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Novel insights into the mechanisms underlying the anti-obesity effects of whey protein

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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: Kanishka N. Nilaweera carried out the animal experiment and he measured body weight, energy intake, RER, VO_2, locomotor activity and hypothalamic gene expression. Raul Cabrera-Rubio carried out the bioinformatic analysis and related statistics.

Chapter 3: Kanishka N. Nilaweera helped to carry out the animal experiment and to collect faecal samples. Raul Cabrera-Rubio carried out the bioinformatic analysis and related statistics. Oleksandr Nychyk helped to perform statistical analysis and to analyse plasma hormones and metabolites.

Chapter 4: Oleksandr Nychyk and Christine Fülling helped to carry out the animal experiment. Oleksandr Nychyk helped to prepare caecal water and faecal DNA for metabolomics and 16S rRNA-based metagenomic analysis, respectively. Anna Golubeva and Joana Pereira helped with the Ussing chambers experiment. Amanda Brechon prepared the 16S rRNA library and carried out the sequencing. Raul Cabrera-Rubio carried out the bioinformatic analysis and related statistics. Thomaz Bastiaanssen helped to prepare the pictures for the metabolomics data.

Signed



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Publications and presentations

Published manuscripts relevant to this thesis

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Boscaini S, Cabrera-Rubio R, Nychyk O, Speakman JR, Cryan JF, Cotter PD and Nilaweera KN, Age- and Duration-Dependent Effects of Whey Protein on High-Fat Diet-induced Changes in Body Weight, Lipid Metabolism and Gut Microbiota in Mice, *Physiol Rep* 2020 August; 2020 8(15): e14523.

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Boscaini S, Cabrera-Rubio R, Golubeva A, Nychyk O, Fülling C, Speakman JR, Cotter PD, Cryan JF*, Nilaweera KN*, Depletion of the Gut Microbiota Differentially Affects the Impact of Whey Protein on High-fat Diet-induced Obesity and Intestinal Permeability. *To be submitted to: Food and Function*

Boscaini S, Skuse P, Nilaweera KN, Cryan JF, Cotter PD, The "Milky Whey" Potentialities: Effect of Milk Proteins on Energy Balance, Gut Microbiota, Satiety and Mood. *To be submitted to: Advances in Nutrition*

Nychyk O, Barton W, Piotrowicz Y, **Boscaini S**, Walsh A, Bastiaanssen TFS, Giblin L, Cormican P, Liang C, Derous D, Fanning A, Yin X, Grant J, Rudolf A, Melgar S, Brennan L, Mitchell S, Cryan JF, Wang J, Cotter P, Speakman JR and Nilaweera KN, Protein Quality and Quantity in High-fat/Low Carbohydrate Diets Affects Weight Gain Via Impacts on Host-microbiota Energy Metabolism. *Submitted to: Nature Communications*

Hoffman Sarda F, Strain C, **Boscaini S**, Fitzgerald P, O'Riordan K, Rea K, Cryan JF, Ross P, Stanton C, Effects of Sugar Substitutes on Microbiome Modulation and Glucose Homeostasis. *To be submitted to: Journal Food Research International*

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Boscaini S, Cabrera-Rubio R, Speakman JR, Cryan JF, Cotter PD, Nilaweera KN, Alpha-lactalbumin influences energy balance and gut functionality without affecting body weight in high-fat diet-fed mice. *Irish Nutrition Society Postgraduate conference*. Belfast (Northern Ireland), February 2018. Speaker

Boscaini S, Cabrera-Rubio R, Speakman JR, Cryan JF, Cotter PD, Nilaweera KN, Dietary Alpha-lactalbumin influences energy balance in association with altered gut microbiota composition and intestinal nutrient transporters without affecting body weight in high-fat diet fed mice. *European Congress on Obesity*. Vienna (Austria), May 2018. Poster

Boscaini S, Cabrera-Rubio R, Nychyk O, Speakman JR, Cryan JF, Cotter PD and Nilaweera KN, Age-dependent effect of whey protein on body weight, lipid, metabolism and gut microbiota in high-fat diet fed mice. *MicrobiotaMi*. Milano (Italy), November 2018. Speaker (flash presentation) and Poster

Boscaini S, Cabrera-Rubio R, Nychyk O, Speakman JR, Cryan JF, Cotter PD and Nilaweera KN, Age-dependent effect of high-fat diet containing whey protein on body weight, lipid metabolism and gut microbiota in C57BL/6J mice. *APC Microbiome Ireland Symposium*. Cork (Republic of Ireland), October 2019. Speaker (flash presentation) and Poster

Boscaini S, Nychyk O, Fölling C, Golubeva A, Cotter PD, Nilaweera KN & Cryan JF, Role of the gut microbiota in whey protein-mediated changes in adiposity, metabolism and gut permeability in high-fat diet-fed mice. *New Horizons Conference*. Cork (Republic of Ireland), December 2019. Poster

Abbreviation list

^{13}C	13-carbon isotope
16S	Small Subunit 16
5-HT	Serotonin; 5-hydroxytryptamine
5-HTP	5-hydroxiltriptophan
AA	Amino acids
ABX	Antibiotics
ACC	Acetyl-CoA carboxylase
ACE	Angiotensin-converting enzyme
ACLY	ATP-citrate lyase
Actb	Actin beta
Adipoq	Adiponectin gene
Adrb3	β-3 adrenergic receptor
AgRP	Agouti-related protein
AI	Augmentation index
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
APC	Antigen-presenting cells
ApoE null	Atherosclerosis-prone apolipoprotein E-deficient
ARC	Arcuate nucleus
ATGL	Adipose TAG lipase
ATP	Adenosine triphosphate
B^0AT1	Neural amino acid transporter 1
BAMLET	Human Alpha-lactalbumin Made LEthal to Tumor cells
BAT	Brown adipose tissue
BBB	Blood brain barrier
BCAA	Branched-chain amino acids
BH	Benjamin Hochberg
BMI	Body mass index
BSA	Bovine serum albumin
BSH	Bile salt hydrolase
CA	Cholic acid
Ca++	Calcium ++
$CaCl_2$	Calcium chloride
Caco-2	Colorectal adenocarcinoma
$CaCO_3$	Calcium carbonate
cAMP	Adenosine 3’,5’-cyclic monophosphate
CART	Cocaine- and amphetamine- regulated transcript
CAS	Casein

CaSR	Calcium sensor receptor
Cb1	Cannabinoid receptor 1
CCK	Cholecystokinin
CD	Crohn's disease
CD	Cluster of differentiation
CDCA	Chenodeoxycholic acid
cDNA	Complementary DNA
cGMP	Guanosine 3,5-cyclic monophosphate
ChREBP	Carbohydrate response element-binding protein
CNS	Central nervous system
CO_2	Carbon dioxide
CoA	Coenzyme A
Cp	Crossing point
CPT	Carnitine palmitoyltransferase
CRH	Corticotropin-releasing hormone
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats-Cas9
Ct	Cycle threshold
DAG	Diacylglycerol
Db/Db	Leptin receptor gene deficiency
DC	Dendritic cells
DCA	Deoxycholic acid
DGAT	Diacylglycerol acyltransferase enzyme
DGGE	Denaturant gradient gel electrophoresis
DIO	Diet-induced obesity
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPPIV	Dipeptidyl peptidase IV
DSS	Dextran sulfate sodium
EC	Enterochromaffin cell
EDTA	Ethylenediaminetetraacetic acid
EEC	Enteroendocrine cell
ELISA	Enzyme-linked immunosorbent assay
ENS	Enteric nervous system
EPEC	Enteropathogenic *Escherichia coli*
ER	Endoplasmic reticulum
ETC	Electron transport chain
EU	Europe
eWAT	Epididymal white adipose tissue
F/B	Firmicutes/Bacteroides
FABPpm	Plasma membrane fatty acid binding protein
FACS	Fatty acyl-CoA synthase
$FADH_2$	Flavin adenine dinucleotide

