels after injection of *B. infantis*.
All of these studies suggest that an immunotherapy associated to use of psychobiotics in mental illness can be considered as a possible future prospect.

Table 1.0.2 Efficacy of probiotics on various mental disorders (summary of paragraph above).

Mental disorder	Probiotics	Results	Study	
Depression	*B. infantis*	displays depressive-like behaviours	(Desbonnet *et al.*, 2010)	Mice
Depression	*L.helveticus*	Amilorate cognition increasing the bdnf expression and the central 5-HT system	(Liang *et al.*, 2015)	Mice
Stress	*L. reuteri*	increases the hippocampal *bdnf* in stressed mice	(Jang, Lee and Kim, 2019)	Mice
Stress	*L.longum*	reduces anxiety-like behaviour	(Kazemi *et al.*, 2019)	Humans
Stress	*L.helveticus*	acts in the immune system increasing anti-inflammatory cytokines. It improves memory and cognitive performance	(Liang *et al.*, 2015)	Rats

Inflammation	*L. rhamnosus*	elevates the production of *gaba* via glutamate decarboxylase	(Jang, Lee and Kim, 2019)	Mice
Inflammation	*L. paracasei*	Reduces the concentration of *tnf-α*	(Huang *et al.*, 2018)	mice
Inflammation	*B. infantis*.	reduce plasma IL-6 levels		humans
Anxiety	*L.plantarum*	Stressed adults experienced a significant decrease in stress/anxiety symptoms	(Chong *et al.*, 2019)	humans

1.2.4.2 Cardiovascular disease and Probiotics

Cardiovascular disease can be caused by multiple factors but especially in the developed world by atherosclerosis. The formation of atherosclerotic plaque which gradually occludes the arteries and lead to heart attack or stroke is the consequence of many risk factors such as high blood pressure, high blood cholesterol, smoking, obesity, physical inactivity, diabetes and stress. These factors often contribute to a group of conditions common to metabolic syndrome and characterized by chronic inflammation which contributes to the onset of cardiovascular disease (Lopez-Candales *et al.*, 2017). In fact, metabolic syndrome is based on chronic low-grade inflammation (Sharma, 2011), impaired glucose tolerance, and obesity that increases an individual's chance to aggravate cardiovascular disease (Alshehri, 2010).

Emerging data indicate the relationship between gut microbiota and ischemic disease, Karlsson *et al.*, 2012 discovered that intestinal microbial communities in patients with atherosclerosis were different from those in healthy controls. In particular, another the gut bacteria in patients with symptomatic atherosclerosis show the changes in composition where the family of Enterobacteriaceae but also Streptococcus spp increase (Jie *et al.*, 2017).

Some studies indicate that modifying the intestinal microbiota with probiotics may impact the metabolic syndrome and related cardiovascular disease (Thushara *et al.*, 2016). Studies in rodents showed the capacity of probiotic therapy to attenuate heart failure (Moludi *et al.*, 2019). *Lactobacillus rhamnosus* administered as oral probiotic was able to attenuate the left ventricular hypertrophy, and improved systolic and

diastolic left ventricular function (Gan *et al.*, 2014). Another Lactobacillus, *L. plantarum,* improved vascular endothelial function and interestingly decreased systemic inflammation in men with cardiovascular disease (Malik *et al.*, 2018).

Most probiotics are involved in host immune modulation and several rodent studies found about a thousand bacteria species and their related properties in relation to inflammation diseases such as diabetes mellitus, cardiovascular disease and obesity (Yoo and Kim, 2016). Proper supplementation with certain probiotics is able activate the immune system and control of inflammation. Strains of Lactobacillus, Bifidobacterium, Lactococcus, Streptococcus and Enterococcus have been studied (Markowiak and Śliżewska, 2017). Certain probiotics may be effective in treating cardiovascular disease, but large scale mechanistic studies are required to produce definitive evidence (Liu, Alookaran and Rhoads, 2018). Furthermore, in-depth studies on the metabolism and the action of prebiotics in carbohydrate and lipid metabolism could help to broaden the knowledge of chronic inflammatory systemic diseases.

1.3 Microbiota and body metabolism

Metabolism is a process that describes all chemical reactions involved in maintaining the living state of the cells and the organism. The pathways of metabolism rely upon nutrients that they breakdown in order to produce energy. In the recent decades the intestinal bacteria got a predominant role in the body health of the host and it also has an important role in regulating body metabolism (Devaraj, Hemarajata, 2013). The study of metabolism through the microbiota started with the observation of germ-free animals. Mice lacking in microbiota are usually lean compared to normal type and recolonization leads to an increase of the body weight with an effect on adiposity (Bä ckhed *et al.*, 2004). It is well documented that the gut microbiota increases the host's ability to harvest energy and produces metabolites that are signalling molecules which modulate energy uptake, energy storage and also gut motility and appetite (Bauer, Hamr and Duca, 2016). This was well illustrated in a study of faecal transplantation where the transfer of microbiota from lean and obese human twins into germ-free mice, resulting in the conveyance of the metabolic phenotype (VK *et al.*, 2013). In a clinical study, patients with Metabolic Syndrome after faecal transplantation from lean and healthy donors showed an increase in insulin sensitivity (Vrieze *et al.*, 2012). These results suggest that microbiota can be considered as an additional organ that can

exert a positive or negative effect on cholesterol, glucose and lipid metabolism (Figure1. 0.6).

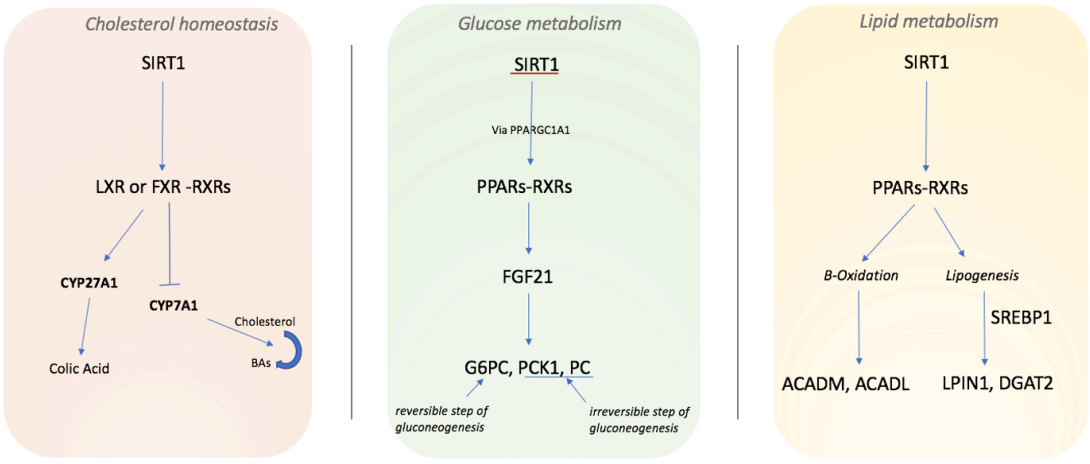


Figure1. 0.6 Cholesterol, Glucose and lipid metabolism on the liver.

Red box: Sirt1 stimulates the Lxr or Fxr -Rxrs and it going to blocks the Cyp7A1 (enzyme that catalyses the cholesterol in bile acid (BAs)) or Cyp27A1 that catalyses oxidation to produce Colic acid. Green box: shows the normal glucose metabolism way where Sirt1 activates the association between heterodimers Ppars- Rxrs that trigger the gluconeogenesis. Yellow box: the lipid metabolism is showed through the Sirt1 that stimulates the aggregation of Ppars and Rxrs family that stimulates the enzymes involved in the B- Oxidation (Acadm, Acadl) and the lipogenesis (Lpin1, Dagt2) (Blue arrow).

1.3.1 Cholesterol homeostasis and microbiota

Cholesterol is an important element for mammalian cell and human health and its homeostasis is tightly regulated. Disturbance of cellular cholesterol homeostasis is linked to atherosclerosis and cardiovascular diseases. Atherosclerosis, coronary heart disease, and stroke are characterized by high cholesterol level in particularly LDL (Levine, Keaney and Vita, 1995). Cholesterol is a complex molecule that is produced in the liver and brain (Simons and Ikonen, 2000) but it can also derive from the diet and be absorbed in the intestine (Orth and Bellosta, 2012). So, the plasma cholesterol level is a result of those strategies where the mechanism is not totally understood.

It is well known that the development of atherosclerosis is associated with gut dysbiosis (Drosos, Tavridou and Kolios, 2015; Gregory et al., 2015; Jie et al., 2017) and many studies have been attempted to discover strategies to reduce cardiovascular disease risk and probiotics have demonstrated to be an efficient strategy (Ebel et al., 2014). Many probiotics belonging to the genus Lactobacillus have demonstrated to have positive effect on patients with hypercholesterolemia. For example,

Lactobacillus plantarum has showed to reduce the circulating cholesterol levels and inhibits the formation of atherosclerotic plaques (Fuentes *et al.*, 2013). *L. plantarum* MA2 (1011 CFU/day) also showed its utility in controlling cholesterol homeostasis reducing not only the total cholesterol level in the serum but also total liver cholesterol and triglycerides (Wang *et al.*, 2009). Another bacteria such as *Lactobacillus reuteri* after 5 weeks of oral treatment, reduced LDL and total cholesterol levels in the serum compared to subjects given placebo capsules (Jones, Martoni and Prakash, 2012). Kawase *et al.*, observed an increase in HDL (+28%) and a decrease in triglyceride concentration (−20%) in rats supplemented with *L. casei* (~2.4×10^{11} CFU/day) and Streptococcus thermophilus (~10^{10} CFU/day) for 4 weeks. Moreover, a positive effect on LDL concentration has been observed after the treatment with *Bifidobacterium longum* (Xiao *et al.*, 2003).

Table 1 0.3 The effect of most used probiotics in cardiovascular disease (summary of paragraphs above)

Probiotics	Results	Study	
L. rhamnosus	attenuates the left ventricular hypertrophy, and improved systolic and diastolic left ventricular function	(Gan *et al.*, 2014)	Rats
L. plantarum	improves vascular endothelial function and decreases systemic inflammation	(Malik *et al.*, 2018)	Humans
	reduces the circulating cholesterol levels and inhibits the formation of atherosclerotic plaques	(Fuentes *et al.*, 2013)	Humans
L.reuteri	restores cardiac function by reverse the level of caspase 3/e Poly polymerase involved in the apoptosis in the cardiomyocytes	(Liao *et al.*, 2016)	Rats
	reduces LDL and total cholesterol levels in the serum	(Jones, Martoni and Prakash, 2012)	Humans
L.mucosae	Reduce cholesterol and lipid profile on cardiovascular disease model	(L. E. London *et al.*, 2014)	Mice
L.acidophilus	inhibites intestinal cholesterol absorption and reverse cholesterol transport in macrophages	(Yoshida, Yamashita and Hirata, 2018)	Mice

L. casei	decrease in triglyceride concentration	(Kawase *et al.* 2000)	Rats
B. longum	total cholesterol, low-density lipoprotein cholesterol, and triglycerides	(Xiao *et al.*, 2003)	Rats and Human

However, the mechanism of action of these probiotics is unclear. Indeed, the cholesterol homeostasis is a very complex process and the level of cholesterol in the body is governed by a delicate balance that occurs between production *de novo* in the liver and intestinal absorption. Recently, the impact on bile acids (BAs) has been investigated (Kriaa, Bourgin, Potiron, *et al.*, 2019). Cholesterol is the initial step of BAs synthesis in the liver (Figure1. 0.7) that involved mainly three cytochrome P450 family enzymes cyp7a1, cyp27a1 and CYP 8 (Chiang, 2013).

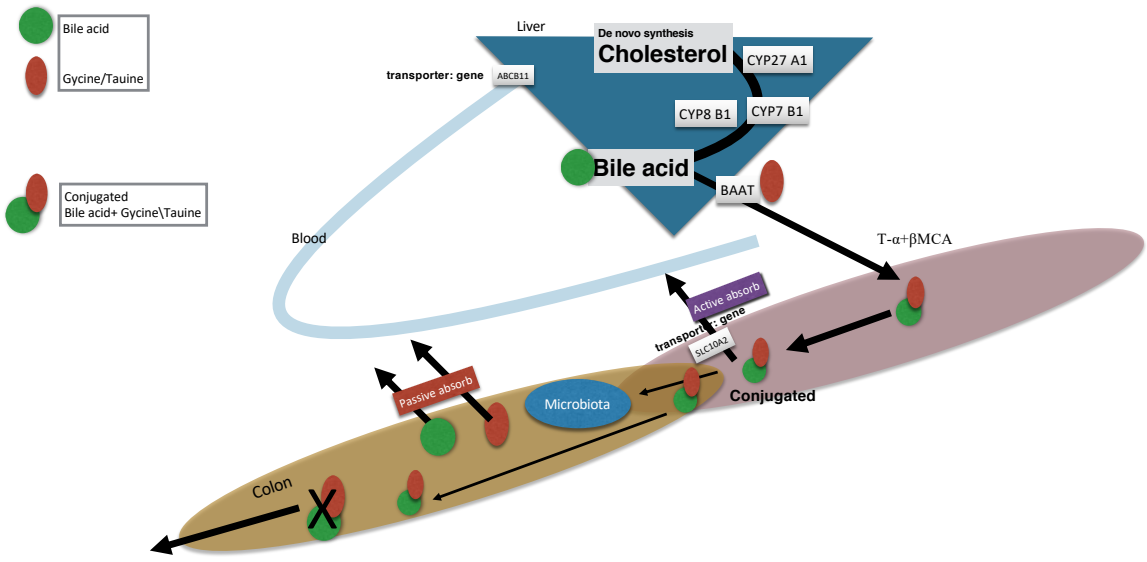


Figure1. 0.7 Bile acid pathway and microbiota. In the liver, cholesterol is transformed into bile acid by 7α-dehydroxylase CYP A1 and CYP 7 B1 enzyme. Bile acids travel via the bile duct to the intestine and they are conjugated to glycine and taurine before being secreted into the duodenum. In the distal part of the small intestine, they are usually absorbed in their conjugated form via active transport such as sodium dependent BA transporter encoded by Slc10a1 gene (SLC10A2) or via passive diffusion in the de-conjugated form. In this way the majority of bile acids are recycled through the portal circulation.

Following synthesis of the new bile acid, they are conjugated to glycine and taurine before being secreted into the duodenum along with the bile flow, which plays a major role in fat emulsification, the release of fat-soluble vitamins and the regulation of cholesterol metabolism (Barnard and Heaton, 1973). BA composition depends on the diet and individual genetic characteristics, but usually the composition includes over 50% BAs, over 20% fatty acids and cholesterol, and lower amounts of other molecules such as bilirubin (Farina, Dumonceau and Lescuyer, 2009). During gastrointestinal transit, most BAs and cholesterol are reabsorbed (~95%) in the distal small intestine (Hegyi et al., 2018) and transported back to the liver via the portal circulation (Figure1. 0.7). Only ~5% of bile salts are lost through the faeces (Islam et al., 2011). To be reabsorbed the bile acids conjugated require an active transporter as Na+-dependent SLC10A transporter. The recycling of BAs allows the regulation of the synthesis of the BAs through a feedback mechanism. Bile acids work as signalling molecules that activate nuclear hormone receptors such as farnesoid X receptor alpha (*fxrα*, NR1H4) that forms a heterodimer with the Retinoid X receptor (*rxr*), a member of the nuclear receptor family. *fxrα* regulates the enterohepatic synthesis of BAs by controlling the expression of *cyp27a1* and inhibiting *cyp7a1* genes.

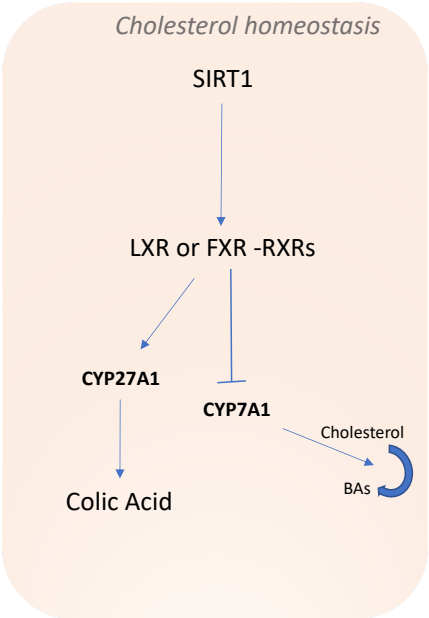


Figure1.0.8 **SIRT1 regulates transcriptional factors and genes that encode enzymes involved in cholesterol metabolism in the liver.** Sirt1 stimulates the *Lxr* or *Fxr -Rxrs* and it going to blocks the *Cyp7A1* (enzyme that catalyses the cholesterol in bile acid (BAs)) or *Cyp27A1* that catalyses oxidation to produce Colic acid.

However, bile acids and consequently cholesterol to be efficiently recycled are required to be in unconjugated so passive absorption can occur all along the intestine, including the colon. The host microbiota interacts with cholesterol reabsorption by deconjugating the BAs thus facilitating passive transport. The ability to deconjugate BAs has been catalysed by bacterial bile salt hydrolases which are attributed to different genera such as *Lactobacillus*, *Bifidobacterium, Clostridium*, *Enterococcus* and *Bacteroides* (Jones *et al.*, 2013). The capacity to increased levels of deconjugated bile acids by microbiota was demonstrated in a rodent study where mice, treated with three days of antibiotics, increased biliary bile acid output threefold while faecal output decreased by 70% (Miyata *et al.*, 2009). The probiotic approach to cardiovascular disease has been considered in the last decade as a method to compensate for side effects but also limitations in the effectiveness of the drugs used so far such as statins or cholesterol absorption inhibitors (Jones *et al.*, 2013). The inefficient of these treatment are evidenced by the failure to reach the goal on pharmacological monotherapy where only the 38% of patients treated for dyslipidaemia and only 18% treated for LDL- cholesterol reached the goals (Pearson *et al.*, 2000). As matter of fact, in a recent study of comparison between different cardiovascular disease treatments, statins (atorvastatin) showed a mild protective effect compared to probiotic and prebiotic treatments with no atherogenic plaque development or reduction of triglycerides (Ryan *et al.*, 2017). Previous work failed to demonstrate an ability of a Simvastatin-based intervention to prevent plaque development in a murine model (Bea *et al.*, 2002). However, simvastatin is an example of a cardiovascular disease drug which modulates gut microbiota composition and whose efficacy appears to rely on a suitably responsive microbiome (Pearson et al., 2000). Therefore, further research is necessary to deepen knowledge about the microbiota in the treatment of cardiovascular diseases and the use of drugs must be considered in conjunction with the effects on the microbiota.

Microbiota is directly involved on the cholesterol metabolism. Some of them are able to use host cholesterol as a source of carbon and energy or degrade it for the synthesis of new steroid derivatives (Tak, 1942; Fernández de las Heras *et al.*, 2009; Ge *et al.*, 2011). The cholesterol oxidases that producing coprostanol from cholesterol, is the most common enzyme in the microbiota and it is frequently found in the bacterial genomes and faecal metagenomes (Molinero *et al.*, 2019). The conversion of

cholesterol in coprostanol gives rise to its direct excretion in faeces, a reduction in cholesterol absorption with a consequently a decrease of total cholesterol concentration in the body(Ooi and Liong, 2010).

Particularly, the cholesterol oxidases gene was found in *bacteroidetes, proteobacteria* and *actinobacteria* but not in Firmicutes phyla, more abundant in the humans (Molinero *et al.*, 2019). *In vitro* study, *Bifidobacterium, Clostridium*, and *Lactobacillus* were reported to reduce cholesterol to coprostanol (Lye, Rusul, 2010). High level of coprostanol was associated to *Lachnospiraceae* and *Ruminococcaceae* families in healthy humans (Kriaa, Bourgin, Mkaouar, *et al.*, 2019). In fact, coprostanol production was associated to the strains of lactobacilli in particularly to *Lactobacillus casei* (Watson *et al.*, 2016). Coherently, *L. casei* is used as probiotic to prevent and treat cardiovascular disease (Yap *et al.*, 2016; Vasquez *et al.*, 2019). An earlier study, evaluating microbial changes in relationship to antibiotic use, decreased conversion of cholesterol to coprostanol (Gustafsson, Lund-Tønnesen and Midtvedt, 1999). It seems that the role of microbiota is essential for cholesterol homeostasis in all body but the data are still insufficient to understand the strategies of the microbiota on the metabolism (Kriaa, Bourgin, Mkaouar, *et al.*, 2019).

1.3.2 Glucose metabolism and microbiota

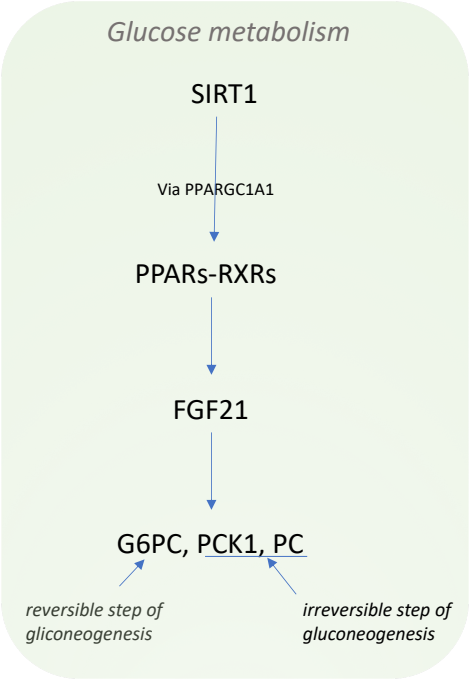


Figure 1.0.9 **SIRT1 regulates transcriptional factors and genes that encode enzymes involved in glucose metabolism in the liver**. *via Ppargc1a1*, *Sirt1* activates the association between heterodimers *Ppars-Rxrs* that trigger the gluconeogenesis. *Sirt1*=sirtuin (silent mating type information regulation 2 homolog) 1; *Ppargc1a1*= peroxisome proliferator-activated receptor gamma coactivator 1-alpha; PparS=peroxisome proliferator-activated receptors; *rxrs*= retinoid X receptors; *fgf21*= Fibroblast growth factor 21; *G6pc*= Glucose-6-phosphatase, catalytic subunit; *pck*1= Phosphoenolpyruvate carboxykinase 1; PC= Pyruvate carboxylase.

Glucose metabolism is regulated by transcriptional factors in the liver as the peroxisome proliferator-activated receptors (*Ppar*) group (Figure 1.0.9). External factors such as diet, fasting and stress can induce modifications in these molecules (De Cosmo and Mazzoccoli, 2017). The nuclear hormone receptor *Ppar* heterodimerizes with the retinoid X receptor (*Rxr*) leading the transcription of genes related to maintaining adequate lipid and glucose homeostasis (Krey et al., 1997). Often, this process is mediated by the NAD-dependent deacetylase sirtuin (encoded by Sirt1), a protein with a central role in energy metabolism (Sedding and Haendeler, 2007). Sirt1 supports gluconeogenesis via peroxisome proliferator-activated receptor (Ppar) γ and has an effect on *G6pc, Pck*1, *Pgc1* genes (Cyphert et al., 2012). It also forms a dimer with *Pparα* and stimulates *Fgf21* which is essential for maintaining glucose

homeostasis (Planavila, Redondo-Angulo and Villarroya, 2015). An over expression of *Sirt1* gene can give benefits in the body such as glucose tolerance, reduction of inflammation but also a protection against hepatic steatosis (Lakhan and Kirchgessner, 2011); consequently, Sirt 1 has an important role in metabolic syndrome including obesity and diabetes and it can be considered as a target for new strategies against metabolic disfunctions (Y *et al.*, 2012). Microbiota is also well-known to be involved in metabolic dysfunction (Devaraj, Hemarajata, 2013) but there are few studies that investigates the role of microbiota in the regulation of Sirt 1. For example, Chen at al. showed the capacity of *Lactobacillus mali* to reduce the lipid accumulation in the liver by regulating the gene expression of Sirt1 but also having an effect on *Pgc-1α* level involved in the gluconeogenesis (Chen *et al*, 2018). However, few studies investigated the role emergent role of microbiota in the intestinal gluconeogenesis (de Vadder, 2018),(Mithieux, 2018). Microbiota metabolites such as small products deriving from protein digestion or short-chain fatty acids (SCFA) from bacterial fermentation activate the intestinal gluconeogenesis, that contributes to glucose homeostasis. In particular, SCFAs such as propionate act as glucose precursors in the gut, which lead to the activation of a portal glucose sensor (Delaere *et al*., 2013), resulting in improved glucose tolerance and insulin sensitivity (F *et al*., 2014). Succinate, in particular, is an intermediate in the production of propionate from the microbial fermentation of polysaccharides and oligosaccharides(JH *et al*., 1987) and it was identified as a substrate for intestinal gluconeogenesis (IGN), controlling the glucose homeostasis (De Vadder *et al*., 2016). A diet rich in fibre increases the production of succinate and consequently improving glucose and insulin tolerance (De Vadder *et al*., 2016). The major producers of succinate in the gut are bacteria from the phylum Bacteroidetes (Miller and Wolin, 1979). In particular, it was observed that diet rich in polyphenols *Prevotella copri* contributes to an improvement in glucose control through succinate-dependent mechanisms (Kovatcheva-Datchary *et al.*, 2015). *Akkermansia muciniphila* belonging to the *verrucomicrobia* phylum, is another bacteria that demonstrated to have beneficial effect on glucose homeostasis (Everard *et al.*, 2013). This human intestinal mucin-degrading bacterium, was able to decrease G6pc mRNA expression to counteract fasting hyperglycaemia in the mouse model (Everard *et al.*, 2013) ameliorating the type 2 diabetes (Shin *et al.*, 2014).

1.4 Microbiota and Ageing

The gut microbiota is a complex ecosystem that changes considerably with individuals and time, making age-related modifications difficult to measure. However, during the life span every stage does show differences in gut microbe composition.

The microbial human colonization begins at birth. However, recently a few controversial studies report the presence of bacteria in amniotic cavity, umbilical cord, and meconium supporting the theory that the dynamic of colonization have already started in the womb (Collado *et al.*, 2016; Nagpal *et al.*, 2016).

After that, the microbiota is drastically shaped by diet and environment especially during the first year before to be converged to adult microbial composition (Palmer *et al.*, 2007). An interesting report suggested that the microbiota population evolves towards an adult-like configuration within the 3-year period after birth (Yatsunenko *et al.*, 2012). Although the individual gut microbiota is largely unstable in the early years of life (Cheng *et al.*, 2016), it become more stable during adulthood (Stewart *et al.*, 2018) and then it undergo dramatic changes in diversity and composition upon onset of ageing (Yap *et al.*, 2016). A detailed study on bacterial composition across all stages of life (Odamaki *et al.*, 2016) reported that the gut microbiota evolves until 20 years old and then, the composition is stable until 30-40 years old (middle age) where it changes again into the elderly type highlighting 3 more different elder stages 60, 70-80, 90-100. In particular, *Bacterioides* increase around 70 years and then decreased again towards the age of 80; at the contrary *Firmicutes* increase around 60 years old but reduce at 70 to raise again after 90. *Actinobacteria,* which are abundant in the first 2 years of life, decrease at 20 to increase in the middle age and drop again in ageing. *Proteobacteria* are abundant from birth to the first 2 years decreasing in the middle age and then from 70 upwards again (Odamaki *et al.*, 2016).

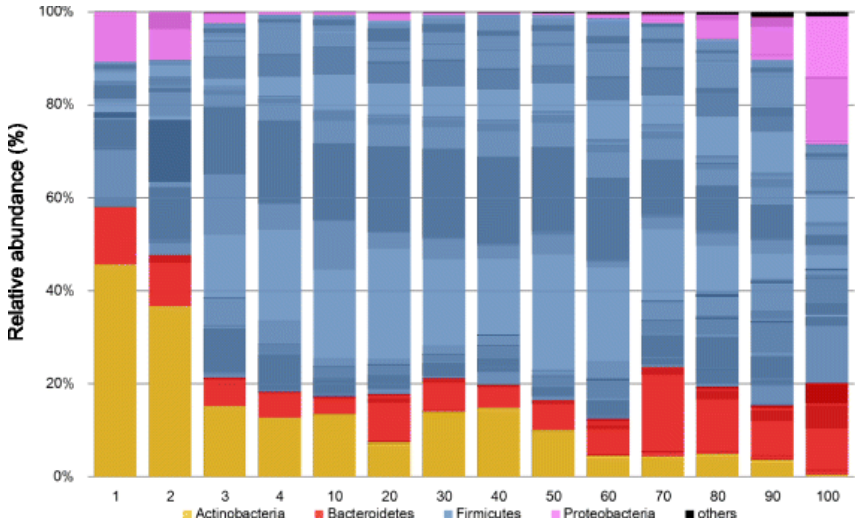


Figure1. 0.10 Gut microbiota composition in different stages of life. Overview of phylum/genus composition. Orange, Actinobacteria; Blue, Firmicutes; Red, Bacteroidetes; Pink, Proteobacteria; Black, sum of other phyla. Each component of the cumulative bar chart indicates a genus. Groups:1= Preweaning, 2 = Weaning, 3 = Weaned-3 years old, 4 = 4–9 years old, 10 = 10–19 years old, 20=20–29 years old, 30 = 30–39 years old, 40 = 40–49 years old, 50 = 50–59 years old, 60 = 60–69 years old, 70 = 70–79 years old, 80 = 80–89 years old, 90 = 90–99 years old, 100 = Over 100 years old (Odamaki *et al.*, 2016).

If we look in detail where the phylum of *Bacterioides* increase at the expense of *Firmicutes* and *Actinobacteria* (Ottman *et al.*, 2012) there is important increasing of the proportion of *Bacteroides, Eubacterium and Clostridiaceae; Enterobacteriaceae* are only enriched in infant and elderly (Yatsunenko et al., 2012). It is interesting to note that ageing is characterized by a modification of the *Lactobacillus* population (Mueller *et al.*, 2006). Particular strains of lactobacillus are abundant only in old people such as *L. paracasei* and *L. plantarum* that are predominantly found in elderly whereas *L. salivarius* and *L. helveticus* that are in higher levels in adults. (Le Roy *et al.*, 2015). It is interesting to note, that as the Bifidobacterium population generally decreases in elderly, the microbiota composition in institutionalized elderly is diverse and different for each individual and Bifidobacterium longum, *B. adolescentis* and *B. bifidum* were the common species (Ouwehand *et al.*, 2008). *Akkermansia muciniphila* decrease in old people and it is negatively correlated to a reduction of antimicrobial factors Ang4 and lysozyme (Sovran *et al.*, 2019).

However, the senescence microbiota profile seems very similar to a clinical picture of some disease characterized by dysbiosis for abundance of pro-inflammatory pathobionts (Maynard and Weinkove, 2018). It remains unknown if this gut dysbiosis

is a natural cause or consequence of ageing and senescence-associated inflammatory disorders; although it has been clearly demonstrated in a germ-free mice study that microbiota from old (18-22 months old that correspond to 56-69 year old in humans (Flurkey, M. Currer and Harrison, 2007)), but not young, mice promoted systemic inflammation in germ-free mice after co-housing increasing pro-inflammatory cytokine levels (Thevaranjan *et al.*, 2017).

During the senescence, chronic inflammation called Inflammageing by Franceschi *et al.*, 2000 develops, which contributes to the pathogenesis of age-related diseases.

The entire organism is involved in a gradual deterioration due to the slowing down of cellular metabolic functions. Systemic inflammation appears and basal metabolism decreases, the ability to absorb nutrients is reduced, abdominal fat increases to the detriment of muscle mass (Huffman and Barzilai, 2009). Antioxidant systems lose their effectiveness and there is an increase in lipid peroxidation in both blood and liver (Honma *et al.*, 2011). The immune system becomes slower to respond (Immunosenescence) (Cannizzo *et al.*, 2011). The metabolic slowdown and the diffuse inflammation appear to be the basement of the risk of majority of the elderly diseases such as diabetes, cardiovascular disease, cancer but also mental degeneration such as Alzheimer or Parkinson disease (Shimokata and Kuzuya, 1993; Grant and Dixit, 2013; Costantini, D'Angelo and Reale, 2018).

1.4.1 Animal Models involved on ageing study

The majority of the study that investigates the relationship between microbiota and the elderly has been performed on mice, even if, only a few years ago, it was more common to use a different animal model for studies on ageing, age-related diseases and mechanisms of longevity, such as *C. elegans. C.elegans is* a nematode that lives for about three weeks and showed a progressive decline among the ageing in cytosolic phosphoenolpyruvate carboxykinase (PEpck-C), and a reciprocal increase in pyruvate kinase (PK) that reduces efficiency and total energy production (Yiyuan Yuan *et al.*, 2016).

The advantage of using C. elegans is that they present a short lifespan (~3 weeks) and their small size is favourable for the screening of anti-aging drugs due to the reduced experimental costs and lack of ethical concerns (Won, 2015). The problem with using C.elegans is that they are lack certain anatomical features of mammals, for example a blood transport system, a blood-brain barrier, a first-pass metabolism process in the

liver and also their lack of long-range transcriptional regulation makes them unadaptable for studying the relevant mechanism in other animal species (Zhang *et al.*, 2020).

Currently, mice are the most attractive model for studying mammalian biology, as they present genetic manageability of their genome, ease of breeding, and the large amount of available baseline phenotypic data (Kõks *et al.*, 2016). However, the simplest and best model for an ageing study is an old organism but studies on old mice require more time and this significantly increases the cost. Therefore, it is tempting to study ageing using mice with accelerated ageing and/or reduced lifespans (Vanhooren and Libert, 2013). In preclinical ageing research, genetically-engineered mice models are based on model trying to accelerate ageing by recreating the most typical disease of old age. For example, IL-10 deficient mice (IL-10tm/tm), an ageing model based on the presence of chronic low-grade of inflammation (inflammaging), were used to accelerate delayed ageing phenotypes and those mice presented premature age-related changes such as skeletal changes, increased cellular senescence and an abondance of tissue inflammation and central nervous system gliosis (Bernal *et al.*, 2014).

The most commonly used mutant animal is ApoE deficient mice that develop severe hypercholesterolemia and have lesions that progress from fatty streaks to fibrous plaques distributed in lesion-prone areas throughout the aorta. Lesions develop in apoE-deficient mice on a regular chow diet and will occur faster on a diet higher in cholesterol. However, we have to consider some physiological differences as the lipid profile in the mice is very different from that in humans, who carry about 75% of their plasma cholesterol in LDL. Mice carry most of their cholesterol in high-density lipoprotein (HDL), which in humans is considered protective. However, the lesion progression and cell types involved in ApoE deficient mice are similar, as is the presence of oxidised lipoproteins. In fact, ApoE-knockout mice are considered to be one of the most relevant models for atherosclerosis (Lo Sasso *et al.*, 2016).

Another animal model for ageing studies is β-cell specific Wfs1gene deficiency mice that have a reduction of body weight and a low insulin level at almost in 12 weeks of life and they are used to study the elderly and diabetes (Riggs *et al.*, 2005). Similarly, a mouse lacking the wolframin gene (*Wfs1-LacZ*) is characterised by diabetes but also psychiatric symptoms and growth retardation (Noormets *et al.*, 2011).

However, studies on mutant mice should be interpreted with care. Those mice might show typical features of ageing, but they often exhibit features not seen in normal old

mice. Consequently, all available mouse models of accelerated ageing do not fully represent normal ageing in mice (Miller, 2004). Moreover, the recent ageing studies need to investigate the impact of comorbidity on different factors, for example, life quality and longevity. Therefore, animal models are a useful tool to investigate possible treatments in the short time.

1.4.2 Ageing Metabolism

The body metabolism is able to maintain the homeostatic processes in balance to keep the cells and consequently all organism alive. During ageing, this process is gradually deteriorated leading the body to a functional decline and increased risk of disease and death. The ageing is characterized metabolically by impairment in lipid and glucose homeostasis that lead to most common elderly diseases such as hepatic gluconeogenesis, adipose lipogenesis, defective glycogen synthesis and, increase in insulin resistance (Barzilai *et al.*, 2012b). The cholesterol metabolism is also altered which coincides with the onset of risk in cardiovascular disease including atherosclerosis, hypertension, and stroke (North and Sinclair, 2012).

1.4.2.1 Ageing hepatic metabolism

The liver is a fundamental organ which plays a central role in metabolism of nutrients, synthesis of glucose, lipids, cholesterol, and detoxification of drugs and xenobiotics (Chiang, 2014). During ageing, the physiological slowdown of bodily functions may compromise the major pathways in the liver such as glucose, fatty acids, cholesterol, and bile acid synthesis.

The first macroscopic change that can be seen between a young and an old liver is the volume and the weight (Katayama *et al.*, 1990). Studies in aged mice and rats have provided evidence of significant declines in hepatic weight similar to those observed in humans (Modi, Katyare and Patel, 2008). The reduction of hepatic volume is more marked in woman (up to 44%) then in men (around 20%-40%), (Schmucker, 2005) and it is associated with hepatic and sinusoidal dysfunction, with elevated hepatic vascular resistance and increased portal pressure (Maeso-Díaz *et al.*, 2018).

Microscopically, elderly subjects have elevated numbers of hepatocytes with a decreased numbers of mitochondria compared to young (Schmucker and Sachs, 2002). In fact, mitochondrial dysfunction has been also implicated as a major causative factor underlying the decline in physiologic functions associated with the process of ageing. Substantial evidence in humans and rodents shows that ageing induces the loss of mitochondrial function in liver (Muller-Hocker *et al.*, 1997). An early study has shown that hepatic expression of various nuclear receptors involved in mitochondrial metabolism declined in senescent rats (Pallet *et al.*, 1997).

The peroxisome proliferation-activated receptor family (*Ppars*) are a group of **nuclear receptor proteins** that act as transcription factors for gene expression, in particular *Ppar*δ is contributes to the regulation of glucose and lipid metabolism and is involved in regulating mitochondrial metabolism and fatty acid β-oxidation (Zardi *et al.*, 2013). In 100 week old animals, peroxisomal β -oxidation and catalase activities were significantly lower in livers of the old animals compared with young groups. In the senescent group, the level of decline in both peroxisomal enzyme activities of 30% was surprisingly similar to the decline observed in the hepatic expression of the rxra protein. (Chao *et al.*, 2002). The level of *Ppars* were associated to longevity in a study of Ppar-null mice altered expression of *Ppars* and it was correlated to increased oxidative stress with age (Sung *et al.*, 2004).

Another hepatic group of transcriptional regulation factors are *Lxrs* (or *Nr1h3*) that act as cholesterol sensor and thereby regulate cholesterol homeostasis (Millatt *et al.*, 2003). *Nr1h3* is also involved in specific processes that increase resistance to pathogens regulating innate immunity with the activation of macrophages for cytokines production (Joseph et al., 2003); and in fact it is considered a mortality-related marker as it decrease with life-span extension (Mooijaart *et al.*, 2007).

Fibroblast growth factor 21 (*Fgf21*) is a member of the endocrine *Fgfs* subfamily that regulates liver and extrahepatic tissue glucose and lipid metabolism (Liu *et al.*, 2020). Circulating fgf21 levels in humans increase with age from 5 to 80 years in healthy individuals (Hanks *et al.*, 2015). In contradiction, low levels of *fgf21* are related to healthy ageing in centenarians (Sanchis-Gomar *et al.*, 2015), in fact endurance

exercise in elderly individuals reduces *fgf21* levels (H *et al.*, 2016). *fgf21* was considered as a pro-longevity hormone (Y. Zhang *et al.*, 2012).

Together with nuclear receptors, the majority of P450s enzymes (*fxr, Cyp7a1*, *Cyp7b1. Cyp8b1, Cyp27a1)* maintained relatively high levels throughout the adulthood and decreased quickly around 24-26 month of age in mice (Xu *et al.*, 2019). The *Cyp7* families, together with *Cyp8* and are important for cholesterol and bile acid metabolism (Liu *et al.*, 2014). However, it is not clear yet the trend of these enzymes specifically during ageing, although an early study found a different in composition of the bile acid and the cholesterol level in the liver in old rats (24 months) compared to young (8 weeks) or middle age animals (12 months); in particularly cholic acid increased and β-muricholic acid decreased by ageing without altering the quantity production. However, in the same study, aged germ-free rats have been shown to reduce bile synthesis, suggesting that bile acid production is linked to the microbiota even in old age (Uchida *et al.*, 1990). In fact, it is well documented the interaction between cholesterol, bile and microbiota (Valdivieso *et al.*, 1978), (Bertolotti, et al., 2007). Although further studies would be needed to elucidate the underlying mechanisms.

Ageing is frequently characterized by glucose intolerance (Chia, Egan and Ferrucci, 2018). In some the progressive decline in glucose tolerance is evident from the third decade through the ninth decade of age. An early study established in particular that the 2h plasma glucose level during an oral glucose tolerance test rises on average, 5.3 mg/dl per decade and the fasting plasma glucose rises on average, 1mg/dl per decade (Davidson, 1979). Only few studies investigated the hepatic glucose metabolism in elderly (Feng *et al.*, 2016). The most frequently used genetic model of ageing is *C. elegans* a nematode that lives about three weeks and showed in ageing a progressive decline in cytosolic phosphoenolpyruvate carboxykinase (*Pepck*-C), and a reciprocal increase in pyruvate kinase (PK) that reduce the efficiency of and total energy production (Yiyuan Yuan *et al.*, 2016).

In rodents, caloric restriction seems to postpone the onset of age-associated pathologies modifying activity of key hepatic enzymes involved with gluconeogenesis (Dhahbi *et al.*, 1999; Spindler, 2001; Tollet-Egnell *et al.*, 2001; Yamaza *et al.*, 2002) However, rodents are a poor model for investigate metabolic

syndrome (O'Donovan *et al.*, 2020) and human clinical data are scarce. More data are necessary to understand the levels of regulatory enzymes of metabolic energetic path at different ages.

1.4.2.2 Probiotics and Prebiotics effect in aged liver

The main aim of ageing research is to improve longevity and delay as much as possible the slow but progressive deterioration of the body of the elderly. The most effective extrinsic factors have been identified such as life-style and diet, which drives into microbiota changes (Salazar *et al.*, 2017).

Since it is difficult to change the lifestyle and diet completely in elderly individuals with well-established habits, one of the relevant nutritional strategies is the use of probiotics and prebiotics.

Probiotics and prebiotics can be considered therapeutic alternative interventions in age-related liver degeneration as a strategy to improve the quality of life against unhealthy ageing. In general, some probiotics which have been suggested for the treatment of chronic liver damage, operates mostly thought decreasing inflammation, and stimulation of host immunity (Cesaro *et al.*, 2011; TH, JK and CJ, 2011).