FASN	Fatty acid synthase
FATP	Fatty acid transport protein
FDR	False Rate Discovery
Fe^{2+}	Iron ++
FFA	Free fatty acids
FFAR	Free fatty acids receptor
FITC	Fluorescein isothiocyanate
FKBP5	FK506 binding protein 5
FMT	Faecal microbiota transplantation
FOS	Fructooligosaccharides
Foxp3	Forkhead box protein 3
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GC-MS	Gas chromatography – mass spectrometry
GF	Germ-fee
Ghrl	Ghrelin gene
GHSR	Growth hormone secretagogue receptor
GIP	Gastric inhibitory polypeptide
GIT	Gastrointestinal tract
GLP	Glucagon-like peptide
GLP-1R	Glucagon-like peptide 1 receptor
GLUT	Glucose transporter
GMP	Glycomacropeptide
GOS	Galactooligosaccharides
GPCR	G protein-coupled receptor
GR	Glucocorticoid receptor
H_2O_2	Hydrogen peroxide
HFD	High-fat diet
HO_2SCN	Cyanosulphuros acid
HO_3SCN	Cyanosulphuric acid
HOSCN	Hypothiocyanous acid
HPA	Hypothalamic-pituitary gland-adrenal
HSD	High-sucrose diet
HSE	Health Service Executive
HSL	Hormone sensitive lipase
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
IEL	Intestinal intraepithelial lymphocyte
Ig	Immunoglobulin
IL	Interleukin
INFγ	Interferon γ
IR	Insulin receptor

Irs-1	Insulin receptor substrate gene
JAK-Stat	Janus kinase signal transducer and activator of transcription
JAM	Junctional-adhesion molecule
K^+	Potassium +
KCl	Potassium chloride
LAB	Alpha-lactalbumin
LABH	Hydrolysed alpha-lactalbumin
LAG	Beta-lactoglobulin
LAT4	L-type amino acid transporter 4
LBP	Lipopolysaccharide binding protein
LCA	Lithocholic acid
LCFA	Long chain fatty acids
LC-MS	Liquid chromatography–mass spectrometry
LepRb	Leptin receptor
Lf	Lactoferrin
LFD	Low-fat diet
Lp	Lactoperoxidase
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
LSD	Low-sucrose diet
Ly	Lysozyme
MAG	Monoacylglycerols
MARVEL	MAL and related proteins for vescicle trafficking and membrane link
mAT	Mesenteric adipose tissue
MC3/4R	Melanocortin receptors 3 and 4
MCF-7	Michigan Cancer Foundation-7
MCP-1	Monocyte chemioattractant protein 1
Mg^{++}	Magnesium ++
$MgCl_2$	Magnesium chloride
MGL	Monoglyceride lipase
Mn^{++}	Manganese ++
mRNA	Messanger RNA
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
mTOR	Mammalian target of rapamycin
Na^+	Sodium +
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver diseases
NaH_2PO_4	Monosodium phosphate
$NaHCO_3$	Sodium bicarbonate

NF-kB	Nuclear factor kB
NGS	Next generation sequencing
NMDA	N-methyl-D-aspartate
NPC1L1	Niemann-Pick C1-Like 1
NPY	Neuropeptide Y
NS	Non significant
NTS	Nucleus of the solitary tract
Ob	Leptin gene
Ob/Ob	Leptin gene deficiency
$OSCN^-$	Hypothiocyante
OTU	Operational taxonomic unit
PAI	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PEPT1	Proton-coupled oligopeptide transporter
PERMANOVA	PERmutational Multivariate Analyses Of Variance
PF	Phyto-oestrogen free
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator 1
PGM	Personal genome machine
PiCrust	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PKA	cAMP-dependent protein kinase
PKG	cGMP-dependent protein kinase
POMC	Pro-opiomelanocortin
PP	Pancreatic polypeptide
PPAR	Peroxisome proliferator-activated receptor
PYY	Peptide tyrosine tyrosine
QC	Quality control
QIIME	Quantitative insights into microbial ecology
rAT	Retroperitoneal adipose tissue
RDP	Ribosomal Database Project
RER	Respiratory exchange ratio
rhLf	Recombinant human Lf
RMR	Resting metabolic rate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SAM	Sequence Alignment/Map
SAS	Statistical analysis system
SCAP	SREBP cleavage-activating protein
SCD1	Stearoyl-CoA desaturase-1
SCFA	Short chain fatty acids

SCN^-	Thiocyanate anion
SEM	Standard error of the mean
SGLT1	Sodium-glucose transport protein 1
Slc6a19	Methionine transporter gene
SNS	Sympathetic nervous system
SOCS3	Suppressor of cytokine signalling 3
SPF	Specific pathogen-free
spp.	Species
SPSS	Statistical Package for the Social Sciences
SREBP	Sterol regulatory element-binding protein
sWAT	Subcutaneous white adipose tissue
T1R1/T1R3	Umami taste receptor
T1R2/T1R3	Sweet taste receptor
TAG	Triacylglycerol (triglyceride)
TAMP	TJ-associated MARVEL proteins
TCA	Tricarboxylic acid cycle
TJ	Tight junction
TLR4	Toll like receptor 4
TNFα	Tumor necrosis factor alpha
UC	Ulcerative colitis
UCP	Uncoupling protein
UK	United Kingdom
UPLC	Ultra performance liquid chromatograpy
USA	United States of America
VCO_2	Carbon dioxide production
VLDL	Very-low-density lipoprotein
VO_2	Oxygen consumption
$WACO_2T$	White adipose tissue
WHO	World Health Organization
WP	Whey protein
WPC	Whey protein concentrate
WPI	Whey protein isolate
YWHAZ	14-3-3 protein zeta/delta gene
Zn^{++}	Zinc ++
ZO	*Zonula occlundes*
α-MSH	α-melanocortin stimulating hormone
β3-AR	β3-adrenegenic receptor

Units

˚C	Centigrade
µg	Microgram
µL	Microlitre
µm	Micrometer
bp	Base Pair
cm	Centimeter
dL	Decilitre
g	Gram
h	Hour
Kcal	Kilocalorie
KDa	Kilodalton
Kg	Kilogram
KJ	Kilojoule
L	Litre
Mb	Megabase pair
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
ng	Nanogram
nm	Nanometer
nt	Nucleotide
xg	Relative centrifugal force

Abstract

Obesity has become one of the major health problems worldwide, due to modern eating habits and an increase in sedentary lifestyles. Dietary strategies represent one among different therapeutic approaches employed to ameliorate, treat or prevent the obesity epidemic.
In this thesis, we focused on bovine whey protein, selected for their ability to influence energy balance, satiety hormones production and glucose tolerance. In particular, using male C57BL/6 mice fed with high-fat diet (HFD), we investigated the effect of whey protein isolate (WPI) and the whey protein component alpha-lactalbumin (LAB) on body weight, adipose tissue, intestinal-related functions and gut microbiota.

LAB given with HFD increased energy intake without affecting body weight. We observed that this energy mismatch was, in part, due to a change in the gene expression of nutrient transporters within the small intestine. This was accompanied by an increase in relative abundance of *Parabacteroides*, *Bifidobacterium*, *Parvibacter* and *Lactobacillus* in the gut.

We next investigated the effect of HFD supplemented with WPI given at two different ages and with different WPI intake durations. We observed that, only in younger mice, WPI decreased body weight and adiposity, which might be caused by an increase of adipose tissue catabolism. In addition, the gut microbiota of younger mice fed with HFD-WPI showed a different gut microbiota composition, including an increased relative abundance of *Lactobacillus murinus*.
In another experiment, we observed that, even during microbiota depletion, WPI still caused a decrease in HFD-induced body weight gain, adiposity and tissues inflammation. On the contrary, the beneficial effect of WPI on HFD-induced gut permeability disruption is abolished during gut microbiota depletion.

Overall, these results provide novel insight on the effects of whey protein on energy balance, adiposity, intestinal functionality and gut microbiota in the presence of HFD-induced obesity.

Chapter 1

General

Introduction

Introduction

According to the most recent World Health Organization (WHO) estimation, 1.9 billion adults (over 18) worldwide were overweight, of which nearly 600 million were obese (WHO 2016). Obesity is a multifactorial condition which primarily arises as a result of a long-term positive energy balance, whereby the energy extracted from the food consumed is greater than what is used by the body. This leads to an increase of adipose tissue mass and, consequently, weight gain. Importantly, obesity leads to a chronic low-grade inflammation and insulin resistance. For these reasons, obesity can be also considered as a metabolic disease. As such, it is considered as one of the major health problems in the world because it increases the risk of development of several clinical conditions, including cardiovascular disease, stroke, hypertension, steatohepatitis, type 2 diabetes and different kind of cancers such as colon-rectal, breast, pancreas, liver, gallbladder and gastric (Calle & Kaaks 2004, O'Neill & O'Driscoll 2015).

During the last 25-30 years, the incidence of obesity has risen dramatically (WHO 2016). While there are many reasons for this, there is a growing recognition that the main driver of obesity is the change in our dietary habits, which have impacted on our ability to regulate the balance between energy intake and energy expenditure. Notably, in the last century, there has been a growing trend towards an increased intake of high energy diets coupled with an increasingly sedentary lifestyle. Alongside this, obesity is characterized by deregulation of the production or action of many hormones, some produced by the gut and others by internal organs such as the adipose tissue (e.g., leptin). This, coupled with increased tissue growth (including the intestine) and altered intestinal nutrient absorption, affects the gut-hypothalamic-adipose cross-talk in the control of appetite and energy balance regulation (Klok et al 2007). In the last few years, it was shown that the composition, diversity and the functions of the gut microbiota can also play important roles in the aetiology of obesity (Cani 2013, Clarke et al 2012, Torres-Fuentes et al 2017). Despite continuous medical and scientific effort, long-term strategies aimed at attenuating or reversing the obesity epidemic are still to be developed. Hence, there is a growing interest to identify safe therapies, in particular those involving nutrients, such as proteins, which can reduce weight gain

and prevent the development of obesity or help already obese people to lose weight and sustain this effect long term.

In the following sections, I will provide an overview of the structure and functions of the above named key tissues important for energy balance regulation, the role of gut microbiota, and how each of these are affected during the progression of weight gain towards and/or following development of obesity. In this regard, the focus would be on the impact of high-fat diet (HFD). In the final part of the introduction, I will provide the background to a potential dietary solution tested in this thesis with a view to the management of HFD-induced weight gain (rather than weight loss), namely whey protein, which is a dietary protein sourced from milk. Ultimately, I will highlight unanswered questions that formed the basis for undertaking the project. In essence, the research project uses mouse models to better understand the impact of whey protein on the gut environment including the microbiota, and their influence on energy balance with specific reference to changes in adipose tissue and the hypothalamus.

1.1 Energy balance homeostasis: organs and hormones involved

Energy balance is defined as the difference between energy absorbed by the intestine following ingestion of food (assimilated energy) and energy used by the body tissues to carry out any kind of activity. The main factors that influence energy balance are, indeed, energy intake, intestinal energy absorption, energy expenditure and energy storage. Through the routinely consumption of macronutrients (such as fats, carbohydrates and proteins), the mammalian body gets energy that serves as fuel to execute all the processes that maintain the body alive. For instance, the energy from food is consumed by metabolic activities, including the resting metabolic rate (RMR), thermogenesis and physical activity (Hill et al 2012). RMR is the largest component of energy spent daily, accounting for 60% of daily energy expenditure in humans. It corresponds to energy expended by the body during a rest condition, in order to maintain vital organ functions and for thermogenesis. Thermogenesis is the autonomic

process that uses energy to produce and release heat (McMurray et al 2014, Rosenbaum & Leibel 2010). Under the influence of environmental and dietary factors, energy intake, intestinal energy absorption, energy expenditure and energy storage fluctuate and change dynamically, resulting in positive or negative energy balance, and where they match, energy homeostasis is achieved. A situation of positive energy balance derives from an energy intake and assimilation that surpasses the energy requirements of physical and physiological energy expenditures. The excess of energy is deposited within the body fat, which is the primary energy store in humans (and other mammals) and, consequently, the body weight and body fat mass of the individual increase. Conversely, a negative energy balance comes from an excess of energy expenditure over intake or following a reduction in intake below basal energy requirements such as during food deprivation or restriction, leading to body weight loss (Hill et al 2013). A long-term disruption of energy homeostasis leads to the development of chronic disorders including obesity (long-term positive energy balance) but also anorexia and undernutrition (long-term negative energy balance) (Kurpad et al 2007, Uauy & Diaz 2005). A practical way to determine energy balance dynamics both in humans and rodents involves carrying out different metabolic measurements. For rodents, a special cage equipped with several chambers and analysers, called metabolic cage, is widely used. Some important metabolic parameters detected are: oxygen consumption (VO_2) carbon dioxide production (VCO_2), respiratory exchange ratio (RER, VCO_2/VO_2) and locomotor activity (Speakman 2013).

To impede energy imbalance and its consequences, there are some strategies that the organism adopts. The most important one is the control of satiety, defined as the physiological state at the end of a meal when further eating is inhibited by ‘fullness’(Amin & Mercer 2016). In this context, there exists a fine regulation of hunger and fullness state based on energy availability of the individual. This is brought about by organs cooperating and communicating within each other to maintain energy homeostasis. The protagonist organs in this process are: gastrointestinal tract (GIT), adipose tissue and the brain, followed by liver, pancreas and skeletal muscle (Boguszewski & van der Lely 2014, Roh et al 2016). They establish a crosstalk with

each other through the production of hormones and neuropeptides and, consequently, act through specific receptors expressed on the cellular surface. These receptors activate a specific cascade of regulation in response to molecules produced by other organs.