A well-known mixture of probiotics called VSL#3 (*Streptococcus salivarius* subsp. *Thermophilus, Bifidobacterium breve* , *B. infantis* , *B. longum*, *Lactobacillus acidophilus , L. plantarum , L. casei , and L. delbrueckii subsp. Bulgaricus*) has been widely investigated and it demonstrated to have positive effect on the liver and immunity, in particularly it was able to improved liver histology, reduced hepatic total fatty acid content, decreased serum alanine aminotransferase (ALT) levels, and reduced the activity of proteins involved in regulating *tnfα* and insulin resistance (Li et al., 2003). In mouse model of non-alcoholic steatohepatitis with methionine-choline-deficient diet, the same VSL#3 treatment failed to prevent steatosis or inflammation but ameliorated liver fibrosis by decreasing expression of procollagen and matrix metalloproteinases (Velayudham *et al.*, 2009). VSL#3 treatment in HFD rats fed the probiotics mixture reduced the expression of lipid peroxidation markers in particular Pparα expression was greater in treated group by reducing inflammatory response (Esposito *et al.*, 2009). In clinical studies, VSL#3 improved oxidative/nitrosative stress parameters in patients with NAFLD and patients with alcoholic liver cirrhosis, but no effects were observed in patients with chronic hepatitis (Loguercio *et al.*, 2005; Dhiman *et al.*, 2014).

However, only recently few research have investigated the specific effect of probiotics on the hepatic enzymes and metabolism without dwelling only on generic anti-inflammatory aspects. A recent study investigated the transcription of genes involved in the *Lxr* axis in the liver, a single probiotic (*Lactobacillus rhamnosus* LV108) upregulated *Lxrα, Lxrβ, Abca1, Abcg1, Scd1*, and Pparα gene transcription levels and downregulated *Srebp-1C* and *Fas* gene transcription levels was more effective than by the combined probiotic (*L. rhamnosus LV108, Lactobacillus casei, and Lactobacillus fermentum*) (Wa *et al.*, 2019).

In fact, in another research*, L.rhamnosus* GG, after 12 weeks of treatment, increased the hepatic *Pparα* expression and faecal butyrate concentration associated with increased hepatic *Fgf21* mRNA expression and circulating *Fgf21* protein levels in mice with metabolic syndrome exposed to chronic intermittent hypoxia (Liu *et al.*, 2020). In obese mice a mixed lactobacilli supplementation (*Lactobacillus plantarum KLDS1.0344 and L. plantarum KLDS1.0386*) significantly improved blood lipid levels and liver function, and alleviated liver oxidative stress. Moreover, the mixed lactobacilli supplementation significantly inhibited lipid accumulation in the liver (Li et al., 2020). Also the treatment of single lactobacillus showed positive effect on the liver metabolism, it was demonstrated that obese mice fed with HFD for 8 weeks and treated with *Lactobacillus plantarum* CQPC02 up-regulated mRNA and protein expression of lipoprotein lipase, Ppar-α, cholesterol 7 alpha-hydroxylase (*cyp7a1*), but down-regulated peroxisome proliferator-activated receptor-gamma (*Ppar-γ*) and *Ccat* enhancer-binding protein alpha (C/ebp-α) expression in the liver (Zhao *et al.*, 2019). Another study using ageing animal model as a premature senescent Sprague-Dawley rats induced by D-galactose (D-gal), showed that *Lactobacillus fermentum* DR9 (LF-DR9), *Lactobacillus paracasei* OFS 0291 (LP-0291), and *Lactobacillus helveticus* OFS 1515 (LH-1515) together where able to activate the 5' AMP-activated protein kinase, *Ampk* that plays a critical role in cellular energy homeostasis (Hor *et al.*, 2019). Perhaps, most of these studies worked in animal models of ageing. Only few researches are focusing in elderly people or old animals. It was demonstrated in patients with Parkinson's disease that the probiotics such as supplementation for 12 weeks upregulated proliferator activated receptor gamma (*Ppar-γ)* in peripheral blood mononuclear cells (Borzabadi *et al.*, 2018). More clinical and animal studies in elderly needed to understand the direct effect of probiotics in liver metabolism.

In recent decades some nutrition supplements and functional foods with prebiotic function have been investigated in ageing in particularly in elderly chronic disease models, unfortunately there are no many studies that use old animals (Ky *et al.*, 2014) and this can be caused by the difficulties encountered raising elderly animals due to high mortality in laboratory conditions.

A growing body of evidence demonstrating therapeutic effects of black garlic, including anticancer, anti-obesity, immunomodulatory, hypolipidemic and antioxidant (Tran, Pham and Trinh, 2020). In an ageing model of chronic alcohol-induced liver disease, the effect of black garlic was investigated in adult male Sprague–Dawley rats for 4 weeks of treatment and its hepatoprotective effect on chronic alcohol-induced liver injury was underlined by 55% reduction of *P450 2e1* expression in the liver (Kim *et al.*, 2011). Grape fruit is well known to be a protector against cardiovascular diseases and cancers (Dresser, Bailey and Carruthers, 2000) but it also can cause inhibition of *Cyp 3a4* and thus serves to increase the bioavailability of the drug by decreasing its pre-systemic metabolism (Kiani and Imam, 2007). Another important hepatoprotective food is grape pomace that demonstrated a positive effect on lipid metabolism and hepatic morphology of adult rats fed with high fat diet (Han'guk, Xian-Hua and Seo, 1996). The peculiarity of these exceptional foods is their richness in polyphenols. Resveratrol is a well-known polyphenol nonflavonoid compound present in strongly pigmented vegetables and fruits and its strong effect is well documented. Faghihzadeh *et al.*, 2014 observed in patients with non-alcoholic fatty liver disease a reduction in weight, body mass index, waist circumference, liver enzymes, and steatosis degree, while resveratrol was associated with lifestyle changes and particularly with physical activity. *In vitro* the direct effect on the liver was investigated in senescence-accelerated mouse P10 hepatocytes Resveratrol (0.04%) for 20 weeks caused reduction of lipid droplets, mitochondria were increased in number and acetyl-CoA carboxylase and the expression of both the mitochondrial ATP synthase β subunit and Mn *Sod2* were increased (Shiozaki *et al.*, 2011), suggesting the enhancement of lipolysis through the activation of mitochondrial functions. In vivo, rats with dimethylnitrosamine (*Dmn*)-induced liver fibrosis showed an antifibrotic role with to block the development of liver fibrosis (Abdu and Al-Bogami, 2019). The coadministration of resveratrol (100 mg/kg/day) with a probiotic

such as *B. longum* further enhanced the efficacy to alleviate non-alcoholic fatty liver and obesity (Hu *et al.*, 2020).

In patients from aged 29-70 years, with an average weight of 84.6 kg, resveratrol reduced the liver fat, as well as decreased hepatic enzymes, serum glutamate pyruvic transaminase (SGPT) and gamma-glutamyl trans- peptidase (g-GT) but also, insulin resistance (Theodotou *et al.*, 2019).The oat beta-glucan, another prebiotic, showed an effect in reducing the insulin resistance too and in addition, it was observed that hepatic glucokinase activity was markedly enhanced both in low-dose and high-dose groups compared with that of control groups (Zheng *et al.*, 2013).

Plant sterols (phytosterols) have also an effect on the lipid and cholesterol metabolism, in particular *Lxrα* antagonism of dietary phytosterols contributes to sterol selectivity and reduces fractional cholesterol absorption and preloading of nascent HDL with dietary cholesterol (Brauner *et al.*, 2012).

In a faecal transplantation of inoculated to antibiotic-pre-treated mice (*hum-ob* mice; humanized obese mice) the stools from four obese donor study, inulin treatment reduced hepatic lipid content in mice by regulating the expression of genes involved in lipogenesis and fat acid oxidation such as ppargc1a mRNA(Rodriguez *et al.*, 2020).

1.4.3 Ageing Immune system

Immunosenescence is a series of age-related changes that include persistent low-grade inflammation (Inflammageing), decreased ability to fight infection, impaired ability to effectively respond to new antigens, and increased incidence of autoimmunity (Goronzy and Weyand, 2013). In detail, there is a decline in the adaptive immune system that decreases its ability to effectively contain viral infections and to eliminate other antigenic challenges (Deeks, 2011), thereby extending the duration of innate immune response and lead to complications typically associated with ageing, including cardiovascular disease, Alzheimer's disease, diabetes mellitus type II, osteoporosis, and other end-organ diseases (Deeks, 2011). The common denominator of these senescence disease is inflammation. Generally, in the blood circulation there is an increased levels of the C-reactive protein (*Crp*) and pro- inflammatory cytokines (Tang *et al.*, 2017). A recent clinical research found that high IL-6 and Crp concentrations were associated with a higher risk of mortality and corelated with fat metabolism where in obese aged patients, the level of IL-6 was higher than in normal weight or overweight individuals (Puzianowska-Kuźnicka *et al.*, 2016). It is well

documented that Inflammageing can be driven by an increase in adipose tissue (Gregor and Hotamisligil, 2011) where the leptin drives the production of IL-6, IL-12 and *Tnfα* (La Cava and Matarese, 2004), (Park and Camilleri, 2005).

Substantial evidence in humans and rodent shows that ageing induces alterations in adaptive immune system (Weiskopf, Weinberger and Grubeck-Loebenstein, 2009), and it is characterized by a decrease in the number of naive *Cd8+ T* cells, an increase in the number of memory-like precursor cells and a moderate reduction in the diversity of the T-cell receptors. During the primary response to intracellular pathogens in the aged environment, dendritic cells exhibit impaired maturation, lower antigen uptake and reduced production of signal 3 (cytokines) (Nikolich-Žugich, 2014). However, the alteration of the immune system response can be influenced by the translocation of microbial products from the gut to the circulation as was observed in patients with HIV (Ortiz and Brenchley, 2018) where aberrant immune activation in immunodeficiency is caused by decrease in the integrity of the intestinal epithelial barrier. As a consequence, Antimicrobial Paneth cell factors Ang4 and lysozyme are significantly reduced in the gut. Transcriptomics revealed age-associated decreases in the expression of immunity and other genes in intestinal mucosal tissue, including decreased T cell-specific transcripts and T cell signalling pathways (Sovran *et al.*, 2019). The close relationship between the intestinal microbiota and the host immune system is well documented (Wu and Wu, 2012; Belkaid and Hand, 2014; Lazar *et al.*, 2018); The intestinal microbes plays a fundamental role on the induction, training and homeostasis of the host immune system. In return, the immune system has evolved as a means to maintain the symbiotic relationship of the host with these highly diverse and evolving microbes (Malys, Campbell and Malys, 2015). In fact, in germ-free models, animals are reared in a sterile environment and thus have never been exposed to any microorganisms, show extensive deficits in the development of the gut-associated immune system such as lymphoid tissues (GALT), defects in antibody production and smaller Peyer's patches (Round and Mazmanian, 2009). So, the combination of ageing microbiota modification and the constant senile inflammation can represent a key node for medical interventions aimed at improving the quality of life in the elderly.

1.4.3.1 Probiotics and Prebiotics effect

The beneficial effects of probiotics and prebiotics have been demonstrated in many diseases. One of the major mechanisms of probiotic and consequently, of prebiotics is through the regulation of host immune response acting directly on the gut-associated epithelial and innate immune cells for example, through the Toll-like receptors (Pujari and Banerjee, 2021). However intervention studies of probiotics and prebiotics in ageing are not very abundant (Pérez Martínez, Bäuerl and Collado, 2014) However, few study have been proven to be beneficial when administered to aged people especially in the gut immunity. For instance, a treatment with *Bifidobacterium lactis* HN 019 to aged individuals led to the recovery of granulocyte and NK cell activities (Gill *et al.*, 2001). An oral intake of *L. pentosus* strain b240 has been shown to augment sIgA secretion in elderly. Moreover, *L. pentosus* b240 was able to reduce frequency of common cold in aged individuals, likely acting via mucosal immunity (Shinkai *et al.*, 2013). A supplementation with *B.longum* Bar33 and *L.helveticus* Bar13 mixture improves immunity in elderly humans (over 75 years) and aged mice (Finamore *et al.*, 2019). However, not all lactobacillus strain are efficacious, for example *L. johnsonii* La1 decreased phagocytic activity of neutrophils showing a negative effect in the elderly, where a daily consumption of *B. lactis* Bi-07 improved the phagocytic activity of monocytes and granulocytes in healthy old adults (Maneerat *et al.*, 2013). Moreover, *B. lactis* HN019 supplementation was highly efficacious in increasing polymorphonuclear phagocytic capacity but moderately efficacious in increasing natural killer cell tumoricidal activity (Miller, Lehtoranta and Lehtinen, 2017).

Also *B. infantis* 35,624 exerts beneficial immunoregulatory effects; in patients with ulcerative colitis but also in patients affected by psoriasis, *B. infantis* 35,624 (1×10^{10} CFU) for 6-8 weeks, was able to reduce the plasma levels of C-reactive protein and the pro-inflammatory cytokines such as *Tnf-α* and interleukin-6 compared to the placebo groups (Groeger *et al.*, 2013).

Thirty-one healthy elderly volunteers aged from 72 to 103 after consumption of probiotic cheese enriched with 10^{9} CFU day−1 of *Lactobacillus rhamnosus* HN001 and *Lactobacillus acidophilus* NCFM for 4-week, resulted a significantly increase the cytotoxicity of NK cells (Ibrahim *et al.*, 2010). On the other hand, lactobacillus and Bifidobacterium have the ability to increase the number of Bifidobacterium in the gut, which in the elderly, is reduced due to advancing age. It has been shown that *B. longum*

2C (DSM 14579) administrated for 3-6 month in elderly population increased significantly in the gut, the abundance *of B.adolescentis e B.catenulatus. B.animalis* and it was correlated to a lower level of the regulatory cytokine IL-10 (Ouwehand *et al.*, 2008). A similar effect emerged only with Oat drink that changed the level of bifidobacterial in the faeces corelated to cytokine anti-inflammatory TGF-b1, in fact the prebiotic effect of oat fibres increased the abundance of B. bifidum and B. longum in aged patients (Ouwehand *et al.*, 2008). The synergetic association of prebiotic and probiotic can be an efficient way to modify the gut microbiota in elderly. In a double-blind trial *B. lactis* BL-01 and *B. bifidu*m BB-02 along with inulin increased the numbers of *B. bifidum* and in general, the total amount of *bifidobacteria* and Lactobacilli in healthy subjects aged 62 years (Bartosch *et al.*, 2005). In another study the association of *L. rhamnosus* and oligofructose increased serum levels of IL-1, IL-6, and IL-8, while reduced basal levels of IL-12 (Amati *et al.*, 2010). Another symbiotic combination that could improve health is *B. lactis* HN019 and GOS which together increased the number of bifidobacteria and to improved phagocytic activity in elderly adults (Vulevic *et al.*, 2008).

Polyphenols, considered as a strong prebiotic present in most of the fruits and vegetables, have been shown to influence the composition of the gut microbiota.

For example, six week consumption of a wild blueberry (rich source of polyphenols) *Bifidobacterium* spp. significantly increased with *Lactobacillus acidophilus* (Vendrame *et al.*, 2011), but also grape juice (Jacobs *et al.*, 2008) and red wine or gin (Queipo-Ortuño *et al.*, 2012) respectively, mainly increased *Bifidobacterium* spp. in faeces of human volunteers. Therefore, intake of polyphenols in the elderly may beneficially act either on microbiota homeostasis or, consequentially, on attenuation of chronic inflammatory conditions.

1.4.4 Intestinal barrier in elderly

The gastrointestinal mucosa harbours one of the largest luminal interface between the environment and the internal host milieu. It constitutes a critical barrier where millions of microbes and environmental antigens come in close contact with the host immune system. The epithelium maintains its selective barrier function through the formation of complex protein-protein networks that mechanically link adjacent cells and seal the intercellular space. The protein networks connecting epithelial cells form three

adhesive complexes: desmosomes, adherent junctions and tight junction. In particularly, tight junction (TJ) are under a constant threat from proinflammatory mediators, pathogenic viruses and bacteria, aiding inflammation and the development of disease (Bhat *et al.*, 2019). Inflammatory bowel disease, irritable bowel syndrome , obesity, non-alcoholic fatty liver disease and type 1 diabetes, reveal loss of TJ barrier function, increased levels of proinflammatory cytokines, and immune dysregulation (Lee, Moon and Kim, 2018). Age-related structural changes of the intestine were observed in mammalian, underlining a progressive degeneration of the barrier function, where the inflammation is a common denominator. In aged rats was observed an increase of endothelial gap in the villous plexus correlate with increase of permeability (Chen, Zhang and Duan, 2003). Data from two independent non-human primate studies suggest the expression of molecular components of tight-junctions (*Zo-1*, *occludin, Jam-A*, *claudin-2* expression) was decreased and correlated to systemic inflammation in the intestinal epithelia of aged baboons (Tran and Greenwood-Van Meerveld, 2013) and aged Vervet monkeys (Mitchell *et al.*, 2017). The same age-effect was observed in the same tight junction expression *Zo-1, Jam-A* and *Claudin-2* in aged rats (Ren *et al.*, 2014). These studies suggest that ageing is associated with significant intestinal barrier dysfunction and this apparent dysregulation in intestinal permeability contributes to the low-level chronic inflammation (Inflammageing) (Mabbott *et al.*, 2015). Unfortunately, so far just few clinical studies in humans have investigated the effects of ageing on intestinal barrier function finding not great differences between young and elderly subjects. The use of lactose or mannitol absorption technique to test the intestinal permeability (Camilleri *et al.*, 2010) did not show an increase in gut permeability of ageing human (> 60 year) compared with young controls (Saltzman *et al.*, 1995).

However, the limitations of this technique must be considered. The orally administered gut permeability markers (such as lactose and mannitol) must be excreted by kidneys to be quantified in urine and cannot be assessed reliably in subjects with altered glomerular filtration rates (Edelblum and Turner, 2015) and silent kidney diseases are very common in elderly (P. K.-T. Li *et al.*, 2017). Moreover, as well the TJ expression showed any difference in aged patients; *Zo-1*, *Occludin*, or *Jam-A1* expression did not change in ileal terminal biopsies sample of 67–77 year old patient although, elevated *claudin-2* expression was only associated with increased IL-6 (Man *et al.*, 2015). A

recent clinical study investigated the *vivo* and *ex vivo* gut permeability using Ussing chamber technique associated to immunofluorescent staining and mRNA expression gene showed no significant differences in permeability between healthy elderly and young adults after sigmoid biopsy (Wilms *et al.*, 2020). It is interesting to note that ageing animal studies and human studies present discordant results.

This could be the result of the difficulty of taking samples in humans with invasive techniques such as biopsy which leads to the use of different sampling sites. Biopsies in humans are done in the sigmoid of the distal colon after a colonoscopy, while in animals, it tends to be taken ex vivo at the point of the proximal colon near the cecum. Different points of the intestine have different characteristics and the composition of the proximal and distal colon can explain the differences between animals and humans results and difference in species should not be underestimated, moreover the use of sucrose.

1.4.4.1 Probiotic and prebiotic interventions in ageing

Growing evidence supports a role of microbiota on the modulation of the gut permeability. Changes in microbiota after antibiotics treatment presented higher intestinal permeability in rats (García-Lafuente *et al.*, 1998). However, the colonization of probiotic can reverse the antibiotic effect reducing the permeability as happened in rats treated with *L. brevis* (García-Lafuente *et al.*, 2001). Several studies have shown that some specific probiotics may ameliorate the intestinal permeability corelated to the inflammation. A study done on obese patients who had a high intestinal permeability, highlighted the capacity of *Bifidobacterium lactis* BB-12, after 3 weeks of consumption, to improve in colonic permeability by measuring sugar recovery in urine (Krumbeck *et al.*, 2018). Moreover, in the same study a similar effect was obtained by the administration of the prebiotic galactooligosaccharides (GOS), but interestingly, the combination of this prebiotic with the probiotic did not show a synergetic effect confirming that the pro- and prebiotic components by themselves improved markers of colonic permeability better then together (Krumbeck *et al.*, 2018).

Lactobacillus rhamnosus GG (LGG) was able to inhibit systemic inflammation and maintain the mucosal barrier function in a mouse model of sepsis where though the histopathological analysis Occludin was much higher compared with the control (Chen *et al.*, 2019).

The powerful effect of human-origin *Lactobacillus paracasei* D3-5 strain (D3-5), was demonstrated in an ageing animal model extends life span of Caenorhabditis elegans; in addition, if it is feeding to old mice (> 79 weeks) can decreases leaky gut and inflammation, improves physical and cognitive functions concurrently an increase of *Akkermansia muciniphila* (mucin-degrading bacteria) (Wang *et al.*, 2020). *A. muciniphala* that decrease with the advancing age (Sovran *et al.*, 2019) can be used as probiotic. This probiotic prevented the age-related decline in thickness of the colonic mucus layer and attenuated inflammation and immune-related processes at old age (Wang et al., 2020). However, in an accelerated ageing mouse model (Ercc1−/Δ7 mice), supplemented with *A. muciniphila* for 10 weeks was able to prevention a decreased mucus layer thickness and reduced the inflammation on ileum but unexpectedly without influence the tight junction gene expression (van der Lugt *et al.*, 2019). In general tight junctions may change with inflammation and *A. muciniphila* regulates the intestinal tight junction in adult (Kuipers *et al.*, 2016),(Bhat *et al.*, 2019) as well win type 2 diabetes animal models (Chelakkot *et al.*, 2018). Although, in ageing the mechanisms involved it is not clear and need further investigation to be clarified.

Evidence that increasing the consumption of polyphenol-rich food can positively affect intestinal microbial ecosystem resulting in reduced intestinal permeability. The 8-week polyphenols interventions in older people (≥ 60 y) showed that the intestinal permeability was decreased evaluating by quantifying serum *Zonulin* concentrations (Guglielmetti *et al.*, 2020).

However, application of probiotics and prebiotic specifically on ageing animal model has limited due to the difficult and expensive breeding.

Table 1. 0.4 Efficacy of probiotics on various Aging disorders (summary of paragraphs above).

Aging disorder	Probiotics	Results	Study	
Metabolic Syndrome	*L. rhamnosus*	upregulates Lxrα, Lxrβ, Abca1, Abcg1, Scd1, and Pparα. Downregulates Srebp-1C and Fas gene transcription	(Wa *et al.*, 2019)	Rats
Metabolic syndrome	*L.rhamnosus GG*	increases Pparα and Fgf21 mRNA expression	(Liu *et al.*, 2020).	Mice
Obese syndrome	*L. plantarum KLDS1.0344 and KLDS1.0386*	improves blood lipid levels and liver function. alleviates liver oxidative stress	(Li et al., 2020)	Mice
Obese syndrome	*L. plantarum CQPC02*	up-regulates mRNA and protein expression of lipoprotein lipase, Ppar-α, cholesterol 7 alpha-hydroxylase (cyp7a1)	(Zhao *et al.*, 2019)	Mice
Parkinson's disease	*L.acidophilus, B. bifidum, L. reuteri, and L. fermentum*	upregulates proliferator activated receptor gamma (Ppar-γ) in peripheral blood mononuclear cells	(Borzabadi *et al.*, 2018)	Humans
Parkinson's disease (PD)	*L.fermentum DR9,L. paracasei OFS , and L. helveticus OFS 1515*	activates the 5' AMP-activated protein kinase on cellular energy homeostasis	(Hor *et al.*, 2019)	Rats
Ulcerative colitis, psoriasis	*B. infantis* 35,624	reduces the plasma levels of C-reactive protein and Tnf-α and interleukin-6	(Groeger *et al.*, 2013)	Humans
Sepsis	*L. rhamnosus* GG	inhibits systemic inflammation and maintains the mucosal barrier function	(Chen *et al.*, 2019)	Mice
type 2 diabetes	*A. muciniphila*	regulates the intestinal tight junction	(Chelakkot *et al.*, 2018)	Humans

Healthy elderly	B. lactis HN 019	Increases in the proportions of total, helper (CD4(+)), and activates (CD25(+)) T lymphocytes and natural killer cells	(Gill *et al.*, 2001)	Humans
Healthy elderly	L. pentosus b240	augments sIgA secretion	(Shinkai *et al.*, 2013)	Humans
Healthy elderly	*B.longum* Bar33 and *L.helveticus* Bar13	increases naive, activates memory, regulatory T cells, B cells, and natural killer activity	(Finamore *et al.*, 2019)	Humans

1.5 Brain in Elderly

Ageing entails many physical, biological, chemical, psychological changes and the brain is one of the organs that can suffer more damage and compromise the health of the whole organism. These alterations render the ageing brain vulnerable to dementia and other neurobiological disorders as impaired neuroplasticity, aberrant neuronal network activity, the accrual of oxidatively modified molecules, inflammation that may drive to Alzheimer's, Parkinson's diseases and stroke (Mattson and Arumugam, 2018).

Brain volume and Gray matter. The first modification that can be observed between a young and an old brain is weight. Every 10 years the brain weight decreases by 5% after 40 years (Svennerholm, Boström and Jungbjer, 1997) which seems to worsen after 70 years. The reduction of cerebral weight with age was confirmed in rodent study, more in detail, wild-type C57BL / 6 mice present a reduction in hippocampal volume and striatum after 23-28 months of age (Lessard-Beaudoin *et al.*, 2015). The reduction of cerebral weight was negatively correlated to the necroptosis, an inflammatory cell death presents also into Alzheimer's disease (Caccamo *et al.*, 2017). Often Alzheimer's patients show a reduction of the total cerebral volume especially on the hippocampus (Kaur *et al.*, 2014). In particular, it seems that the Gray matter is reduced in old people with Alzheimer (Taki *et al.*, 2011) and interestingly, it seems to start to decrease several years before the first clinical symptoms in patients with family genetic predisposition (Weston *et al.*, 2015). In fact, the degeneration of the Gray matter can be used as an early marker for cognitive impairment diseases, such as

Parkinson where it decrease in the early stages of the disease in amygdala (X. Li *et al.*, 2017) and in the hippocampus (Chen *et al.*, 2016).

Brain inflammation. Just like in all the body, ageing leads to a chronic low-grade inflammation in the brain (Frank-Cannon *et al.*, 2009). This contributes to decrease pre- and postsynaptic densities, as well as reduce the synapses and dendritic spines overall (Yankner, Lu and Loerch, 2008) resulting in cognitive impairments and memory loss in elderly. Additionally, inflammation is a common hallmark observed in many neurodegenerative diseases, suggesting that both normal ageing and pathological alterations converge on common proinflammatory pathways.

Microglia. Microglia are the primary immune cells of the brain and, in elderly, exhibits shorter telomeres and exaggerate production of proinflammatory cytokines such as TNF α, IL 1β and IL-6 (Bachstetter *et al.*, 2011).

Astrocytes. In recent decades, changes in astrocytes in relation to old age have been studied (Palmer and Ousman, 2018) and these key cells of the brain that control and regulate the BBB but also occupy a strategic position between capillaries and neurons (Lécuyer, Kebir and Prat, 2016) it was found to change morphology and increase the glial fibrillary acidic protein (GFAP) expression progressively with the age in humans and rodents (Middeldorp and Hol, 2011; Chisholm and Sohrabji, 2016). In conjunction with astrocytes, the permeability of the BBB may be compromise in elderly as happened in the gut with a decrease expression of the tight junctions (Enciu, Gherghiceanu and Popescu, 2013, 2013).

Blood brain barrier. The Deterioration of BBB in ageing is believed to be a correlating factor in neurodegenerative and age-associated diseases such as type 2 diabetes mellitus (Magnusson, Brim and Das, 2010), Alzheimer's (Zenaro, Piacentino and Constantin, 2017) and schizophrenia (Zenaro, Piacentino and Constantin, 2017).

Changes in TJ protein expression have been identified at preclinical stages in animal models of neurodegenerative diseases, in particularly on the occluding expression pointing towards the BBB as an early target in the disease (Morgan *et al.*, 2007).

In a rodent's study, old mice (24mo.) showed a BBB disruption with a low Occludin expression, in conjunction with the leakage of IgG into the parenchymal space of the cerebral cortex and hippocampus when compared with young controls (Elahy *et al.*, 2015).Molecular mechanisms of BBB disruption in ageing (mice of 16-22 mo.) have been identified, and include declined expression of *sirtuin-1* (Elahy *et al.*, 2015).

The neuronal plasticity. Neuroplasticity is the capacity of the brain to optimize the neural networks during phylogenesis, ontogeny and physiological learning.

Cellular and synaptic plasticity involved the excitatory neurotransmitter glutamate system (glutamate + receptors + transporters such as *nmda* (N-methyl-D-aspartate) and the brain derived neurotrophic factor (*bdnf*) system (*bdnf* + receptors) (Gulyaeva, 2017). Gamma aminobutyric acid (*Gaba*) is a naturally occurring amino acid that works as a neurotransmitter in brain and also plays a major role in brain plasticity. In elderly, the basic *Gaba* levels were observed to be lower in older as compared to young adults (Chalavi *et al.*, 2018). However, the same study where the motor learning task were tested and the patients were trained for 3 days, ageing people showed an amplified positive modulation in *Gaba* promoting mechanisms of long-term potentiation, cortical plasticity and learning better than in young people (Chalavi *et al.*, 2018), in spite of reports of age-related decline in motor performance and skill acquisition have been reported (Seidler *et al.*, 2010). To measure the neuroplasticity, it is possible to analyse the gene expression and the level of proteins involved in neurogenesis. The serum level of *bdnf* has been related the gene expression on the brain and it is used in clinical study as a neuroplasticity marker (Seidler *et al.*, 2010). In ageing people, *bdnf* serum level was reduced and it was associated to Gray matter volume loss and poorer memory performance (Erickson *et al.*, 2010). It is interesting to note in ageing that *bdnf* level increased or decreased differently in brain regions, for example *bdnf* raised in murine hippocampus and cerebral cortex during normal ageing, but not during ageing of mice with pathological changes (Katoh-Semba *et al.*, 1998). This suggest that the different brain region need to be analyse separately and in different conditions.

Instead N-methyl-D-aspartate (*nmda*) seems to show a clear declines in binding densities and functions with increasing age in many species (reviewed in Magnusson, Brim and Das, 2010).

Rats have provided a rich array of details on age-related changes in *nmda* receptor binding and related changes in behaviour, including memory (Magnusson, Brim and Das, 2010). The *GluN2B* subunit is the component of the *nmda* receptor that is most affected by the ageing process, showing greater declines in mRNA and protein expression than the *GluN1 or GluN2A* subunits(Magnusson, Brim and Das, 2010). *GluN2B* shows dramatic declines in mRNA expression across development from birth to adulthood (Magnusson, Brim and Das, 2010).

1.5.1.1 Prefrontal cortex

The Prefrontal cortex (PFC) region is implicated in planning complex cognitive function, working memory and decision making. Advancing age was strongly associated with decreases in the volume of the whole frontal cortex without obvious neurological disease (Elahy *et al.*, 2015). It is well-known that age affect memory capacity and transfer of learning skill acquisition (Kausler, 1994) and they are related to the neuronal plasticity (Park and Bischof, 2013). In fact, genes involved in synaptic function and plasticity were among those most affected in the ageing cortex; an exhaustive ageing study in humans, reported significantly reduced expression of many genes related to synaptic plasticity in the PFC after age 40, such as *the GluR1 AMPA* (a-amino-3-hydroxy-5-methyl- 4-isoxazole propionic acid) receptor subunit, the *nmda* (N- methyl-D-aspartate) R2A receptor subunit, and subunits of the *Gabaa receptor* (Lu *et al.*, 2004). Another human research, using microarray data from orbitofrontal cortex of young and aged subjects (16–96 years), confirmed an age-related downregulation of *bdnf* by an initial effect on GABA presynaptic genes in this cerebral region (Erickson *et al.*, 2010). In rodents, age has a very strong impact causing significant morphological changes in PFC; important modification in microglia cells was observed in a rat ageing study which the increased volume of microglia was correlated to a decrease of spine density in old rats (20 mo.) (Chan *et al.*, 2018). However, no alteration in astroglia were observed in ageing. The initiation of a chronic inflammatory response in microglia during ageing can explain these results. Chronic inflammation in the dorsal PFC has been suggested as a possible neurobiological basis of cognitive deterioration (Chan *et al.*, 2018). Although inflammation-related gene downregulation was observed in a study in elderly patients with schizophrenia (López-González *et al.*, 2019). Elderly mice subjected to an induction of chronic inflammation, showed dystrophic microglia and a high level of proinflammatory cytokines and oxidation with cognitive impairments compared to young mice (d'Avila *et al.*, 2018); this suggests that aged mice have increased susceptibility to episodic systemic inflammation and the capacity to return at a base normal state is compromised compared to the young.

1.5.1.2 Hypothalamus

The hypothalamic region has the function to link the nervous system to the endocrine system via the pituitary gland and it controls metabolism, reproduction, circadian rhythm, and homeostasis of the entire body. The ability of the hypothalamus to responds to inflammation though the nutrient status, may enable the organism to adapt rapidly to physiological perturbations as it takes part of the hypothalamic, pituitary, adrenal (HPA) axis. High fat diet is considered an metabolic and nutritional stressor (Kamara, Eskay and Castonguay, 1998) where the hypothalamic microglia are the protagonists which orchestrate both the immunologic and physiologic responses to dietary excess and instruct the hypothalamic control of food intake, energy expenditure, and body weight (Valdearcos *et al.*, 2017). Inflammageing is also present on the hypothalamus even as few proinflammatory markers was found increased such as IL-1 ß (Tha *et al.*, 2000) in adult mice. Nevertheless, inflammation and cognitive decline has been associated to this region in ageing conditions (Zhang *et al.*, 2017).

Differently from the other brain regions, it seems that hypothalamus does not change in volume or number of neurons with advancing age and only in certain circumstances such as Alzheimer's disease (Lacalle, Iraizoz and Gonzalo, 1993) or osteoporosis (Roberts, Killiany and Rosene, 2012).

Few studies investigated the genes expression modification in elderly on the Hypothalamus. An interesting hypothalamic study showed synaptic modification due to age such as postsynaptic density protein-95, which plays a role in *nmda*-dependent signalling, decreased 16-fold. Conversely, expression of the *Gaba*(A) receptor decreases only in PFC but not in Hypothalamus of aged mice (Jiang *et al.*, 2001).

The role of *bdnf* in hypothalamus is quietly different from the other cerebral regions and is an important component of the hypothalamic pathway that controls energy homeostasis (Xu and Xie, 2016). In healthy ageing, *bdnf* signalling is reduced and it is associated with most of ageing neurodegenerative disorders including Alzheimer' s, Parkinson's and Huntington's disease (Tapia-Arancibia *et al.*, 2008). In a recent research, *bdnf* gene transfer induced, was able to prevent the development of ageing-associated metabolic declines such as decline of brown fat activity, increasing adiponectin while reducing leptin and insulin in circulation suppressing inflammatory genes in the hypothalamus and adipose tissues in adult mice (McMurphy *et al.*, 2019).

1.5.1.3 Hippocampus

Hippocampus has an important role in learning and memory consolidation but also mood regulation correlated to neurogenesis and structural plasticity (Bettio, Rajendran and Gil-Mohapel, 2017). Structural and functional changes to the hippocampus are related to the severity and development of neurodegenerative disorders associated with cognitive decline. In fact, many of the cognitive deficits observed with ageing can be mirrored in animal models that show hippocampal damage (Geinisman *et al.*, 1995). In elderly, the hippocampal volume decreases and it has associated to a low level of *bdnf* (Erickson *et al.*, 2010). In humans, circulating levels of *bdnf* decline with advancing age, and *bdnf* has been related to grey matter volume loss in ageing people (Erickson *et al.*, 2010). The loss of grey matter seems to be correlated with the decline of neurogenesis in adulthood and in elderly even if it is difficult to establish a clear mechanism in humans due to the clear methodological limitations associated with evaluating this process across the lifespan of human subjects (Bettio *et al.*, 2017). However, the evaluation of doublecortin, a neurogenesis marker, has estimated in post-mortem tissue thought the lifespan of humans and this is corelate to the linear age-related decline of the neurogenesis that was demonstrated to be similar to rodents models (Knoth *et al.*, 2010). Even in expression of neurotransmission-regulating proteins was observed a progressive reduction from young-adult (3 months), Adult (12 months) to Aged (26 months) rats (such as the vesicle-associated protein synaptophysin (Syp), the postsynaptic density protein *Psd95*, and the *Snare* proteins *vamp2*, syntaxin 1 (*Stx1*), and *Snap25*) (VanGuilder *et al.*, 2010).

The hippocampus appears to be more affected by age then the other cerebral regions and in particularly, the BBB structure is the first to breakdown in hippocampus during ageing process (Montagne *et al.*, 2015). This deterioration may be related to the massive presence of microglia cells in this region compared to the others (Lawson *et al.*, 1990). On the other hand, a prolonged activation of the microglia during a chronic inflammation (Lawson *et al.*, 1990) as it happens in old age, may be the cause of deterioration of the BBB and therefore all the age-dependent cognitive decline.

In fact, it has been observed that microglial dysfunction increased with age in humans and rodents (Griffin *et al.*, 2006) concurrently with an up-regulation of pro-inflammatory genes in hippocampus (Cribbs *et al.*, 2012).

1.5.1.4 Amygdala

Fear, anxiety and aggression are regulated by the amygdala that also it has a primary role in the processing of memory, decision-making. Emotional regulation is a characteristic of elderly that improve in their ability to manage emotional responses, particularly to negative situations (Charles and Piazza, 2009), (Zanchi *et al.*, 2017). Furthermore, whereas episodic memory typically deteriorated, emotional memory appeared to be well preserved during ageing (Ritchey *et al.*, 2011). Conversely, older adults showed decreased functional connectivity with posterior brain regions, likely reflecting reduced perceptual processing. (Xiao *et al.*, 2018).

The gray matter of the amygdala is decreased with age which, in conjunction with the reduction of the hippocampal volume, favours the cognitive decline typical of the latter season of life (Lawson *et al.*, 1990).

The slow reaction of the body during elderly is possible to appreciate in this cerebral region. The diffused inflammatory effect of HFD in all the body is reflected also in the amygdala. HDF was able to activate microglia markers and increased proinflammatory cytokines only in aged and not in young rats; interestingly, physical exercise that usually reduce inflammation in adult, did not reduce amygdala IL1 and in addition, did not prevent HFD-induced amygdala memory decline (Spencer *et al.*, 2017).

1.5.2 Psychobiotics

Psychobiotics have opened a very interesting scenery that changes the current paradigm of symbiosis between bacteria and humans. The introduction of probiotic in the gut can have an impact to the regulation of the neuroimmune axes (hypothalamic pituitary adrenal (HPA)-axis and to diseases that involve the nervous system, but they can also influence to memory, learning and behaviour (Bermúdez-Humarán *et al.*, 2019).

Lactobacillus strains are the most powerful probiotics that effects on behaviour. For instance*, Lactobacillus plantarum* PS128 ameliorated opposition/defiance behaviours on boys with autism spectrum disorder (Liu *et al.*, 2019). Also, *L. plantarum* 299v demonstrated to improve cognitive function in patients with major depression decreasing kynurenine concentration (Rudzki *et al.*, 2019). Through studies on rodent

behaviour, it was demonstrated that *L. plantarum*, has the capacity to ameliorate memory and learning tasks as happened after administration of *L. Plantarum* CCFM639 for 14 weeks, restoring the learning and memory deficits in mice (Yu *et al.*, 2017). However, a new *Lactobacillus plantarum* 286 isolated from the fermentation of cocoa exerted anti-depressant- and anxiolytic-like effects but not in learning and memory performance after 30 days of administration in male Swiss mice (Barros-Santos *et al.*, 2020). Instead, another *L. plantarum* ATCC 8014 was able to ameliorate learning and memory tasks and it seems to be correlated with the capacity of this particular *L.plantarum* to increase the abundance of Lactobacillus family on the gut while a dramatically reduction of *Clostridiales* and *Bacterioidales*. In the same study, it was observed an important increased level of Glutathione peroxidase (*Gpx*), superoxide dismutase (*Sod*), and total antioxidant capacity (TAC) in the PFC, together with the reduction of malondialdehyde (*Mda*) in the PFC. These results underline the powerful effect of this psychobiotic on the brain (Morshedi, Saghafi-Asl and Hosseinifard, 2020).

1.6 Aims and Objectives

There is growing evidence of the important role played by the gut-brain axis in the regulation of physiological and metabolic parameters across the life cycle of animals. Despite efforts to understand how the advancing age affects the microbiota changes and the cognitive and metabolic parameters, are currently not explored in detail and new therapeutic strategies need to be deeply investigate. In this thesis we examine the complex effects of probiotic and prebiotic treatment in the body in different age bracket.

Aim 1: Do microbiota treatments affect the behaviour and intestinal permeability in aged animals thought the change of microbial diversity?

Polyphenols have previously been reported to modulate the gut microbiota in mice (Rastmanesh, 2011), (Chacar *et al.*, 2018), (Van Hul *et al.*, 2018). Ageing is associated with a decrease in gut microbiota diversity (Biagi *et al.*, 2010; Claesson *et al.*, 2012) and may contribute to the progression of a number of age-related disorders. With a focus in behaviour and intestinal barrier, we wanted to investigate whether polyphenols could influence microbiota diversity, gut permeability, peripheral and central inflammation, and cognitive function, social and anxiety-like behaviour in aged mice (**Figure 1.0.11**).

Aim 2: How metabolism change in relationship with microbiota in young and middle-age animals after microbiota treatments?

The middle-age represents the first step of physiological decline before elderly where metabolism, the immune system and a cognitive decline becomes more common (Barzilai *et al.*, 2012). We hypothesise that inulin, well studied prebiotic, can have a preventive impact on the metabolism, cholesterol and glucose homeostasis.

Aim 3: Do microbiota treatments affect the brain in a model of premature ageing?

In an attempt to understand the mechanisms through which microbiota-targeted interventions exert the anti-aging effects, we used a model of premature ageing (ApoE

deficient mice). The effect of two different strains of lactobacillus *(L.reuteri* and *L.mucosae*) and two prebiotics derived from plants (plant sterol esters and oat β-glucan) were examined. Neural plasticity was restored in the Prefrontal cortex after 24 weeks of prebiotic administration and the level of claudin 5 increased in the hippocampus following consumption of *L. mucosae*.

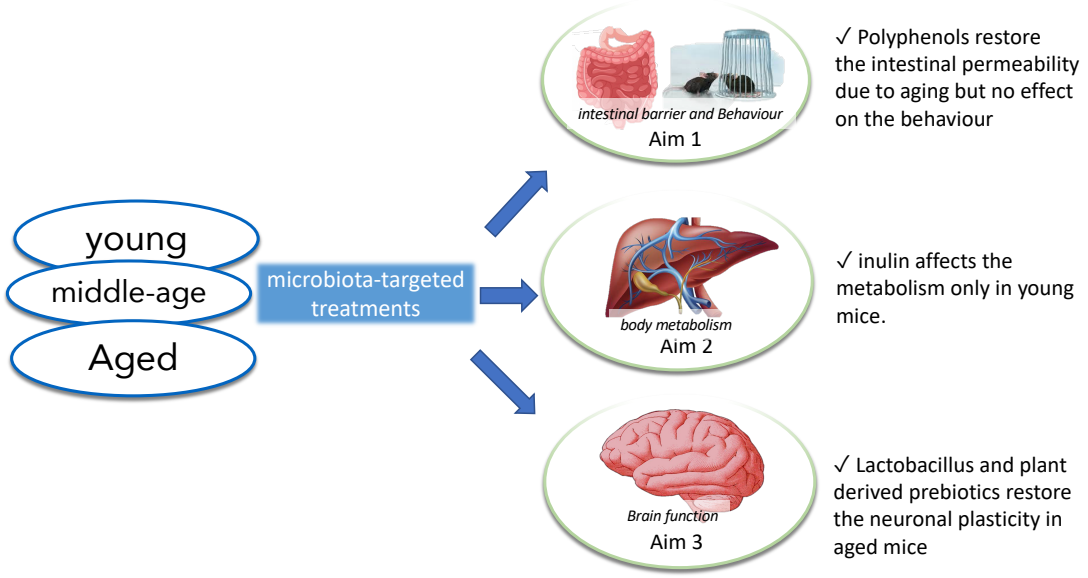


Figure 1.0.11 Diagram representing the aims of the thesis. Aim 1 (Chapter 2) investigates the effect of polyphenols treatment on microbiota diversity, intestinal permeability and on behaviour in old mice. Aim 2 (Chapter 3).

Chapter 2

Grape Pomace and Rafuma extracts improve gut barrier in aged mice

Animal trial was run in conjunction with Karen Scott

2.1 INTRODUCTION

There is a growing emphasis on the relationship between the complexity and diversity of the commensal microorganisms that inhabit our gut (human gastrointestinal microbiota) and healthy ageing. The maintenance of healthy microbiota-gut-brain communication is governed by tissues and organs including the brain, glands, gut, immune cells and gastrointestinal microbiota. Ageing is associated with a decrease in gut microbiota diversity (Biagi *et al.*, 2010; Claesson *et al.*, 2012) and may contribute to the progression of a number of age-related disorders by altering physiological processes including hypothalamic-pituitary-adrenal (HPA) axis activation, gut physiology and function, neurotransmitter systems, immune function and inflammatory response (Cryan *et al.*, 2019). Furthermore, focus on the role of microglia and neuroinflammation in ageing, and associated changes in behaviour has intensified (Tian *et al.*, 2017). A recent study providing evidence for microbiota, and bacterial by-products (short chain fatty acids) in the maturation and activation of central microglia (Erny *et al.*, 2015). reinforces a critical role for gastrointestinal microbiota in healthy ageing of the brain.

The elderly account for approximately 12% of the global population, with projected numbers expected to surpass 20% over the next two decades (Partridge, Deelen and Slagboom, 2018). Common ageing-related ailments include frailty, cognitive decline and gastrointestinal complaints, often present with concomitant chronic low-grade inflammation and altered HPA activity. As the treatment of many ageing-related disorders has profound socio-economic implications, much work has gone into dietary means to promote healthy ageing. One such dietary intervention is the use of

polyphenolic compounds primarily with the aim of alleviating gut disorders and reducing low grade inflammation.

It has been suggested for many years that a diet rich in polyphenols can protect against a variety of age-related disorders (Vauzour *et al.*, 2010) including neurodegenerative and neuropsychiatric disorders (Matarazzo, Toniato and Robuffo, 2018)(Solanki *et al.*, 2015). There is also evidence that dietary polyphenols promote the growth of beneficial gut bacteria, which may contribute to healthy ageing (Cardona *et al.*, 2013; Espín, González-Sarrías and Tomás-Barberán, 2017). It is plausible that the neuromodulatory potential of polyphenols and their ability to protect vulnerable neurons and improve neuronal function and regeneration (Spencer, 2008) may be mediated through microbiota or their metabolites. In particularly, the antioxidant and anti-inflammatory effect of polyphenols was suggested by a research studying the grape pomace diet in obese mice (Hogan *et al.*, 2010). Rafuma extract polyphenol rich plant, was implicate in antidepressant studies (Li *et al.*, 2018) and recently it showed antioxidant properties (Du *et al.*, 2020).

We have previously demonstrated that age-related deficits in cognitive performance are associated with changes in microbiota, gut permeability and peripheral inflammation in mice (Boehme *et al.*, 2019), (Scott *et al.*, 2017). Moreover, we have found that changes in the composition of the caecal microbiota were correlated with anxiety-like behaviours, providing further evidence of the critical relationship between the brain and gut.

In this study we assessed the effect of two polyphenol-rich, naturally occurring products, grape pomace and rafuma extract, on gut microbiota, gut permeability, peripheral and central inflammation, and cognitive function, social and anxiety-like behaviour in aged mice.

2.2 MATERIAL AND METHODS

Ethical statement and assurances

All animal protocols were approved by the Animal Experimentation Ethics Committee at University College Cork (UCC) and by the Health Products Regulatory Authority (HPRA) of Ireland, in accordance with EU Directive 2010/63/EU.

Animals and housing

Young and aged male C57BL/6J mice were obtained from Charles River (UK) and housed individually prior to start of the studies. All mice were housed in the same temperature and humidity-controlled animal room with a 12 h light:dark cycle starting at 8am.

Aged (20 month old) male C57BL/6 mice (n= 10-11) were assigned to one of 3 diets, 1) standard, nutritionally complete control chow (CON) (18% crude protein, 5.8%, fat, 76.2% carbohydrate) or standard control chow with 2) grape pomace (GP) (0.07% w/w) from Nippon Flour Mills Co. Ltd. (Tokyo, Japan) or 3) rafuma (Raf) extracts (0.07% w/w) from TOKIWA Phytochemical Co., Ltd. (Chiba, Japan). Young (4-month-old) mice (n=12) were used as an additional control and were maintained on standard control chow. The treatment was given for 11weeks. All diets were prepared by ssniff Specialty Diets (Soest, Germany). Behavioural test was performed as in figure **Errore. L'origine riferimento non è stata trovata.** and further explanatory notes for behaviour are provided in the supplementary material.

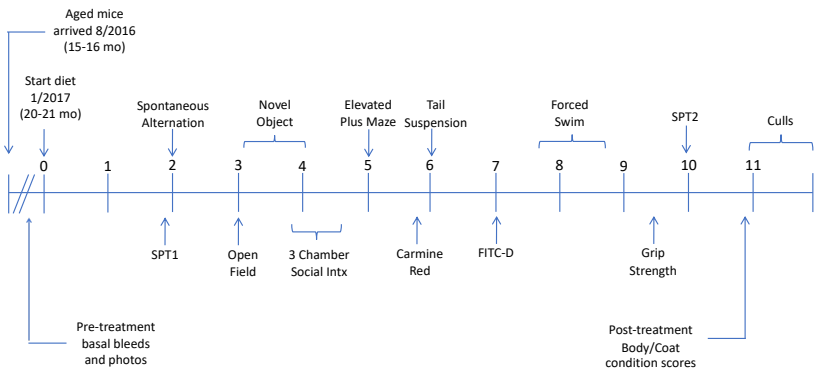


Figure 2.1 **Experimental timeline.** the diets started at the time poin 0 and finished after 11 weeks. *Abbreviations: SPT1 as Sucrose Preference Test*, FITC-D as fluorescein isothiocyanate dextran.

Behavioural Tests

Sucrose preference test (SPT)

Mice were assessed for reward-seeking behaviour as previously conducted (O'Leary *et al.*, 2014).Mice were given access to 2 water bottles in their home cage for at least one week prior to the sucrose preference test (SPT) to habituate. The SPT occurred on 3 consecutive days, where one bottle contained tap water and the other bottle contained a 1% sucrose solution. The position of the bottles was changed each day to limit the effects of side preference. Sucrose preference is presented as the total percentage of sucrose solution consumed over the 3-day testing period.

Spontaneous alternation in the Y-maze

The Y-maze is a test of spontaneous alternation, used to assess exploration, cognition and hippocampal-dependent memory. It is based on the premise that mice will alternate between arms visited when exploring a new environment. The test was performed as previously described with minor adaptation (Scott *et al.*, 2017).

Novel Object/Object Displacement test

The Novel Object Recognition (NOR) test was used to assess cognitive function, including and spatial working memory and is based on the premise that mice will spend more time interacting with a novel object in comparison with a familiar object. Exploration of an object was defined as orientation of the mouse with its nose 2 cm or closer to the object as described previously (Scott *et al.*, 2017).

3-chamber test of social behaviour

Sociability was assessed using the 3-chamber social interaction test (SIT), in which time spent interacting with a novel conspecific mouse is compared to time spent with a novel object or familiar conspecific mouse. The 3-chamber social interaction test was performed as previously described (O'Tuathaigh *et al.*, 2007; Desbonnet *et al.*, 2014; Scott *et al.*, 2017).

Open field test

The open field (OF) test was used to assess locomotor activity and anxiety-like behaviour and is based on the expectation that mice will avoid the center of the test arena, which are anxiety-provoking and will spend more time near the walls (Scott *et al.*, 2017).

Elevated Plus Maze

The elevated plus maze (EPM) was used to assess anxiety-like behaviour (Scott *et al.*, 2017). The percentage of time spent in open arms was calculated and used as an index of anxiolysis.

Tail Suspension Test

The tail suspension test (TST) was used to assess antidepressant-sensitive behaviours and is based on the premise that immobility, is a marker of depressive- or despair-like behaviour. Using tape, the mouse was suspended by its tail for 5 min and the time spent immobile was recorded.

Forced swim test

The forced swim test (FST) was used to assess antidepressant-like behaviour. Similar to the tail suspension test, the duration of immobility (the minimum amount of movement to keep one's head above water) is used as an index of depressive-like behaviour (Porsolt *et al.*, 1978; Cryan and Mombereau, 2004).

Grip strength

As ageing is associated with decreased body condition and frailty, grip strength was assessed using a grip strength meter (Ugo Basile). The mouse was allowed to grip onto a bar attached to the meter and was gently and steadily pulled away from the meter by the tail. The average of 6 trials was taken, with 1 minute of rest time between each trial.

Physiological Measures

Carmine red test of gut motility

Gastrointestinal motility was assessed as previously described (Golubeva *et al.*, 2017). On the morning of the trial, mice were moved to a clean cage with minimal bedding with *ad libitum* access to chow and water. Following 3h of habituation to the new cage, mice were gavaged with 200 µl of 6% carmine (prepared in 0.5% methylcellulose in 0.01 M phosphate buffered saline). Cages were checked every 10 min and the first appearance of a red fecal pellet was recorded.

In vivo intestinal permeability (FITC-D)

Intestinal barrier function was assessed using fluorescein isothiocyanate-labelled dextran (FITC-D) (FD4, Sigma Aldrich, Ireland). Mice were fasted overnight, prior to the study, and in the morning (9.00), they were gavaged with FITC-D (600 mg/kg). Two hours following gavage, mice were placed in ventilated plastic restrainers and a basal blood sample was taken, with another blood sample being collected 1 h post-restraint. A separate aliquot of plasma was collected at each time point (0, 30, 60, 90 120, 150, 180 minutes) to assess basal and stressed corticosterone levels.

Corticosterone response to acute stress

Plasma corticosterone (CORT) levels prior to, and following, exposure to forced swim and restraint stress were used to assess hypothalamic-pituitary-adrenal (HPA) axis activity. Blood samples were also taken before and 30, 60, and 120 min following the onset of the FST to assess peak and recovery CORT levels. Plasma CORT was assessed by ELISA, following vendor instructions (ENZO Corticosterone ELISA, ADI-900-097, Enzo Life Sciences, Exeter, UK).

Flow Cytometry

Blood (60 µl) was collected during the forced swim test at baseline and 30 minutes after the forced swim test, as measure of a response to acute stress as previously describe (Boehme *et al.*, 2020). The plasma was used for corticosterone analysis, and the pellet after centrifugation was used for flow cytometry. At the end of the study, mesenteric lymph nodes (MLNs) were collected in sterile RPMI media and processed by mechanical dissociation when animals were euthanized (RPMI-1640 medium with L-glutamine and sodium bicarbonate, supplemented with 10% FBS and 1% Pen/strep). Briefly, cells were stained for 30 minutes with difference mixes of antibodies to assess peripheral inflammatory state, and then fixed with 4% PFA. Blood

aliquot 2 and MLNs underwent intracellular staining using the eBioscience™ Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher, 00-5523-00), according to the manufacturers' instructions, using antibodies for intracellular staining (2 µl FoxP3-APC (ThermoFisher, 17-5773-82) and 5 µl Helios-PE (ThermoFisher, 12-9883-42)). Fixed samples were resuspended in staining buffer and analysed the subsequent day on the BD FACSCalibur flow cytometry machine. Data were analysed using FlowJo (version 10).

Ex vivo intestinal permeability (FITC-D)

Macromolecular intestinal permeability in Ussing chambers ex vivo was carried out essentially as described previously (Golubeva *et al.*, 2017). Distal ileum (a 2.0 cm segment taken ~2.0 cm proximally to the caecum) and middle colon (a 2.0 cm segment) samples were mounted in Ussing chambers with exposed tissue area of 0.12 cm^2 (Harvard Apparatus, Kent, UK) . FITC flux was calculated as an increment in fluorescence intensity vs. baseline fluorescence in the serosal compartment, and presented in ng/ml.

Reverse Transcription Quantative Polymerase Chain Reaction (RT-qPCR)

Ileum, colon and brain tissues were rapidly hand-dissected and stored at − 80 °C until tissue processing. RNA was isolated as per instructions using mirVana miRNA isolation kit (ThermoFisher Scientific, AM1560). A Nanodrop 1000 (Thermo Scientific, UK) was used to determine RNA concentration. cDNA was then synthesized using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, 4368814) in a G-storm thermocycler (G-storm, Surrey, UK). cDNA was diluted to 10ng/µl using RNase free water. 2 µl of the cDNA is diluted with 8 µl Mastermix. Mastermix was prepared from Taqman® Universal Mastermix II, no UNG (ThermoFisher Scientific, 4440040). Gene expression analysis was finally carried out by quantitative real-time polymerase chain reaction (qRT-PCR) using various probes designed by Applied Biosystems and Integrated DNA Technologies. RT-qPCR was performed with Roche LightCycler 480 for PCR**.** All samples were measured in triplicate. Cycle threshold (Ct) values were normalized to that of the housekeeping gene, β-actin (ACTB), and transformed using the 2 delta Ct method (Livak and Schmittgen, 2001). The data were then assessed and

presented as the fold change in mRNA expression from the control group (Young-Vehicle Treated).

Table 2.1 List of probes used in the study.

Gene	Common gene name	Sequence (5′ → 3′) left	Sequence (5′ → 3′) right
IL-6	Mus musculus interleukin 6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
IL-10	Mus musculus interleukin 10	GACAACATACTGCTAACCGAC	ATCACTCTTCACCTGCTCC
Tnfa	Mus musculus tumor necrosis factor	aagcctgtagcccacgtcgta	ggcaccactagttggttgtctttg
IL-1beta	Mus musculus interleukin 1 complex	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
Gfap	Mus musculus glial fibrillary acidic protein	AGCTGTCCCTTTGAACTGAAAC	CCCCAACAAGAGTAGGAGCA
Tlr4	Mus musculus toll-like receptor 4	GGAACAGCAACGAGCTAAGG	TGATGATCCGATTCACCAGA
Cd68	Mus musculus CD68 antigen	GGCCAAGCTATTGCGACATG	CCGAACACAGCGTAGATAGAC
Cd11b	Mus musculus integrin alfa M	ATGGACGCTGATGGCAATACC	TCCCCATTCACGTCTCCCA
Cldn1	Mus musculus claudin 1	CTTGACCCCCATCAATGC	CACCTCCCAGAAGGCAGA
Cldn2	Mus musculus claudin 2	GTTTAAGGATGGAGGTGTAGTTAG	AAACCAAATAACCTATAAAACCTCTAAT
Cldn3	Mus musculus claudin 3	GCAAGCAGACTGTGTGTCGT	TACCGTCACCACTACCAGCA
Ocld	Mus musculus occludin	GCACTCAGGTTAGGCACCTT	TCACTTACAGGTGCGTGCTT

Caecal Microbiota Analysis

DNA extraction

Caecal contents were snap frozen at the end of the study and stored at -80°C until the samples could be processed. DNA was extracted and quantified as before (Scott *et al.*, 2017).

The V3-V4 regions of the 16S rRNA gene were then amplified and prepared for library preparation. The sample pool was prepared following Illumina guidelines and sequenced on the MiSeq sequencing platform in Clinical Microbiomics, Denmark using standard Illumina sequencing protocols.

Bioinformatic Analysis

Paired-end reads were prefiltered based on a quality score threshold of > 28 and trimmed, filtered for quality and chimeras using the DADA2 library in R (version 3.6.3). Taxonomy was assigned with DADA2 against the SILVA SSURef database release v138. Parameters as recommended in the DADA2 manual were adhered to unless mentioned otherwise. Amplicon Sequence Variants (ASVs) were cut off at genus level, those that were unknown on the genus level were not considered in downstream analysis, as were genera that were only detected as non-zero in five percent or fewer of total samples.

The iNEXT R package was used to for statistical calculations of alpha diversity. Alpha diversity was calculated using the Chao1, Simpson and Shannon Index, Pairwise Mann Whitney U test was performed to assess significance between groups. Beta diversity was calculated using Aitchison's distance as calculated by taking the euclidean

distance from centered-log ratio (clr) transformed count values generating using the ALDEx2 library in R. Principal Component Analysis (PCA) plots were used to visualize beta diversity. Significance differences of beta diversity between groups was assessed by performing a pairwise implementation of the ADONIS PERMANOVA test found in the vegan library in R.

Statistical Analyses

Data, with the exception of microbiota data, are presented as mean +/- SEM. Data were excluded from analyses if greater than 2 standard deviations from the mean. Naïve control group and Aged control groups were compared using unpaired t-tests or Mann-Whitney U-tests where appropriate. The parametric data were analysed using one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test where relevant and Non-parametric data were analysed with Kruskal-Wallis test followed by post-hoc Dunn's. Outliers were excluded using the Grubbs test (Grubbs, 1950). A pairwise implementation of the ALDEx2 non-parametric t-test was used to assess statistical differences in microbiota differential abundance and p-values were corrected for multiple comparisons using the Benjamini Hochberg (BH) correction (FDR<0.05). Microbiota were analysed by subject rather than cage. Microbiota diversity and correlations were performed and visualized in R (version 3.6.3).

2.3 RESULTS

2.3.1 Behavioural Analyses

2.3.1.1 Cognitive Function

We determined the effect of ageing, and treatment in the ageing groups in the novel object recognition test (**Figure 2.2 A**) and the Y-maze test (**Figure 2.2 B**). There was no apparent effect of ageing on cognitive performance in the NOR ($t_{(19)} = 1.029$, $p = 0.3162$), or Y-maze test ($t_{(20)} = 0.3645$, $p = 0.7193$) respectively. Neither grape pomace nor Rafuma significantly altered cognitive performance in the novel object recognition ($F_{(2,25)} = 0.1246$, $p = 0.8834$) or in the Y-maze ($F_{(2,28)} = 0.8373$, $p = 0.4434$).

2.3.1.2 Social Behaviour

All mice exhibited a pronounced preference for a novel mouse over a novel object, and this preference was significantly higher in Aged-Control mice ($t_{(20)} = 3.004$, $p = 0.007$) compared to Young-Control mice (**Figure 2.2 C**). There was no effect of ageing on social preference (**Figure 2.2 D)** for a novel mouse ($t_{(19)} = 1.962$, $p = 0.0646$). There was no significant effect of either grape pomace or Rafuma on social preference ($F_{(2,28)} = 2.047$, $p = 0.148$) or social novelty ($F_{(2,28)} = 2.165$, $p = 0.1336$) respectively.

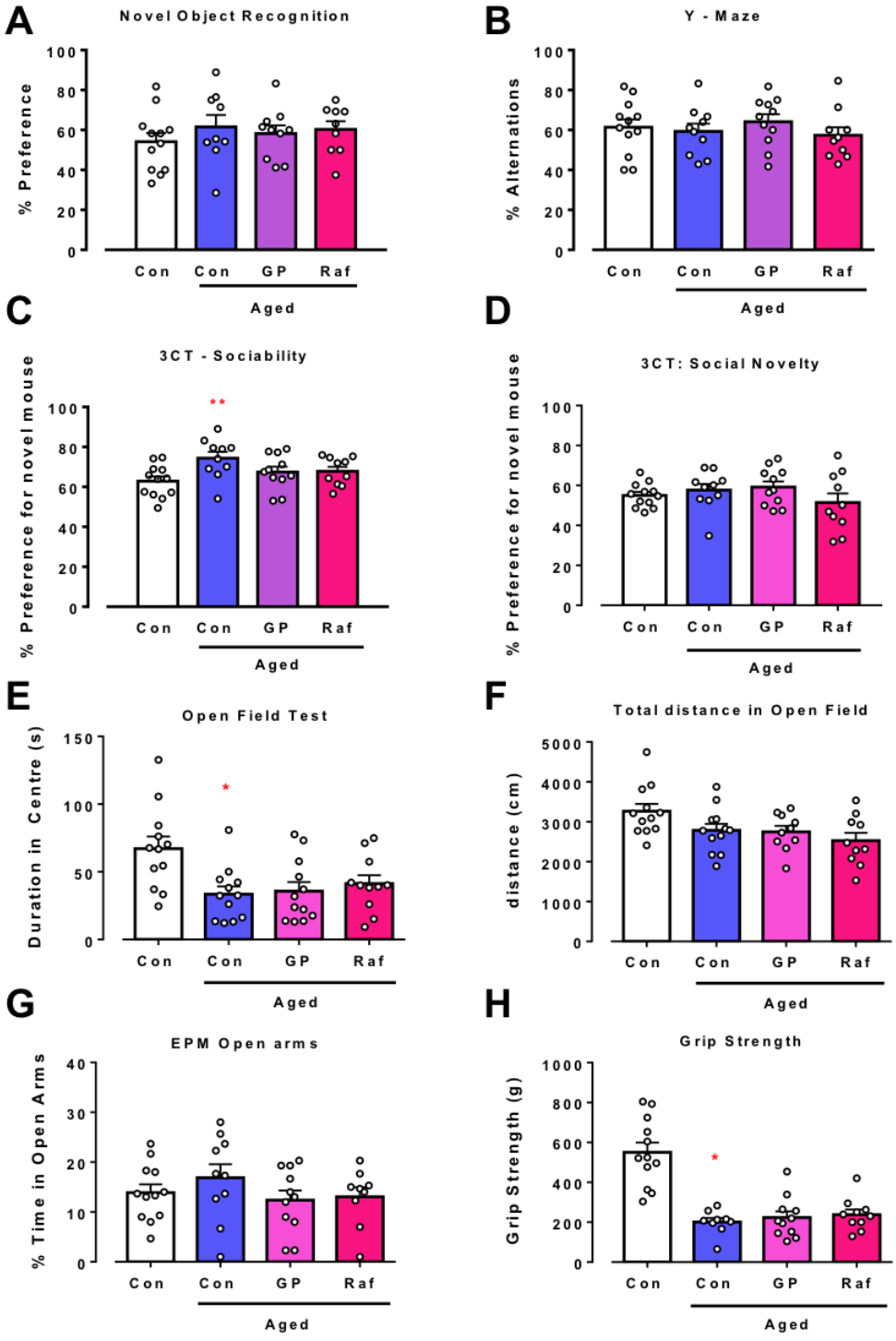


Figure 2.2 Effects of dietary polyphenols on age-related changes in cognition in the novel object recognition test (A) and Y maze (B); Social behaviour in the sociability test (C) and social novelty test of the 3 chamber test (D); Anxiety-like behaviour in the open field test (E) and elevated plus maze (G) and locomotor activity in the open field (F); Frailty as indexed by the grip strength test. All data are expressed as mean ± SEM. * represents significance ($p < 0.05$) against young control counterparts. N = 12 (young control group), n = 9–11 (aged).

2.3.1.3 Anxiety-like Behaviours

Aged mice exhibited an increase in anxiety-like behaviour in the Open Field Test (OF), as indexed by a decrease in duration in the centre zone ($t_{(22)} = 3.182$, $p = 0.0043$) **Figure 2.2 E**), but this may be accounted for by the decrease in general locomotor activity observed in aged mice ($t_{(22)} = 1.953$, $p = 0.0637$) (**Figure 2.2 F**). There was no effect of grape pomace or rafuma on duration in the centre zone ($F_{(2,32)} = 0.3985$, $p = 0.6746$), nor in general locomotor activity ($F_{(2,29)} = 0.6657$, $p = 0.5216$). However, in the Elevated Plus Maze (EPM) ageing had no effect on anxiety-like behaviour ($t_{(20)} = 0.974$, $p = 0.3417$) (**Figure 2.2 G**), and there was no further effect of treatment ($F_{(2,27)} = 1.193$, $p = 0.3189$).

2.3.1.4 Grip Strength

Grip strength was significantly reduced ($p < 0.05$) in aged mice (it corresponds to the amount of force/weight that it takes for the mouse to lose grip when it is holding onto a bar). The aged groups decrease about 63% of the grip strength compared to young group ($t(19) = 5.889$, $p < 0.001$). However, there was no further effect of treatment on grip strength ($F(2,27) = 0.4595$, $p = 0.6364$) (**Figure 2.2 H**).

2.3.1.5 Depressive-like Behaviours

All mice exhibited a preference for sucrose solution in the SPT tests performed at the beginning and end of the study as indicated by percentage consumed as compared to water. Age did not affect hedonic aspects of behaviour as indexed in the sucrose preference tests ($t_{(19)} = 1.463$, $p = 0.8852$; $t_{(20)} = 1.129$, $p = 0.2723$) (**Figure 2.3 A** and **B**) or Tail Suspension Test (TST), ($t_{(20)} = 1.307$, $p = 0.2061$), (**Figure 2.3 D**) but exhibited an decrease (23%) in depressive-like behaviour in the Forced Swim Test (FST) ($t_{(20)} = 2.358$, $p = 0.0287$) (**Figure 2.3 C**). There was no significant effect of treatment in the FST ($F_{(2,27)} = 0.4647$, $p = 0.6333$), TST ($F_{(2,27)} = 1.747$, $p = 0.1934$), or in the respective sucrose preference tests ($F_{(2,27)} = 3.235$, $p = 0.0550$; $F_{(2,28)} = 0.2628$, $p = 0.7708$).

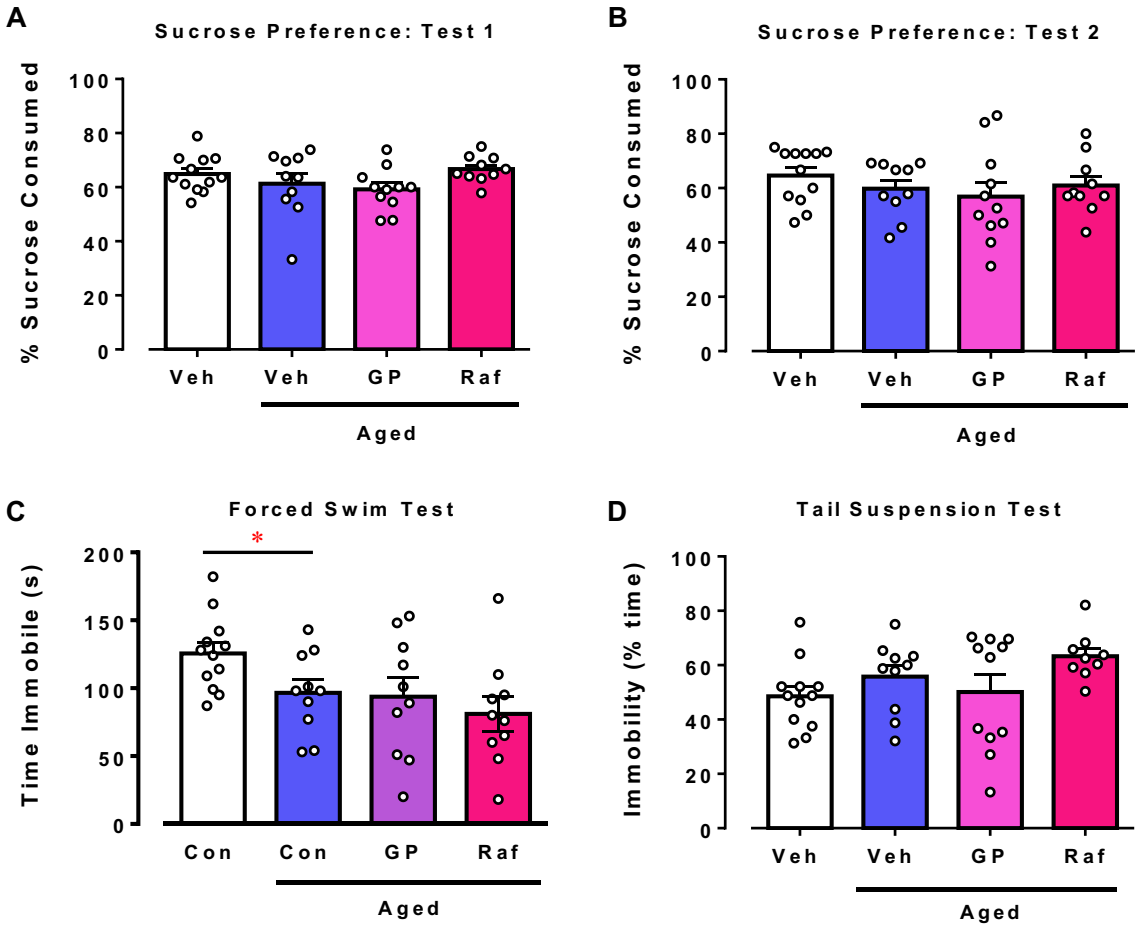


Figure 2.3 Effects of dietary polyphenols on age-related changes in depressive-like behaviour including the sucrose preference test (A) and (B); learned helplessness in the forced swim test (C) and tail suspension test (D). All data are expressed as mean ± SEM. * represents significance ($p < 0.05$) against young control counterparts. N = 12 (young control group), n = 9–11 (aged).

2.3.2 Physiological Analyses

Corticosterone Responses to Acute Stress

Despite having similar basal levels, aged mice exhibit a blunted corticosterone (CORT) response to an acute 1 h restraint (**Figure 2.4**A) ($F_{(1,19)} = 9.731$, $p = 0.006$), but there was no further effect of treatment in aged animals ($F_{(2,26)} = 0.929$, $p = 0.408$). In the forced swim test, there was a strong trend toward a difference between stress response in aged and adult control animals ($F_{(1,19)} = 3.657$, $p = 0.0728$), yet there was no further effect of treatment in the aged animals ($F_{(2,26)} = 0.546$, $p = 0.586$) (**Figure 2.4** B).

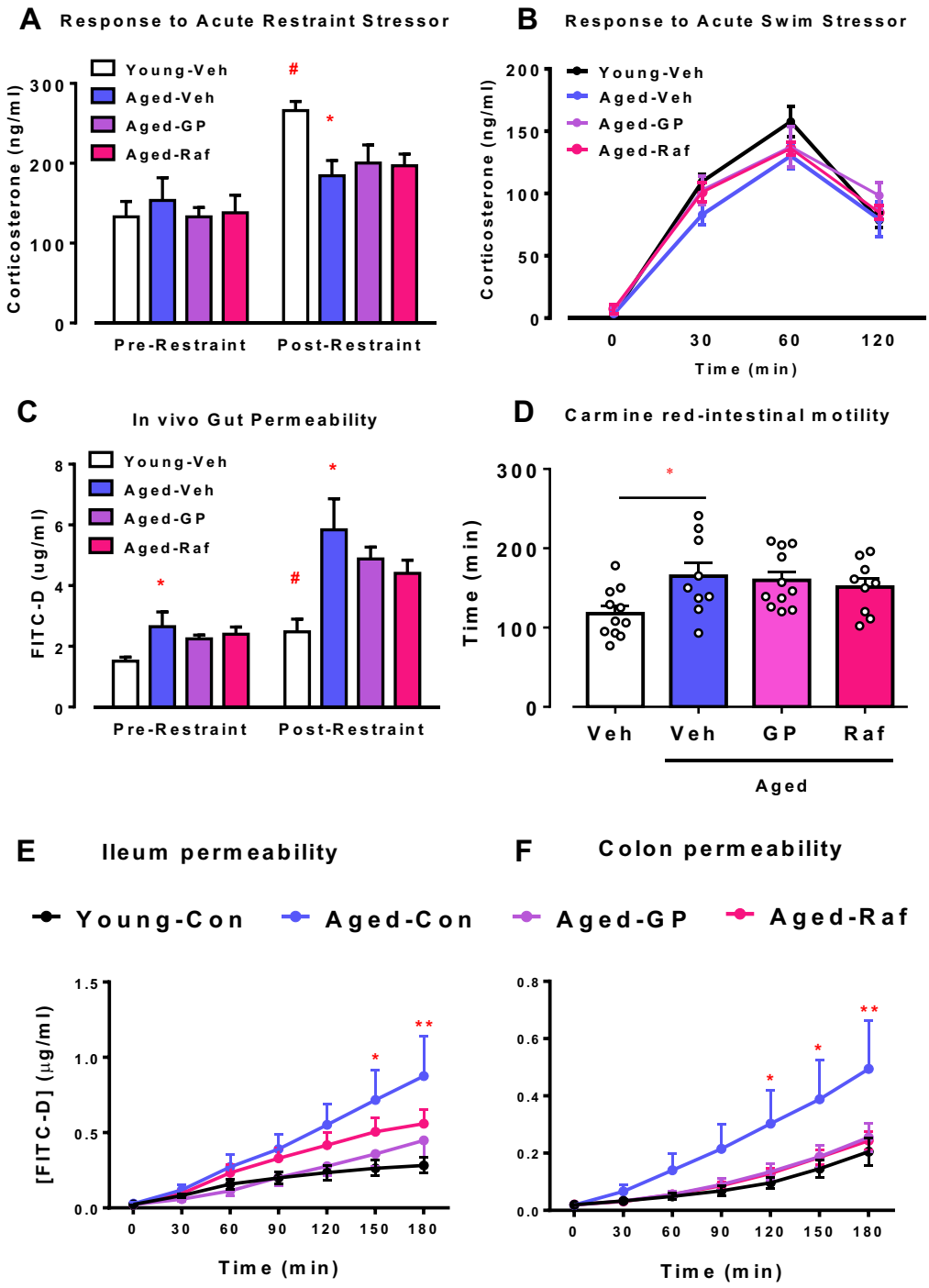


Figure 2.4 **Polyphenols did not reverse age-related changes in stress response in the acute restraint test (A) or forced swim test (B)**. Similarly, polyphenols did not reverse age related increase in *in vivo* gut permeability (C) nor gut motility (D). Polyphenols did not reverse age-related increases in *ex vivo* ileum permeability, but they did reverse age-related changes in *ex vivo* colonic permeability, although there was no significant effect at any distinct timepoint as determined by post-hoc analysis. All data are expressed as mean ± SEM. * represents significance ($p < 0.05$) against young control counterparts at the relevant timepoint. # represents significance ($p < 0.05$) against young controls pre-restraint. N = 12 (young control group), n = 9–11 (aged).

2.3.2.1 Gastrointestinal Function

In vivo gut permeability was significantly higher (increment of 97%) in aged mice ($F_{(1,36)} = 13.7, p = 0.0007$) and also, was further exacerbated in response to stress ($F_{(1,36)} = 11.75$, $p = 0.0015$). (**Figure 2.4 C**). This age-related increase in gut permeability was not attenuated following treatment ($F_{(2,28)} = 1.075$, $p = 0.355$).

Gut motility was assessed by recording the time that it takes for a red faecal pellet to be passed following intragastric gavage with carmine dye. Age significantly increased (48%) the GI transit time ($t_{(18)} = 2.587, p = 0.0186$). Neither Grape Pomace nor Rafuma attenuated the age-related increase in gut motility ($F_{(2,26)} = 0.2662, p = 0.7683$) (**Figure 2.4 D**).

Gut permeability was also assessed ex-vivo at the end of the study to determine whether there are regional differences in intestinal barrier function in the ileum and colon (**Figure 2.4 E** and **F**). Permeability of the ileum was significantly higher in Aged-Controls in comparison with Young-Controls ($F_{(6,114)} = 4.601$, $p < 0.0001$), but there was no further effect with polyphenol treatment ($F_{(12,168)} = 1.396$, $p = 0.172$).

Permeability of the colon was significantly higher in Aged-Controls in comparison with Young-Controls ($F_{(6,108)} = 3.481$, $p = 0.003$) and this effect was attenuated following treatment with the polyphenol treatment groups ($F_{(12,162)} = 2.109, p = 0.019$).

2.3.2.2 Peripheral Inflammation

Regional expression of genes related to inflammation were assed in gut tissue using PCR. In the ileum (**Figure 2.5 A-D**), mRNA expression of the pro-inflammatory cytokines was increased in aged mice: IL-1β ($t_{(18)} = 2.759$, $p = 0.0129$) with an increment of 78% and IL-6 ($t_{(19)} = 3.616, p = 0.0018$) (41%); also, the gene expression of anti-inflammatory cytokine IL-10 ($t_{(20)} = 3.423$, $p = 0.0027$) was rise (80%) compared to young animals. There was no significant increase in TNF-α levels ($t_{(19)} = 1.547, p = 0.1384$).

In colon (**Figure 2.5 E-H**), an age-related effect was only reported for TNF-α ($t_{(19)} = 2.335, p = 0.0307$) with an increment of 88%, with no effect on IL-6 ($t_{(19)} = 1.043, p = 0.3100$), IL-10 ($t_{(20)} = 0.8754$, $p = 0.3917$) or IL-1β ($t_{(19)} = 1.157$, $p = 0.2616$). Grape pomace significantly decreased Il-1 β ($F_{(2,27)} = 3.728$, $p = 0.0372$) and IL-10 ($F_{(2,27)} =$

5.7, p = 0.0086) mRNA expression in the colon of aged animals, but there was no difference in IL-6 ($F_{(2,28)}$ = 1.516, p = 0.2370) or TNF- α ($F_{(2,26)}$ = 0.5409, p = 0.5887).

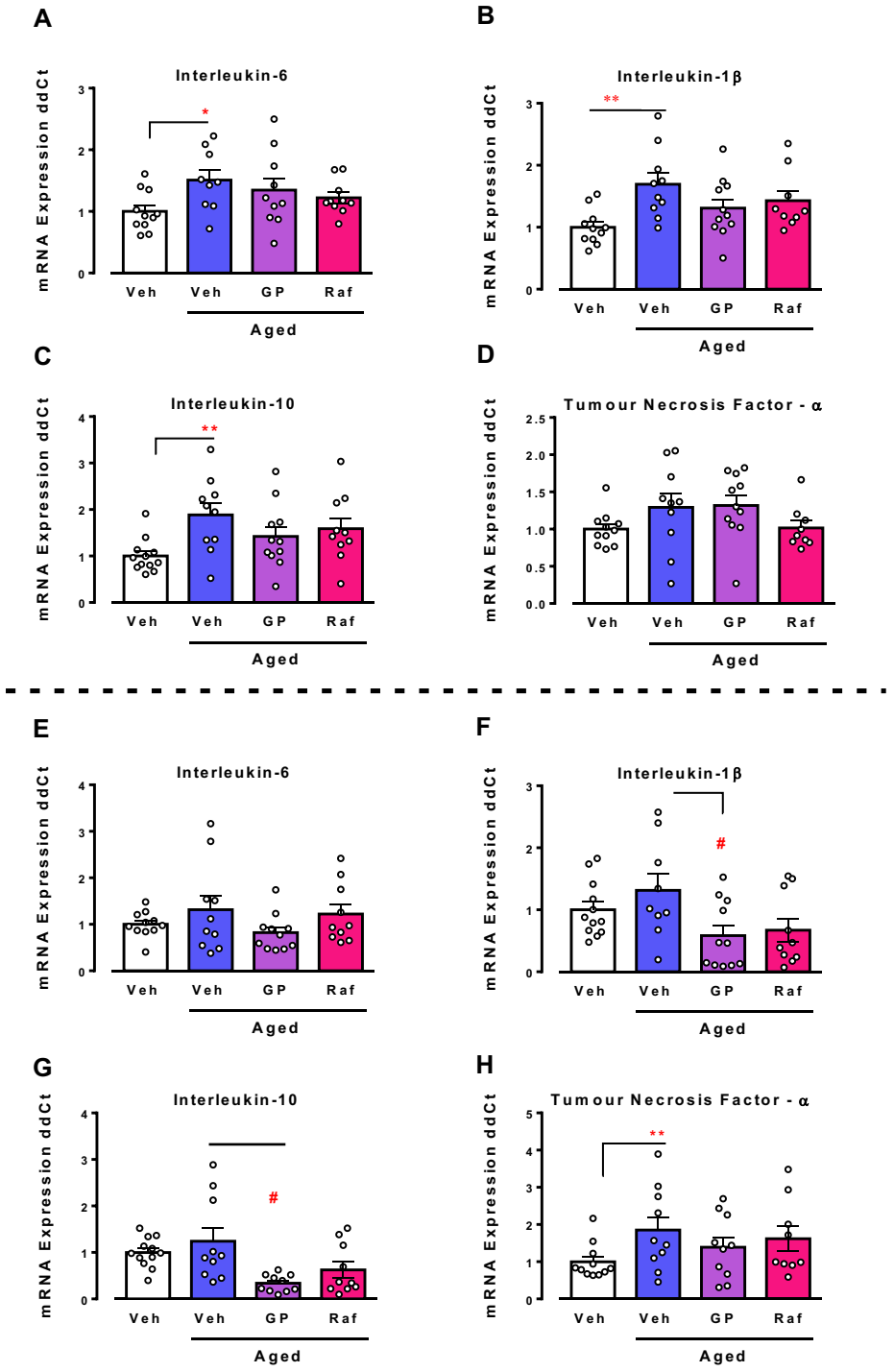


Figure 2.5 Effect of polyphenols on age-related changes in gene expression for interleukin-6, interleukin-1β, interleukin-10 and TNF-α in ileum (A-D) and colon (E-H) respectively. All data are expressed as mean ± SEM. * represents significance ($p < 0.05$) against young control counterparts. # represents significance ($p < 0.05$) against aged control animals. N = 12 (young control group), n = 9–11 (aged).