1.1.1 Brain and hypothalamic control of energy balance

In general, energy balance-related hormones produced in the periphery communicate with the brain through a variety of mechanisms; most notably by the activation of receptors on the vagus nerve and by directly activating brain circuits, especially in the hypothalamus where there is a relatively permeable blood brain barrier (BBB) (Owyang & Heldsinger 2011). Once in the brain, the satiety-related hormones stimulate a specific area of the hypothalamus, the arcuate nucleus (ARC). Notably, the ARC neurons are in close proximity to the circulatory system, and thus can sense not only levels of hormones but also nutrients and thereby coordinates hunger and fullness. The neurons in the ARC are orexigenic (appetite-increasing) or anorexigenic (appetite-decreasing). Under peripheric satiety hormonal stimulation, ARC neurons produce other neuro-hormones, such as the orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgrP), and the anorexigenic pro-opiomelanocortin (POMC) and cocaine- and amphetamine- regulated transcript (CART) (**Fig. 1.1**). POMC is the precursor of all melanocortin peptides, including α-melanocortin stimulating hormone (α-MSH), one of the most important mediators of the catabolic response. To exert its function, α-MSH binds the hypothalamic melanocortin receptors 3 and 4 (MC3/4R) (Shimizu et al 2007). In turn, the hormones produced by ARC stimulate other hypothalamic areas (such as paraventricular nucleus and lateral hypothalamus) and higher centers (such as amygdala, limbic system and cerebral cortex) in order to control feeding behavior (Camilleri 2015). Specifically, second order neurons are activated and, via sympathetic neurons, directly control catabolic and anabolic reactions in the peripheral tissues (Roh & Kim 2016). In summary, in the CNS, multiple inputs from the periphery are integrated and, as a response, outputs are

elaborated and directed back to the periphery. This process regulates energy intake and energy expenditure in order to maintain energy homeostasis.

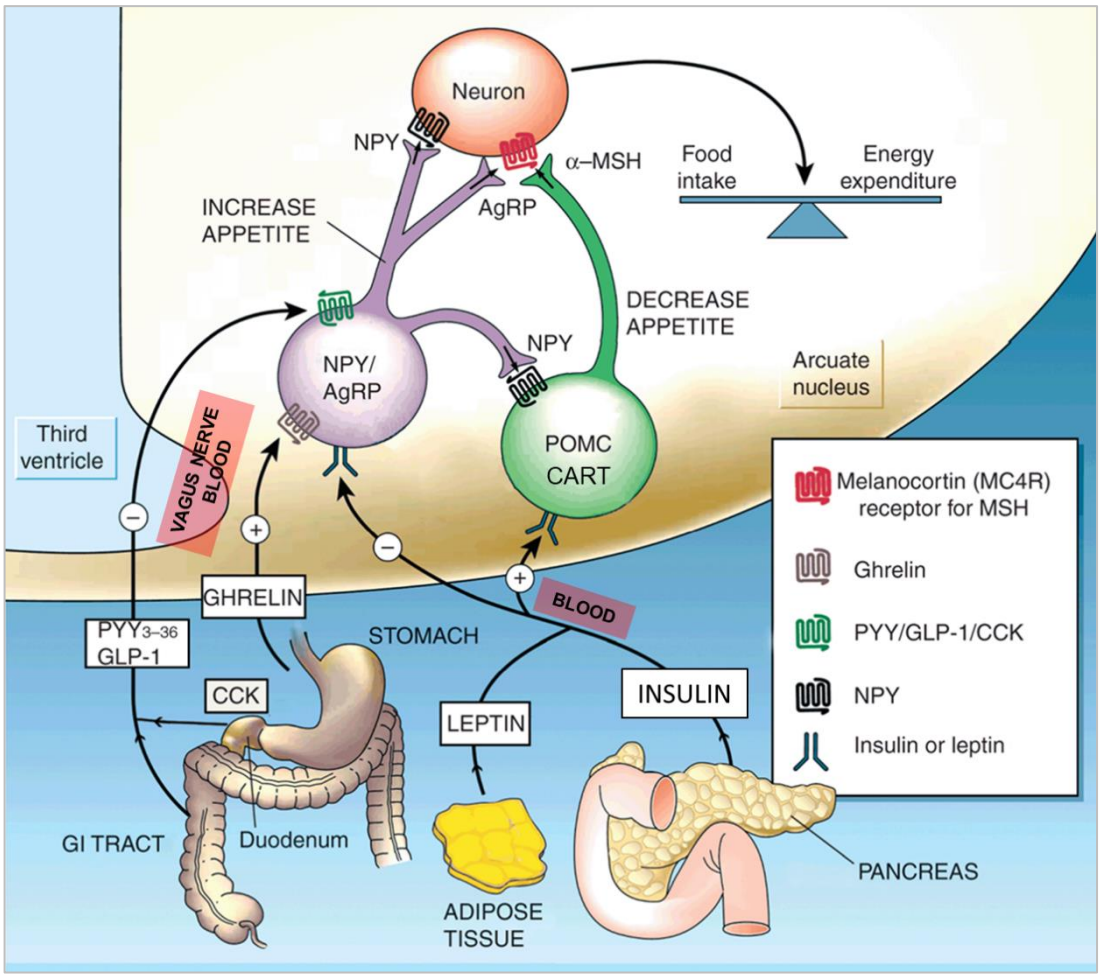


Figure 1.1 Neuroendocrine control of food intake. *Role of the hypothalamic arcuate nucleus in regulating food intake. Leptin and insulin circulate in the blood at concentrations proportional to body fat mass. They decrease appetite by inhibiting neurons that produce the molecules NPY and AgRP, at the same time stimulating α-MSH (POMC) and CART neurons in the arcuate nucleus. NPY and AgRP stimulate eating, and α-MSH inhibits eating, via other neurons (top). Activation of NPY/AgRP-expressing neurons inhibits POMC neurons, either directly through NPY receptors or by AgRP blocking the effects of α-MSH. The hormone ghrelin stimulates appetite by activating the NPY/AgRP-expressing neurons. PYY3-36 and GLP-1, released from the small intestine, inhibit these neurons, and thereby decrease appetite. Abbreviations: AgRP, agouti-related peptide; CART, cocaine- and amphetamine-regulated transcript; CCK, cholecystokinin; GI, gastrointestinal; GLP-1, glucagon-like peptide-1; MSH, melanocyte-stimulating hormone; NPY, neuropeptide Y; POMC, proopiomelanocortin; PYY, peptide YY. Figure adapted from (Wilkinson & Imran 2019).*

1.1.2 Small intestinal morphology and nutrient absorption

The gastrointestinal tract (GIT) is responsible for the ingestion and digestion of foods and liquids and, subsequently, for the absorption of nutrients important for individual survival. Food digestion starts in the mouth, continues in the stomach, and concludes in the small intestine.

The small intestine is the portion of the GIT where nutrient absorption primarily occurs. The small intestine is divided into duodenum, jejunum and ileum, forming, respectively, the proximal (just after the stomach), central and distal parts (before the colon). The duodenum receives chyme from the stomach and contributes to digestion by the action of pancreatic enzymes and bile acids from the liver. The jejunum and ileum are the regions in which the absorption of nutrients (i.e., fats, amino acids, sugars, vitamins and minerals) mainly occurs (Collins & Badireddy 2020). The intestinal epithelium contains a large variety of transporters (active and passive) that are responsible for the translocation of the nutrients into the bloodstream (Kiela & Ghishan 2016). The density of these transporters changes across the small intestine (Spiller 1994), reflecting the diverse nutrient absorptive capacities of the intestinal regions.