Flow cytometry was used to assess inflammatory markers from peripheral blood mononuclear cells taken prior to, and 30 min after, the Forced Swim Test (FST) (Figure 2.6 **A, B, C, D**). All effects observed were related to age, and no effects were observed in response to treatment. PBMCs ($F_{(1,39)} = 17.72$, $p = 0.0001$), inflammatory monocytes ($F_{(1,38)} = 26.96$, $p < 0.0001$), T-helper cells ($F_{(1,38)} = 71.59$, $p < 0.0001$) and granulocytes ($F_{(1,38)} = 30.22$, $p < 0.0001$) were all increased in aged animals, while stress decreased levels of granulocytes only ($F_{(1,39)} = 17.83$, $p = 0.0001$). Treatment had no further effect on age-related changes in PBMCs ($F_{(2,56)} = 10.37$, $p = 0.9017$), inflammatory monocytes ($F_{(2,54)} = 0.01326$, $p = 0.9868$), T-helper cells ($F_{(2,54)} = 0.007293$, $p = 0.9927$) or granulocytes ($F_{(2,54)} = 36.12$, $p = 0.6985$).

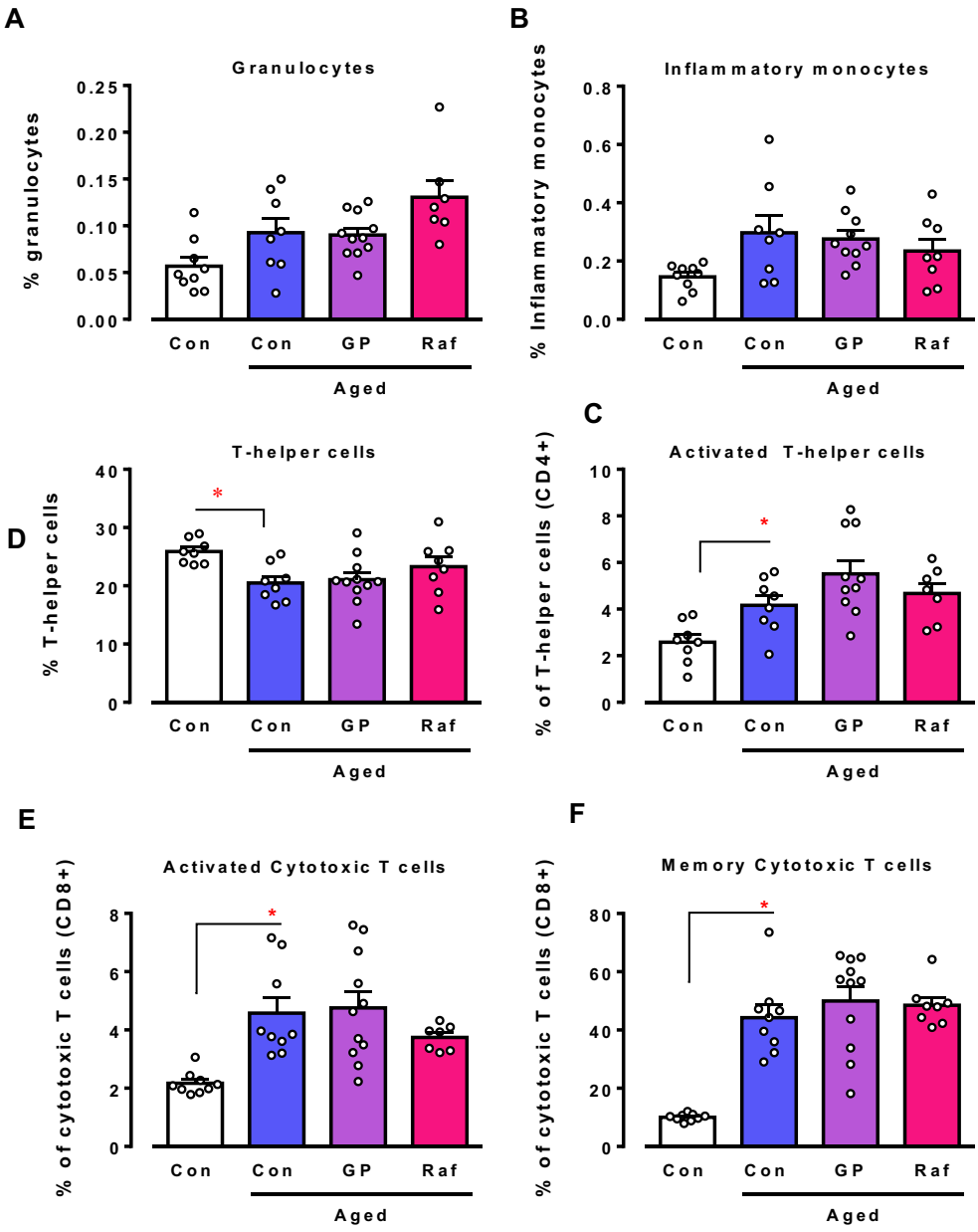


Figure 2.6 **Polyphenols did not attenuate age-related changes in systemic inflammatory markers** including granulocytes (A), inflammatory monocytes (B), T-helper cells (C), activated T-helper cells (D), activated cytotoxic T-cells (E) or memory cytotoxic T-cells (F) from mesenteric lymph nodes. All data are expressed as mean ± SEM. * represents significance ($p < 0.05$) against young control counterparts. N = 12 (young control group), n = 9–11 (aged).

Similarly to the observations in PBMCs, we determined an age-related profile for inflammatory markers in MLNs (**Figure 2.7 A-D**) using t-tests or Mann Whitney U-tests where appropriate, that was not reversed with dietary polyphenols as determined by one way ANOVA for granulocytes ($t_{(15)} = 2.08$, $p = 0.055$; $F_{(2,23)} = 2.864$, $p = 0.078$),

inflammatory monocytes (U = 16, p = 0.0592; $F_{(2,23)}$ = 5.102, p = 0.607), T-helper cells ($t_{(14)}$ = 4.017, p = 0.0013; $F_{(2,24)}$ = 1.115, p = 0.3442).

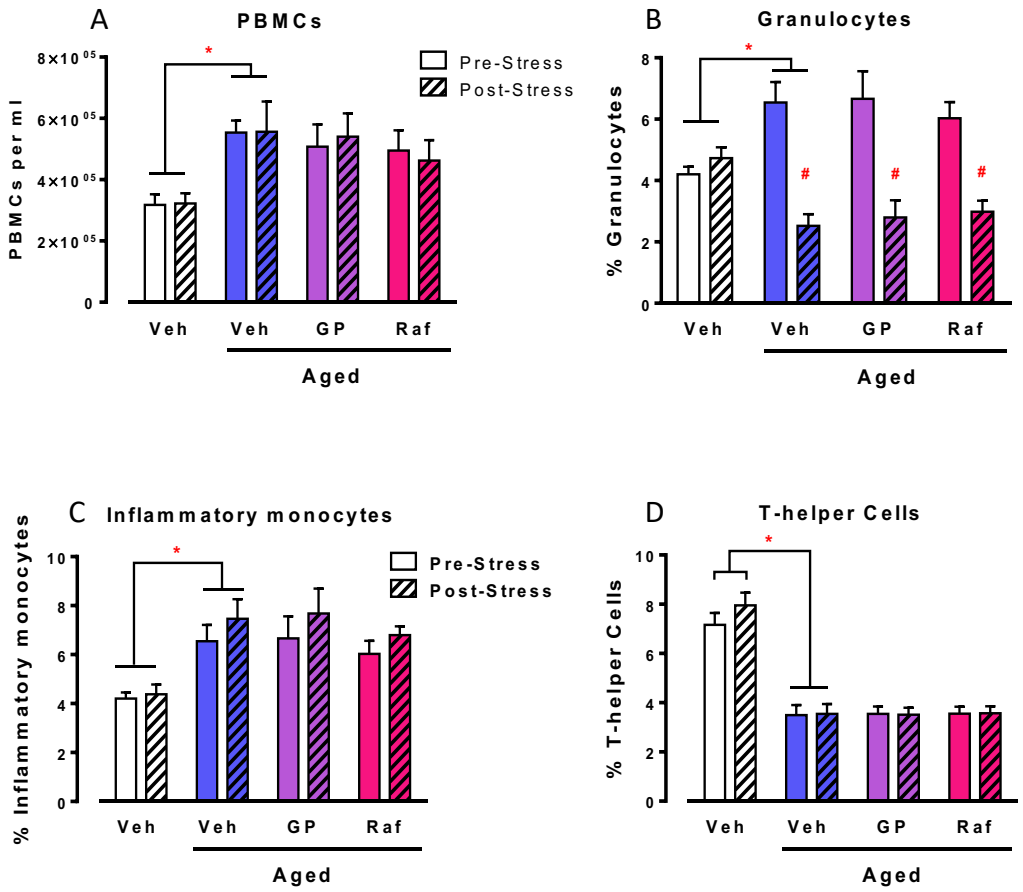


Figure 2.7 Polyphenols did not reverse age-related changes inflammatory markers from peripheral blood mononuclear cells taken prior to, and 30 min after, the acute forced swim test stressor. There was an increase in PBMCs (A), granulocytes (B) and inflammatory monocytes (C) with a decrease in T-helper cells (D). Granulocyte levels were decreased post-stress in aged animals but not young controls. All data are expressed as mean ± SEM. * represents significance ($p < 0.05$) against young control counterparts. # represents significance ($p < 0.05$) against pre-restraint controls. N = 12 (young control group), n = 9–11 (aged).

2.3.2.3 Central Inflammation

Markers of inflammation were also assessed within the hippocampus of mice using qPCR. Age was associated with robust increases in expression of genes associated with inflammation, however polyphenol treatment did not reverse any of these age-related changes in inflammatory mRNA expression. Hippocampal expression of genes encoding the proinflammatory cytokines IL-1β ($t_{(18)}$ = 3.584, p = 0.0021), IL-6 ($t_{(20)}$ =

5.9520, $p < 0.0001$) and TNF-α ($t_{(19)} = 5.112$, $p < 0.0001$) were significantly elevated in hippocampus of aged mice (respectively of 350%, 58%, 210%) (**Figure 2.8 A**, **B** and ***C***,). Similarly, there was a significant increase (47%) in expression of toll-like receptor 4 (TLR4) in Aged-Controls ($t_{(19)} = 2.292$, $p = 0.0335$) (**Figure 2.8 D**), Glial fibrillary acidic protein (GFAP), an astrocytic marker, was also elevated (49%) in aged mice ($t_{(20)} = 7.221$, $p < 0.0001$), as were CD68 (48%) ($t_{(19)} = 6.612$, $p < 0.0001$) and Cd11b (36%) ($t_{(20)} = 5.407$, $p < 0.0001$), markers of microglial activation (**Figure 2.8 E**, **F** and **G**,).

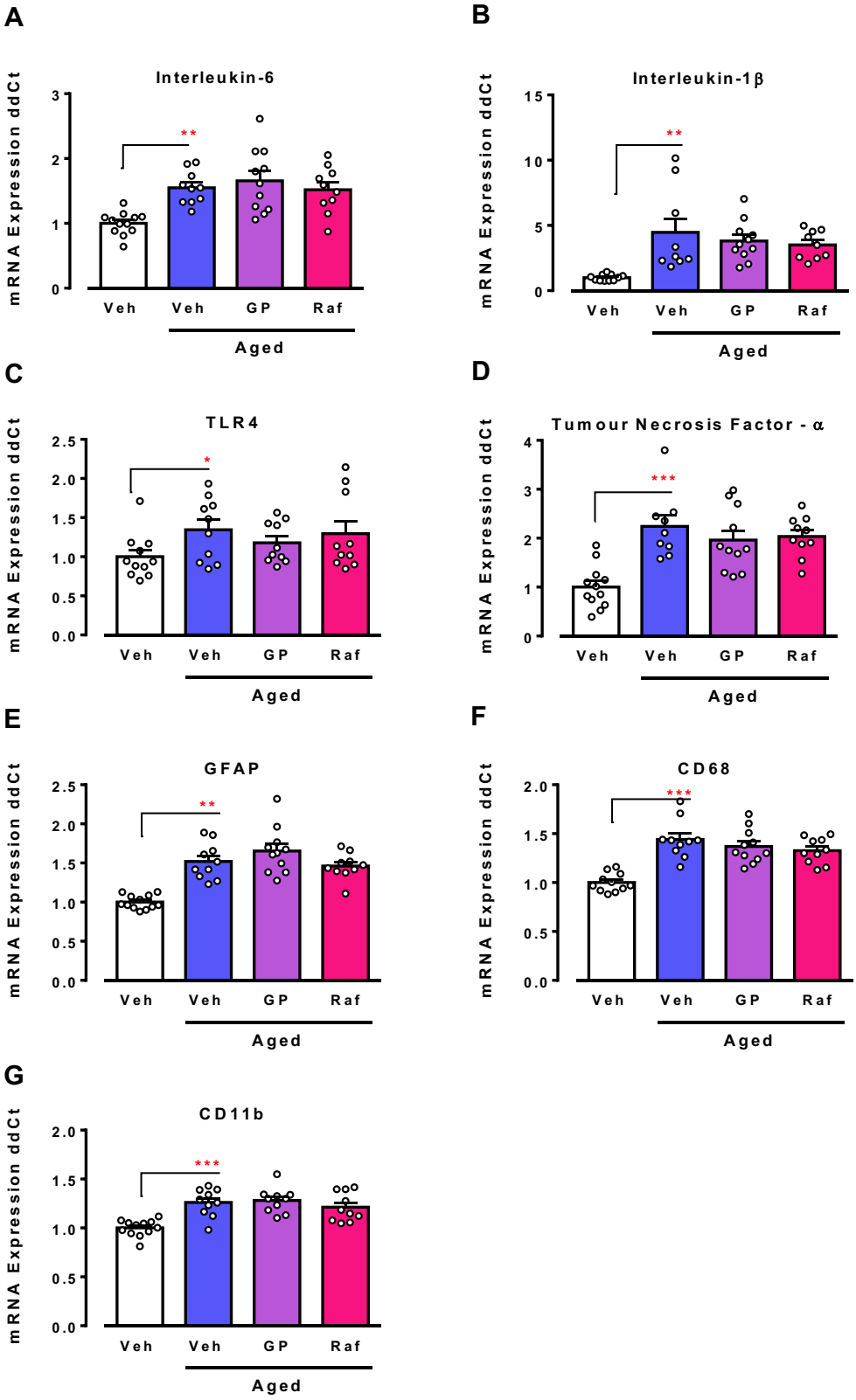


Figure 2.8. Dietary polyphenols did reverse age-related increases in inflammatory tone in hippocampus in interleukin-6 (A), Interleukin-1β (B), toll-like receptor 4 (C), TNF-α (D), GFAP (E), CD68 (F) and CD11b (G). All data are expressed as mean ± SEM. * represents significance ($p < 0.05$) against young control counterparts. N = 12 (young control group), n = 9–11 (aged).

2.3.2.4 Caecal Microbiota Analyses

We determined an effect of ageing in gut microbiota when assessing the levels of alpha diversity using Chao1 (p = 0.0119), and Shannon Diversity (p = 0.0035) and with pairwise Mann-Whitney U test (p = 0.004), but not with Simpson Diversity (p = 0.46) indices (**Figure 2.9A**). There was no effect of polyphenol on age-related changes in alpha diversity.

A Principal component Analysis (PCA) was used to visualize beta diversity as defined by Aitchison's distance (**Figure 2.9 B**). Although there were no significant differences in overall bacterial composition across all groups (PERMANOVA followed by pairwise PERMANOVA; p > 0.05), there was a significant difference between the composition of Young-Control and Aged-Control mice (PERMANOVA; p = 0.004). No significant differences in beta diversity between Aged-Control and Aged-Grape Pomace (PERMANOVA; p = 0.6) or Aged-Control and Aged-Rafuma (PERMANOVA; p = 0.552) were detected.

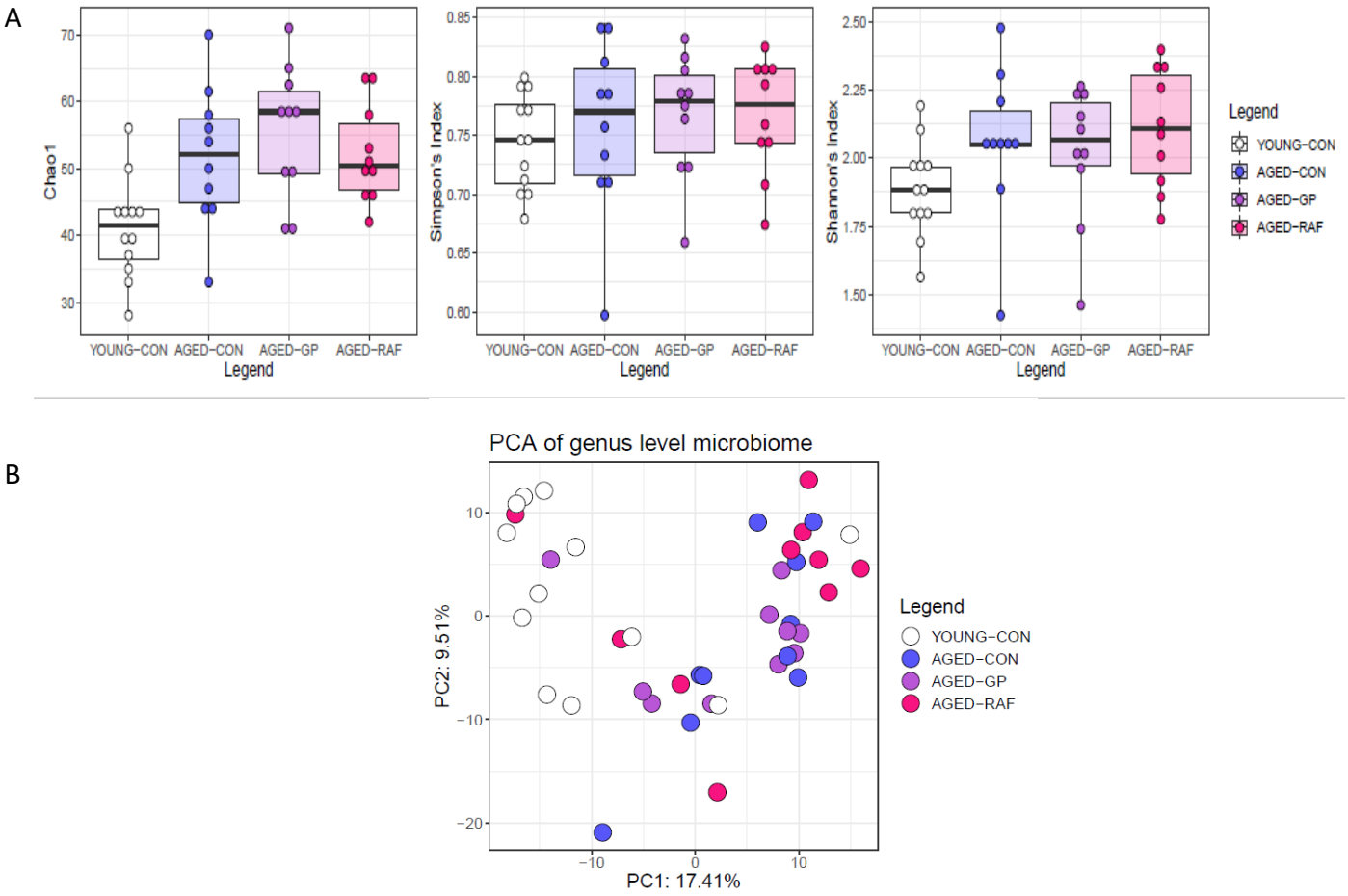


Figure 2.9 Dietary polyphenols did not reverse age-related changes in alpha (A) or beta (B) diversity of gastrointestinal microbiota. For alpha diversity, data are shown as a box and whiskers plot and data are analysed by Mann-Whitney U tests. * represents significance ($p < 0.05$) against young control counterparts. N = 12 (young control group), n = 9–11 (aged).

2.4 DISCUSSION

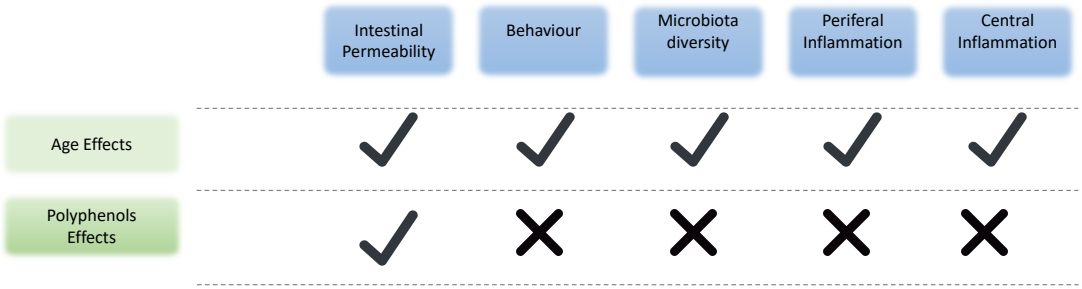


Figure 2.10 Summary of the Findings. Age-related changes are evident in the intestinal permeability analysis, in the behavior tests, in the microbiota diversity (beta and alpha), in the peripheral inflammation and in the central inflammation markers. Instead, polyphenols only have an effect on the intestinal permeability. No changes are observed in all other parameters.

Population ageing is a powerful and transforming demographic force, and we are only just beginning to comprehend its impacts at the national and global levels. According to the World Health Organization, the number of individuals over 65 is expected to almost treble in the next 30 years, resulting in a significant social and economic impact on healthcare and wellbeing (Who). Recent studies suggest that the gut-brain axis and its regulation by the microbiota (commensal microbes that inhabit our gastrointestinal tract) play a key role in healthy ageing, and dysfunction of this axis may contribute to the pathophysiology of age-related disorders (Maynard and Weinkove, 2018). Most information on the role of diet in healthy ageing comes from observational studies, while information on nutrients and food-borne non-nutrients comes from both observational studies and intervention trials. However, comprehensive double-blind placebo-controlled studies on dietary interventions are limited in elderly subjects (Claesson *et al.*, 2012). As with previous studies from our lab and elsewhere (Boehme *et al.*, 2019, Scott *et al.*, 2017, Thevaranjan *et al.*, 2017) we determined an age-related alteration in behaviour and physiology of aged mice, namely increases in anxiety-like behaviours and increased frailty, with altered HPA axis activity, altered gut permeability and gut motility, alterations in caecal microbiota and increases in peripheral and central inflammation. In general, there was no effect of polyphenol-rich grape pomace or Rafuma extract treatment on any of these age-related changes except for the attenuation of age-related increases in gut permeability. It may be that Grape Pomace and Rafuma may need to be fed at a higher dosage in order for their phenolic

compounds and metabolites to be effective. More likely, grape pomace and Rafuma may have more beneficial effects as a prophylactic, rather than as a treatment in mice that were already over 20 months old.

From a behavioural standpoint, it is difficult to disentangle our observed changes in affective responding from natural ageing. In this study we noted a decrease in time in the centre of the open field and a decrease in immobility in the forced swim test, which classically would relate to changes in anxiety-like and depressive-like behaviour. However, there are concomitant decreases in locomotor activity and increase in frailty associated with ageing. It is possible that these age-related changes in muscle strength may confound the content validity of affective responding that may account for our findings. Regardless, the polyphenol supplementation had no effect in reversing these age-related changes in behaviour.

What is of interest in this study is the age-associated impairments in gut function, under homeostatic conditions and in response to stress, and the concomitant changes in microbiota composition and immune response. Numerous factors including histological changes within the structure of the gut, including decreased expression of tight junction proteins, altered morphology of intestinal villi (Ren *et al.*, 2014), (Tran and Greenwood-Van Meerveld, 2013). The pro-inflammatory cytokines IL-6, TNFα, and IL-1 contribute to inflammageing in healthy elderly individuals, as well as in many age-related diseases. A great deal of evidence supports a link between systemic low-grade inflammation and cognitive decline in both normal and pathological ageing (Schram *et al.*, 2007). Our results demonstrated an increase in gene expression for the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α in both ileum and colon of aged mice, with a concomitant increase in the anti-inflammatory cytokine IL-10 possibly to compensate for the increase in pro-inflammatory cytokines. Using flow cytometry, we similarly reported an age-related increase in blood immune markers including granulocytes, PBMCs, inflammatory monocytes and T-helper cells. Of note, the granulocytes were profoundly attenuated in response to restraint stress, an effect which was no longer apparent in non-aged animals. Similarly, we reported an age-related increase in inflammatory tone in the mesenteric lymph nodes that line the intestinal tract. Finally, we investigated if these age-related effects of gut function, microbiota, stress response and systemic immune activation also contributed to immunoregulatory events in the brain. Hippocampal expression of genes encoding the proinflammatory

cytokines IL-1β, IL-6 and TNF-α, toll-like receptor 4 (TLR4), the astrocytic marker glial fibrillary acidic protein (GFAP), CD68 and Cd11b as markers of microglial activation were all significantly elevated in hippocampus of aged mice. Collectively, these results support previous findings demonstrating age-related changes in microbiota, gut permeability, gut motility and a dysfunctional response to stress, that contributes to inflammatory status in the gut, periphery and brain.

Our hypothesis was that these age-related effects could be attenuated with polyphenol administration. The attenuation of gut permeability in polyphenol-treated aged animals did not positively correlate with changes in changes in microbiota, gut function, or changes in stress response, nor the consequential changes in immune response. The lack of treatment effects in caecal microbiota composition, suggest that the beneficial effects of polyphenols may occur further down the GI tract. Indeed, many studies suggest that metabolism of polyphenols occurs by bacteria within the colon (Boussenna *et al.*, 2016). Further work with polyphenol treatment from earlier in life as a prophylactic treatment may yield stronger effects on behaviour, gut physiology and function, as well as endocrine and immune readouts in the periphery and brain.

The gastrointestinal microbiota has recently emerged as a key facilitator of behavioural response, physiological systmes, stress adaption and immune response in the body (Cryan *et al.*, 2019). The evidence presented herein suggests that age-related susceptibility to stress- and immune-related dysfunction may be dependent on the diversity and complexity of gastrointestinal microbiota. Many studies investigating the influence of gastrointestinal microbiota on age-realted behaviour, stress and immune responding have been largely preclinical in nature, with the findings in clinical cohorts being diagnostic in nature, and further studies looking at microbiota-focussed interventions, particularly dietary changes are warranted. Ultimately, prophylactic management of microbiota diversity and complexity, a targetable and flexible biosystem, may contribute to healthier ageing and have a positive prospective on mental health.

2.5 Supplemental Material

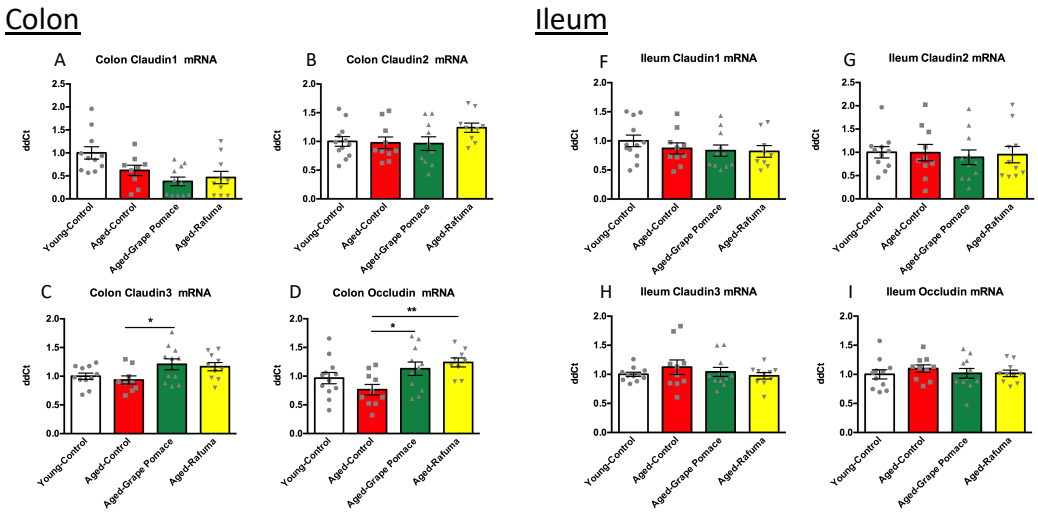


Figure S 2.1 Polyphenol treatment increased claudin3 and occludin expression in colon of aged mice, possibly contributing to decrease in permeability. No changes in genes associated with barrier function were observed in ileum. * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) represent significant differences when compared against HFC in one-way ANOVA. PostHoc Bonferroni. Plots depict individual replicates with mean and SEM.

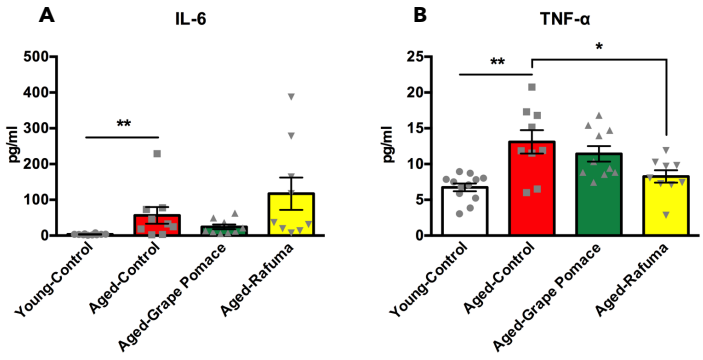


Figure S 2.3 Blood cells

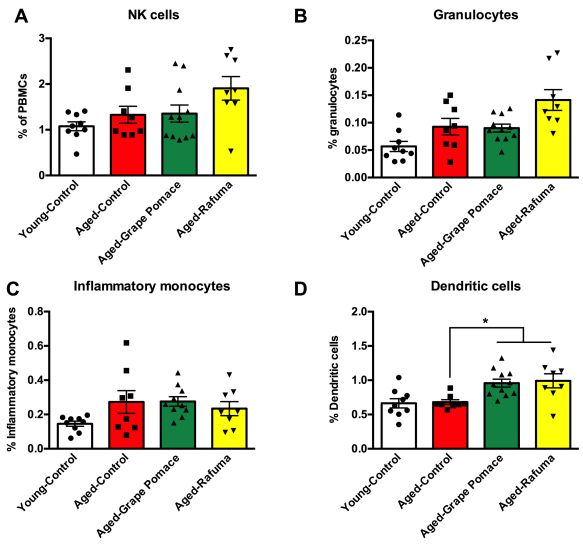


Figure S 2.2 Polyphenols did not significantly affect NK cells in aged mice in comparison with Aged-Controls, although Aged-Rafuma mice have an increase in NK cells when compared to Young-Controls. B) Polyphenol treatment did not significantly affect granulocytes in aged mice, although they were elevated in Aged-Rafuma compared to Young-Controls. C) Inflammatory monocytes were unaltered in aged mice. D) Levels of dendritic cells did not differ between Young- and Aged-Controls. Polyphenol treatment resulted in a significant increase in the number of dendritic cells in aged mice. Y-C n=8, A-C n=8, A-GP n=11, A-R n=8.

Chapter 3

Investigating the metabolic effects of prebiotic inulin in middle-age (10-14mo) Vs young (2-6mo) mice

Animal trial was run in conjunction with Marcus Boehme.

3.1 INTRODUCTION

The gut microbiota is one of the most important systems of the human body widely studied in recent years. An alteration of intestinal bacteria can lead to important changes in the intestinal tract and brain manifesting through modification of metabolism and behavioural aspects amongst. The microbiota is influenced by lifestyle, diet , disease, sex and age (Chaddha *et al.*, 2016). During the lifespan of humans and animals, the proportion of bacterial families changes and each age is characterized by a different microbiota composition that has a profound influence on the individual health (Kelly *et al.*, 2017).

Ageing is plausibly the most universal contributor to the brain and the body decline and related diseases, including dementia, diabetes and hepatic steatosis (Dinan *et al.*, 2017).

In the brain, the reduction of neuronal connections and alterations of endocrine receptors on the neuronal membrane can manifest with advancing age in deficit of memory (Wahl, Anderson and Le Couteur, 2019), depression and anxiety may increase (Bishop, Lu and Yankner, 2010). In the body, the major impairments are metabolic alterations where adipose lipogenesis, hepatic gluconeogenesis, cholesterol homeostasis and immune system are imbalanced (Perna *et al.*, 2016). The middle-age represents the first step of this physiological decline. As metabolism changes, the immune system responds in a different way and a cognitive decline becomes more common (Barzilai *et al.*, 2012). In middle-aged rodents subjected to a high fat diet,

metabolism deceleration was detected through the observation of a low tolerance of the body to stressful conditions more pronounced than in young animals (Nagpal *et al.*, 2018). Also an increase of anxiety-like behaviour driven by cognitive decline in adult middle-age animals was observed (Tschen *et al.*, 2009). In the last few decades, the role of the intestinal microbiota in the physiology of both body and brain diseases is becoming increasingly recognized (Ennaceur *et al.*, 2008; Shoji *et al.*, 2016). Different diets and nutrients have already been demonstrated to influence the microbiota and projects the effect on the brain through the gut-brain axis (Quigley, 2017; Codagnone *et al.*, 2019). The host-indigestible dietary fibres called "prebiotics" have the capacity to modify the microbiota composition and be of benefit to the brain and consequently on the behaviour. The capacity of prebiotics to reduce depression- and anxiety like behaviour in mice is well demonstrated (van de Wouw *et al.*, 2017; Boehme *et al.*, 2019). Depression and anxiety are the phenotypic result of a dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis (Burokas *et al.*, 2017) and modifications of this axis result in alterations of the glucose and lipid metabolism in the liver and in adipose tissue (Menke, 2019). The microbiota plays an important role in the manipulation of functional gene pathways involved in HPA axis regulation (Spencer and Deak, 2017) and consequently is reflected in the glucose metabolism and in lipid homeostasis (Vodička *et al.*, 2018). Pre-clinical evidence from germ-free mice suggests that the microbiota can markedly alter the metabolism with significant modification of farnesoid X receptor (*Fxr*)/retinoid X receptor (*Rxr*) activation, hepatic cholestasis, steroid synthesis, and fatty acid metabolism pathways (van Olden, Groen and Nieuwdorp, 2015). In fact, expression of *Sirt1* a key protein involved in metabolism, decreases during ageing (Swann *et al.*, 2011), and in mice can be affected by the administration of probiotics as *Bifidobacterium* and prebiotics such as blueberry juice (Yujia Yuan *et al.*, 2016).

Inulin is a polysaccharide produced by many types of plants. In the gut it is fermented by specific bacteria leading to short-chain fatty acid (SCFA) production, which is thought to improve colonic and systemic health (Ren, Huang and Cheng, 2014). Numerous studies have shown the healthy proprieties of inulin on the body (Spencer and Deak, 2017). For example, inulin as a prebiotic can confer beneficial effects to the host improving the mood and the behaviour (Hiel *et al.*, 2019), in addition, it has been reported to enhance the lipid profile decreasing in gene expression of hepatic enzymes responsible for de novo synthesis of lipids and encourage the lipoprotein lipase

enzyme in the muscle (Smith, Sutherland and Hewlett, 2015). However, the mechanism behind the metabolic interaction of inulin is not entirely clear in young or in elderly subjects.

In this study we would like to extend the knowledge of the metabolic effect of inulin on the young mice and in the middle age animals as a first step towards ageing in three of most important metabolic organs of the body: the brain, liver and fat. In the brain, we focus on the hypothalamus, amygdala and prefrontal cortex as brain metabolic centres which are also involved in the HPA axis. From different adipose tissues we have chosen to analyse the mesenteric fat as one of the most important metabolic tissue positioned in a strategic location, between the liver and the gut. Overall, our results show an unexpected inulin effect only in the liver and brain of young animals. A decreased level of the glucocorticoids *(Nr3c1*) and corticotrophin-releasing hormone (*Crhr1*) receptors was limited to the amygdala area. However, the liver showed changes in lipid and glucose metabolism directly through key enzymes as *Sirt1* without the involvement of transcriptional factors. At the end, we were able to clarify the basic metabolic mechanism of inulin and its side effect on immune modulation.

3.2 MATERIAL AND METHODS

3.2.1 Animals and prebiotic treatment

Young eight-weeks old male C57BL/6J mice (n = 40; Harlan, Cambridgeshire, UK) and middle age of 40 weeks old male C57BL/6J mice (n=37) were used in this experiment. Groups of four mice were housed in standard holding cages in a light-controlled (12-hour light/dark cycle; lights on at 7.45 am), temperature-controlled (21 ± 1 °C) and humidity-controlled (55± 10%) environment. Food and water were given *ad libitum*. All experiments were conducted in accordance with European Directive 86/609/EEC, Recommendation 2007/526/65/EC and approved by the Animal Experimentation Ethics Committee of University College Cork (B100/3774).

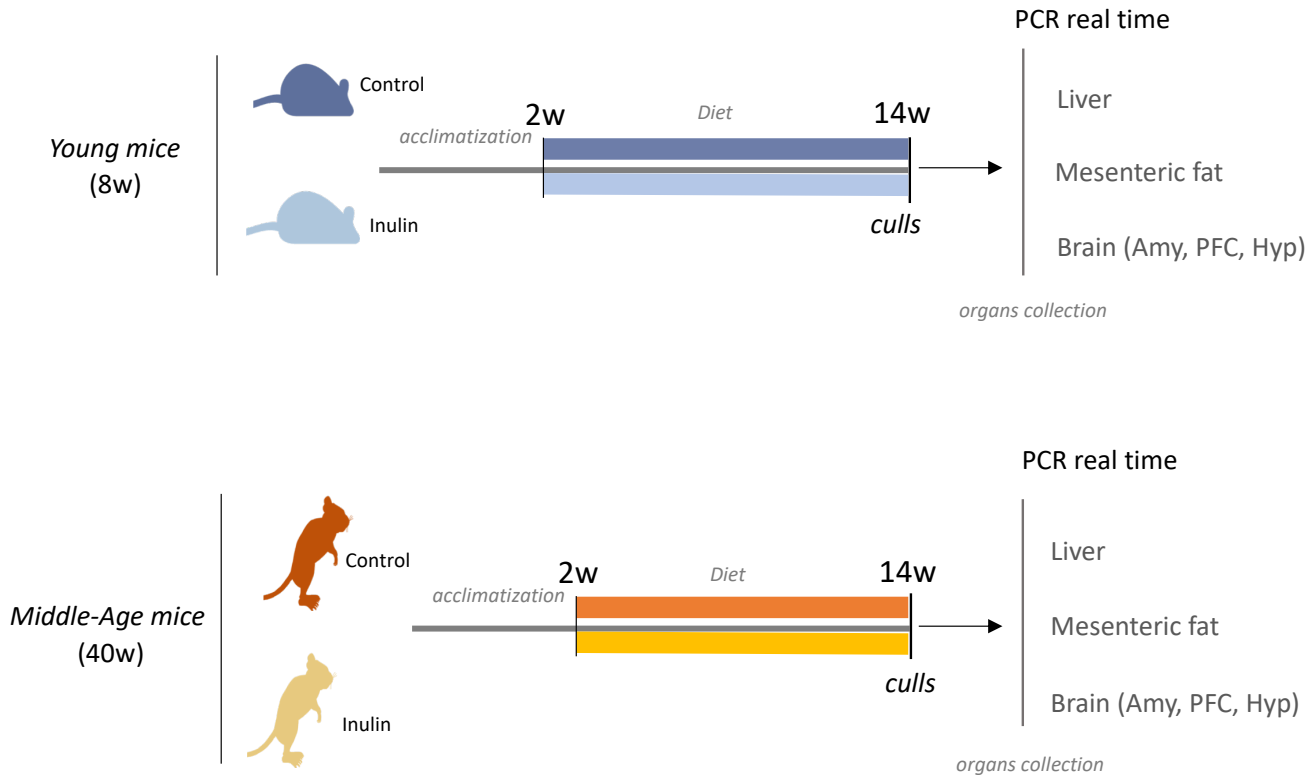


Figure 3.1 **Study design and experimental timeline**. Young 8 weeks old male C57BL/6J mice (n = 40) and middle age of 40 weeks old male C57BL/6J mice (n=37) were divided into four separate groups after two weeks of acclimatization. Two control groups: the young adult control: n = 10 (the dark blue mouse) and middle-aged: n = 10 (red mouse) received standard chow; two prebiotic treatment groups young adult n = 10 (light blue mouse) and middle-aged: n = 10 (yellow mouse) received chow with 10% FOS-Inulin for 14 weeks. At the end of the 14 weeks all animals were sacrificed and the Liver, mesenteric fat and brain in particularly amygdala, prefrontal cortex and hypothalamus were collected. W=weeks.

After two weeks of acclimatization mice were divided into four separate groups. For more detail see the experimental time-line (**Figure 3.1** Study **design and experimental timeline**. Animals received standard chow (2018S Teklad Global 18% Protein Rodent Diet) or chow (ssniff-Spezialdiäten GmbH, Soest, Germany) enriched with 10% FOS-Inulin (mixture of 92 ± 2% Inulin and 8 ± 2% Fructoligosaccharide, Orafti®Sy- nergy1; BENEO-Orafti N.V., Tienen, Belgium (Aparecida dos Reis *et al.*, 2015)). Two control groups (young adult: n = 10 [control] and middle-aged: n = 8 [control]) were compared with two prebiotic treatment groups (young adult n = 10 [inulin]and middle-aged: n = 10 [inulin]). After 14 weeks of diet, animals were sacrificed using cervical dislocation and individual body weight and organs weights

were recorded. We focused on the brain, in particular we analysed the prefrontal cortex, hypothalamus, amygdala, liver and mesenteric fat.

3.2.2 Tissue extraction and RNA sequencing

Brain tissue was dissected at 4 °C, stored in RNA Later (Ambion, Warrington, UK) and subsequently was stored at -80 °C until the analysis. The prefrontal cortex, hypothalamus and amygdala, the three regions associated to the metabolism were dissected. The coordinates of the brain regions were selected according to the ‘‘The Mouse Brain in Stereotaxic Coordinates, 3rd Edition’’(Messaoudi *et al.*, 2005). The liver and the mesenteric fat were extracted from the body immediately after the sacrifice and all tissues were processed in RNA Later RNAlater® Solution (Thermo Fisher Scientific Inc., Waltham, MA, USA). Total RNA was extracted using the mirVana™ miRNA Isolation kit (Thermo Fisher Scientific, AM1560), followed by DNase treatment using the TURBO DNA-free™ Kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA AM2238) according to the manufacturer’s instructions. RNA concentration and quality were determined using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA ND2000). RNA integrity number (RIN) was determined using the AgilentTM Bioanalyzer (Agilent, Stockport, UK). RNA samples that satisfied criteria (RIN value > 7) were reverse transcribed to cDNA using the High Capacity cDNA kit (Applied Biosystem, Warrington, UK. 4368814) according to manufacturer’s protocol.