This intestinal tract is composed of three layers. The external layer, serosa, is constitute mainly by mesothelium and endothelium. The middle layer, muscularized, consists of two smooth muscle layers, a thin outer longitudinal layer that shortens and elongates the gut, and a thicker inner circular layer of smooth muscle which causes constriction (Collins et al 2020). Nerves belonging to the enteric nervous system (ENS) are located between the two muscle layers (Nezami & Srinivasan 2010). The inner layer, divided into submucosa and mucosa, is composed by innervated connective tissue irrorate by blood vessels and, on the lumen side, it accommodates different cell types:

- Enterocytes: cells organized in a monolayer that forms a polarized epithelium. Their plasma membrane is characterized by basal, lateral and apical domain. Microvilli with a brush border appearance protrude from the apical domain and facilitate nutrient absorption. The enterocytes plasma membrane is equipped

with different enzymes and transporter proteins that control metabolism, absorption and/or secretion of nutrients, metabolites and electrolytes (Overeem et al 2016). A comprehensive list and description of all nutrient transporters within the small intestine is provided in the work of Kiela and Ghishan (Kiela & Ghishan 2016).

- Goblet cells: cells organized in exocrine glands able to secrete mucins, which are large molecular weight glycoproteins. Mucins are the main component of the mucus, which provides mucosal protection from dehydration and mechanical damage. In addition, it constitutes a physical barrier between the intestinal epithelium and luminal contents, including pathogenic microbes (Kim & Khan 2013).

Table T1.1 Enteroendocrine cells (ECCs): location within the gastrointestinal tract (GIT) and hormones secreted. *Abbreviations: CCK, cholecystokinin; 5-HT, serotonin; GIP gastric inhibitory polypeptide; GLP-1 and GLP-2, glucagon-like peptide 1 and 2; PYY, peptide YY.*

EECs kind	Location within the GIT	Hormones secreted
A (X-like)	Stomach	Ghrelin
G	Stomach, Duodenum	Gastrin
D	Stomach, Small intestine	Somatostatin
I	Proximal small intestine	CCK, 5-HT
K	Proximal small intestine	GIP, 5-HT
L	Distal small intestine, colon	GLP-1, GLP-2, PYY, 5-HT
Enterochromaffin	Stomach, small intestine, colon	5-HT

- Crypts of Lieberkühn: glands lined with the intestinal epithelium. They are home to:
    - Stem cells: cells in charge of epithelial layer renewal every few days.
    - Paneth cells: specific cells that assist undifferentiated columnar cells and rapidly amplify cells in the regeneration of absorptive and secretory cell types. In addition, Paneth cells are the primary producers of antimicrobial peptides in the intestinal tract (Bevins & Salzman 2011). Thus, they are involved in shaping the composition of the gut microbiota (Gassler 2017).
    - Enteroendocrine cells (EECs): cells able to secrete specific hormones in response to stimuli. They are present also in the stomach and in the colon. The EECs are divided in subcategories detailed in **table T1.1** (Latorre et al 2016).
- Peyer's patches: aggregations of lymphoid follicles which represent the gut-associated lymphoid tissue. In the Peyer's patches are present B-cells, T-cells, macrophages and dendritic cells (DCs). They are considered the immune sensor of the intestine and they are more abundant in the ileum (Jung et al 2010).

1.1.3 The gastrointestinal tract controls energy balance *via* communication with the brain

The GIT acts as an essential regulator of energy homeostasis in response to the availability or demand of food. This is facilitated by the presence of diverse cell types mentioned above, which regulate nutrient absorption and production of several satiety and metabolism-related hormones, such as ghrelin (produced by the stomach), cholecystokinin (CCK, produced by duodenum and jejunum), glucagon-like peptide 1 and peptide YY (GLP-1 and PYY respectively, both produced by distal ileum and colon) (**Fig. 1.1**) (Boguszewski & van der Lely 2014, Castagneto Gissey et al 2019, Murphy & Bloom 2004). Such hormones produced by GIT are defined as "short-term energy balance signals", which relay a sense of fullness or starvation in order to limit

the size of individual meal and change the time of intake of the next meal through the communication with the brain in a short period of time (Havel 2002).

1.1.3.1 Ghrelin

Ghrelin is a small protein composed of 28 amino acids, which is produced mostly by endocrine cells in the stomach and, in small part, by the small intestine cells and pancreas (van der Lely et al 2004). Ghrelin is encoded by the same gene (i.e., *Ghrl*) that encodes obestatin, another satiety-related hormone, functions of which are still under investigation (Grönberg et al 2008). Initially, the *Ghrl* gene is translated into a longer peptide, called preproghrelin that undergoes cleavage, becoming proghrelin. After a proteolysis process, proghrelin is converted to ghrelin through a post-translational modification at a serine residue with an octanoyl group, a specific kind of acylation (Sato et al 2011). The addition of octanoyl groups is essential because it allows ghrelin to be active, to bind its receptor and to exert its function. Ghrelin is denominated the "hunger hormone" for its ability to stimulate appetite and increase food intake and fat storage. It is secreted before a meal, during fasting, and its production decreases after feeding (Ibrahim Abdalla 2015). After secretion, both blood circulation and vagus nerve afferent neurons have been identified as routes for conveying ghrelin's signal from the periphery to the brain (Date et al 2002, Schaeffer et al 2013). Once in the brain, ghrelin is able to cross the BBB and bind a specific isoform of growth hormone secretagogue receptor (GHSR) expressed in the hypothalamus and in the pituitary gland (Howard et al 1996). The activation of GHSR in the ARC neurons of the hypothalamus stimulates the production of the orexigenic neuropeptides NPY and AgRP (Guan et al 2010), promoting food intake and suppressing thermogenesis (Wren et al 2000, Yasuda et al 2003). It was shown that the orexigenic effect exerted by ghrelin is impeded in mice with a deletion or mutation in *Ghsr*. Moreover, antagonists of this receptor have been designed to regulate food intake as a possible strategy to ameliorating eating disorders (Howick et al 2017, Zigman et al 2005).

1.1.3.2 Cholecystokinin

Cholecystokinin (CCK) is produced mainly by intestinal I cells in the duodenum and jejunum and, in a small part, in the CNS. Different active CCK isoforms (with a specific number of amino acids) can be obtained by processing of pro-CCK, the CCK precursor (Rehfeld 1998). CCK is released during food ingestion and the principal stimulators of its secretion are dietary protein and fats. CCK has an anorexigenic effect and acts as a satiety-stimulator in two ways: by suppressing gastric emptying and transit through a direct action on the stomach and by inhibiting food intake through the communication with the brain (Beglinger 1994, Matzinger et al 1999). The CCK-1 receptors are present on the afferent fibres of the vagus nerve as well as in the nucleus of the solitary tract (NTS) in the hind brain and in the hypothalamus (Wank 1995). The NTS is a region in the brain considered as gateway for meal-related signals entering the brain from the periphery. An increase of post-prandial CCK stimulates the NTS region and the upregulation of CART in the hypothalamic neurons, promoting an anorexigenic response (D'Agostino et al 2016, Maletínská et al 2008). Vagotomy or the removal of the NTS region abolishes CCK satiety effects downstream (Edwards et al 1986, Shillabeer & Davison 1985). Agonists of CCK receptors have been developed and have been demonstrated to have different beneficial effects, such as increase of insulin-sensitivity and regulation of adipokines production in the adipose tissue (Cawston & Miller 2010, Lin et al 1990, Plaza et al 2019).

1.1.3.3 Glucagon-like peptide 1

Glucagon-like peptide 1 (GLP-1) is a 30 amino acids hormone produced by intestinal L cells found in the distal ileum and colon. In response to a meal, intestinal L cells are promoted to activate proglucagon gene expression, which is the same gene that encodes glucagon in the pancreas. Following the cleavage of proglucagon, GLP-1 and GLP-2 are the two isoforms produced (Donnelly 2012, Janssen et al 2013). GLP-1 secretion is higher in the presence of a meal rich in carbohydrates and fats (Gibbons et al 2013, Yoder et al 2009). Recently, it was showed that GLP-1 release is also controlled by intestinal intraepithelial lymphocyte (IEL) within the intestine (He et al 2019). GLP-1 is considered an incretin hormone due to its ability to stimulate the

proliferation of the insulin-secretor β-pancreatic cells and, thus, to enhance the secretion of insulin. In addition, GLP-1 inhibits the pancreatic secretion of glucagon (Ramracheya et al 2018). Accordingly, GLP-1 secretion is correlated with a decrease of blood sugar levels (Ramracheya et al 2018, Shigeto et al 2015). In parallel, GLP-1 acts as an anorexigenic molecule, able to reduce food intake. As in the case of other satiety-regulator hormones, GLP-1 exerts its anorexic function by binding its specific receptor (i.e., GLP-1R) present on the vagus nerve, NTS and in hypothalamic ARC, both on POMC and NPY/AgRP neurons (Baggio & Drucker 2014). Here, simultaneously, GLP-1 stimulates the activation of POMC neurons and inhibits NPY/AgRP neurons and, then, those anorexogenic signals are integrated to elaborate the output (Bugarith et al 2005, Seo et al 2008). From a study conducted in humans, it was shown that GLP-1 can also influence energy expenditure in that GLP-1 injection in healthy humans resulted in decreased diet-induced thermogenesis and carbohydrate oxidation during the post-prandial period (Flint et al 2000). In rats, GLP-1 receptor has been observed to be involved in the control of energy expenditure during chronic HFD-feeding (Krieger et al 2018).

1.1.3.4 Peptide YY

Peptide tyrosine tyrosine (PYY) is a 36 amino acids protein secreted by L-type endocrine cells along the GIT, found at a higher concentration in the terminal ileum and colon. PYY belongs to pancreatic polypeptide-fold family of peptides, together with NPY and the pancreatic peptide PP. Like GLP-1 and CCK, PYY is an anorexigenic hormone and is secreted as PYY (1-36) in response to calorie increase. PYY (1-36) becomes active, termed PYY(3-36), after a cleavage performed by the enzyme dipeptidyl peptidase IV (DPPIV) (Ballantyne 2006). The active form PYY(3-36) has satiety effects. A peripheral administration of PYY(3-36) results in a strong food intake inhibition both in human and rodents (Batterham et al 2003, Batterham et al 2002). PYY(3-36) binds Y2 receptor, which is expressed both in the periphery and in different areas of the brain, such as the NTS, hypothalamic ARC, medial nucleus of the amygdala, substantia nigra, and parabrachial areas (Dumont et al 1998). In the hypothalamus, PYY stimulates and represses the expression of POMC and NPY, respectively (Challis et al 2003). In addition, considering that Y2 receptors are

expressed in parts of the limbic system, including the amygdala, it is possible that PYY participates in the modulation of hedonic pathways involved in food intake control (Münzberg et al 2016). Regarding energy expenditure, it was observed that mice overexpressing PYY showed an enhanced lipogenic capacity, and an increase in thermogenesis and post-prandial insulin action as well as a reduction in food intake (Shi et al 2012, Sloth et al 2007).

1.1.3.5 Regulation of gastrointestinal hormones by nutrient availability: an overview

GIT hormones in charge of the regulation of energy balance are primarily regulated by the nutrients that are present in the intestinal tract. The response of EECs, which result in hormone production, can be various, depending on which nutrient is more available and its capacity to stimulate different EECs.

- Proteins are broken down by stomach proteases into oligopeptides and amino acids. It was demonstrated that I and L cells secrete CCK and GLP-1, respectively, in response to oligopeptides availability. Indeed, those cells express the proton-coupled oligopeptide transporter PEPT1 (Diakogiannaki et al 2013, Liou et al 2011a). In addition, single amino acids can stimulate gut hormone release, specifically through the activation of different G protein-coupled receptors (GPCRs). Different amino acids stimulate the production of different gut hormones. The I cells express the calcium sensor receptor CaSR and the taste receptor T1R1/T1R3 on their surface, which bind tryptophan and leucine, respectively (Liou et al 2011b, Tian et al 2019). Thus, tryptophan and leucine stimulate CCK secretion. The CaSR is present also on L and K cells, promoting the production of GLP-1 and GIP, upon tryptophan stimulation (Diakogiannaki et al 2013, Mace et al 2012). In addition, GIP production is stimulated by arginine and lysine, which activate the basic amino acid sensor GPRC6a present on K cells (Wellendorph et al 2005).

  It was shown that dietary proteins can control gut hormone biosynthesis, such as GLP-1 and ghrelin, through the activation of mTOR, a key regulator of cellular anabolism (Li et al 2019b, Xu et al 2015).

- Lipids are present in the intestinal lumen as free fatty acids (FFAs) and monoacylglycerols (MAG) after completion of digestion (paragraph 1.2.1). There are evidences that show the presence of FFAs receptors (FFARs) on different EECs, which stimulate CCK, GLP-1 and GIP secretion upon lipids ingestion (Isaacs et al 1987). Moreover, a intraduodenal lipid injection enhanced the expression of FFAR1 in K cells, which was positively correlated with GIP secretion (Cvijanovic et al 2017). Interestingly, it was observed that L cells express FFAR1, which is located on the basolateral membrane of the cells and not on the apical side, as previously thought (Christensen et al 2015). In addition, through the interaction with the lipid amine receptor GPR119, MAG is able to stimulate the production of GLP-1 and GIT, but not CCK or PYY (Chu et al 2008, Hansen et al 2011, Parker et al 2012).

- Carbohydrates are sensed by most of EECs. In the presence of glucose, ghrelin secretion is suppressed and 5-HT, CCK, GIP and GLP-1 secretions are increased (Gribble & Reimann 2016, Muller et al 2015). Specifically, it was shown that GLP-1 secretion by L cells is driven by glucose uptake from SGLT1 transporter and fructose uptake by GLUT5 (Gorboulev et al 2012, Reimann et al 2008). Enterochromaffin cells (ECs) are able to sense glucose and fructose, and consequently, produce 5-HT, through the expression of different glucose transporters such as SGLT1, GLUT2, GLUT5 and the sweet taste receptor T1R2/T1R3 (Martin et al 2017a, Martin et al 2017b, Young et al 2018). In addition, K cells are also able to sense glucose via metabolism-dependent mechanism and produce GIP but, despite their ability to express GLUT5 on their surface, there are no evidence *in vivo* that GIP is produced by fructose presence in the lumen (Kuhre et al 2014, Parker et al 2009).