3.2.3 Quantitative real-time PCR (qRT-PCR)

All real-time RT-PCR experiments were performed using specific TaqMan and SYBR green Gene Expression Assays on the LightCycler® 480 System (Roche, Basel, Switzerland). The reactions were conducted in technical triplicates. Data were analysed with the comparative cycle threshold (Ct) method. Expression levels were calculated as the averaged Ct values for each target gene were normalized using housekeeper *Actb* and *GAPDH* as endogenous control and transformed using the $2^{-\Delta\Delta CT}$ method (Livak KJ, 2001). The data are presented as a fold change vs. young control.

Table 3.1 List of probes used in the study

Gene	Common gene name	Sequence (5' → 3') left	Sequence (5' → 3') right
PPARa	Mus musculus peroxisome proliferator activated receptor alpha (Ppara)	cacgcatgtgaaggctgtaa	gctccgatcacacttgtcg
PPARg	Mus musculus peroxisome proliferator activated receptor gamma (Pparg)	gaaagacaacggacaaatcacc	gggggtgatatgtttgaacttg
RXRa	Mus musculus retinoid X receptor alpha (Rxra)	acatgcagatggacaagacg	gggtttgagagccccttaga
LXRa	Mus musculus nuclear receptor subfamily 1, group H, member 3 (Nr1h3)	agagcctccagggtgagg	agccctggacattaccaaga
FXR	Mus musculus nuclear receptor subfamily 1, group H, member 4 (Nr1h4)	caaaatgactcaggaggagtacg	tccttgatgtattgtctgtctgg
SREBF1	Mus musculus sterol regulatory element binding transcription factor 1 (Srebf1)	ggttttgaacgacatcgaaga	cgggaagtcactgtcttggt
SREBF2	Mus musculus sterol regulatory element binding factor 2 (Srebf2)	acctagacctcgccaaaggt	gcacggataagcaggtttgt
FGF2	Mus musculus fibroblast growth factor 2 (Fgf2),	cggctctactgcaagaacg	tgcttggagttgtagtttgacg
SIRT1	Mus musculus sirtuin 1 (Sirt1)	cagtgagaaaatgctggccta	ttggtggtacaaacaggtattga
G6PC	Mus musculus glucose-6-phosphatase, catalytic (G6pc)	tctgtcccggatctaccttg	gaaagtttcagccacagcaa
PCK1	Mus musculus phosphoenolpyruvate carboxykinase 1, cytosolic (Pck1)	atgtgtgggcgatgacatt	aacccgttttctgggttgat
PC	Mus musculus pyruvate carboxylase (Pcx)	tccgtgtccgaggtgtaaa	caggaactgctggttgttga
ACADL	Mus musculus acyl-Coenzyme A dehydrogenase, long-chain (Acadl)	gcttatgaatgtgtgcaactcc	ccgagcatccacgtaagc
ACADM	Mus musculus acyl-Coenzyme A dehydrogenase, medium chain (Acadm)	agtaccctgtggagaagctgat	tcaatgtgctcacgagctatg
Lpin1	Mus musculus lipin 1 (Lpin1)	cgcaacatcttgccaaact	cccacgtaattcattgtctgaa
DGAT2	Mus musculus diacylglycerol O-acyltransferase 2 (Dgat2)	gctggtgccctactccaag	ccagcttggggacagtga
Cyp27A1	Mus musculus cytochrome P450, family 27, subfamily a, polypeptide 1	cctcacctatgggatcttcatc	tttaaggcatccgtgtagagc
CYP27A1	Mus musculus cytochrome P450, family 7, subfamily a, polypeptide 1	ggagcttatttcaaatgatcagg	ttggccagcactctgtaatg
HMGCR	Mus musculus 3-hydroxy-3-methylglutaryl-Coenzyme A reductase	cctcacctatgggatcttcatc	tttaaggcatccgtgtagagc
Tlr4	Mus musculus toll-like receptor	agcttgaatccctgcatagat	gttctcctcaggtccaagttgccgtttc
TNF	Mus musculus tumor necrosis factor	aagcctgtagcccacgtcgta	ggcaccactagttggttgtctttg
Ccl2	Mus musculus chemokine	GCTACAAGAGGATCACCAGCAG	GTCTGGACCCATTCCTTCTTGG
Cdkn1	Mus musculus cyclin-dependent kinase inhibitor 1A	TCGCTGTCTTGCACTCTGGTGT	CCAATCTGCGCTTGGAGTGATAG
Acta2	Mus musculus actin, alpha 2, smooth muscle, aorta	CTATGCCTCTGGACGCACAACT	CAGATCCAGACGCATGATGGCA
Tgfb1	Mus musculus transforming growth factor, beta 1	CCATGGATGCCGCCCTCGGG-	AAGCTTTTAAACCTTATCGTCGTCATCCTTG
Timp1	Mus musculus tissue inhibitor of metalloproteinase 1	CGCCTACACCCCAGTCATG	TGCGGTTCTGGGACTTGTG.
Cox 4|1	Mus musculus Cytochrome C Oxidase Subunit 4I1	TTTCACCGCGCTCGTTAT	CTTCATGTCCAGCATCCTCTT
Cycs	Mus musculus, cytochrome c, somatic	CTTGACCCCCATCAATGC	CACCTCCCAGAAGGCAGA
Sod2	Mus musculus, superoxide dismutase 2, mitochondrial	ACCCAAACCGAAGTCATAGC	ACAGGTGCCATCAGAGCAGT
Cyp2E1	Mus musculus, cytochrome P450, family 2, subfamily e, polypeptide 1	ATGGCCAACCTCCTACTCCT	GCGGTGATGTAGAGGGTGAT
Nr3c2	Mus musculus, nuclear receptor subfamily 3, group C, member 2	TCTCTGGGCCGATACACTGA	CGGAACTACTGCCCTGTCTG
Nr3c1	Mus musculus, nuclear receptor subfamily 3, group C, member 1	agccagaacaccagttatcctgtct	gctgaacttggacgcagctgatt
Ucp1	Mus musculus, uncoupling protein 1	TCTCTGGGCCGATACACTGA	GTCATCATCAAGGGCACTGTTTC
Adipoq,	Mus musculus, adiponectin, C1Q and collagen domain containing	CGATGGAAGACGGGTTCTACGAAG	TTCAGTTTGCTGGACCAAC
Pparg	Mus musculus, peroxisome proliferator activated receptor gamma	CGTGGCCGCAGATTTGAA	CTTCCATTACGGAGAGATCCAC
Hsd11b1	Mus musculus, hydroxysteroid 11-beta dehydrogenase 1	CGGTTTCAGAAGTGCCTTG	GGTTCAGCTGGTCGATATCAC
Lipe	Mus musculus, lipase, hormone sensitive	GAAGCGATTCTAGGGAGCA G	GGAGCAGCGATTCTGAGTAGA
CRH	Mus musculus, corticotropin releasing hormone	ATCTCTCTGGATCTCACCTTCC	CCCGATAATCTCCATCAGTTTCC
Crhr1	Mus musculus, corticotropin releasing hormone receptor 1	CTCCAGGGTTGTCTTCATCTAC	CGGACCTCACTGTTCAGAAA
Crhr2	Mus musculus, corticotropin releasing hormone receptor 2	TACAGAGAGTGCCTGGAGAA	ATGAGGGCGATTCGGTAATG
Nr3c1	Mus musculus, nuclear receptor subfamily 3, group C, member 1	TCTCTGGGCCGATACACTGA	CGGAACTACTGCCCTGTCTG
Nr3c2	Mus musculus, nuclear receptor subfamily 3, group C, member 2	agccagaacaccagttatcctgtct	gctgaacttggacgcagctgatt
CYP46A1	Mus musculus, cytochrome P450, family 46, subfamily a, polypeptide 1	TGTCATCGCTGGCTTTTCAG	GACGATGGTAGTTGTGGTGATAGC
UCP2	Mus musculus, uncoupling protein 2	AAAATCAGTATGCGGCCGCCTTCTGCACTG	ATGAAGCTTTCAGAAAGGTGCCTCCC

3.2.4 Statistical analysis

Statistical analyses ware conducted using SPSS 25 (IBM Corp., Armonk, NY, USA) and Graphpad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). All datasets were checked for the normality with the Shapiro-Wilk test and for equality of variances using the Levene' s test. All organs weight as adrenals, spleen and caecum weights were normalized to body weight. Normally distributed data were presented as mean ± SEM. Non- parametric data were analysed with Kruskal–Wallis test followed by post hoc Dunn's. Two-way analysis of variance (ANOVA) post hoc Bonferroni was used in parametric data and are shown as mean ± SEM. MWM learning was analysed using two-way-repeated measurement (RM)-ANOVA post hoc Sidak. Correlation analyses were performed using Spear- man's correlations for non-

parametric data. Outliers were excluded using the Grubbs test. Statistical significance was set at $p \leq 0.05$.

3.3 RESULT

3.3.1 Organs weight

3.3.1.1 Inulin treatment reduced the body and organs weight in young mice

The inulin diet fed for 14 weeks had an influence on the body weight (**Figure 3.2 Inulin diet reduced the body weight in young and aged mice**.). The young mice under inulin administration were leaner than those on a basic diet. In adult mice, the effect of inulin was stronger with a p value of 0 ($F_{(3,35)}$= 0.47).

The effect of the microbiota metabolism in the body can be appreciated through macroscopic changes in bowel length and caecum size where most of the fermentation happened. In this study, we have measured the colon length and interestingly, we found that the inulin diet increases ($F_{(3,34)}$=3.1, p= 0.04) the colon length in young animals (increment of 13%) **(Figure 3.2A**) while middle age mice did not show any change. The caecum showed an increase in weight young (78%) and also in middle (82%) age mice treated with inulin **(Figure 3.2B**).

Adrenal glands are one of the most important nodes of the HPA axis and interestingly, the weight of the adrenal glands (left and right together) was decreased only in young animals treated with inulin, while there were no changes observed in adult mice (**Figure 3.2F**). If we look at the individual weight of the glands **(Figure 3.2 D and E**) we notice that it is the right adrenal gland that is increased (p= 0.011).

In the current study, three types of fat were weighed (**Figure 3.2 G, H, I**). The inulin diet lightly decreases of 24% (p=0.01) the weight of mesenteric fat (mWat) only in the adult mice while there is no effect of inulin diet on the young animals. Inulin diet did not change the weight of retroperitoneal fat (RPAT) or the weight of subcutaneous fat (subcut).

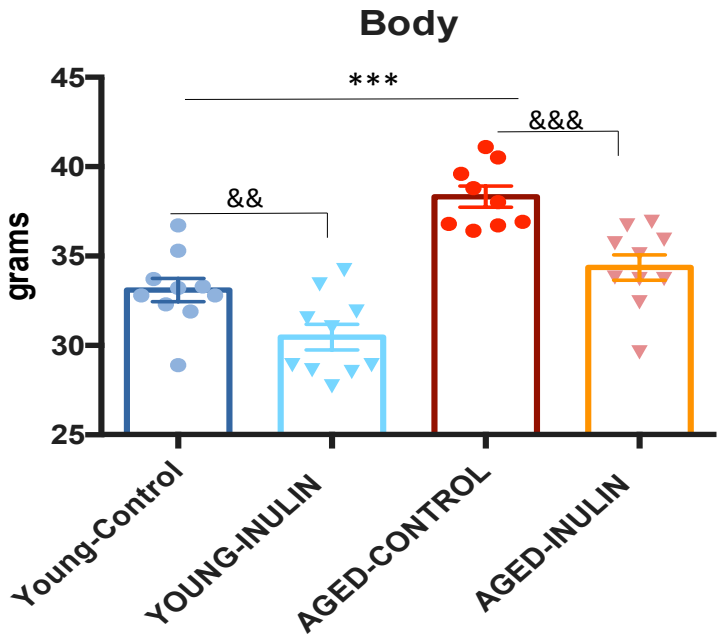


Figure 3.2 **Inulin diet reduced the body weight in young and aged mice**. The body weight of young animal was reduced to young control and in middle age animal (aged) compared to middle aged control. Statistical analysis: data are presented as Mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni, vs. young control: *$p < 0.05$, ***$p < 0.001$, vs. control groups: $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$.

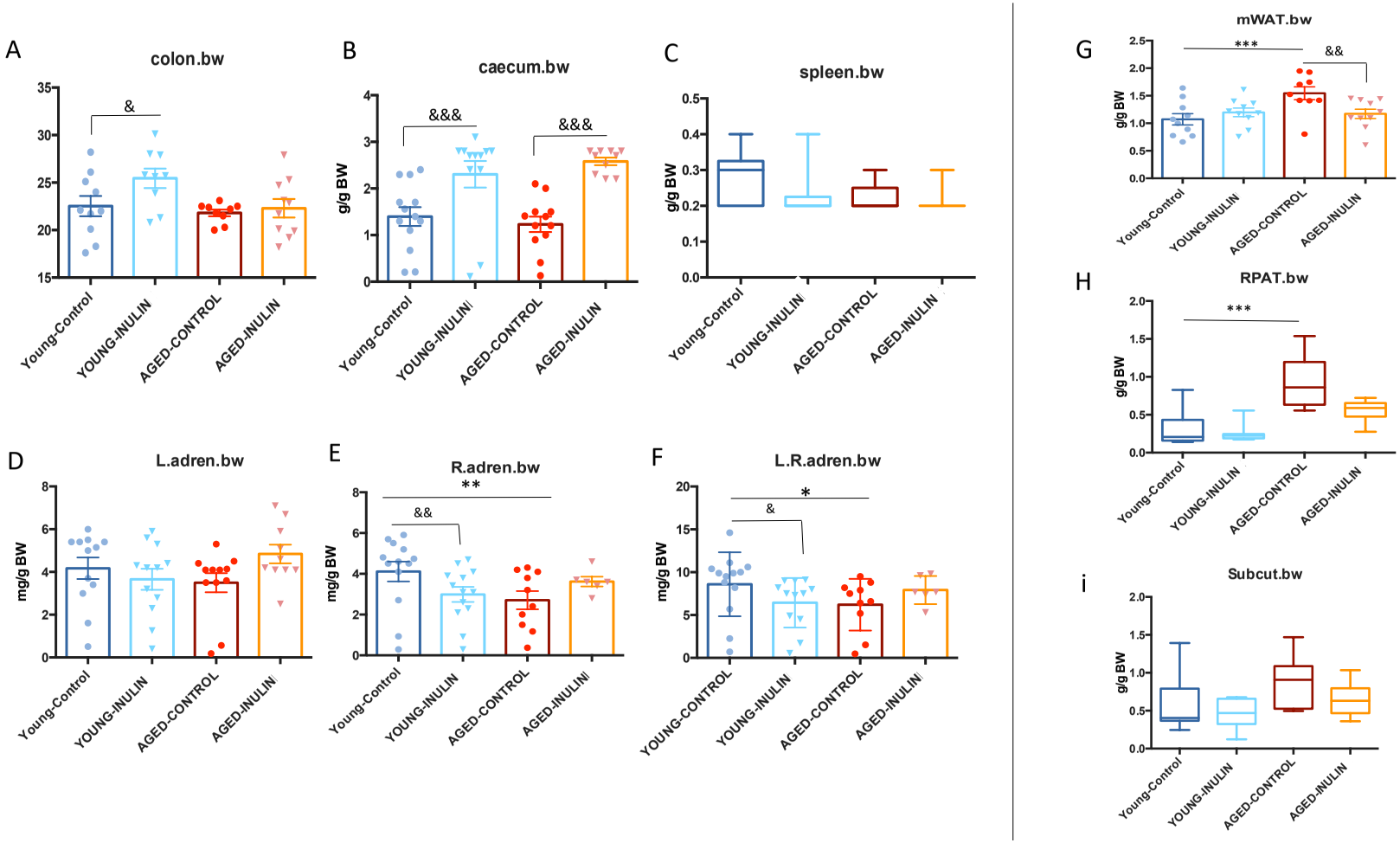


Figure 3.3. **Inulin increased the length of the colon and the caecum weight but reduced the mesenteric fat and the adrenal gland weight. A**, young inulin increased the length of the colon compared to young control. **B**, inulin increased the ceacum weight in young and in aged group compared to the controls. **E**, Inulin decrease the right adrenal gland weight on young group and there is a reduction of the weight between the two control groups. **F**, the weight of the adrenal glands (right and left toghether) decreased in young inulin group and in aged control groups. **G**, mesenteric fat (mWAT) reduced the weight in aged mice treated with inulin compared to the control but it increased in aged control compared to young control group. **C**, spleen weight, **D**, left agrenal gland, and **I**, subcutaneus fat did not change compared to the control groups. Statistical analysis: data are presented as Mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni , vs. young control: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs. control groups: $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$

3.3.2 Liver

Liver metabolism requires strictly controlled changes in gene expression. Thus, we have analysed basic metabolism through a number of different metabolic genes

- Transcriptional regulation factors (*Rxrα and Pparα, Fxr, Lxrα, Pparδ*), sterol regulatory element-binding factor (*Srebfs*) and fibroblast growth factors (*Fgf21*) and, silent information regulator 1 (*Sirt1*)
- Glucose metabolic enzymes (*G6pc, Pc, Pck1*)
- Lipid metabolism: (*Acadm, Acadl*, *Lpin* and *Dgat2*)
- Cholesterol homeostasis (bile metabolism *Cyp27A1* and *Cyp7A1*, cholesterol metabolism *Hmgcr*)
- Immunity system (*Tlr4, TnfF, Ccl2, Cdkn1* and apoptosis markers as *Acta2, Tgfb1* and *Timp1*)
- Metabolism related to reactive oxygen species (*Cox 4|1, Cycs, Sod2* and *Cyp2E1*)

3.3.2.1 Inulin diet and age factor manipulate the cell transcriptional regulation in the liver.

There is growing evidence to suggests that members of the nuclear hormone receptor (NR) superfamily are involved in the coordinated expression of genes (Aranda and Pascual, 2001). We looked at the RNA expression of Nuclear receptors (**Figure 3.4**) as *Rxrα(rxrA*) and its heterodimeric partners *Pparα* (PparA), *Fxr* (NR1H4), *Lxra* (NR1H3) and *Pparδ* (PparG). There was no difference in *Rxrα(RxrA*) expression (**Figure 3.4 B**) ($F_{(3,32)}$= p=0.541) in the liver . Two-way ANOVA revealed an effect of inulin ($F_{(3,36)}$ = 6.35, p = 0.0017) (**Figure 3.4 A**) between aged and young mice treated on the RNA expression of Pparα. The analyses revealed an effect of age ($F_{(3;30)}$= 3.82 p= 0.029) in the RNA expression of Pparδ in the control group compared to the young mice (**Figure 3.4 D)** that present an increment of 84%.

However, the inulin treatment showed a slight restoration of 24% (p=0.057) the level of *Fxr* (*Nr1h4*) in middle age mice and a not significant reduction duo to the age (p=0.075) (**Figure 3.4 E)**.

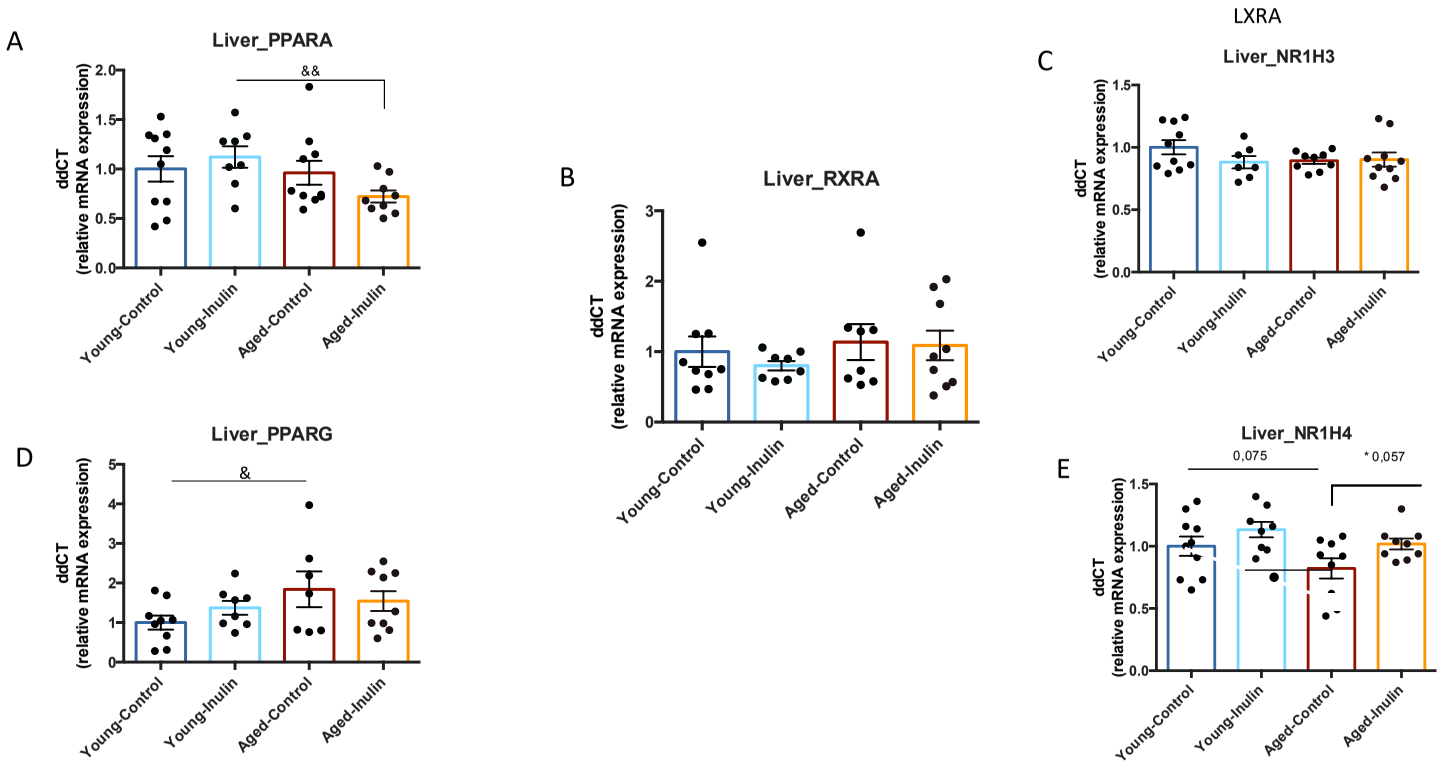


Figure 3.4 **Inulin diet and age factor manipulate the cell transcriptional regulation mRNA expression in the liver. A**, The mRNA expression of the PparA decrease in aged inulin mice compared to young inulin mice. **B** and **C**, the mRNA expression of Rxrα and of Lxrα were unaffected by the inulin or age effect. **D**, Age effect is showed by the increase of mRNA expression of Pparδ in aged control mice compared to young control group. **E**, the change of Nr1h4 mRNA expression was statistically not relevant and it showed a trend of an increase mRNA level in aged inulin compared to the aged control group and a trend of reduction in aged control group compared to young control group. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni, vs young inulin: $^{\&}p < 0.05$. vs. young inulin: $^{\&\&}p < 0.01$.

However, there was no age effect (($F_{(3,32)}$= 0.67, p=0.41), nether prebiotic treatment ($F_{(3,32)}$= 1.176, p=0.28), as well as an interaction of both ($F_{(3,32)}$= 1.51, p=0.22) in *Lxrα (Nr1h3*) another heterodimeric partner of *Rxrα* (**Figure 3.4 C).**

Intracellularly, levels of cholesterol and fatty acids are controlled through a feedback regulatory system mediated by a transcription factors family: sterol regulatory element-binding factors (*Srebf*) (Brown and Goldstein, 1997). *Srebf1*, is more active in driving transcription of genes involved in fatty acid biosynthesis. While *Srebf2* is more active in stimulating transcription of genes involved in cholesterol synthesis. In this study, RNA expression of Srebf1 was increased (38%) in middle age mice control group compared to young animals ($F_{(3,32)}$= 17.89, p=0.007). Also, there is a raise in inulin adult mice compared to the young treatment group (p=0.004) **(Figure 3.5)**.

However, the level of *Srebf2* did not show a statistically significant difference. A tendency was only observed in this gene involved in cholesterol homeostasis where the inulin slightly increased in adult mice ($F_{(3,33)}$= 4.28, p=0,089) and in treatments groups ($F_{(3,33)}$= 3.41, p=0,073).

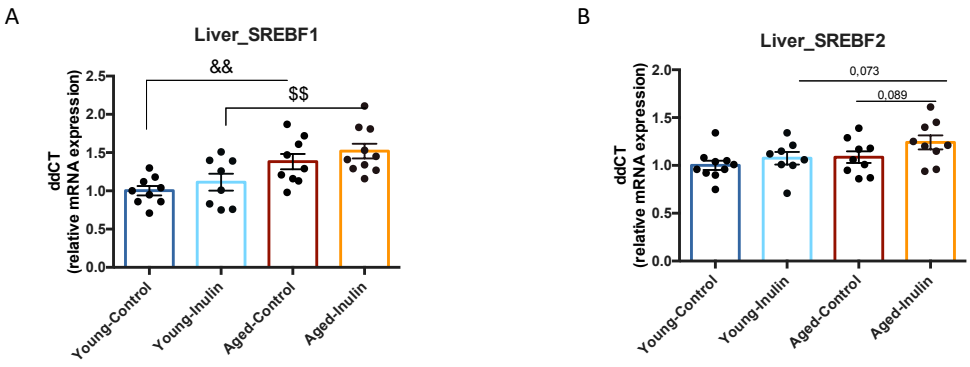


Figure 3.5 ***Srebfs* were increased by the age effect on the liver**. **A**, the mRNA expression of Srebf1 increased in aged control group compared to young control group and in aged inulin group compared to young inulin group. **B**, no change statistically relevant were observed in the mRNA expression of *Srebf2*, however aged inulin group compared to aged control group showed a trend to increase the mRNA level. Another trend was observed in Aged inulin group. Statistical analysis: data are presented

as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni , vs. young inulin: $^{\$\$}p < 0.01$, vs. young control: $^{\&\&}p < 0.01$.

Next, we examined fibroblast growth factors *Fgf21* an hepatokine involved in regulation of sugar intake **(Figure 3.6 A)** and we found that expression increased (51%) with the inulin diet but only in young animals p (=0.047). In addition, we found a similar result in another important enzyme Sirt1 in young mice ($F_{(3,31)}$= 3.60, p= 0.037). This gene is also involved in silencing and suppresses recombination of rDNA in sugar metabolism and insulin resistance (**Figure 3.6 B**).

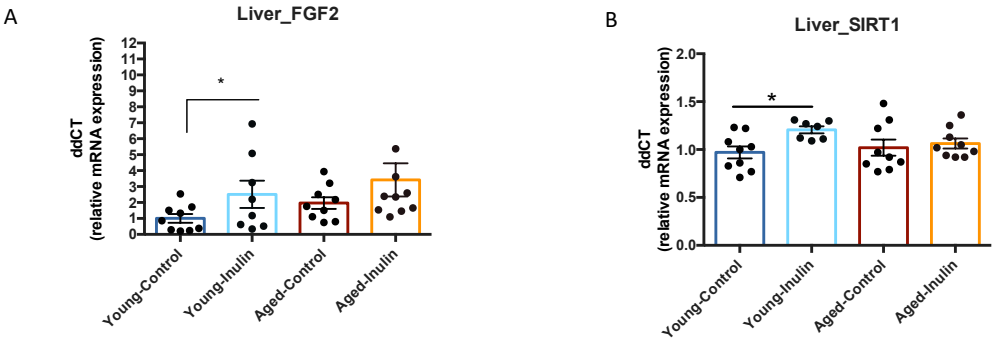


Figure 3.6 **Inulin increased the mRNA expression of fibroblast growth factors and Sirtuin1 in young mice on the liver**. **A**, inulin increased the mRNA level of *Fgf2* in young inulin group compared to young control group. **B**, young inulin group increased the mRNA expression of *Sirtuin 1* (*Sirt1*) compared to the young control group. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni, vs. young control: $^{*}p < 0.05$ vs. young control: *p < 0.05.

3.3.2.2 Inulin diet maneuvered the glucose metabolic enzymes

Gluconeogenesis is the reverse pathway of the glycolysis, however there are irreversible steps catalysed by three important enzymes: glucose 6-phosphatase (*G6pc*), pyruvate carboxylase (*Pc*) and phosphoenolpyruvate carboxykinase (*Pck1*).

In the liver of mice involved in this study, the *G6pc* RNA expression changed only in young animals **(Figure 3.7 A)**: inulin diet is able to increase the level of this enzyme that catalyses the hydrolysis of D-glucose 6-phosphate to D-glucose ($F_{(3,31)}$=5.35, p=0.017). No effect on aged mice was observed. Instead, we observed an increment of 55% in RNA expression of *Pck1* in aged mice ($F_{(3,33)}$= 4.44, p=0.014) but not in young subjects (**Figure 3.7 B)**. Also, we found a difference in RNA expression between young animals and aged animals fed with inulin diet (p= 0,011).

Two-way ANOVA did not revealed any effect of prebiotic treatment on *Pc*, a mitochondrial enzyme ($F_{(3,36)}$= 2.58). There is only a tendency of the enzyme to increase in young animals (p=0,082) and decrease in aged animals (p=0,080) by the effect of inulin (**Figure 3.7 C**).

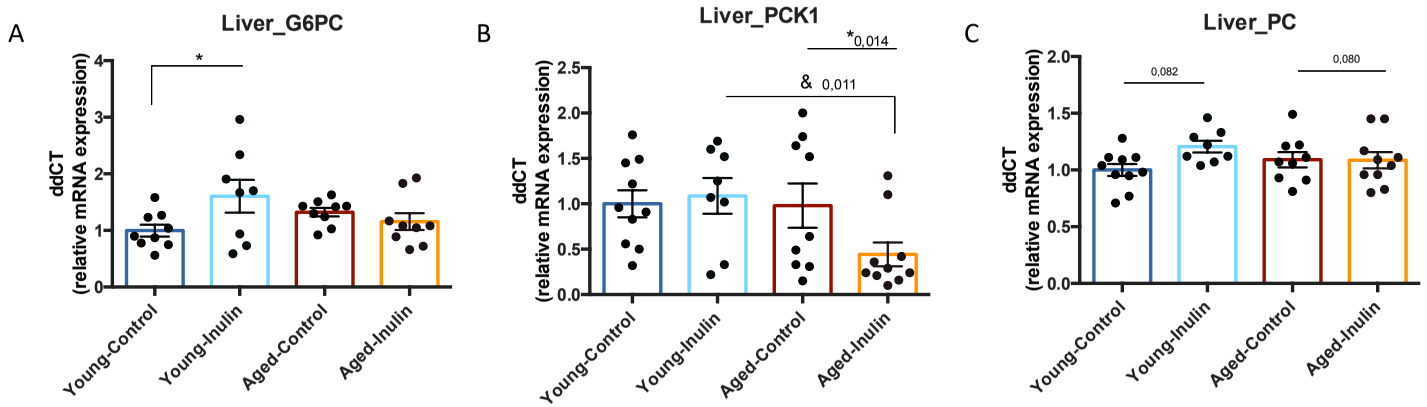


Figure 3.7 **Inulin increased the mRNA expression of glucose 6-phosphatase in young mice on the liver. A**, young inulin group showed an increase of mRNA level of G6pc compared to the young control group. B, an age effect on Pck1 was observed in aged inulin group compared to the young inulin group. A reduction by inulin was found in aged group compared to the control but it was not statistically significant. **C**, we reelevated a trend to decrease the mRNA level of pyruvate carboxylase (Pc) on the young inulin group compared to the control and a trend of reduction in aged inulin mice compared to the aged control groups. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni vs. young control group: $^{*}p < 0.05$, vs. young inulin: $^{\&}p < 0.05$.

3.3.2.3 Inulin effects the young mice in mitochondrial lipid metabolism

Lipid metabolism in the liver is characterized by the synthesis and degradation of lipids, involving the storage and breakdown of fats for energy. To study energy, we have focused our attention on the genes involved in the mitochondrial B-oxidation pathway. Long-chain acyl-CoA dehydrogenase (*Acadl*) and medium-chain acyl-CoA dehydrogenase (*Acadm*) is primarily responsible for beta-oxidation of fatty acids and they are essential for converting these particular fatty acids to energy. In this study, the *Acadl* level was increased (33%) in the young animals treated with inulin compared to the controls ($F_{(3,32)}$=10.49) **(Figure 3.8A**). No effect was observed in middle age mice. Likewise, we observed the RNA level of *Acadm* that was significantly increased ($F_{(3,31)}$= 6.011, p=0.001) in young mice fed with inulin diet compared to the control (**Figure 3.8 B**). The middle age control animals showed a slight increment of16% (p=0.045) in *Acadm* RNA expression among the inulin treated mice. However, we did not find differences in RNA levels of the two genes involved in the hepatic *de novo* lipogenesis *Lpin1 and Dgat2* (**Figure 3.8 C and D**).

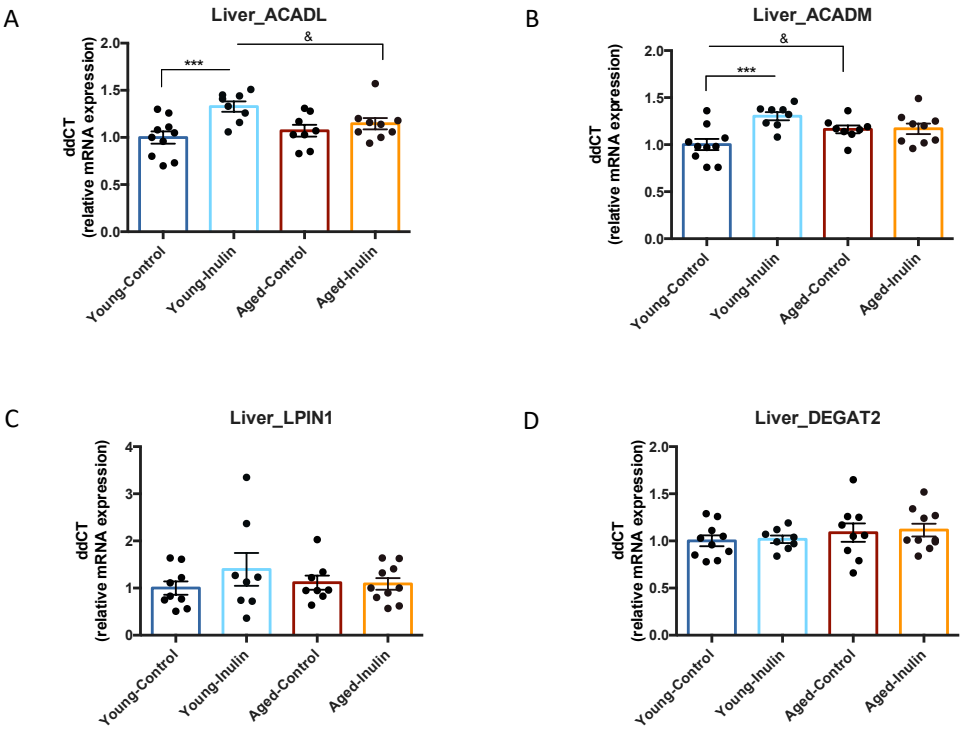


Figure 3.8 **Inulin increased the mRNA expression of Mitochondrial B-oxidation enzymes in young mice on the liver. A**, inulin increased strongly the mRNA expression of Acadl in young mice compared to the young control group. More, inulin aged mice decrease the mRNA level compared to young inulin group. **B**, inulin increased strongly level of Acadm RNA expression in young mice compared to the young control group. Here, a decreased of the enzyme (Acadm) was observed also in aged mice control compared to the young control group. **C** and **D,** no changes were observed between groups in the mRNA expression of Lpin1 and Degat2. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni vs. young control group: ***$p < 0.001$, vs. young inulin: $^{\&}p < 0.05$.

3.3.2.4 Cholesterol homeostasis is affected by Inulin in young mice

Cholesterol homeostasis is achieved through a tight regulation between synthesis, dietary absorption, utilization of bile salts, and excretion. Hmgcr is the rate-limiting enzyme for cholesterol synthesis on the melanovate pathway. We found an increase of the RNA expression in middle age mice fed with inulin diet compared to young animals treated with inulin ($F_{(3,29)}$=8.54, p=0.17)). There were no changes between controls groups (**Figure 3.9 A**). Bile metabolism enzyme is regulated by two key enzymes, *Cyp7A1* and *Cyp27A1* **(Figure 3.9 B and C**). we observed an increase of the *Cyp7A1* level (58%) in young animals fed with inulin diet compared to the controls ($F_{(3,31)}$= 3.79, p= 0,04) while no change was observed in *Cyp27A1* expression (Age $F_{(3,30)}$= 1.47, p=0.23, treatment $F_{(3,30)}$=0.004 , p=0.95) (**Figure 3.9 C)**.

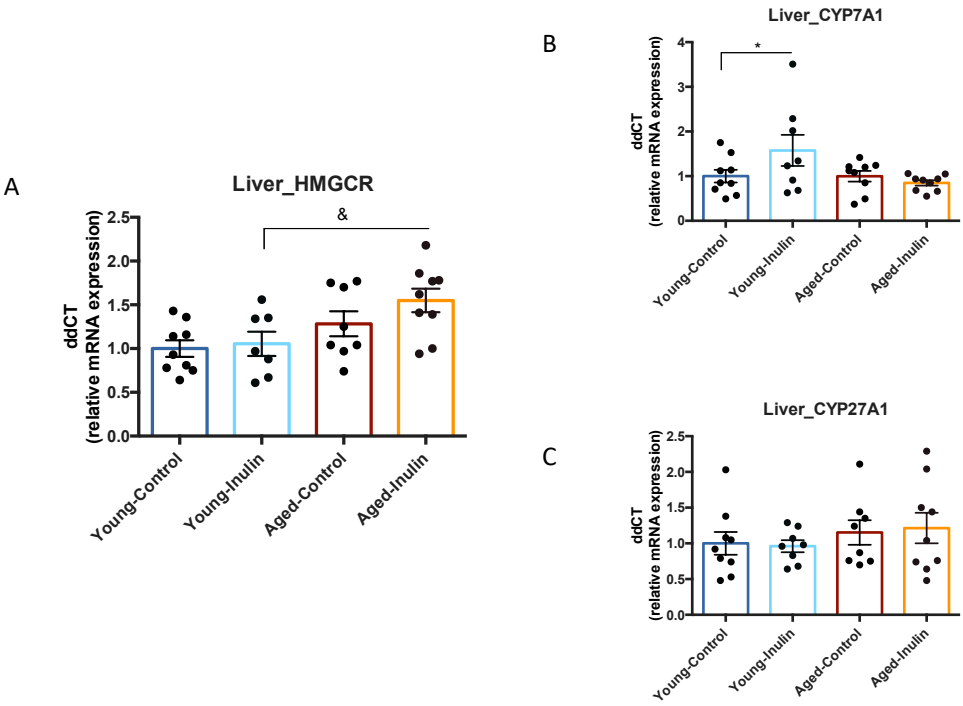


Figure 3.9 **Inulin increased the mRNA expression of Cyp7A1 in young mice on the liver**. **A**, aged inulin group increased the expression of HMG-CoA reductase (Hmgcr) compared to young inulin group. **B**, Inulin increased the mRNA expression of *Cyp7A1*compared to the young control. **C**, no change was relevated in the RNA gene expression of *Cyp 27A1*. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni vs. young control: *$p < 0.05$, vs. young inulin: $^{\&}p < 0.05$.

3.3.2.5 Changes in liver immunity by inulin treatment

Fibrosis, apoptosis and inflammation are parameters that may be influenced by the diet and age progression. However, in this study we only found minor changes in RNA expression of the biomarkers involved in immune system. We have looked at *Tlr4*, *Tnfα, Ccl2* and *Cdkn1* **(Figure 3.10A, B, C, D).** We found an increase of TNFα RNA expression in aged control mice compared to the young animals ($F_{(3,30)}$= 3.316, p=0.053), though not significant statistically. In addition, in young subject inulin treatment increased (40%) the TNFα level compared to the control ($F_{(3,30)}$= 6.89, p= 0.018) **(Figure 3.10A**). Cdkn1, a gene on chromosome 6p21.2 that encodes a cyclin-dependent kinase inhibitor was decreased (36%) in middle age mice treated with inulin compared to young inulin treated mice. No changes were found in the gene expression of *Tlr4 or Ccl2* (Figure 3.10**B and C**).

Interestingly, there was an increase of the Acta2 level in inulin middle age mice compared to young subjects ($F_{(3,33)}$= 2.25 p= 0.032)(**Figure 3.10E**). Apoptosis markers as *Tgfb1* and *Timp1* were not affected by age or inulin treatment **(Figure 3.10 F,G**).

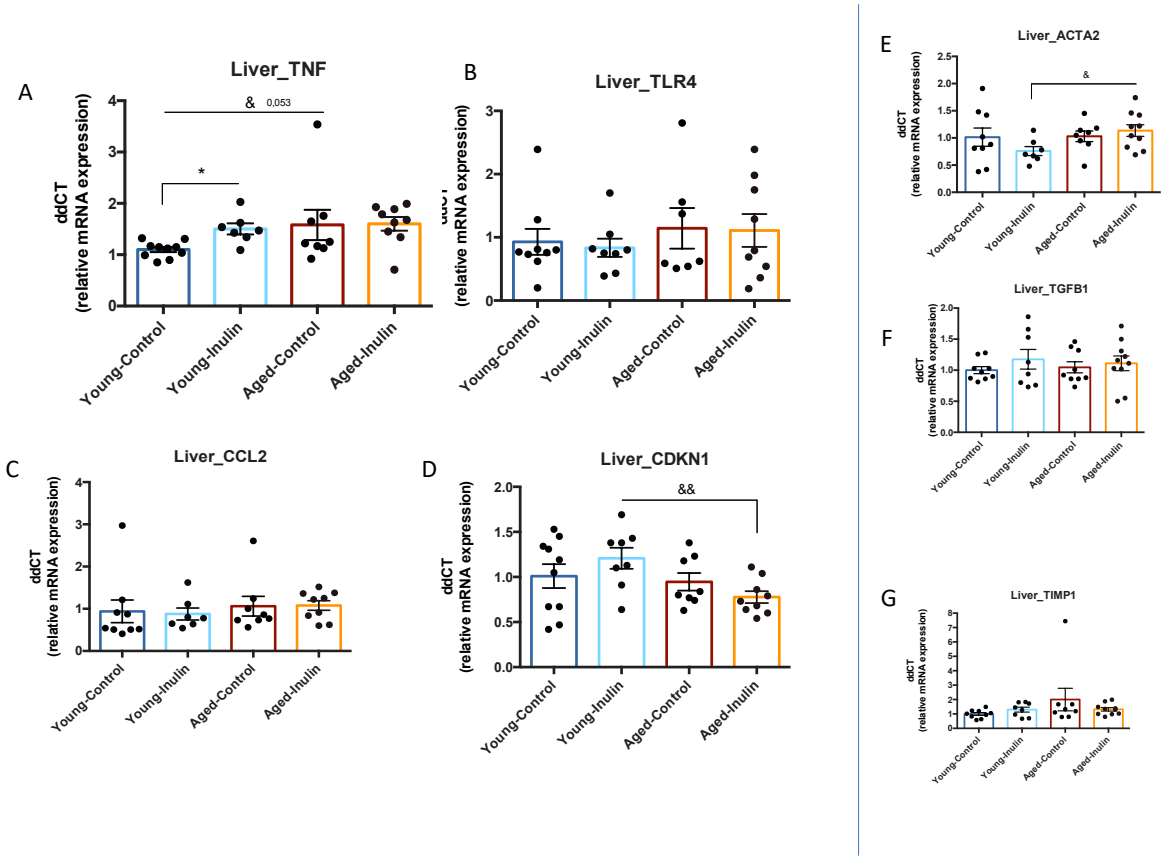


Figure 3.10 **Inulin increased the level of TNFα mRNA in the liver. A**, TNFα mRNA expression increased in young inulin group compared to the young control. Aged control group increased the mRNA expression of TNFα compared to the young control but the result is no statisticly significant. **D**, Aged inuln group decreased the mRNA expression of Cdkn1 compared to the young inulin group. **E**, an increase of the Acta2 expression in aged inulin group was observed compared to the young inulin group. **B**, **C**, **F** and **G,** no chenges were releveted in the gene expression of *Tlr4, Ccl2* and *Tgfb1*. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni vs. young control: *p < 0.05, vs. young inulin: $^{\&}p < 0.05$ $^{\&\&}p < 0.01$.

3.3.2.6 Mitochondrial oxidative phosphorylation and antioxidant defence were no effected by Inulin or age

The mitochondrial oxidative phosphorylation system is involved in the generation of cellular energy and reactive oxygen species (ROS) production. Cytochrome *c* (*Cycs*) and cytochrome *c* oxidase (*Cox*) represent the terminal step of the electron transport chain, catalysing the reduction to water from molecular oxygen (Franklin and Paxinos, 2008). In this study, inulin did not have any effect on Cox 41 ($F_{(3,30)}$= 0.017, p=0.89) and *Cycs* ($f_{(3,29)}$= 0.001, p= 0.97) RNA expression and there was no change between the young and the middle age mice (**Figure 3.11 A and B**). However, no changes were observed Sod2 expression level ($F_{(3,30)}$= 0.23, p= 0.63), an important enzyme involved in the defence of all cells exposed to reactive oxygen species (**Figure 3.11C**).

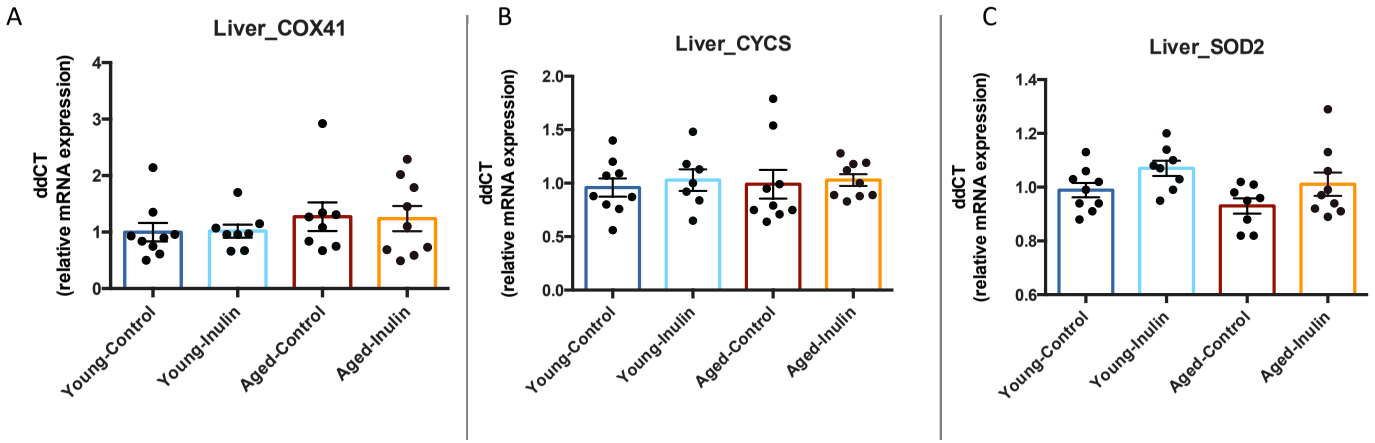


Figure 3.11 **Mitochondrial oxidative phosphorylation and antioxidant defense mRNA expression were not affected by Inulin or age in the liver. A**, cytochrome *c* oxidase (Cox) was not different between groups. **B**, No changes were observed in Cytochrome *c* (Cycs)mRNA expression. **C**, difference between groups were increased in the mRNA expression of Sod2. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni

Cytochrome P-450 2E1 (Cyp2E1) is an important enzyme which is involved in the metabolism of xenobiotics in the body and can produce ROS (Hüttemann *et al.*, 2012). In the current study no changes were observed in the RNA expression of Cyp2E1 ($F_{(3,32)}$= 0.79, p= 0.38) (**Figure 3.12**)

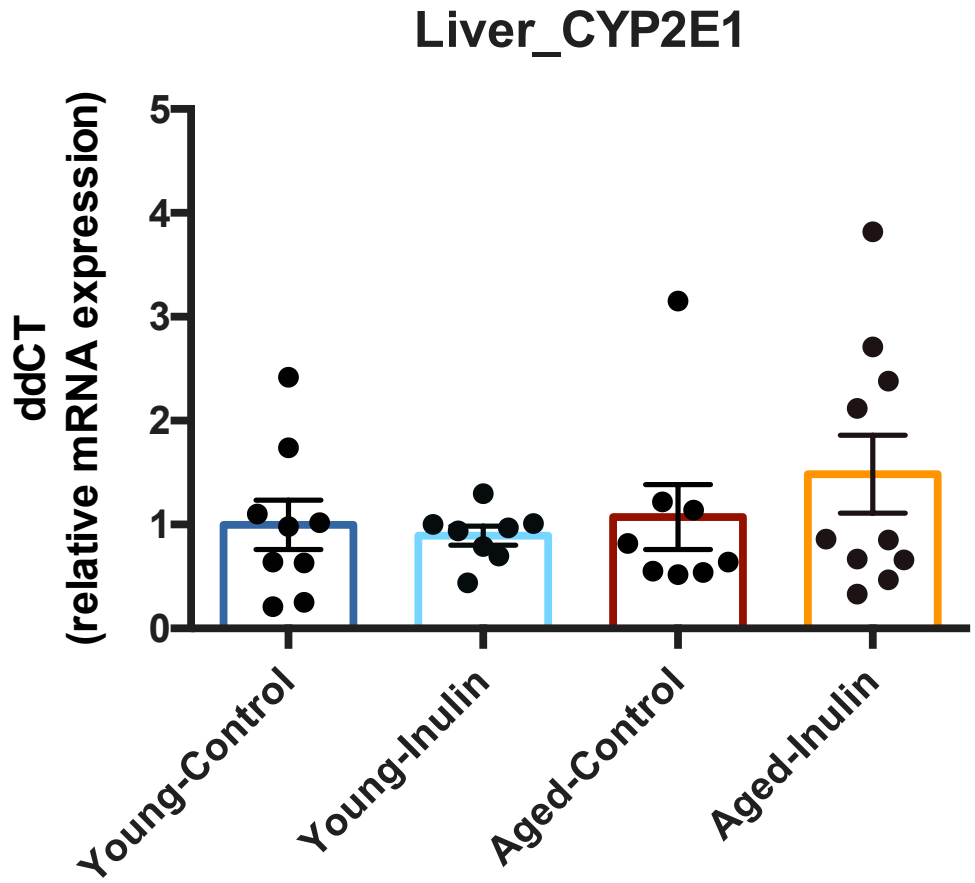


Figure 3.12 **Inulin does not effect expression of Cyp2E1 in the liver**. There are not significant differences between groups in expression of the Cyp2E1 enzime. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni.

3.3.3 Mesenteric fat

We focused on the mesenteric fat, an adipose tissue that not only serves as a site for fat storage of triglycerides but also act as a metabolically active endocrine organ that synthesizes and secretes hormones (Linhart, Bartsch and Seitz, 2014). This study analysed the RNA expression of mineral- and gluco-corticoid receptors (Nr3c2 and Nr3c1), energy metabolic marker (Ucp1), glucose and fatty acid metabolic biomarkers (Adipoq, Pparg and Hsd11b1), and lipid metabolic enzyme (Lipe).

3.3.3.1 Mineralocorticoid receptors increased in the middle age animals

Glucocorticoid and Mineralocorticoid receptors are mainly cytoplasmic at the basal state. Once activated by the ligand, they translocate to the nucleus to regulate their target genes. Cytosolic Mineralocorticoid receptors MR (Nr3c2) are activated by both aldosterone and cortisol in mice (Bjørndal *et al.*, 2011). In our study the NR2C2 RNA expression show an increment in middle age mice compared to young animals ($F_{(3,31)}$ =3.6, p= 0.051) (**Figure 3.13B**). However, the RNA expression of glucocorticoid receptor (NR2C1) did not show significant differences between young and adult mice ($F_{(3,30)}$=0.017, p= 0.9) (**Figure 3.13 C**). In summary, inulin diet did not change the level of both cytosolic receptors compared to the control diet.

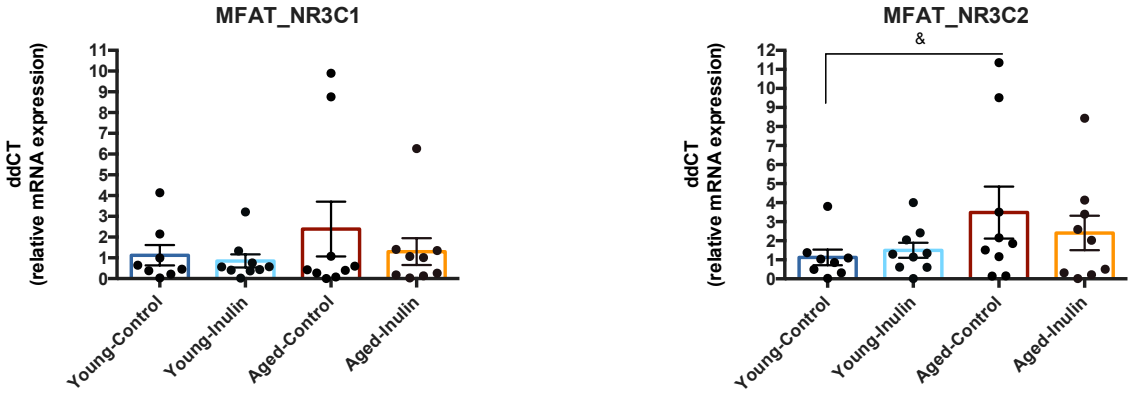


Figure 3.13 **The mRNA expression of mineralocorticoid receptors NR3C2 increased in the mesenteric fat of aged mice. A,** there was no difference between the groups in the mRNA expression of glucocorticoid receptor (Nr3C1) compared to the control groups. **B**, aged control group increase the mRNA level of Nr3c2 compared to the young control. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni vs. young control: $^{\&}p < 0.05$.

3.3.3.2 Lipid metabolic biomarkers does not suffer variations by Inulin

The 11β-Hydroxysteroid dehydrogenase type 1(Hsd11b1) is an important enzyme that reduces cortisone to cortisol and consequently activates glucocorticoid receptors. No changes were found in RNA expression of Hsd11b1in this study ($F_{(3,32)}$= 0.01, p=0.97) (**Figure 3.14A and C**) similarly, the expression of Hormone-sensitive lipase (Lipe) in the mesenteric fat was unchanged. Nevertheless, a trend towards increased the levels of Uncoupling Protein 1 (Ucp1) in middle age mice was found compared with young animals ($F_{(3,31)}$= 3.07 P=0,060), **(Figure 3.14 B**).

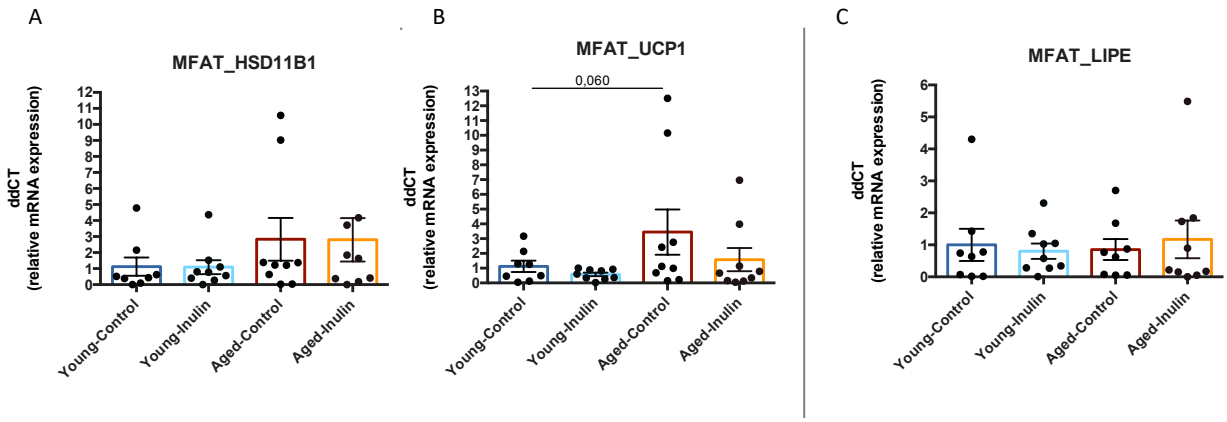


Figure 3.14 **No statistical difference were observed in the mRNA expression of genes involved in lipid metabolism on the mesenteric fat**. **A**, the mRNA expression of HSD11B1 did not chenged between the groups. **B**, Ucp1 mRNA expression showed a trend to increase the level in aged control group compared to young control group. **C**, no relevant difference were observed in the mRNA expression of LIPE between all groups. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post-hoc Bonferroni.

3.3.4 Brain

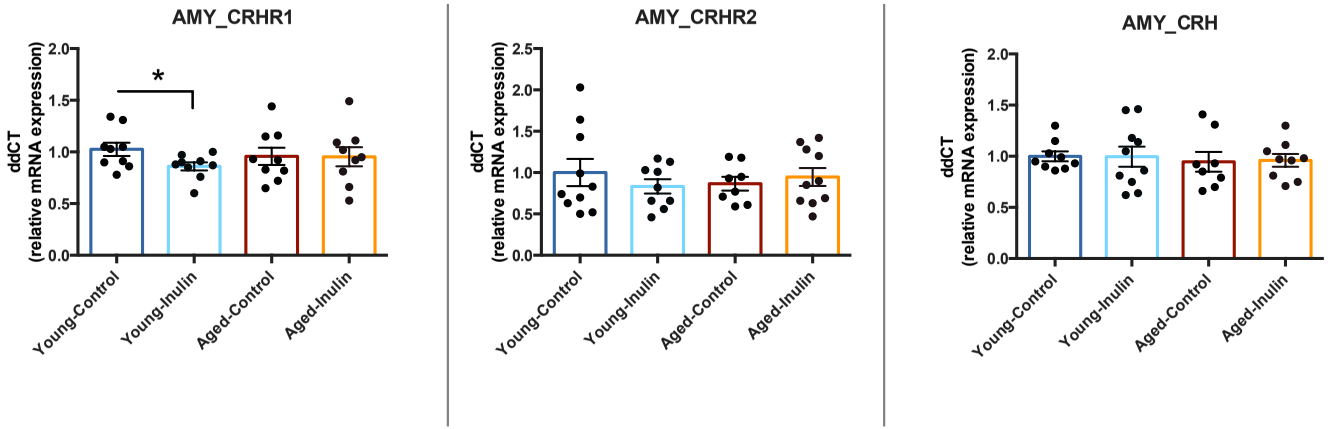
In this study, we also measured gene expression in the amygdala, hypothalamus and prefrontal cortex. We examined RNA expression of gene markers that are part of the HPA axis including Crhr1, Crhr 2 and Crh, Nr3c1and Nr3c2, and markers of cholesterol metabolism (Cyp46A1) and energy metabolism (Ucp2) respectively.

3.3.5 Amygdala:

3.3.5.1 Inulin diet decreases the Crh and glucocorticoid receptors in young mice.

The involvement of Crhr1 and Crhr2 in controlling HPA axis activity is extremely complex and its important role has been shown recently in the stress response where the increase of the Crh receptors was associated to a hypercortisolism in anxiety and affective disorders (Gomez-Sanchez, 2015). This study showed an effect of inulin in

the Crhr1 RNA expression (**Figure 3.14**). The inulin treatment was able to decrease the level of the Crh receptor in young animals compared to the control while there was no change in middle age mice. This effect is only present in Crhr1($F_{(3,32)}$=2.98, p=0.22) and not in Crhr2 ($F_{(3,31)}$= 0.133, p=0.717). Similarly, the level of the glucocorticoid receptor (Nr3c1) is reduced (22%) by the inulin in young animals only (**Figure 3.16 A**)($F_{(3,32)}$= 6.039, p=0.016). . No modification in the level of Nr3c2 was observed and interestingly, we did not find changes in the RNA expression of Crh at all ($F_{(3,30)}$= 0.012, p=0.91) (**Figure 3.16 B**).



iFigure 3.15 **Inulin decrease the mRNA expression of Crh receptor 1 in young mice in amygdala**. **A**, young inulin group decrease the mRNA level of CrhR1 compared t ɔung control group. B and C, there was no difference between groups in the mRNA expression of CrhR2 and Crh. Statistical analysis: data are presented as mean ± SEM. n = ɣoung adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni vs. young control: $^{*}p < 0.05$.

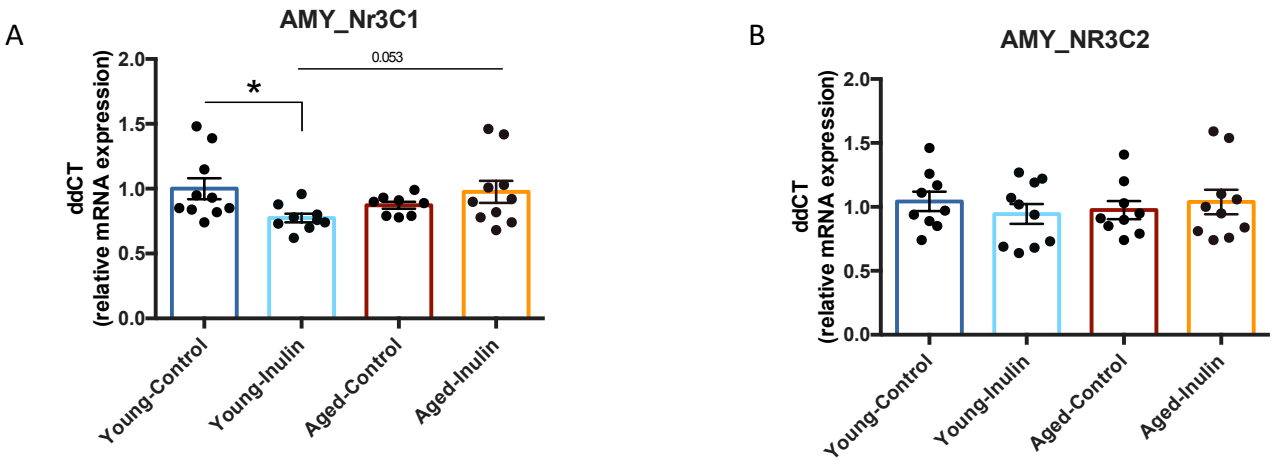


Figure 3.16 **Inulin decreased in young mice the glucocorticoid receptor mRNA expression on amygdala**. **A**, mRNA level of Nr3C1 decreased by inulin in young mice compared to the control. **B**, No difference were observed on the mRNA expression of Nr3c2 between groups. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post-hoc Bonferroni vs. young control: $^{*}p < 0.05$.

3.3.5.2 No changes in cholesterol metabolism by Inulin

The brain has the capacity to produce and metabolize cholesterol as this molecule cannot pass the blood brain barrier (BBB). Cholesterol 24-hydroxylase enzyme (Cyp46A1) is able to control the cholesterol homeostasis in the brain. The enzyme converts the cholesterol to 24S-hydrocholesterol and allowing it to overcome the BBB. In this work, the inulin diet and the age effect were not able to influence the RNA expression of Cyp46A1 in amygdala ($F_{(3,32)}$= 0.038, p=0.84). (**Figure 3.17**).

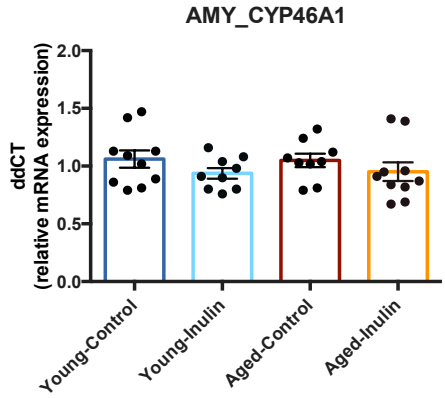


Figure 3.17 **No change was observed on mRNA expression of Cyp46A1 in amygdala**. There was no statistically change between groups of the Cyp 46A1mRNA expression. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post-hoc Bonferroni.

3.3.6 Hypothalamus:

3.3.6.1 Crh is increased in middle age mice fed with inulin.

The corticotrophin-releasing hormone gene (Crh) encodes a peptidehormone involved in the stress response as a part of the HPA axis. Our results showed an increase of Crh RNA expression in middle age mice treated with inulin compared to young mice ($F_{(3,33)}$= 4.32, p=0.026) (**Figure 3.18 A**). However, inulin diet seems to not influence the gene expressions of Crhr1 ($F_{(3,32)}$= 0.35, p=0.55), neither Crhr2 in the hypothalamus $F_{(3,33)}$= 0.022, p=0.88). (**Figure 3.18 B and C**). Glucocorticoid receptors (Nr3c1 and Nr3c2) did not change in this region (respectively $F_{(3,33)}$= 1.316, p=0.26 and $F_{(3,32)}$= 0.017, p=0.89) (**Figure 3.18 D and E**).

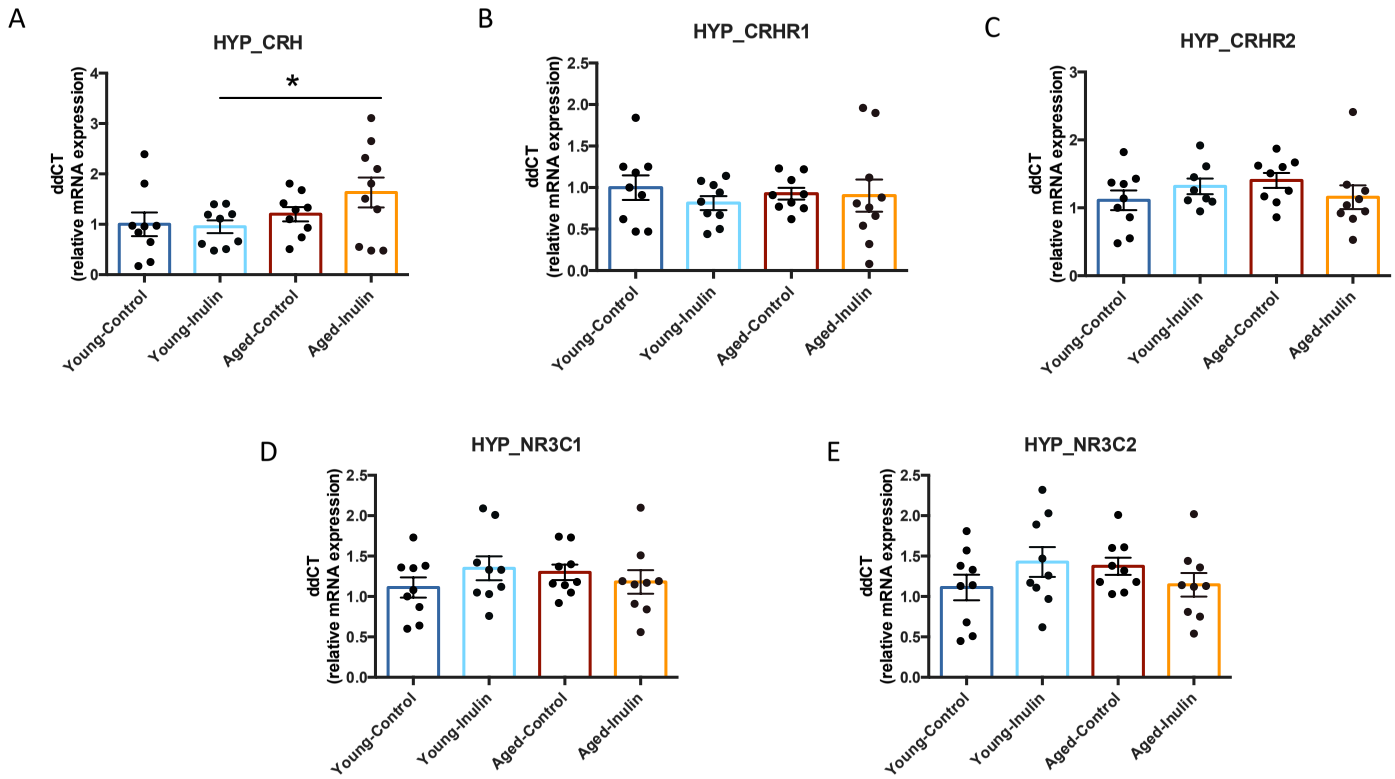


Figure 3.18 **Crh mRNA expression increased in aged inulin treated mice on the hypothalamus**. **A**, the mRNA expression of Crh increased in aged inulin group compared to young inulin group. **B**, **C**, **D** and **E,** no changes were observed between groups in the mRNA expression of CrhR1, CrhR2, NR3C1 and NR3C2. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni vs. young inulin: $^{*}p < 0.05$.

3.3.7 Prefrontal cortex:

3.3.7.1 Inulin does not influence the genes expressions in the prefrontal cortex.

The prefrontal cortex is the fulcrum of cognitive abilities and in this experiment, we analysed the RNA expression of the genes involved in the HPA axis. There were no changes in glucocorticoids receptors neither in Crhrs (**Figure 3.19 C and D; A and B**). Inulin, also, did not show expression changes in cholesterol metabolism through the gene that encode the enzyme Cyp46A1 (**Figure 3.19 E**). There were no considerable modifications by age effect between young and middle age mice.

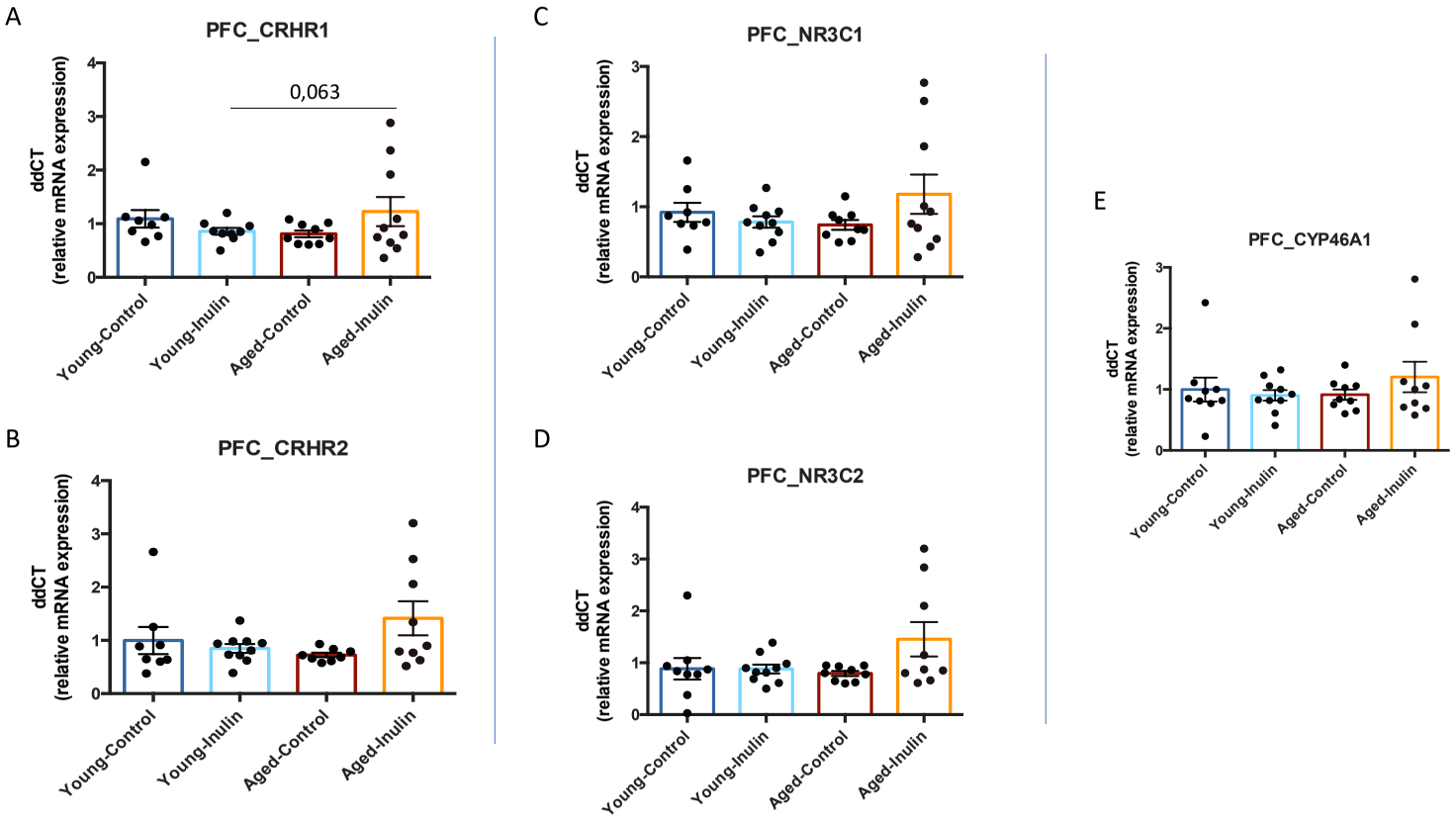


Figure 3.19 **No change was observed of mRNA expressions in Prefrontal cortex**. **A**, the mRNA expression of CrhR1 was not statistically changed between groups, only a trend was observed in aged inulin group compared to young inulin group. **B**, **C**, **D** and **E**, no changes were observed between groups in the mRNA expression of CrhR2, NR3C1, NR3C2 and Cyp46A1. Statistical analysis: data are presented as mean ± SEM. n = 10 (young adult), n = 9–10 (aged). Two-way analysis of variance (ANOVA) post hoc Bonferroni.

3.4 DISCUSSION

During ageing, the last step of life, the metabolic changes, in combination with microbiota modifications have been widely considered as important in recent years (Donner *et al.*, 2016). Through the use of Inulin, an important prebiotic able to induce positive changes in the microbiome composition (Le Bastard at *al.*, 2020), we have looked how the metabolism in the body changes in the initial phase of senescence, the middle adult age compared to youth. From different adipose tissues we have chosen to analyse the mesenteric fat as one of the most important metabolic tissue positioned in a strategic location, between the liver and the gut.

We decided to look at nuclear hormone receptors in the liver (such as Rxrα and its heterodimeric partners Pparα , Fxr, Lxra and Pparδ) that are also involved in the metabolism because, as a part of family of ligand-regulated transcription factors, they can directly translate simple chemical changes into distinct physiologic effects (Kuipers *et al.*, 2016). Once activated, nuclear receptors directly regulate the transcription of genes that control the metabolism (Figure 3.20), such as glucose (G6pc, Pc, Pck1), lipid (Acadm, Acadl, Lpin and Dgat2) and cholesterol (Cyp27A1 and Cyp7A1) metabolic enzymes.

Therefore, our research sought to analyse the metabolism at different levels to get a broader view of possible changes.

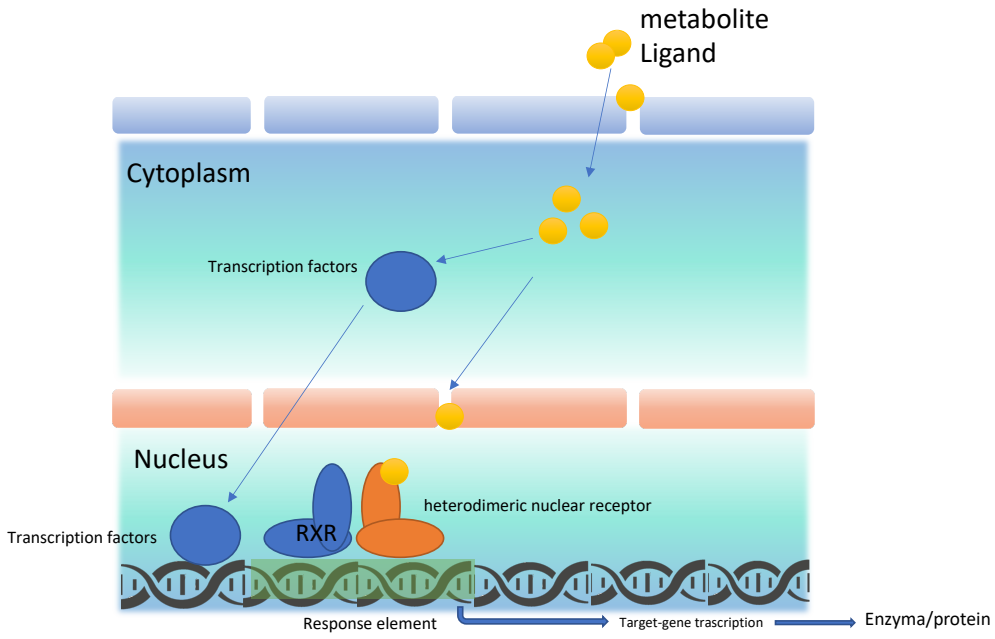


Figure 3.20 **Nuclear receptor transcriptional activation.** When a ligand or a metabolite binds the receptor at the plasma membrane initiates enzymatic phosphorylation cascades culminating with transcription factor translocation into the nucleus or Type II heterodimeric nuclear receptors bind constitutively to DNA with RXRs as obligate partners. Ligand binding causes a conformational change and stimulate the production of specific proteins or enzymes. Adapted from Avior *et al.*, 2013.

We have found modification in gene expressions of glucose and lipid metabolism, changes relative to cholesterol homeostasis, immune imbalance and alteration of HPA axis were observed in young animal's after inulin treatment (**Figure 3.21**).

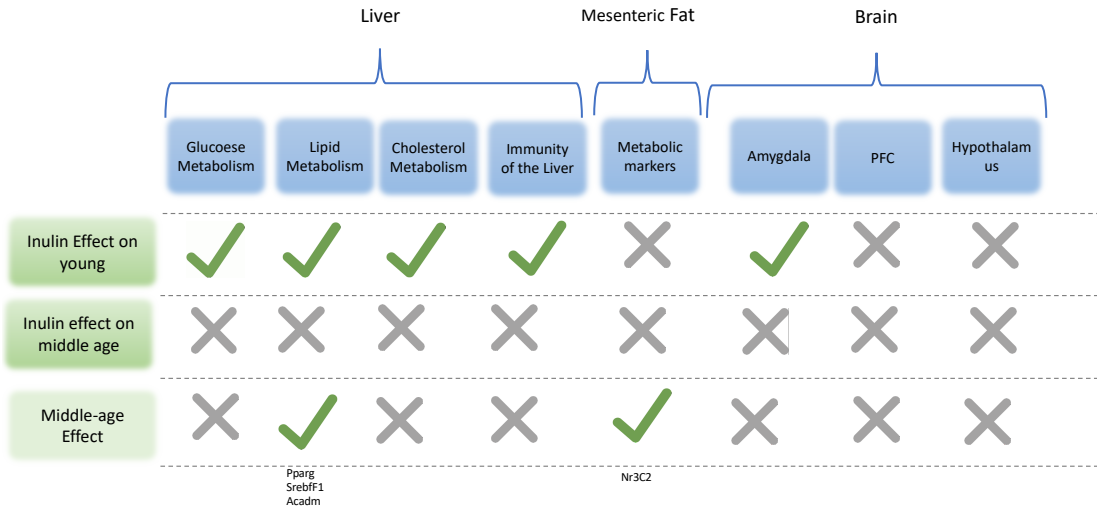


Figure 3.21. Summary of the findings. In the liver, we found that inulin can influence the metabolism and the inflammatory markers in young mice only; Middle age animals presented changes in the lipid

metabolism compared to young animals. No effect was observed on middle age animals treated with inulin. On the mesenteric fat there were no changes except the age effect on the Nr3c2. In the brain the changes were observed only in young animals treated with inulin.

3.4.1 Glucidic and lipid metabolic stimulation by inulin in young mice through Sirt1

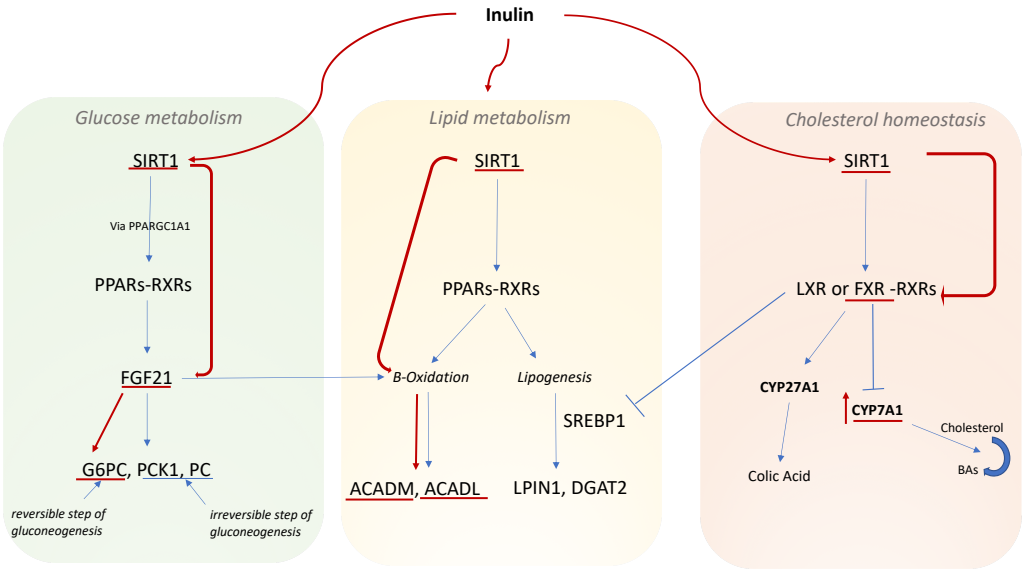


Figure 3.22 **Inulin impact on transcriptional factors and genes that encode enzymes involved in the glucose, lipid and cholesterol metabolism on the liver (red arrows). Green box:** shows the normal glucose metabolism way where Sirt1 activates the association between heterodimers Ppars-Rxrs that trigger the gluconeogenesis (blue harrow). The red arrow shows the way of how inulin stimulates the gene expression of Sirt1 and enzymes as Fgf21 and G6pc without passing *via* PparGC1A1. **Yellow box**: the lipid metabolism is showed through the Sirt1 that stimulates the aggregation of Ppars and Rxrs family that stimulates the enzymes involved in the B- Oxidation (Acadm, Acadl) and the lipogenesis (Lpin1, Dagt2) (Blue arrow). The red harrow indicates that inulin has an effect on Sirt1 that stimulated directly the b-oxidation. **Red box:** Sirt1 stimulates the Lxr or Fxr -Rxrs and it going to blocks the Cyp7A1 (enzyme that catalyses the cholesterol in bile acid (BAs)) or Cyp27A1 that catalyses oxidation to produce Colic acid (blue arrow). The red arrows show how Inulin, through Sirt1, stimulates Fxr but instead blocks Cyp7A1, increasing the level of this enzyme.

Glucose metabolism is regulated by transcriptional factors in the liver as the peroxisome proliferator-activated receptors (Ppar) group (**Figure 3.22 green box, blue arrow**). This nuclear hormone receptor heterodimerizes with the retinoid X receptor (Rxr) leading the transcription of genes related to maintaining adequate lipid and glucose homeostasis (Nagpal *et al.*, 2018; Baiden-Amissah and Tuyaerts, 2019; Ticinesi *et al.*, 2019). External factors such as diet, fasting and stress can induce modifications in these molecules (Krey *et al.*, 1997). Often, this process is mediated by the NAD-dependent deacetylase Sirtuin (encoded by Sirt1), a protein with a central

role in energy metabolism (De Cosmo and Mazzoccoli, 2017). Sirt1 supports gluconeogenesis via peroxisome proliferator-activated receptor (Ppar) γ and has an effect on G6pc, Pck1, PGC1 genes (Sedding and Haendeler, 2007). It also forms a dimer with Pparα and stimulates Fgf21 which is essential for maintaining glucose homeostasis (Cyphert *et al.*, 2012). In our study, the Sirt 1 is increased by the effect of inulin in young mice (**Figure 3.6 B**), this leads to a cascade of events that triggers the modification of Fgf21, G6pc and Pck1 (**Figure 3.7**). However, it is interesting to note that all these modifications do not occur through a Pparα dependent mechanism. In our study no changes were observed in Ppar (α and γ) or in Rxr. Inulin appears to act directly on Fgf21 e G6pc without the need for Ppar α -Rxr (**see red harrow in Figure 3.22**). Rxr gene expression did not change and Pparα is modified only in the groups treated with inulin showing a reduction in the middle age adult mice **(Figure 3.4 A**). This direct effect of inulin departs from the kinetics of other prebiotics that normally act through Ppar-Rxr. Curcumin has the capacity to manipulate Fgf21 gene expression in the liver through Pparα dependent mechanism (Planavila, Redondo-Angulo and Villarroya, 2015) and Sirt1 can be positively increased by the ingestion of polyphenols (Jin, 2018). Ellagic acid has a positive effect on Ppar γ and Pparα (Sarubbo *et al.*, 2018). G6CD can be increased by administration of polyphenols too, giving a protective effect against toxic substances (García-Niño, 2015). However, our result is in agreement with previous work (Muthumani and Prabu, 2014; Nazimabashir, Manoharan and Miltonprabu, 2015) where rats fed with inulin showed no change in Rxr expression.