1.1.4 Adipose tissue control of energy balance

The adipose tissue, despite its simple cellular structure compared to other organs, is involved in different important functions such as storage, thermogenesis and endocrine capability, because of its ability to secrete different metabolically active components important for the energy homeostasis (Booth et al 2016). In humans and rodents, the two most important kinds of adipose tissues are: white adipose tissue (WAT) and brown adipose tissue (BAT).

The WAT is subcategorized based on its location: it can be found underneath the skin (subcutaneous) and all around the internal organs (mesenteric, retroperitoneal, perirenal, omental, and epididymal in rodents). The BAT is localized in the cervical-scapular zone in rodents, and in the clavicular-paravertebral zone in humans (Choe et al 2016). WAT is responsible for fat storage regulation in response to dietary fat supply. Excess of fat is stored in the adipocytes mainly as triacylglycerol (TAG). The synthesis or breakdown of TAG within the WAT is dynamic: they are assembled from lipoproteins into the adipocytes and hydrolyzed and released into the bloodstream as free fatty acids (FFAs) with different chain length and degree of saturation (detailed description of adipose tissue metabolism: paragraph 1.2) (Fernandez-Quintela et al 2007).

In the meantime, based on the fat deposition, adipose tissue growth and peripheric hormonal network, WAT releases specific hormones denominated adipokines. The adipokines are involved in the regulation of energy balance, metabolism and inflammation through the communication with the CNS and peripheric organs (Khan & Joseph 2014). Leptin and adiponectin are two hormonal adipokines involved in energy homeostasis (leptin description: paragraph 1.1.4.1). The production level of adiponectin, encoded by the gene *Adipoq*, is inversely correlated with adiposity, leptin levels and insulin resistance (Robinson et al 2011) and mainly affects liver, endothelial cells, blood vessels and skeletal muscle (de Oliveira Leal & Mafra 2013). In addition, other adipokines involved in inflammation are secreted by the adipose tissue, such as resistin, chemerin, apelin, visfatin, plasminogen activator inhibitor 1 (PAI1), monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor alpha (TNFα) and interleukin 6 (IL6) (Fasshauer & Blüher 2015).

BAT is a particular type adipose tissue able to dissipate energy (heat) to ensure correct thermoregulation, using glucose and fat as metabolic fuels. This ability relates to the fact that BAT adipocytes are enriched in mitochondria that express uncoupling protein 1 (UCP1). This protein, present in mitochondrial inner membrane, allows the production of non-shivering heat by uncoupling the fatty acids oxidation from adenosine triphosphate (ATP) synthesis in order to dissipate energy (Nicholls et al 1978, Zhang & Bi 2015). UCP1 expression depends on brown adipocytes state of differentiation and on the different peripheral induction or inhibition signals (Villarroya et al 2017). Despite the two different approaches, both BAT and WAT participate in the regulation and maintenance of energy balance.

1.1.4.1 Leptin

The gene encoding for leptin (i.e., *Ob*) was observed for the first time by chance in the Jackson Laboratory in 1950. Mice with a mutation in *Ob* gene, in homozygosis, manifested in obesity with rapid progression, increased energy intake, infertility, diabetes and neuroendocrine dysfunctions (Ingalls et al 1950). Later on, through the examination of two severely obese children, a homozygous frame-shift mutation in the gene encoding for leptin was identified. These individuals had a very low level of circulating leptin despite the large amount of fat mass. This finding is considered to be the first genetic evidence to establish leptin as an important regulator of energy balance in humans (Friedman 2019, Montague et al 1997). Leptin is a protein composed of 167 amino acids and is secreted into the bloodstream mainly by the WAT in proportion to the fat mass and based on the nutritional state. For instance, after a meal or during fasting, circulating leptin increase and decrease, respectively, and it is considered as a long-term acting energy balance-related signal (Ahima et al 1996). Additionally, leptin secretion can be controlled by insulin and glucocorticoids (Lewandowski et al 2001). Leptin was found both in circulation and in cerebrospinal liquid and, thanks to its ability to cross the BBB, is able to directly target the CNS, specifically the hypothalamic ARC (Peng et al 2015). Here, leptin binds the long form of the leptin receptor (LepRb), which belongs to the JAK-Stat family. LepRb is regulated by different signalling proteins such as a suppressor of cytokine signalling 3 (SOCS3). In the presence of leptin, SOCS3 is activated and, with a negative feedback

action, decreases the leptin signalling cascade (Mori et al 2004). Leptin signalling activates anorexigenic neurons expressing POMC and CART and inhibits orexigenic neurons expressing AgRP and NPY. Downstream signalling then results in a satiety effect (Park & Ahima 2015). In addition, leptin was reported to act on the hippocampus, where it seems to exert an antidepressant effect (Lu et al 2006). BAT is mainly innervated by the sympathetic nervous system (SNS) and it was shown that leptin signalling through the hypothalamus is able to increase BAT sympathetic tone, regulating thermogenesis (Enriori et al 2011). In addition, SNS activation by leptin modulates energy expenditure by elevating heart rate and arterial pressure (Haynes et al 1997, Shek et al 1998). Finally, leptin has a critical role in the control of glucose homeostasis and insulin sensitivity. For this reason, it is not surprising that the two metabolic diseases, obesity and diabetes, are very frequently related to each other (Amitani et al 2013). Obesity-related leptin dysfunctions and consequences are detailed in the paragraph 1.3.1.

1.1.5 Pancreatic control of energy balance

Energy homeostasis is also controlled by the endocrine compartment of the pancreas, called the islets of Langerhans. These areas are composed of different cell types, which are able to secrete different hormones: β-cells for insulin, α-cells for glucagon, δ-cells for somatostatin, ε-cells for ghrelin and PP-cells for pancreatic peptide (Mastracci & Sussel 2012). The exocrine portion of the pancreas, populated by acinar cells, plays a role in the digestion through the production of digestive enzymes (Pandiri 2014). In what follows, particular focus will be given to insulin.

1.1.5.1 Insulin

Insulin, together with leptin, is considered a long-term acting signal as its secretion is related to blood glucose level and to the adipose tissue mass within the body. Since 1886, it was noticed that a total pancreatectomy caused severe diabetes and, in the following years, insulin was identified as the hormone directly involved in this metabolic disease (Bliss 1993, Vecchio et al 2018). Insulin is encoded as pre-proinsulin, that becomes proinsulin after the cleavage of the signal peptide. Proinsulin undergoes other modifications by the action of endopeptidases and, after the formation of three disulphide bonds and the cleavage of C-peptide, insulin is complete and active (Fu et al 2013). During a meal, insulin is secreted into the bloodstream as a 51 amino acids dimer by β-cells of the pancreas in response to glucose uptake and through the action of the intestinal incretin hormones GLP-1 and glucose-dependent insulinotropic peptide (GIP) (Yabe & Seino 2011). Subsequently, insulin reaches its target organs (liver, adipose tissue, CNS and skeletal muscle) and binds insulin receptor (IR) (Escribano et al 2017). This activates a signal cascade, which promotes membrane expression of glucose receptors (GLUT) (Furtado et al 2002). For instance, insulin promotes the uptake of glucose into the adipocytes through the transporter GLUT4. Once in the adipocytes, glucose is converted to TAG by *de novo* lipogenesis (paragraph 1.2.2) (Smith & Kahn 2016). In the skeletal muscle, insulin promotes the conversion of glucose into glycogen and, in the liver, promotes the biosynthesis of TAG, through glycolysis and lipogenesis (Jensen et al 2011, Saltiel & Kahn 2001). Additionally, insulin crosses the BBB and interacts with areas in the CNS that express IR, such as the hypothalamus, cerebral cortex, cerebellum and the hippocampus. In the hypothalamus, insulin stimulates POMC neurons and inhibits NPY signalling in related neurons, inducing an anorexigenic response downstream (Benoit et al 2002, Schwartz et al 1996).