The consistent increased expression of Fgf21, G6CD, Pck1 and Sirt1 due to the effect of inulin only in young animals has led us to realize that young animals are more receptive to dietary interventions than middle-aged animals and that there is a positive effect of inulin in glucose metabolism in the liver. With advancing age Sirt1 has the tendency to decrease (Chang and Guarente, 2014), but in this study, we did not notice this tendency in the middle age mice and it may suggest that Sirt1 decline in Sirt1 expression starts after 40 weeks of age.

In lipid metabolism (**Figure 3.22 yellow box, blue arrow**), Pparγ -Rxr is promoted also by the Sirt1 stimulation that usually, leads to manipulation of lipogenesis (driven by LIPIN1 and DEGAT) and to beta oxidation stimulation increasing the expressions

of Acadl and Acadm (Chang and Guarente, 2014). Moreover, a rise of Ppar expression influences positively the body weight by reducing the fat mass and the cholesterol metabolism with triglycerides (Wagner *et al.*, 2010). Even then, our results showed a direct effect of inulin on these enzymes without the involvement of Ppar γ. This is in accordance with previous work (Domínguez-Avila *et al.*, 2016) where a diet enriched with Yamabushitake mushroom increased the Acadm and Acadl gene expression with no change in Pparα gene expression in the liver.

The cholesterol metabolism (**Figure 3.22 red box, blue arrow**) is regulated via Lxr (NR1H3) and Fxr (NR1H4), (Ipsen *et al.*, 2019) and in this study, the inulin does not alter gene expression of Lxr and the expression of Fxr was slightly increased (P=0.057) only in middle age mice although not significant . It is interesting to note that the Cyp7A1 gene expression increased in young animals suggesting that there is an increase in the endoplasmic reticulum membrane enzyme that catalyses the first step of the conversion of cholesterol to bile acids with a positive effect on the body. In fact, a lack of this enzyme leads to hypercholesterolemia (Ipsen *et al.*, 2019) and an overexpression of Cyp7A1 can reduce the body weight and protects from glucose intolerance and insulin resistance (Pullinger *et al.*, 2002). In our study, the body weight is reduced by inulin in young and middle age mice (**Figure 3.2**). An upregulation of Fgf21 similar to our data, was already associated with a reduction of body mass in the obesity (Li *et al.*, 2010). However, the reduction of mesenteric fat associated with an increase of Fgf21 expression in the liver (Z. Li *et al.*, 2019) is in contrast with our result where the mesenteric fat was decreased only in middle adult treated and no changes were observed in retroperitoneal fat or in subcutaneous fat mice **(Figure 3.2 G, H and I**). The change of body weight can however be justified by the epididymal fat that was not taken into consideration in this study, which in studies investigating obesity is the one that is most easily modified based on the diet (Yamazaki, Okawa and Takahashi, 2016).

It is interesting to note that there is an increase in the caecum weight and colon length **(Figure 3.2B and A**) that is correlated with microbiota modification (Chang *et al.*, 2013; Lee *et al.*, 2015). Intestinal microbiota, through the gut-brain axis, is able to drive the energy metabolism associated with a reduction in body weight (Kuo, 2018; Wei *et al.*, 2018).

Previous data from our lab, involving inulin treatment (Boehme *et al.*, 2019) elucidated the effect of inulin in mice and how it effects on the microbiota. In this study young mice treated with inulin showed a reduction in the *Ruminococcaceae* family on the microbiota composition analysis. A decline of *Rumincoccaceae* abundance, has already been associated with a reduction of the body weight in mice treated with oligofructose (Boehme *et al.*, 2019) and also demonstrated an inverse relationship with Cyp7a1 expression and *Rumincoccaceae* after hepatectomy (Huazano-García, Shin and López, 2017). However, in contrast, Boehme *et al.*, 2019 found an abundant presence of the *Prevotellaceae* family after inulin treatment but this family is usually associated with an increase of the body weight in obese mice (Liu *et al.*, 2016). In contrast, *Bacteroides* genus, that in this case is abundant in young and adult mice anther inulin effect looks to protect from weight gain as is associated with lean mice (Hjorth *et al.*, 2018). Therefore, despite the presence of *Prevotellaceae*, mice are probably thinner due to the effect of *bacterioides.* Also, inulin fed mice showed an increased abundance of *bifidobacteriaceae* (Boehme *et al.*, 2019). *Bifidobacteria*, in addition to the countless beneficial properties for the organism, have bile salt hydrolases (BSH) (Boehme *et al.*, 2019) and they can deconjugate bile acids and increase the Cyp7A1 expression on the liver (Urdaneta and Casadesús, 2017).

Another not coherent result is about the abundance of *Porphyromonadaceae* only in young inulin mice (Degirolamo *et al.*, 2014). It was reported that a high presence of this family in the intestinal gut was associated with mice with hepatic steatosis, obesity and glucose intolerance (Boehme *et al.*, 2019) and this is in contrast with our results.

All these results highlight the necessity to investigate the relationship between metabolism and gut microbiota. Further investigation into how the microbiome shifts as metabolism changes could help determine the closer communication between the host and the gut microbiota.

3.4.2 Inulin increase TNFα in young mice in the liver: a possibly side effect

It is well acknowledged that inulin can have a the positive effect on the body (Henao-Mejia *et al.*, 2012), in particular on the lipid profile (Hiel *et al.*, 2019)and its impact on metabolism (Aparecida dos Reis *et al.*, 2015). It was demonstrated that inulin is able restore the leptin related pathways in the glucose and lipid metabolism

disorders(Aparecida dos Reis *et al.*, 2015). Other works showed the ant-inflammatory effect of inulin in metabolic disease as type-2 diabetes in mice (Song *et al.*, 2019) and in women (K. Li *et al.*, 2019).

However, controversial data have emerged about inflammation, recently, (Dehghan *et al.*, 2014) showed the capacity of inulin to modulate functions of the immune system activating immune cells in Peyer's patches including IL-10 production and natural killer (NK) cell cytotoxicity. Moreover, very little research has elucidated the negative effect of inulin as its capacity to accelerate the atherosclerosis process in mice increasing the inflammation (Hoving *et al.*, 2018), or the pro-inflammatory cytokine (TNFα) level increased in monocytes in *vitro* following inulin administration (Hoving *et al.*, 2018).

Unexpectedly, our data provided an unexpected result in the liver of young mice, we found that TNFα RNA expression increased in the young animals treated with inulin (

Figure 3.10 **A**). This can be related to our previous report (Boehme *et al.*, 2019) where the level of corticosterone in plasma after stress remained high only in the young adult treated with inulin. It is known that corticosterone can stimulate the production of TNFα (Capitán-Cañadas *et al.*, 2014). Our results underline one of the negative effects of inulin. The stimulation of the gene expression encoding the proinflammatory cytokine TNFα only in young subjects let us to realize that the administration of inulin has different effects in different stages of the life. In fact, there were no changes in middle age mice. Further work is required to elucidate this aspect, in order to clarify whether the side effect of inulin can be influenced by age and general health.

3.4.3 Inulin effect on the HPA axis: amygdala involvement

One of the main efferent routes of communication between brain and gut is represented *via* the HPA axis. External stimuli such as stress leads the production of Crh and activation of its receptors in the hypothalamus; through the HPA axis, the adrenal cortex releases glucocorticoids (cortisol in man and corticosterone in rodents) that stimulate the changes in the metabolism, preparing the body to react to the stressor. Glucocorticoids are normally regulated by a feedback inhibition where they act in a feedback loop on the brain through glucocorticoid receptors (Nr3c1), (Liao *et al.*, 1995). The hypothalamic area is central to this process while recently the indirect

influence of amygdala in HPA axis was also demonstrated (Uschold-Schmidt *et al.*, 2013). The amygdala, an important part the limbic system, has a crucial role in the emotional memory such as fear and also it is involved in anxiety and depression (Smith and Vale, 2006). It is interesting to note that in our results we observed that inulin has an effect only in the amygdala of young animals and in particularly inulin decreased level of Crhr1 (**Figure 3.16 A**). It is well known that Crh and its receptors are highly implicated in the regulation of anxiety related behaviour and plays a central role in depressive disorders (He *et al.*, 2019). In our previous study (Boehme *et al.*, 2019), inulin has the capacity to reduce the fear and anxiety like behaviours in young mice demostrated through an increased time spent in the open arms in the Elevated Plus-Maze test. Indeed, a study on Crh1-deficient mice reported that the Crh1 receptor is predominantly involved in the control of anxiety-like behaviour (Paretkar and Dimitrov, 2018). Interestly, we found that inulin decreased the RNA level of Crhr1 (**Figure 3.16 A**) in amygdala but no changes in Crhr2 (**Figure 3.17 A and B**) was observed. This may be explained by the high affinity of Crhr1 to bind to Crh, in contrast to Crhr2 where the involvement of Crhr2 starts only when the production of Crh is higher (Smith and Vale, 2006). In fact, we found that Crh gene expression was unchanged (**Figure 3.17C**).

Glucocorticoid receptors are also implicated in anxiety and depressive behaviour (Reul and Holsboer, 2002) and are involved in the HPA-axis (Wiley, Higgins and Athey, 2016). In amygdala, we found a reduction of the glucocorticoids receptors (Nr3c1) but the level on mineralocorticoids receptors (Nr3c2) was unchanged **(Figure 3.16**). In previous studies it was demonstrated that stress can alter the levels of Nr3c1 in CNS, in particular, in the amygdala the exposure to elevated glucocorticoid levels can be associated with a significant reduction in Nr3c1 receptor expression; however as in our study no changes in MR expression was observed (Garabedian, Harris and Jeanneteau, 2017). Usually, Nr3c2 has higher affinity to corticosteroids than the Nr3c1 (Wiley, Higgins and Athey, 2016) and the basal levels of corticosterone stimulate only Nr3c2 to the maintain the homeostasis (Kozlovsky *et al.*, 2009). This may indicate that inulin could have an important role in stress and memory as Nr3C1 is also implicated in cognition and memory function (Ronald de Kloet *et al.*, 1993).

In summary it seems that inulin has a positive effect on the HPA axis where in addition to the reduction of anxiolytic receptors it also led to a reduction of the weight of adrenal gland (Figure 3.2 E and F) that can be an index of gland activity (Vukojevic *et al.*, 2014). Inulin is a potent prebiotic that is able to manipulate the microbiota (Boehme *et al.*, 2019) and through the intestinal bacteria may act on the HPA axis (Everds *et al.*, 2013). It has already been shown that probiotic treatment can reduced HPA axis-related transcripts in the hippocampus, including Crhr1 and mineralocorticoids (Abildgaard *et al.*, 2017). Moreover, the reduction of Nr3c1 and Crhr1 mRNA expression were found in the amygdala of antibiotic-treated rats in the context of increased depression-like behaviour (Abildgaard *et al.*, 2017). Therefore, this study may suggest the potential role of inulin on the HPAaxis, that it may be able to moderate the stress reactivity on the body and it could be interesting in chronic stress situations.

3.5 CONCLUSION

The present study demonstrates that inulin differentially affects the body and the brain metabolism in young and middle age subjects. Interestingly, the effect of inulin acts directly bypassing traditional pathways and having a direct action on key factors such as Sirt1 in energy metabolism and Crhr receptors together with glucocorticoid receptors in the HPA axis. In addition, some side effects have emerged as the elevation of TNFα but all changes have occurred only in young animals. Together, these data highlight the importance of investigating the impact of inulin on the different stages of the life and in different conditions. So, in conclusion, the gut-brain axis can be perturbed through the use of prebiotics but not always with positive effects. More studies need to be done to fully understand the basic mechanism between microbiota, lifespan and metabolism.

Chapter 4

Effects of Anti-hypercholesterolemic Diets on the Brain in a mouse model of Cardiovascular Disease

4.1 INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of death in almost every region of the world. According to the World Health Organization 2016 statistics, CVDs estimated to account for 31% of all global deaths. An estimated 7.9 millions of these deaths are due to coronary heart disease and atherosclerotic plaques formation (WHO, 2016).

The cause of CVDs is usually a combination of risk factors, such as diabetes and obesity, physical inactivity and harmful use of alcohol, tobacco, male gender, older age, family history of CVDs but also unhealthy diet and psychologic factors as stress and anxiety (Borzabadi *et al.*, 2018)(Forouhi W., Krauss N., Taubes R., 2018). Diet appears to contribute most to the onset of these illnesses (Stewart, Manmathan and Wilkinson, 2017). International comparisons (Dariush Mozaffarian, 2015),(Harika *et al.*, 2013),(Nakamura *et al.*, 2010) and laboratory data suggest that diets, high in animal saturated fat and cholesterol and low in polyunsaturated fat increase the risk of coronary heart disease (Goto *et al.*, 2011). This is caused by the close connection between the diet and the intestinal bacteria (Forouhi *et al.*, 2018). The microbiota leads the bidirectional signalling between the gut and the brain and it is regulated at neural, endocrine, and immune levels and together, they comprise the brain-gut-microbiota axis (Franzosa *et al.*, 2019). Recently, many studies have shown that diet and gut health affects symptoms seen in stress related disorders such as anxiety and depression through changes in the gut microbiota (Grenham *et al.*, 2011), (Kelly *et al.*, 2016),(Foster, Rinaman and Cryan, 2017), (Liu and Zhu, 2018). Benerjee *et al.* estimated that about ~40% of anxieties and ~80% depressive patients have intestinal function disturbances, such as in irritable bowel syndrome (IBS) (Molina-Torres *et al.*, 2019). In fact, the interaction of microbiota with stress, the HPA axis and the immune system is well known (Banerjee *et al.*, 2017), (Baes *et al.*, 2014),(Sudo, 2014). The connection between the brain and the microbiota is specifically demonstrated in works that address neuronal plasticity. Vaughn *et al,* have showed that the Brain-Derived Neurotrophic Factor (*bdnf*) and its relationship with receptors for N-methyl-D-aspartate (*nmda*) could be modulated though the intestinal tract. Moreover, high-fat diets also lead to changes in neuronal plasticity through modifying the microbiota modification (Farzi, Fröhlich and Holzer, 2018b). *nmda* and *bdnf* expression on the

brain can be decreased by HFD and also on stress condition (Vaughn *et al.*, 2017)(Rothman and Mattson, 2013)(Nam *et al.*, 2017).

Over the last decade, new therapeutic strategies as the use of other beneficial bacteria to address intestinal dysbiosis have been studied. Many bacteria have been used as probiotics in many diseases such as mood disorders and mental illnesses (Valladolid-Acebes *et al.*, 2012),(Vitetta, Bambling and Alford, 2014). Probiotic bacteria as a curative strategy has also been introduced in the area of cardiovascular disease (Genedi *et al.*, 2019). Furthermore it has been shown a connection between mental health and cardiovascular diseases (DiRienzo, 2014), for example, since 2014 the American Heart Association has reported that depression can accelerate atherosclerosis and promote the onset and severity of the coronary risk factors of diabetes, hypertension, and high levels of low density lipoprotein (LDL) (Zhang *et al.*, 2019). It has been demonstrated that *Lactobacillus reuteri* has the potential to intervene positively on the heart risk biomarkers (Lichtman *et al.*, 2014), it also has an effect on the *bdnf* RNA expression on the cortex in the brain (Tenorio-Jiménez *et al.*, 2018). Recently, a new bacteria *Lactobacillus mucosae* have been isolated from the intestine of pigs and decreased the serum cholesterol in apolipoprotein-E deficient mice maintained on a high-fat/cholesterol diet (Distrutti *et al.*, 2014). In addition, *Lactobacillus mucosae* seems to have the potential to improve host lipid and cholesterol metabolism through the modification of the bile pool composition (L. E. London *et al.*, 2014b) but, so far, there is no investigation about his effect on the brain. Other cardiovascular disease strategies such as statins are thought to significantly regulate the central nervous system (CNS), (Ryan *et al.*, 2019). Plant sterols is the collective term for free and esterified phytosterols and phytostanols, regardless of biological source have great potential to reduce the atherosclerosis burden by reducing inflammation, LDL cholesterol (LDL-C) and oxidative stress, respectively (Fracassi *et al.*, 2017). However, a study in phytosterolaemia investigated the effect of plant sterols in the brain showing their influence on cholesterol homeostasis but no phenotypic effect in memory or anxiety related behaviour in the hippocampus and cortex (Scolaro, Soo Jin Kim and de Castro, 2018). Instead, Beta glucans, a well-known polysaccharides was shown to influence on the brain, improving the hippocampal synaptic plasticity with its antioxidant properties (Vanmierlo *et al.*, 2011) while also possessing the capacity to interact with lipids and bile salts in the bowel and consequently reduce cholesterol levels in the body (D *et al.*, 2012).

Based on the data of the previous study where they analysed hyporcholesterolemic interventions (Beta glucans, plant sterols, L.mucosae, L. reuteri and statins) in relation to changes in the microbiota and the effect of these changes on the atherosclerotic markers (Sima, Vannucci and Vetvicka, 2018a), in the present study, we would like analyse the effect of these the hyporcholesterolemic treatments on the brain to increase the knowledge about the relationship between mood changes and cardiovascular diseases in a model of premature ageing and thus consolidate probiotic strategies that can intervene simultaneously in the two most studied districts in aged patients: the heart and the brain (Ryan *et al.*, 2017). To this end, we have investigated the six well-documented treatment strategies against cardiovascular diseases and their effects on the brain in particular in those areas involved in memory and depressive behaviours, such as the hippocampus and prefrontal cortex. We used plant sterol ester, oat β-glucan and atorvastatin and three probiotics, *Lactobacillus mucosae*, heat-killed *Lactobacillus mucosae* and *Lactobacillus reuteri* in the apo-$E^{-/-}$ murine model of atherosclerosis (Wu, Kwan and Tang, 1997).

4.2 MATERIAL AND METHODS

4.2.1 Animals and Diets

Four-week-old male ApoE$^{tm1Unc/J}$ mice were purchased from the Jackson Laboratory (JAX). Mice were housed under barrier-maintained conditions within the Biological Service Unit, University College Cork. Following two weeks of acclimatization, the mice were randomly divided into eight separate groups (A -B controls: n=7, C-H treatments: n=7-9) and maintained at 25°C with a 12:12 hour cycle and were fed ad libitum with an atherogenic diet: high fat/cholesterol 42% fat,1,25% cholesterol; (Teklad TD.96121, Harlan Laboratories). Water was available throughout the study at all times. Two groups as control: A) Standard lab chow (Normal) (2018S Teklad Global 18% Protein Rodent Diet) and B) High fat Diet as control (HFD). All other groups (C-H) received HFD diet alongside anti-hypercholesterolemic treatment for a 24-week period. There were six intervention groups: C) *Lactobacillus mucosae* DPC 6426 (HFD+L ~109 cells/mouse.day-1), D) the equivalent dose of heat-killed *Lactobacillus mucosae* DPC 6426 (HFD+LK), E) a bile salt hydrolase-active probiotic *Lactobacillus reuteri* APC 2587 (HFD+LR; ~109 CFU/day), F) plant sterol ester (HFD+P 3.4% w/w; Raisio), G) oat β-glucan (HFD+O; 7% w/w; OatWell) and H) atorvastatin (STAT; 1·5 mg/kg). More details on the flowchart experimental design (Figure 4.1). Use and care of animals was carried out in accordance with protocols approved by the Ethics Committee, University College Cork, under a license issued by the Department of Health and Children (Cruelty to Animal Act 1876, Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes). Following 24 weeks on experimental diets, animals were sacrificed using cervical dislocation. Brain tissue was rapidly hand-dissected at 4°C, stored in RNA Later (Ambion, Warrington, UK) for 24 hours followed by storage at −80°C until tissue processing.

4.2.2 Bacteria and culturing

L. mucosae strains used in this study - DPC 6426 - is of mammalian intestinal origin and was obtained from the Teagasc Moorepark Food Research Centre culture collection. Rifampicin-resistant variants of *L. mucosae* DPC 6426 were activated and propagated three times in de Man, Rogosa and Sharpe (MRS; 288110; Difco

Laboratories, Detroit, MI, USA) containing 5 µg/ml rifampicin (R3501; Sigma-Aldrich, Ireland) at 37°C anaerobically. Fermentation was undertaken in MRS containing 5% w/v sucrose (84100; Simga-Aldrich, Ireland) at 37°C anaerobically for 20 h. Following fermentation, the cultures for heat-killed *L. mucosae* group were heat-treated (121°C, 5 min) and EPS was washed thoroughly from cultures. Cultures were then washed twice in phosphate-buffered saline (PBS, D8537; Sigma-Aldrich, Ireland) and resuspended at a concentration of ~$2x10^{10}$ cells/ml in 15% (w/v) trehalose (10308653; Fisher Scientific Ireland Ltd). Aliquots were freeze-dried (VirTis AdVantageTM Freeze Dryer, SP Industries, NY, USA) as suggested by Wall et al. (Wall R, 2009). Viable cells in freeze-dried powders containing *L. mucosae* DPC 6426 were enumerated weekly for every batch of powder produced. This involved enumeration of *L. mucosae* DPC 6426 on MRS agar supplemented with rifampicin following anaerobic incubation for 72 hours at 37°C. Individual mice consumed ~$1x10^{9}$ cells of either heat-treated or live *L. mucosae* DPC 6426 per day. This was achieved by resuspending appropriate quantities of freeze-dried powder in the water that mice consumed *ad libitum*. Fresh faecal samples were taken from experimental mice of the lDPC group every fortnight and analysed for the presence of *L. mucosae* DPC 6426. Microbial analysis involved enumeration of the strain on MRS agar supplemented with rifampicin following anaerobic incubation for 72 hours at 37°C.

L. reuteri APC 2587 is a bile salt hydrolase-active strain of human origin and was obtained from the APC Microbiome Institute culture collection. A rifampicin-resistant derivative of *L. reuteri* was created and stored in 70% v/v glycerol, at -80 °C. From these stocks, bacteria were routinely cultured anaerobically at 37 °C on de Man Rugosa Sharp (MRS; Difco; 1·5% w/w agar) with the addition of 100 ug/ml w/v rifampicin (MRSr). From these plates, single colonies were inoculated in 5 ml MRSr broth and grown overnight under the same conditions. Bacteria were subcultured twice more prior to freeze-drying. Bacteria were cultured as above until stationary phase and centrifuged (16,900 x *g* for 15 min, at 4 °C; SLA-3000 rotor, Sorvall RC B5-Plus). The resulting cell pellet was washed twice with phosphate buffered saline (PBS; Sigma Aldrich). Cells were then resuspended at ~2 x 10^{10} CFU/ml in sterile 15% (w/v) trehalose (Sigma Aldrich) and 1 ml aliquots were dispensed into sterile 2 ml lyophilisation vials. The vials were lyophilised on a 24-hour program (freeze temperature -40 °C, additional freeze 1 min, condenser set point -60, vacuum set point

600 mTorr; VirTis AdVantage Wizard 2·0) and stored at 4 °C until use. Bacteria were resuspended in distilled water daily in order to deliver 10^9 CFU/day to each animal. Gastric transit of all strains was assessed weekly by culturing serial dilutions of fresh faecal samples on MRSr plates, as described previously.

4.2.3 Tissue extraction and RNA sequencing

The prefrontal cortex and hippocampus were the two regions where most of the analysis is carried out. The brains were removed from the skull and placed with the dorsal side up, on an ice-cooled Petri dish and dissected. For dissection, the coordinates of the brain regions were selected according to the ‘‘The Mouse Brain in Stereotaxic Coordinates, 3rd Edition’’ (Paxinos G, 2007). Total RNA was extracted from the hippocampus and prefrontal cortex using the mirVana™ miRNA Isolation kit (Ambion/life technologies, Waltham, MA, US), followed by DNase treatment using the TURBO DNA-free™ Kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) according to the manufacturer’s instructions. RNA concentration and quality were determined using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). RNA integrity number (RIN) was determined using the AgilentTM Bioanalyzer (Agilent, Stockport, UK). RNA samples that satisfied criteria (RIN value > 7) were reverse transcribed to cDNA using the High-Capacity cDNA kit (Applied Biosystem, Warrington, UK) according to manufacturer’s protocol.

4.2.4 Quantitative real-time PCR (qRT-PCR)

All qRT-PCR experiments were performed using specific TaqMan Gene Expression Assays (on the LightCycler® 480 System (Roche, Basel, Switzerland). PCR was run in triplicate. Data were analysed with the comparative cycle threshold (Ct) method. Expression levels were calculated as the averaged Ct values for each target gene were normalized to the endogenous housekeeping gene β-actin expression and transformed to the relative number of copies with $2-\Delta Ct$ equation (Livak KJ, 2001). We confirmed beforehand that the housekeeper *Actb* is neither changed by atherogenic diet (HFD) nor by treatments Then, relative gene expression levels in HFD control group was normalized to Normal control group and all six treatment groups were normalized to HFD control group.

Gene	Common gene name	Sequence (5' → 3') left	Sequence (5' → 3') right
Grin2a	Glutamate [nmda] receptor subunit epsilon-1	gcgtagaggatgccttggt	cctcccggccttgtagtt
Grin2b	NMDA2B (epsilon 2) (Grin2b)	aatacagagaacgaaacctcg act	aaagcgcattgctttcgtat
bdnf	Mus musculus brain derived neurotrophic factor (bdnf)	gtggtgtaagccgcaaaga	aaccatagtaaggaaaaggatggtc
Htr2c	5-hydroxytryptamine (serotonin) receptor 2C	atcatgtggtgcccgttt	ttacaggccttcccacaaag
Cyp46	Mus musculus cytochrome P450, family 46, subfamily a, polypeptide 1	aacaccctggcaaagttcat	agcagacggatgctctcg
TNF-a	Mus musculus tumor necrosis factor (TNF), transcript variant 2,	tcttctcattcctgcttgtgg	ggtctgggccatagaactga
IL-1b	Mus musculus interleukin 1 beta (Il1b)	tcttcctaaagtatgggctgg a	aaagggagctccttaacatgc
Ccl2	Mus musculus chemokine (C-C motif) ligand 2 (Ccl2)	gagccagacgggaggaag	agctggcttcagtgagagttg
Cldn-5	Mus musculus claudin 5 (Cldn5)	acgggaggagcgctttac	gttggcgaaccagcagag
Ocln	Mus musculus occludin (Ocln), transcript variant 1	gtccgtgaggccttttga	ggtgcataatgattgggtttg
Zo-1	Mus musculus tight junction protein 1 (Tjp1)	aaatcatccgactcctcgtc	cagttggctccaacaaggtaa

Table 4.1 List of TaqMan probes used in the study

4.2.5 Statistical analysis

Statistical analyses were conducted using SPSS 25 (IBM Corp., Armonk, NY, USA) and Graphpad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). All datasets were checked for the normality with the Shapiro-Wilk test and for equality of variances using the Levene's test. Normally distributed data were presented as mean ± SEM. T-test and Mann-Whitney U were performed to compared Normal diet to HFD. In the data relative treatments, the parametric data were analysed using the One-way analysis of variance (ANOVA) with post-hoc Dunnett and Non-parametric data were analysed with Kruskal-Wallis test followed by post-hoc Dunn's. Outliers were excluded using the Grubbs test. A p value <0.05 was deemed significant in all cases. The statistical significance was indicated as follows: * indicates p,0.05; ** indicates p,0.01 and *** indicates p,0.001.

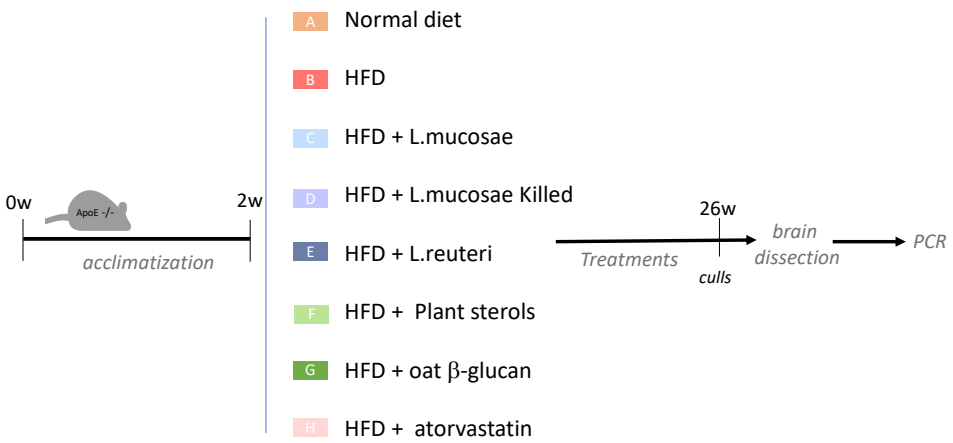


Figure 4.1 Flowchart experimental design: After two weeks of acclimatization, four-week-old male ApoE$^{tm1Unc/J}$ mice were randomly divided into eight separate groups (A -B controls: n=7, C-H treatments: n=7-9) fed with an atherogenic diet: high fat/cholesterol 42% fat,1,25% cholesterol; (Teklad TD.96121, Harlan Laboratories Two groups as control: A) Standard lab chow (Normal) (2018S Teklad Global 18% Protein Rodent Diet) and B) High fat Diet as control (HFD). All other groups (C-H) received HFD diet alongside anti-hypercholesterolemic treatment for a 24-week period. There were six intervention groups: C) *Lactobacillus mucosae* DPC 6426 (HFD+L ~109 cells/mouse.day-1), D) the equivalent dose of heat-killed *Lactobacillus mucosae* DPC 6426 (HFD+LK), E) a bile salt hydrolase-active probiotic *Lactobacillus reuteri* APC 2587 (HFD+LR; ~109 CFU/day), F) plant sterol ester (HFD+P 3.4% w/w; Raisio), G) oat β-glucan (HFD+O; 7% w/w; OatWell) and H) atorvastatin (STAT; 1·5 mg/kg). The animals were sacrificed, and the brains were dissected. From hippocampus and prefrontal cortex, RNA was extracted, and PCR analysis were performed.

4.3 RESULTS

4.3.1 Probiotics, plant sterol ester and oat β-glucan restore the neural plasticity in the PFC

The NR2A (Grin2A) and NR2B (Grin2B) subunits of the N-methyl-D-aspartate (nmda) receptor play a prominent role in the synaptic plasticity, were analysed.

In the PFC, HFD had a negative impact on both subunits, **(Figure 4.2 A and C**). The RNA expression of NR2B (Grin2B) was decreased (p= 0.001) of 65% in HFD compared to the normal diet .The expression of Grin2A subunit present also a redaction of 64% (p= 0.024) compared to the control ($F_{(6,46)}$ =103,5).

The impact of anti-hypercholesterolemic treatments compared to the HFD shows an ability to restore the values of Grin2A (**Figure 4.2 B**) ($F_{(6,46)}$=56.3). *L.reuteri* (increment of 104 %) and heat-killed *L. mucosae* (increment of 61%) had a strong capacity to increase the expression of Grin2A. Interestingly, a less intense but significant impact was observed in HFD+P (plant sterol ester) and HFD+O (oat β-glucan) diet (p= 0,011 with an increase of 54% and p=0,035 with an increase of 70%). However, the Grin2A expression was unchanged for HFD+L. mucosae and HFD+S (atorvastatin). Similarly, the mRNA expression of Grin2B showed in Figure 4.2 D, was increased for HFD+LK (67%), HFD+LR (39%) and HFD+O (56%) compared with the HFD group, but it was not significant for HFD+P (p= 0,23) compared to the control. In addition, in the hippocampus, the expression of *nmda* subunits did not change in the hippocampal area (**Figure 4.3 G, H, I, J**) . In the PFC and hippocampus, Brain-derived neurotrophic factor (*bdnf*) expression was also analysed. No significant changed was noted in RNA expression of *bdnf* in the hippocampus. No difference was noted between HFD and normal diet (**Figure 4.3 K**). Interestingly, in the PFC **(Figure 4.2 E**), *bdnf* RNA expression was significantly lower (redaction of 56%) in HFD group then in the normal group (p=0.002) but no significant alterations in *bdnf* expression was as a result of anti-hypercholesterolemic treatments were observed in either hippocampus **(Figure 4.3 L**) or PFC (**Figure 4.2 L**).

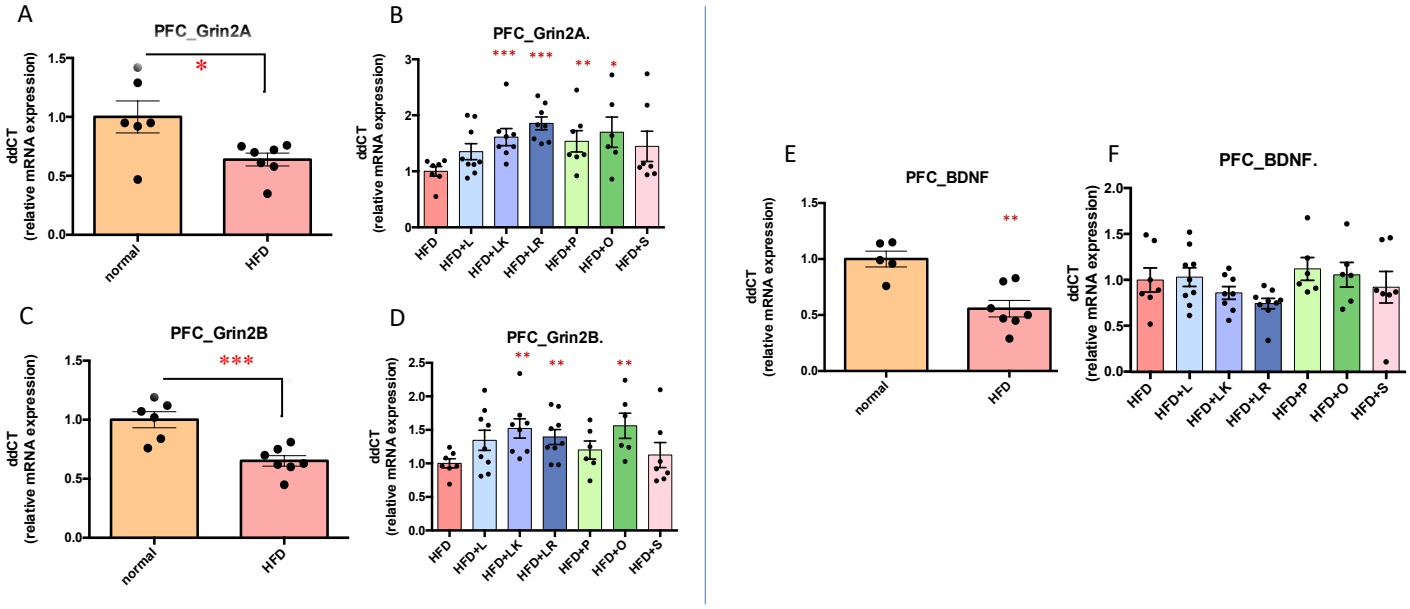


Figure 4.2 **Effect of probiotics, Oat and Plant sterols on neuron plasticity. mRNA expression in PFC.** the RNA expression of the Grin2A and Grin2B (A and C) was reduced by the HFD in the PFC compared to the normal diet. (E) The RNA expression of *bdnf* decreased by HFD compared to the normal diet. (B) Grin2A mRNA expression was increased by heat-killed *L.mucosae* and *L.reuteri,* followed by plant sterol ester and oat β-glucan compared to HFD control. (D) Grin2B mRNA expression was repristinated from the negative effect of HFD by heat-killed *L.mucosae*, *L.reuteri* and oat β-glucan. (F) *bdnf* mRNA expression was unaffected by anti-hypercholesterolemic treatments compared to HFD control. *Lb.mucosae* (HFD+L), heat-killed *Lb. mucosae* (HFD+LK), *Lactobacillus reuteri* (HFD+LR), plant sterol ester (HFD+P), oat β-glucan (HFD+O), atorvastatin (HFD+S). * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) represent significant differences when compared against HFC in one-way ANOVA. Post-Hoc Dunnett. Plots depict individual replicates with mean and SEM.

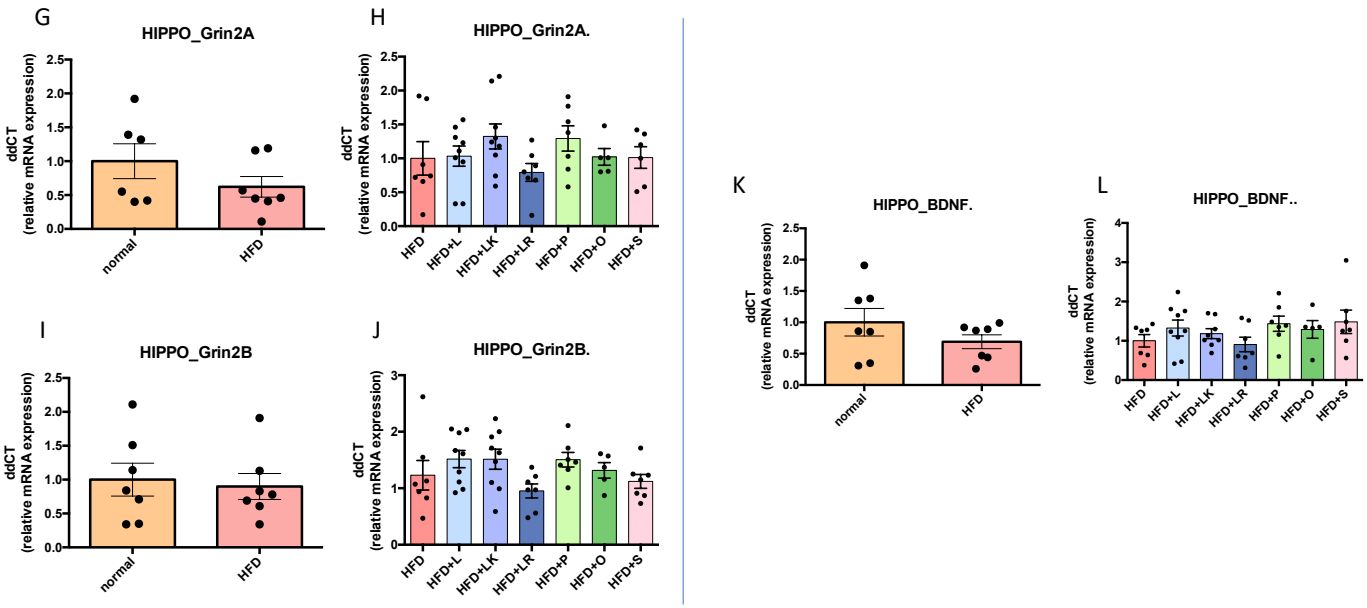


Figure 4.3 **No Effect of probiotics and prebiotics on neuron plasticity. mRNA expression in Hippocampus.** there was no effected in Grin2A mRNA expression (G and I) by the HFD compared to the normal diet. (H and J); Also, it was no change of Grin2A and Grin2B mRNA expression by anti-hypercholesterolemic treatments compared to HFD control. (K) bdnf mRNA expression didn't change in HFD compared to normal diet and (L) anti-hypercholesterolemic treatments have not effect on the HFD. Lb.mucosae (HFD+L), heat-killed *Lb. mucosae* (HFD+LK), *Lactobacillus reuteri* (HFD+LR), plant sterol ester (HFD+P), oat β-glucan (HFD+O), atorvastatin (HFD+S). * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) represent significant differences when compared against HFC in one-way ANOVA. Post-Hoc Dunnett. Plots depict individual replicates with mean and SEM.

4.3.2 L. mucosae influences the RNA expression of Tight junctions in hippocampus

Along with vascular pathology, neuronal dysfunction has been associated with psychiatric illnesses through impaired blood brain barrier function (Shalev H, 2009), (Yonatan Serlin, 2011), (Frey, 2015). In this study we analysed the mRNA expression of three genes part which form part of the blood brain barrier, namely Claudin5, Zo1 (Zonnula occludens-1) and Occludin (**Figure 4.4)**. No difference emerged between HFD and normal diet in all 3 genes (Figure 4.4A, C, E, G). Moreover, all diets with treatments did not show any alteration in mRNA expression compared to the control (**Figure 4.4 D, F**). However, an increase of Claudin5 mRNA expression induced by *L.mucosae* was observed (p=0.018) compared to the HFD control ($f_{(6,43)}$= 4.43) with an increment of 85% (**Figure 4.4B**).

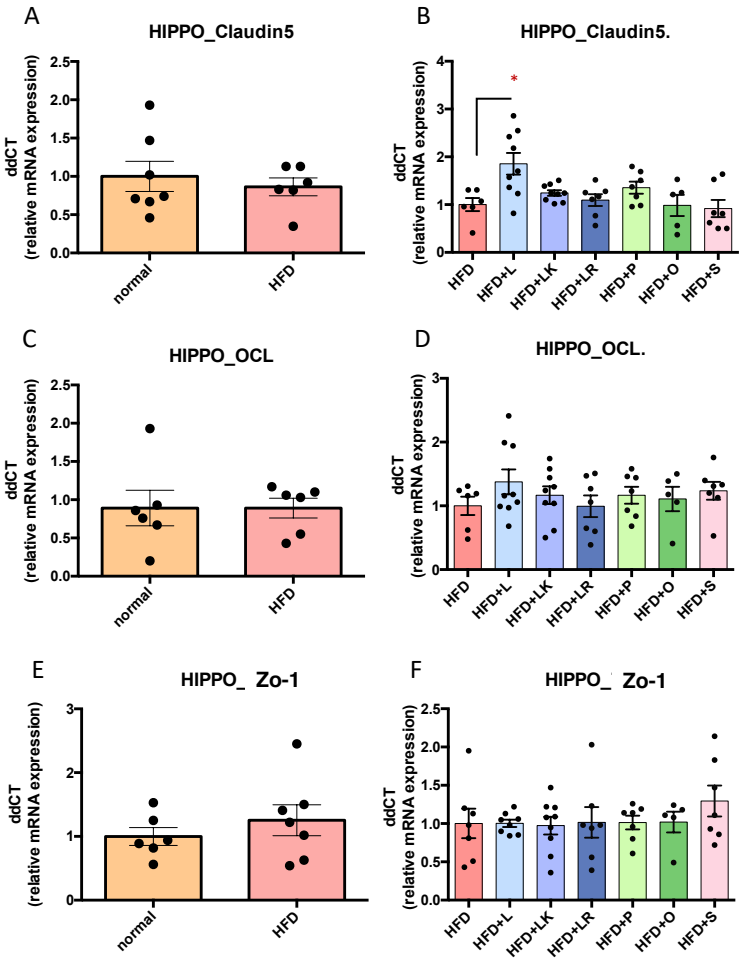


Figure 4.4 **Effect of anti-hypercholesterolemic treatment diets on the host tight junctions mRNA expression in Hippocampus.** The RNA expression of the tight junctions was unaffected by the HFD in the hippocampus (A, C and E) compared to the Normal control group. Only the dietary intervention HFD+L increase 5-HT2C gene expression in PFC compared to HFD (B). HFD added with anti-hypercholesterolemic treatments did not change the expression of Occludin (D) and Zo-1 (F) compared to the HFD control in the hippocampus. * $p < 0.05$. Statistical analysis: data are presented as mean + SEM; n=6 for HFD and n=7 for Normal, n=9 for HFD+L and HFD+LK, n=7 for HFD+P, HFD+LR and HFD+S, n=5 for HFD+O. In Normal vs HFD (A, C and E), one sample T test was used on parametric data and Mannwhitney test on non-parametric data. In HFD vs HFD added with anti-hypercholesterolemic treatments (B, D and F), was used one-way ANOVA with Dunnet's *post hoc* comparisons on parametric and Krusckal wallis with Mann-whineu U test on non-parametric data. HFD+L= High Fat Diet added with *L.mucosae*, HFD+LK = High Fat Diet added with heat-killed *L. mucosae,* HFD+LR= High Fat Diet added with *Lactobacillus reuteri*, HFD+P = High Fat Diet added

with plant sterol ester, HFD+O = High Fat Diet added with oat β-glucan, HFD+S = High Fat Diet added with atorvastatin.

4.3.3 Anti-hypercholesterolemic treatments don't change the Serotonin 2c receptor in PFC and Hippocampus.

To determine central serotonergic receptor expression in relation to cardiovascular disease, hippocampal receptor mRNA expression was analysed using quantitative real-time PCR together with mRNA levels in the PFC. No differential expression of the 5-HT_{2C} receptor was observed between Normal diet and HFD control groups in the two regions assessed (**Figure 4.5A, C**). However, in the prefrontal cortex, no difference of 5-HT_{2C} expression was observed in all diet groups alongside anti-hypercholesterolemic treatment groups compared to HFD control ($F_{(6,43)}$= 0.94 ,p=0,115) (**Figure 4.5B**). Coherently, the data showed no difference between HFD and anti-hypercholesterolemic treatment diets in the hippocampus (**Figure 4.5D**).

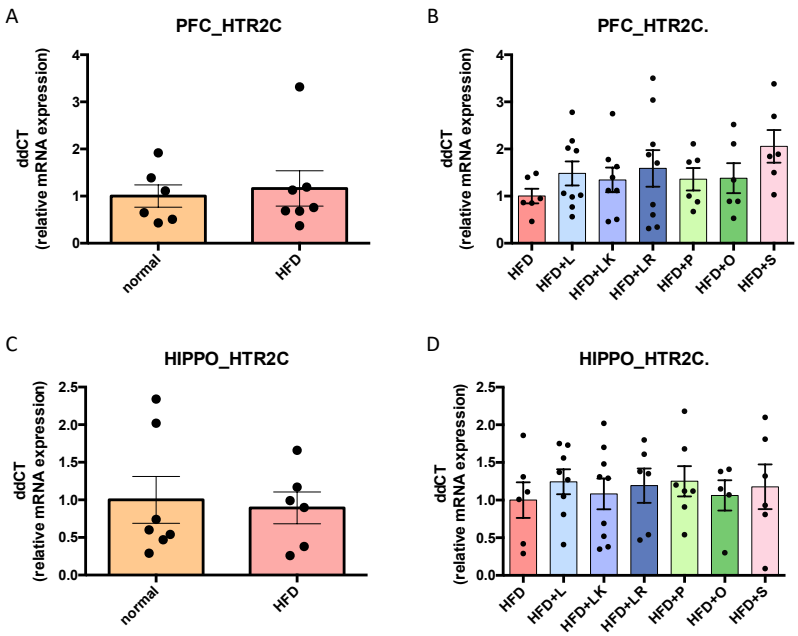


Figure 4.5 **the 5-HT2C receptor was unaffected by HDF in PFC and Hippocampus.** (A) the 5HT2C expression in PFC was similar in the HFD and the normal control. (B) In PFC, HFD added with anti-hypercholesterolemic treatments doesn't present any difference compared to the HFD control. (C) Hippocampus was not affected by HFD compared to Normal diet, (D) neither HFD added with anti-hypercholesterolemic treatments compared to the HFD control. Statistical analysis: data are presented as mean + SEM; n=6 for HFD and Normal, n=9 for HFD+L and HFD+LR, n=8 for HFD+LK, n=6 in

either group for the rest. In Normal vs HFD (A and C), one sample t-test was used on parametric data and Mann–Whitney test on non parametric data. In HFD vs HFD added with anti-hypercholesterolemic treatments (B and D), was used one-way ANOVA with Dunnet's *post hoc* comparisons on parametric and Krusckal wallis with Mann–Whitney U test on non parametric data.

4.4 DISCUSSION

The efficacy of anti-cholesterol treatments, including plant sterol, beta glucans, statins and probiotics, used to improve the conditions of patients with cardiovascular and coronary disease has been consistently reviewed in pharmacological and medical studies (Kolovou *et al.*, 2008). A mutant animal model was study to investigate on cardiovascular disease. Apo-$E^{-/-}$ is one of the most commonly used murine model of atherosclerosis (Wu, Kwan and Tang, 1997). Lesions develop in apoE-deficient mice on a regular chow diet and will occur faster on a diet higher in cholesterol (Lo Sasso *et al.*, 2016). In fact, Apo-E deficient mice are able to develop hypercholesterolemia and aorta fibrous plaques after 3 month of normal diet (Tamminen *et al.*, 1999) and more, in 8 weeks if the animal are exposed to cholesterol diet presenting inflammatory resemble human lesions in their sites of progression to the fibroproliferative stage (Lo Sasso *et al.*, 2016). The animal protocol was followed carefully as described in the previous experiment (Ryan *et al.*, 2017).

This study demonstrates the effect of these treatments on the brain of ApoE deficient mice and in particular the ability to restore the expression of the N-methyl-D-aspartate (nmda) receptor in the PFC in ApoE deficient mice fed with HFD compared (**Figure 4.2 B and D**). Moreover, the capacity of *Lactobacillus mucosae* to increase the hippocampal RNA expression of claudin 5 has been shown (**Figure 4.4B**). However, no changes were found in the serotonin receptor RNA expression. All the changes were highlighted in the prefrontal cortex.

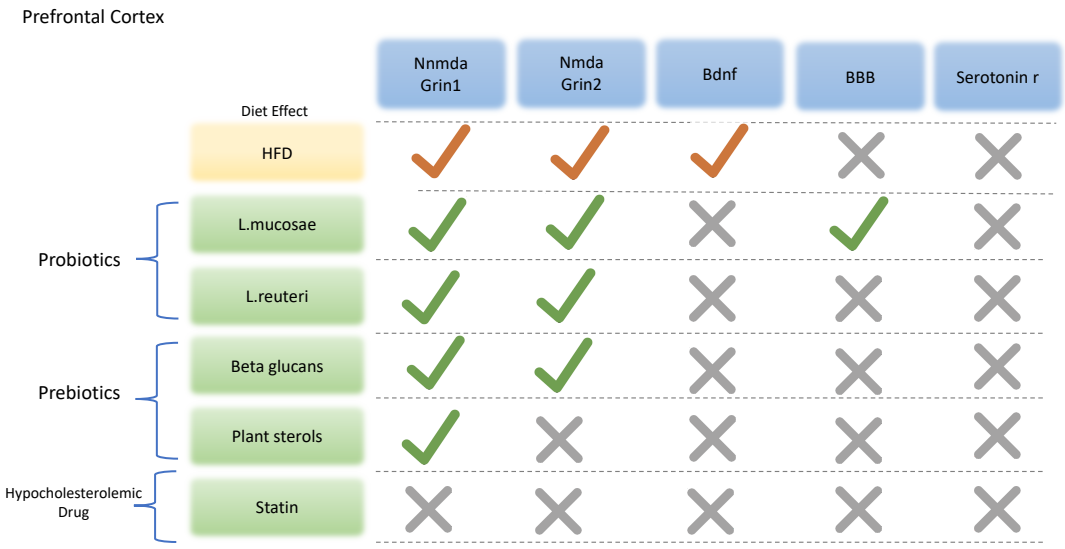


Figure 4.6 Summary of findings: The effect of a HFD and the effect of microbiota treatment intervention on the prefrontal cortex. The HFD changed the neuronal plasticity markers (Grin1, grin2 and Bdnf), but had no effect on the blood brain barrier (BBB) or in the serotonin receptors. *L. mucosae* and *L.reuteri* (prebiotics) had an effect on the *Grin1* and *Grin2* but not on the *Bdnf* . *L.mucosae* had an influence on the BBB (*claudin5*). Prebiotics such as beta glucans and plant sterols had an effect only on the *Grin1*, except beta glucans that showed an effect on *Grin2*. Statins did not have an effect on the neuronal markers, BBB or on the serotonin receptors.

4.4.1 Anti-cholesterol treatments affect on neuronal plasticity

The ability of the brain to change its structure or function in response to internal and external goals is named synaptic plasticity (Avior *et al.*, 2013). In a mature nervous system, the key regulators of neuronal plasticity, long-term memories but also neuronal survival are *Bdnf* and *nmda* (Zimerman and Hummel, 2014).

In the current study, *Bdnf* and *nmda* are affected by the HFD in the prefrontal cortex. Following 24 weeks of hypercaloric diet, the gene expression of both subunits of the N-methyl-D-aspartate (*nmda*) are reduced **(Figure 4.2A and C**). Similarly, the expression of *bdnf* is also reduced in these animals **(Figure 4.2E**). These results show how the diet can be considered as important factor influencing cognition and neuronal function. This is in agreement with (Mu, Tavella and Luo, 2018; Sima, Vannucci and Vetvicka, 2018b; Trautwein *et al.*, 2018) who showed the reduction in *nmda* mRNA levels in high fat diet fed PFC, leading to a compromise of synaptic function. In addition, as has previously been shown in other rodent studies associated with cognitive decline, the reduced levels of *bdnf* in prefrontal cortex and hippocampus can

be caused by high energy diet (Yoon *et al.*, 2019). Indeed, HFD has been linked to cognitive impairment, anxiety and depressive symptoms but also to alteration of behaviour as deficiencies in short and mid-term spatial memories and increases of anxious and depressive- like behaviour (Park *et al.*, 2010; Pistell *et al.*, 2010; Yamada-Goto *et al.*, 2012) (Vinuesa *et al.*, 2016).

However, in the current study, this decline is evident only in the PFC. Unexpectedly no changes in RNA expression were observed in the hippocampus. The reasons for which may include, firstly, the role of the PFC and the mice age.

The diet is an epigenetic factor, that can lead to changes in brain, in particular it was demonstrated that HFD is able to influence the protein expressions involved in impulsive behaviour and synaptic plasticity (Takase *et al.*, 2016). The prefrontal cortex is a brain region that appears particularly sensitive to experience-dependent plasticity throughout life, compared with the hippocampus (Hansen *et al.*, 2018) and this may explain our positive result observed in the PFC but not in the hippocampus. Other studies showed a similar disagreement in the bdnf expression changes of these two brain areas (Basso *et al.*, 2015) and it was explained thought a compensatory mechanism that may leads divergent changes between PFC and hippocampus.

Physiologically, in both the young and adult brain, the PFC and the hippocampus work together, for example to support short and long-term memory processes. However, these two brain areas could show distinct functions in aged animals which may contribute to neural mechanisms related to working memory decline. A recent study in elderly rats demonstrated that this decline in working memory is mediated by the PFC and not by the hippocampus (Mancino *et al.*, 2015). The hippocampus and the prefrontal cortex show distinct patterns of dysfunction in advanced age for example within a subunit of hippocampus there is hyperactivity (McQuail, Frazier and Bizon, 2015), at the contrary the PFC increase the inhibitory tone in electrophysiological data (Yassa *et al.*, 2010). Another study reports a similar pattern caused by a ketogenic diet on aged rats (Carpenter *et al.*, 2016). In the current study the mice were sacrificed at the 27 th week, indicating that at this stage, mice may already present the senescent decline.

Perhaps the most interesting result was the capacity of anti-cholesterol treatments to restore the negative effect of the HFD on the *nmda* RNA expression in the PFC (**Figure 4.2 B and D**). Prebiotics, such as plant sterol ester and beta glucans and probiotics (*L.reuteri,* heat-killed *L.mucosae*) significantly increased the level of the

Nr2a subunit (Plant sterol P= 0,011; b-glucans P= 0,035; l. Reuteri P= 0,001; HK L.mucosae P= 0,001), while live *L.mucosae* and Statins did not effect *Nr2a* expression. A similar result was observed in Nr2b expression where only *L.reuteri,* heat-killed *L.mucosae* and Oat beta glucans showed a change **(Figure 4.2 D**). However, in a previous study (Hernandez *et al.*, 2018) plant sterol ester and beta glucans allowed the regression of atherosclerotic plaque in the aorta artery, and no effect of probiotics was observed.

Prebiotics as plant sterol esters have a positive impact on the body. They are able to stabilize the plasma cholesterol which plays a crucial role in the functioning of immune cells and increases the healthy gut microbial communities (Ryan *et al.*, 2017). Different from cholesterol, they can also cross the blood brain barrier (Cuevas-Tena *et al.*, 2018; Plat *et al.*, 2019) and accumulate in lipid rafts on the cell membranes (Vanmierlo *et al.*, 2012b). They may have the capacity to modify the anchored receptors on the cell surface. Indeed, in this study, plant sterol ester has effect on Nr2a subunit but no effect on the Nr2b subunit of nmda receptors. Interestingly the effect in the serotonin receptors was not found (**Figure 4.5**). This indicates the specificity of the plant sterol effect on the Nr2a subunit in the prefrontal cortex.

The beta glucans, unlike the plant sterol ester, changed the RNA expression in both nmda subunits. Beta glucans are an excellent antioxidant with a strong action were shown previously to have an important anti-inflammatory and immunomodulate effect. They modulate the populations of the beneficial gut bacteria and also have the capacity to decrease aortic plaque by reducing inflammation (Burg *et al.*, 2013) (Ryan et al. 2017) in apoE deficient mice. The reduction of inflammation can be connected with the restoration of the nmda as its activity was linked with neuroinflammation in rats (Sil *at al.*, 2016).

Of further interest was the strong effect of two probiotics on both nmda subunits. *Lactobacillus reuteri* is known to reduce level of lipids in the blood and lower triglycerides production in the liver (Sil, Ghosh and Ghosh, 2016). It also has an anti-inflammatory effect, reducing the production of pro-inflammatory cytokines while promoting T regulatory cell development. Considering that nmda receptors have the tendency to react in inflammatory conditions (DiRienzo, 2014) and cardiovascular disease are a consequence of chronic inflammation (Steullet *et al.*, 2016), the restoration of the levels of the two subunits Nr2a and Nr2b may be the consequence of the anti-inflammatory effect of *L.reuteri* in the intestine. However, a previous study

(Pashkow, 2011) did not show the reduction of the plaque size in the aorta but a modification of microbiota population was observed. In addition, *L.reuteri* has the potential to be a biomarker of a healthy gut status (Ryan *et al.*, 2017).

L.mucosae has been shown to attenuate the gross dyslipidaemia and hypercholesterolemia observed in APO-E deficient mice maintained on a high-fat/cholesterol diet in particular, the level of cholesterol was decreased of 33-50% in serum and 30% in liver (L. London *et al.*, 2014). Strangely, in the current study no effect of live *L.mucosae* on the brain was observed. The heat-killed *L.mucosae* increased *nmda* subunit expression. The heat-killed intervention was intended to deprive the bacteria from exopolysaccharides and their metabolic action. Nevertheless, it appears that these metabolically inactive bacteria mainly confer a T-reg- promoting immunomodulation to the host (L. E. London *et al.*, 2014b). So in this work, heat-killed *L. mucosae* clearly acted only through immune stimulation without interfering with other microbial populations. This is in accordance with a previous study from Ryan *et al.* who observed that live *L. mucosae* changes the microbiota composition and decreases the circulating cholesterol, and that heat-killed *L. mucosae* modifies only the inflammatory markers such as c-reactive protein (CRP), (Martoni *et al.*, 2015).

In this research, the effect of atorvastatin on the brain was not observed, statins, which are well known to reduce the risk of cardiovascular complications, has been proposed as novel agents that may be useful in Alzheimer's disease therapy due to its antioxidant effect on the cortex (Ryan *et al.*, 2019). In another study, it was reported that the protective effect of statins in *nmda* receptor anchored on the lipid rafts but only in the subunit 1(Barone *et al.*, 2011). Future work in this area should look at the effect of statins on the other subunits.

4.4.2 L.mucosae influences the RNA expression of tight junctions in hippocampus

We also investigated the RNA expression of the three more important tight junction proteins, components of the blood brain barrier. *L.mucosae* has the capacity to increase expression of Claudin 5. By limiting the passage of molecules between blood and brain, tight junction proteins guarantee a protective barrier that prevents the passage of unwanted molecules. Claudin-5 is expressed in many tissues with the highest expression observed in the brain especially in the hippocampus. Claudin 5 together

with Occludin protein appears to have a close relationship with nmda receptors. In rodents, the regulation of Claudin 5 and Occludin proteins can occur through the release of glutamate by neurons and astrocytes binding nmda receptors (Ponce *et al.*, 2008). However, in our study, only Claudin 5 was increased. This may be due to an inflammatory state that characterizes these animals subjected to HFD. In fact, Claudin 5 appears to be most sensitive to an inflammatory state as its development is linked to CD146 belonging to the superfamily immunoglobulin (Greene, Hanley and Campbell, 2019). It is interesting to note that only *L.mucosae* improved the expression of the tight junction protein while other treatments also appear to act on inflammation. Other studies are needed to fully understand the action mechanism involved.

4.4.3 Anti-hypercholesterolemic treatments does not change the Serotonin 2c receptor in PFC and Hippocampus.

The relation between depression and cardiovascular disease it is known. At least a quarter of cardiac patients suffer with depression, and adults with depression often develop heart disease (Khandaker *et al.*, 2019). In this thesis, we analysed the serotonin receptors (Htr2c) to investigate the involvement of depression in ApoE deficient mice (Hu *et al.*, 2022) and the possible effect of microbiota treatments.

No changes in gene expression of the receptor Htr2c were seen in the prefrontal cortex or in the hippocampus. The serotonin receptors, in particularly, Htr2c, plays a crucial role in psychiatric disorders such as schizophrenia, depression and anxiety in these brain regions, (J. Chen *et al.*, 2017). While, it was previously demonstrated that HFD might induced depression and anxiety behaviour, the hypercholesterolemic diets had no effect on the mice in this study (**Figure 4.5 A, C).** These data are in agreement with a previous report that showed no changes in hippocampal gene expression of serotonin receptors in rats fed with high fat diets despite the fact that they showed a depressant-like behaviour (Chagraoui *et al.*, 2016).

In the hippocampus, the Htr2c receptor appears to be particularly sensitive to corticosterone levels in the blood especially under chronic stress conditions (Dornellas *et al.*, 2018). This mechanism, together with our results, allow us to hypothesize that the link between depression and cardiovascular disease is not correlated through a stress factor but through an inflammatory cascade as suggested previously (Holmes, French and Seckl, 1997).

In the brain, no changes related to hypocholesterolemic strategies were found. Probiotics did not show any effect on the serotonergic receptor and these results are similar to those reported in a study looking at a cafeteria diet where the 5htr2c and 5htr2a receptors do not change under probiotic administration (Khandaker *et al.*, 2019).

These results, unexpectedly, underscore the fact that serotonin receptors are not easy to manipulate. This may be a protective mechanism of the body for survival as high or low values of these receptors can lead to psychiatric illnesses. New studies would be needed to investigate this link by integrating inflammation analysis and behavioural tests.

4.5 Conclusion

These results enrich the knowledge of the therapeutic effect of hypocholesterolizing treatments used against coronary heart disease on the brain, an area not highly regarded in cardiological studies.

L. reutieri and *L. mucosae* together with plant sterol ester and β-glucans have also shown a positive effect in neuronal plasticity so they may be of interest for a combined therapeutic strategy.

However, intriguing correlations between cardiovascular disease and mental illness must still be clarified. (Beilharz *et al.*, 2018): The most important reason depression increases the risk for, or worsens outcomes in, cardiovascular disease, is its effects on lifestyle. Diet and stress seem to have an important influence on these diseases and also, at the same time on microbiota composition.

The increasingly crucial role of microbiota in heart diseases opens up an important opportunity for therapy. In this way, other part of the body, including the brain, can be reached from the intestine care.

As has been shown in this study, therapeutic diets using probiotics (L.reuteri and L.mucosae) and prebiotics (plant sterol and b glucans) may have important therapeutic consequences on three distinct regions: the gut, the brain, and the heart. Disease manifestation in one part of the body may cause an unbalance in other organs. More studies should be done to increasing the all-round knowledge.

Chapter 5

General Discussion

In this thesis, we have explored the action of microbiota-targeted treatments in young, middle age and aged animals as potential resolutions to slowdown the progress of natural body and brain decline through the gut-brain axis.

Through the manipulation of the microbiota by use of probiotics and prebiotics, we show that ageing can be ameliorate by restoring neuronal plasticity in the brain (Chapter 4) and improve the intestinal permeability (Chapter 2). These findings cumulatively show that diet intervention with prebiotic and probiotic can modulate the microbiota-gut-brain axis (Dinan, *et al.* 2013, Burokas *et al.*, 2017) in elderly.

In the first instance (Chapter 2), two polyphenol rich diets (Grape pomace and Rafuma) administered to aged (20-month-old) C57BL/6 mice, show a notable effect on the intestinal barrier (Figure 2.4). The two polyphenol interventions are able to reverse the damage of the intestinal barrier duo to ageing. The effect is evident in the ileum and more in colon through analysis with FITC-D (Figure 2.4) E and F and the increase of tight junction protein RNA expression (Occludin and claudin 3) (Figure S 2.1 C and D).The reduction of permeability is generally associated with a reduction of systemic inflammation (Kelly *et al.*, 2015) but interesting, Rufuma and Grape Pomace do not have a strong effect on the impaired immune system of the elderly (Figure 2.6) only a significant increase in the number of dendritic cells in aged mice emerged (Figure S 2.2 D) for both diet and Rafuma is also able to reduce the level of TNF-α in the blood (Figure S B). Moreover, the two polyphenols diet did not reverse some of the behavioural deficits associated with ageing (Figure 2.2**)**.
On the other hand, the effect of two polyphenols do not strongly interfere on the microbiota composition, where they do not reverse age-related changes in alpha (A) or beta (B) diversity of gastrointestinal microbiota (Figure 2.9). These data show that it is not so easy to interfere with the microbiota and consequently with the chronic physiologic inflammation that characterizes this last stage of life (inflammageing) (Franceschi and Campisi, 2014). In fact, our inflammatory marker results show a substantial increase of inflammation in hippocampus, in intestine and in blood of old animals (Figure 2.8 and Figure 2.7).
Having shown that prebiotics can have an effect on ageing and intrigued by the mechanism of interaction with the body, we decided to look at whether one of more powerful prebiotic largely used in the last decade, inulin (Watzl, Girrbach and Roller,

2005; Dehghan *et al.*, 2014; Hoving *et al.*, 2018; Zhang *et al.*, 2018). We focused on body metabolism in the middle-age mice (Chapter 3), which is considered as the first step of ageing (Barzilai *et al.*, 2012). Interesting, we found that inulin has metabolic effects only in young mice. Middle-age mice did not show any consequence of inulin treatment in the metabolism or in the immune system, showing that it is not so easy to interfere with the inevitable metabolic decline or with Immunosenescence (Franceschi and Campisi, 2014). However, Inulin had a great impact in young animals and stimulating the lipid and glucose metabolism in the liver increasing the level of SIRT1 without impacting transcriptional factor changes **(** Figure 3.22). Inulin shows also to be able to interact with the hypothalamus-pituitary-adrenal-axis by decreased level of the glucocorticoids (Nr3c1) and corticotrophin-releasing hormone (Crhr1) receptors in the amygdala of young mice. These findings reveal that inulin has a different metabolic effect from young to middle age.

The investigation of prebiotic effect continue in Chapter 4, the effect of two different strains of lactobacillus (L.reuteri and L.mucosae) and two prebiotics derived from plant (plant sterol ester and oat β-glucan) show to be able to restore the neural plasticity on the PFC (Figure 4.2) by increasing the level of nmda rna expression in a mouse model of ageing (ApoE deficient mice). More, L. mucosae that previously demonstrated to have cardio-protective properties (Ryan *et al.*, 2019), increase the level of an important BBB protein such as claudin 5 in hippocampus (Figure 4.4B).

Taken together these data suggest that the microbiota- brain axis as a therapeutic target for metabolic and immune disease react in different way in elderly compared to youth and middle age mice (Figure 5.0.1). These results provide novel insight into microbiota-targeted interventions on age-related alterations in physiology and brain function and suggest that the efficacy of such interventions is at least partly determined

by age.

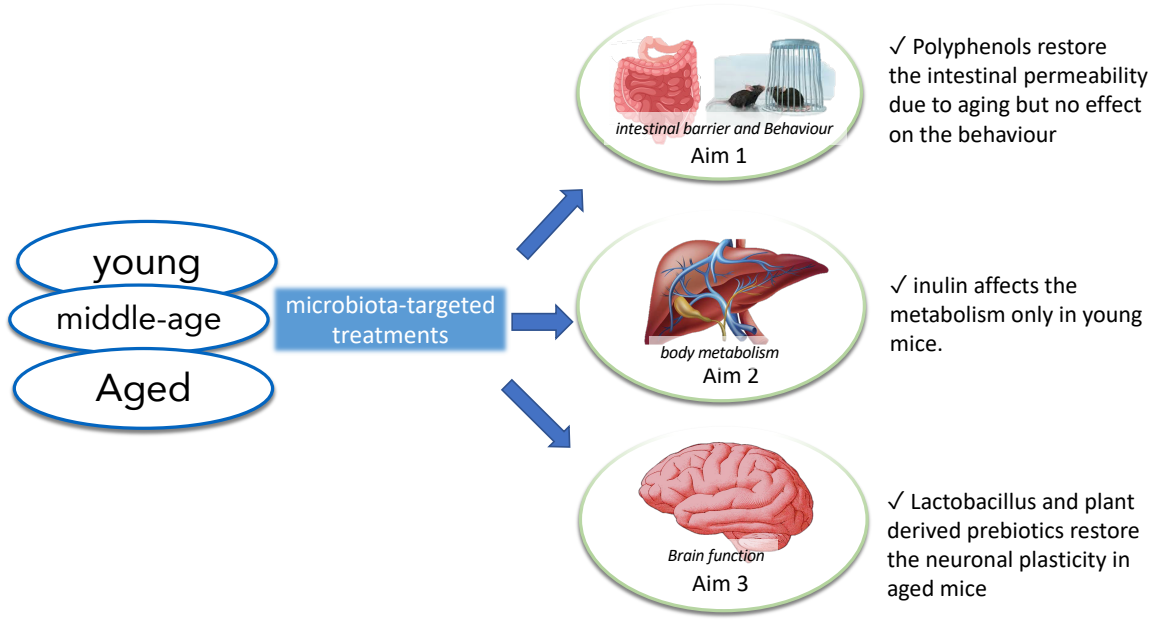


Figure 5.0.1 Summary of the findings of this thesis.

5.1 Interplay between microbiota target treatment and intestinal microbiota: Age-related complications

In this thesis, we show that the polyphenols, inulin, lactobacillus and plant derived prebiotics positively influence on the gut-brain axis. The neuron plasticity of the brain, the general inflammation, the intestinal permeability and the metabolism can be ameliorated by specifics enriched diet. Nevertheless, the microbiota target treatments do not act in the same way across all age groups. This thesis highlights that the effectiveness of these treatments differs in young, middle-aged and elderly individuals. The emerging understanding of the differences in microbiota composition during the life cycle in conjunction with physiological modification could guide future clinical practice and more specific microbiota interventions development, especially for those in the older age bracket. Some of the new avenues for translational applications include implementing knowledge on the physiology of aging, improving prebiotics efficacy, identifying side effects and focusing the interventions on the key points of the organism that can be ameliorated. The implications of our results are discussed in the next section (Figure 5.0.2).

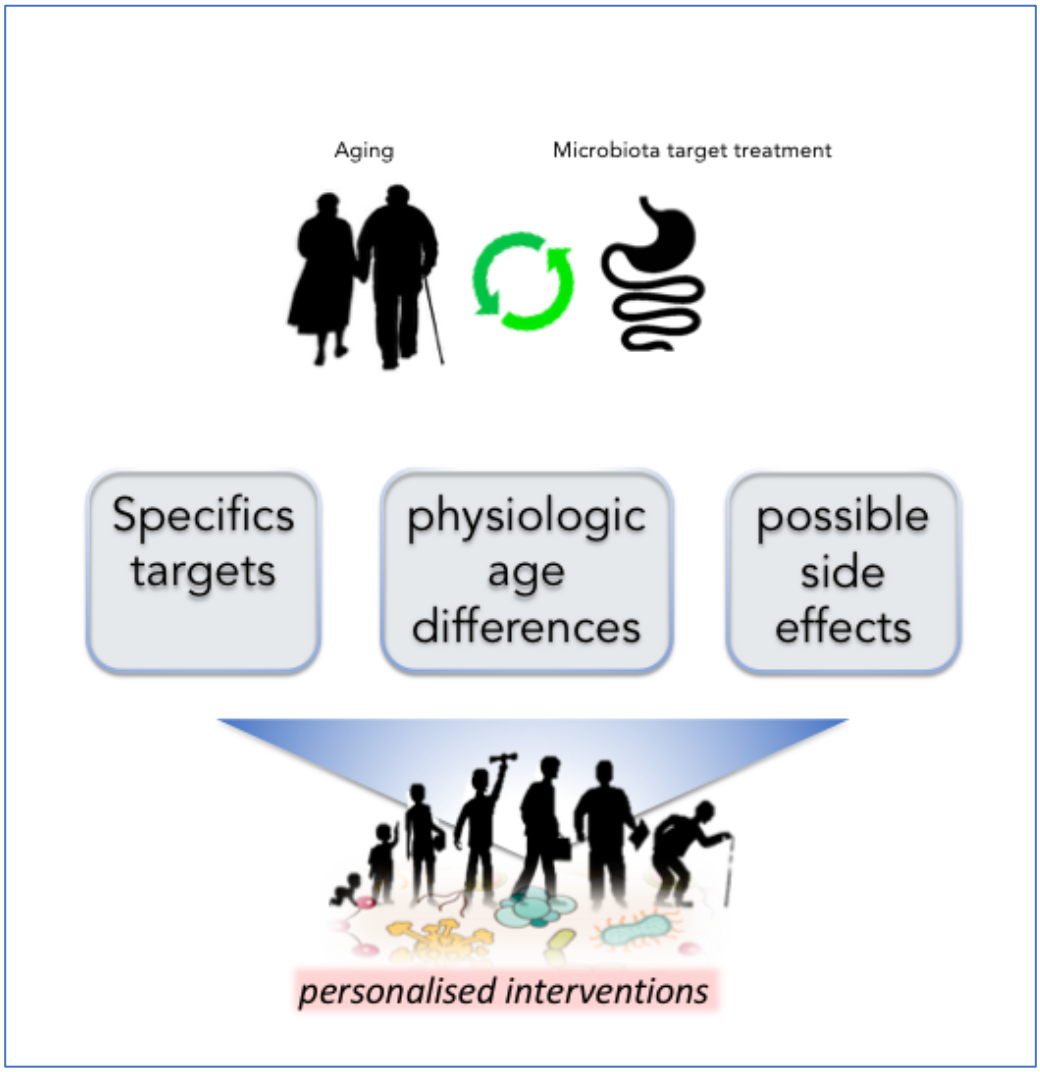


Figure 5.0.2 Implications of relative aging and microbiota interventions.

5.1.1 The gap generation: different microbiota treatments for different age groups

Age is an important factor when considering changes in the trajectory of health. The patient's age can affect the course and progression of a disease (Hasenclever and Diehl, 1998) and influence the optimal treatment strategy. The efficiency of medical treatments depends on the reactivity of the patients that it is different from an individual to another for all the intrinsic and extrinsic factors that we have already described previously. Available evidence suggests that there is an important gap between the physiological responsiveness of the younger and the old patients (Vecht, 1993). Currently, the use of age information in medicine is somewhat simplistic and coarse (Geifman, Cohen and Rubin, 2013) and this is even more evident in the

emerging field of microbiota gut-brain axis research. As we discussed earlier, changes the microbiota community and loss of their relative abundance of microbiota in elderly, can negatively impact health, with further economic and social consequences (Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, 2012).

Chronic inflammation is associated with aging process and as we noted, beneficial commensal microbes are reduced and pro-inflammatory commensal microbes are enriched in older people compared to young and middle-aged adults (Bodogai *et al.*, 2018). Our data demonstrates that microbiome modulation may contribute to the deceleration certain aspect of aging process. We found that polyphenols (i.e. grape pomace and Rafuma) reduced gut and the intestinal inflammation but interesting, not on systemic inflammation. This result it is not congruent with other study where the reduction of systemic inflammation with a similar polyphenols treatment was observed, but in different age bracket (3 mo old mice) (Hogan *et al.*, 2010). The explanation can be that young animals may be more receptive to polyphenols then aged animals. More, in our study, polyphenols did not have impact on cognitive processes. Behavior tests and brain analysis did not show any different on mice treated with polyphenols, although previous studies showed to be able to modulate synaptic plasticity and influence positively the behavior of middle age animals (16 mo) (Bensalem *et al.*, 2018; Tikhonova *et al.*, 2020). It is well known that the therapeutic effects of drugs can be different across different age groups. For example, the effects of fluoxetine in young mice were mediated by neurogenesis, but not in elderly mice (Couillard-Despres *et al.*, 2009). Our data confirmed that inulin does not act in the same way in middle-aged mice and young mice, proving once again that the young are more reactive than older adults. In addition, side effects, such as increasing inflammatory markers expression, only emerged in young animal (Figure 3.10).

Further studies are needed to evaluate precise interaction between the effects of microbiota treatments in different age groups, because identifying ways for optimize precise therapeutic interventions, is a vital objective in the health care.

5.1.2 Side effects of prebiotics

Not all effects of microbiota treatments on the host were positive. Our results showed that prebiotics such as Inulin may increase lightly the production of proinflammatory cytokine. The stimulation of inflammation was observed only in young animals,

suggesting a possible adverse effect. It is interesting to note that inulin had both positive and negative effects only in young animals and no effects on middle age subjects. In our previous study, we observed a similar result where positive and negative effects were found only in young animals and Inulin increased the cortisone level only on stressed young mice (Boehme *et al.*, 2019). For many year prebiotics and probiotics were condisered safe treatment with low risk of side effects. Any potential negative effects were thought to be confined to the gut. Few studies reported evidence of prebiotics induced negative effects in other regions of the body (Lin *et al.*, 2018). Lin and colleagues showed that inulin can stimulate the colonizadion and the traslocation of Salmonella outside the intestine. The American Society for Clinical Nutrition demonstrated that polyphenols could potentially interfere with iron absorption, and thus may induce iron deficiency in certain borderline anaemic subjects (Mennen *et al.*, 2005).

While most of the research to date has been focused on positive effects of prebiotics and probiotics, further studies are needed to investigate the possible side effects in different conditions and in different stage of life.

5.2 Microbiota interventions: A new holy Grail?

The dream of prolonging life is one of the oldest desires of men. Life expectancy has lengthened considerably over the last few centuries and quality of life has improved dramatically. Despite this, old age seems to be an unstoppable process and the emergence of chronic age-related diseases is still a major problem. As the treatment of many aging-related disorders has profound socio-economic implications (Partridge, Deelen and Slagboom, 2018), much work has gone into dietary means to promote healthy aging. Dietary microbiome therapies such as probiotic and prebiotics supplementation may lead to health benefits. However, as we have previously discussed, older people are less responsive to interventions and identifying targeted and efficient treatments is somewhat difficult. Through the gut-brain axis, we have seen that the use of treatments targeted to manipulate the microbiota can be an efficient intervention. In the next section, we analyze the possible targets that emerged from our research.

5.2.1 Intestinal permeability and neuroplasticity. Are these the new key points of intervention in aging?

Several animal studies have shown that gut barrier function is compromised with aging. In particularly, it seems that intestinal permeability may increase, and intestinal mucosal immune function may decline, contributing to the silent low grade inflammation in elderly (Thevaranjan *et al.*, 2017).

Against this background, a restoration of the intestinal barrier could improve the health of the body through the gut-brain axis. Indeed, Matsumoto and collaborators showed that longevity and quality of life of old animals is directly proportional to the restoration of the intestinal barrier (Matsumoto *et al.*, 2011).

It is interesting to note that our data showed that powerful antioxidants such as polyphenols acted exclusively in the intestine in old mice, restoring the intestinal barrier to those of young mice (Chapter 2). Rafuma and Grape Pomace have been shown to modulate the irreversible aging process of the intestine decay. It should be noted, however, that the great effect of these polyphenols was localized exclusively in the intestine as this was the most accessible of all possible target regions. Perhaps it is due to the fact that the intestine is the first organ which it comes in contact with the polyphenols, with the resultant direct action on the mucosa as well as on local intestinal bacteria. Another explanation could be that physiological changes in the elderly are so ingrained in very old mice that they cannot be changed so easily. The situation changes if the treatments are done as a preventive measure. Prebiotics and probiotics treatments have shown a protective effect in long-term therapy where the inflammation was suppressed, longevity was improved and protection from age-induced memory impairment was proved (Kibe *et al.*, 2015).

Therefore, perhaps the intestinal barrier holds the key to slowing the natural aging process.

In contrast to our previous study study (Chapter 4) we did not find any change in neuronal inflammation or brain plasticity. Using an animal model of aging (APO-E deficient mice), prebiotics and probiotics ware able to increase neuron plasticity in the PreFrontal cortex. In the same animals, treated with the same probiotics and prebiotics, a reduction of systemic inflammation was observed with consequent improvement in cardiovascular risk (Ryan *et al.*, 2017).

Since microbiota modification can be profoundly affected by several confounding factors, the interpretation of these data should be conducted with caution.
These results, take together, highlight the importance of starting new research into whether the intestinal barrier can be an important key point of intervention for healthy age.

5.2.2 Cardiovascular disease and brain: two birds with one stone through microbiota manipulation

The heart and brain are the organs most affected during aging process. Damage to these organs can result in dementia and cardiovascular disease. Through the gut-brain axis we have demonstrated in our data how the health of the heart and the brain can be influenced by intestine health in an animal model of aging (Chapter 4). The manipulation of the microbiota through the use of anti-ischemic treatments benefited the heart and have also been successful in the brain. In particular, the diets with probiotics (L. reuteri and L. mucosae) and prebiotics (plant sterol and b glucans) may have important therapeutic consequences on three distinct regions: the gut, the brain, and the heart. The important connection between the heart and the brain and its association with the French paradox (Obrenovich *et al.*, 2020) has come into focus over the last few years (Finsterer and Wahbi, 2014). The French paradox consists of the involvement of gut microbiota, where the effect of polyphenols in the red wine protect the cardiovascular system and the brain. The French diet is rich in lipids and fat but French people have relatively low morbidity and mortality from heart-related disease and Alzheimer risk (Roth *et al.*, 2017). The microbiota gut-brain axis plays an important role in heart health, heart disease and neurodegenerative diseases and can open new frontiers for the treatment of chronic and degenerative diseases that affect the elderly. This is of particular importance given cardiovascular diseases, including heart diseases and stroke, account for nearly one-third of deaths throughout the world (Fuster, 2010). Therefore, the possibility of acting through the gut-brain axis and bringing benefits to different systems of the body could also be the best solution from an economic point of view. The modulation of the microbiota is also a non-invasive

approach, and this can lead to new treatment possibilities in individuals where any invasive intervention could compromise the life of patients as well as in the elderly.
Numerous limitations and challenges, however, still exist within this newly emerging field, which require further investigation and improvement. Most of these limitations are related to research methodology generally applied in these studies. Animal models are an important tool that research can use but with many limitations. Well power clinical studies are needed but we must take into account the individual's history and extrinsic factors (Chapter 1.2.1).

5.2.3 Personalized Medicine. A new frontier

An interesting direction which will possibly influence future patient care is represented by manipulations of the individuals' microbiota. The emergence of the role of microbiota as a biomarker for disease phenotype, prognosis and response to treatment, is well described. The microbiota of each human body organ is unique, and its effects on inflammation and other disease processes as well those disease associated with aging, can help in treatment and diagnosis.
However, the main problem of modern medicine is to understand the potential characteristics of microbiome diversity among individuals (Bielanski and Haber, 2013) and understand why disease, disorder and healing often manifest differently in different patients (Bährle-Rapp, 2007).
Patients are not a homogenous population and, as previously discussed, genetics, age, lifestyle, and other factors can influence the host microbiota and consequently the therapeutic outcomes. Female and male present differences in microbiome duo to hormones, but more important the composition of the human microbiome is unique in each individual. In our studies we realized how a microbiota intervention can have positive effect on an individual and a negative effect on others for example, inulin can have a positive effect on metabolism in young individuals but increase inflammation in other individuals.
In the last few years, a new medical approach has emerged to try to address these issues. Personalized medicine aims to consider individual differences across multiple levels of analysis (e.g. genetic, environmental, lifestyle) to enhance diagnosis and targeted therapies (Maughan, 2017).

The gut microbiome is an additional modifiable system that can be incorporated into a personalized medicine framework. Potential therapeutic agents, such as personalized probiotic and prebiotic supplements, dietary interventions, and fecal microbiota transplantation that can be used to reshape the gut microbiome, represent potential strategies in the era of personalized medicine. In the near future, routine microbiota sequencing may improve the predictions of disease occurrence and may lead to novel therapeutic strategies.

5.3 Overall conclusion

This thesis presents data showing that microbiota-targeted treatments modulate various aspects of the microbiota-gut-brain axis in different age target. We demonstrate that polyphenols reverse the damage of the intestinal barrier duo to ageing in a preclinical model. Moreover, our data show that inulin, the most widely studied prebiotic, has a greater impact young instead middle age animal. It is able to stimulate the lipid and glucose metabolism, increasing the level of Sirtuin1, without impacting transcriptional factor changes in the liver. Also, inulin can uncover a range of changes in the HPA in the amygdala.

In this thesis we also show that two different strains of lactobacillus *(L.reuteri* and *L.mucosae*) and two prebiotics derived from plants (plant sterol esters and oat β-glucan) restored the neuronal plasticity in the Prefrontal cortex the level of claudin 5 increased in the hippocampus in a model of premature ageing.These results and the literature provide convincing evidence for microbiota-targeted treatments to play an important role in degenerative diseases that engage different organs on the body differently in distinct age bracket. The findings presented here might inform future clinical geriatric practice, suggesting that the probiotics and prebiotics should be taken into account during the clinical care, as crucial and not-invasive treatments alongside normal care protocols. This thesis represents an appropriate starting point for a deeper investigation of the impact of a portfolio of microbiota-targeted interventions on age-related alterations in physiology and brain function and suggest the importance of using target-specific therapeutic options during particular time windows for maximum gain.

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