1.2 White adipose tissue metabolism: dietary fatty acids absorption, lipogenesis and lipolysis

1.2.1 Dietary fatty acids absorption

The digestion of dietary fat occurs in the duodenum (**Fig. 1.2**). Initially, dietary fats are present in the small intestine mainly as TAG and phospholipids, which are insoluble. The combined action of hepatic bile acids and pancreatic lipases solubilizes TAG in micelles. Subsequently, intestinal lipases break down TAG-soluble micelles into free fatty acids (FFAs) and monoacylglycerols (MAG) (Wang et al 2013). FFAs and MAGs are imported into the enterocytes by passive diffusion or by protein transporters (i.e., fatty acids transporter proteins, FATPs and cluster of differentiation 36, CD36) present on the brush border of the enterocytes. FFAs and MAGs uptake can occur along the entirety of the small intestine (Abumrad & Davidson 2012). Once in the cytoplasm of the enterocytes, acyl-CoA synthetase converts FFAs into the active form: FFA-CoA. Meanwhile, MAGs are converted into diacylglycerol (DAG). Subsequently, FFA-CoA and DAG are used to re-form TAGs (Mansbach & Nevin 1998). If the level of TAGs is high, they are temporary stored within the enterocytes as cytoplasmatic lipids droplets. Otherwise, from the cytoplasm, TAGs are translocated to the endoplasmic reticulum (ER) and, here, they are assembled with proteins and cholesterol and surrounded by phospholipids. These circular and hydrophilic structures, called chylomicrons, are transported through the Golgi to the basolateral side of the enterocytes and, then, secreted into the lymphatic system and into the bloodstream (Mansbach & Siddiqi 2010). Through the circulation, the chylomicrons reach the organs provided with fat storage or fat oxidation system, such as WAT, BAT, liver, skeletal muscle and the heart.
In this thesis, I will focus on fat uptake, lipogenesis and lipolysis by WAT.

1.2.2 White adipose tissue anabolism

Once chylomicrons and very-low-density lipoproteins (VLDLs; TAG-carrier structures that come from the liver) arrive in the blood vessels in proximity of WAT,

TAGs within those structures are hydrolysed into FFAs by an enzyme called lipoprotein lipase (LPL), which is anchored to the capillary endothelium. Long-chain fatty acids (LCFAs) are part of the FFAs released after LPL action. LPL is encoded and produced by WAT and its activity is very important for fat uptake regulation. In light of this, it is not surprising that LPL is finely regulated at transcriptional and post-transcriptional level (Kersten 2014). On the adipocyte membrane, several transporters involved in FFAs uptake into the cell are present. These are FATPs, CD36, plasma membrane fatty acid binding protein (FABPpm) and caveolin-1 (Thompson et al 2010). The FFAs in the adipocytes are re-converted into TAGs through diacylglycerol acyltransferase enzyme (DGAT), which causes the esterification of FFAs. By this process, TAGs are formed and stored within the adipocytes (Harris et al 2011) (**Fig. 1.2**). The FFAs from dietary fat are not the only FFAs source that results in TAG production within the adipocyte. FFAs can be synthetized also starting from carbohydrates through a metabolic process called *de novo* lipogenesis. Briefly, after a meal, insulin released from the pancreas binds IRs that promote adipocytes intake of glucose through GLUT4. In the cell, glucose is converted in acetyl-coenzyme A (acetyl-CoA) by glycolysis and tricarboxylic acid cycle (TCA cycle). Glycolytic metabolites activate carbohydrate response element-binding protein (ChREBP), which translocates into the nucleus and upregulates the expression of enzyme involved in the lipogenesis, such as ATP-citrate lyase (ACLY), acetyl-CoA carboxylases 1 (ACC1), fatty acid synthase (FASN) and stearoyl-CoA desaturase-1 (SCD1). All these enzymes contribute to the conversion of acetyl-CoA in FFAs (Song et al 2018). Together, FFAs from dietary fat and from *de novo* lipogenesis contribute to the TAG stock in the adipocytes, even if the first case is the most important in normal healthy conditions.

1.2.3 White adipose tissue catabolism

When the energy supply to the organs is not enough, for example during fasting, a metabolic process called lipolysis is activated in the WAT (**Fig. 1.2**). Lipolysis ensures TAGs breakdown into glycerol and FFAs, which will be transported through the bloodstream to the organs in energy deficit. During fasting, different hormones are in

charge of lipolysis activation, such as pancreatic glucagon and catecholamines from the SNS (Carmen & Victor 2006). The catecholamines stimulate β-adrenergic receptors (β-AR). These molecules enhance the production of adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3,5-cyclic monophosphate (cGMP), which activate cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG). In turn, the kinases promote the phosphorylation and, thus, the activation of the enzymes involved in the lipolytic cascade (Langin 2006). In order of action, the lipolytic enzymes are: adipose TAG lipase (ATGL), which converts TAG into diacylglycerol (DAG), hormone-sensitive lipase (HSL), which converts DAG into monoacylglycerol (MAG), and monoglyceride lipase (MGL), which converts MAG into FFAs and glycerol (Fredrikson et al 1986, Haemmerle et al 2002a, Zimmermann et al 2004). Other factors that can promote lipolysis are glucocorticoids, growth hormones and TNFα (Hansen et al 2002, Ryden et al 2002, Xu et al 2009). On the other hand, insulin production and action inhibits this process (Jensen & Nielsen 2007, Nielsen et al 2014).

In the adipose tissue, catabolism also includes oxidation of FFAs. This specific reaction occurs within the mitochondria in aerobic conditions. Through β-oxidation, FFAs (mainly LCFAs) transported within the adipocytes are metabolized into acetyl-CoA units (Houten & Wanders 2010). From the cytoplasm, FFAs are conjugated to a CoA group by fatty acyl-CoA synthase (FACS). At this point, carnitine palmitoyltransferase 1 (CPT1) converts FFA-acyl-CoA into FFA-acylcarnitine (Qu et al 2016). In this form, FFAs can translocate into the mitochondria. Within the mitochondrial inner membrane, carnitine palmitoyltransferase 2 (CPT2) re-converts FFA-acylcarnitine into FFA-acyl-CoA. This molecule undergoes different biochemical reactions that result in the production of acetyl-CoA. Subsequently, acetyl-CoA units join the TCA cycle, promoting the production of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) which, in turn, are used by electron transport chain (ETC) to produce the energy carrier ATP (Adeva-Andany et al 2019). In addition, ACC enzyme plays an important role in the regulation of FFAs β-oxidation. The ACC catalyses the reaction that converts acetyl-CoA into malonyl-CoA, which is an intermediate of FFA biosynthesis described in the

paragraph 1.2.2.. Moreover, malonyl-CoA can interfere with β-oxidation of FFAs by inhibiting CPT1 (Munday & Hemingway 1999).

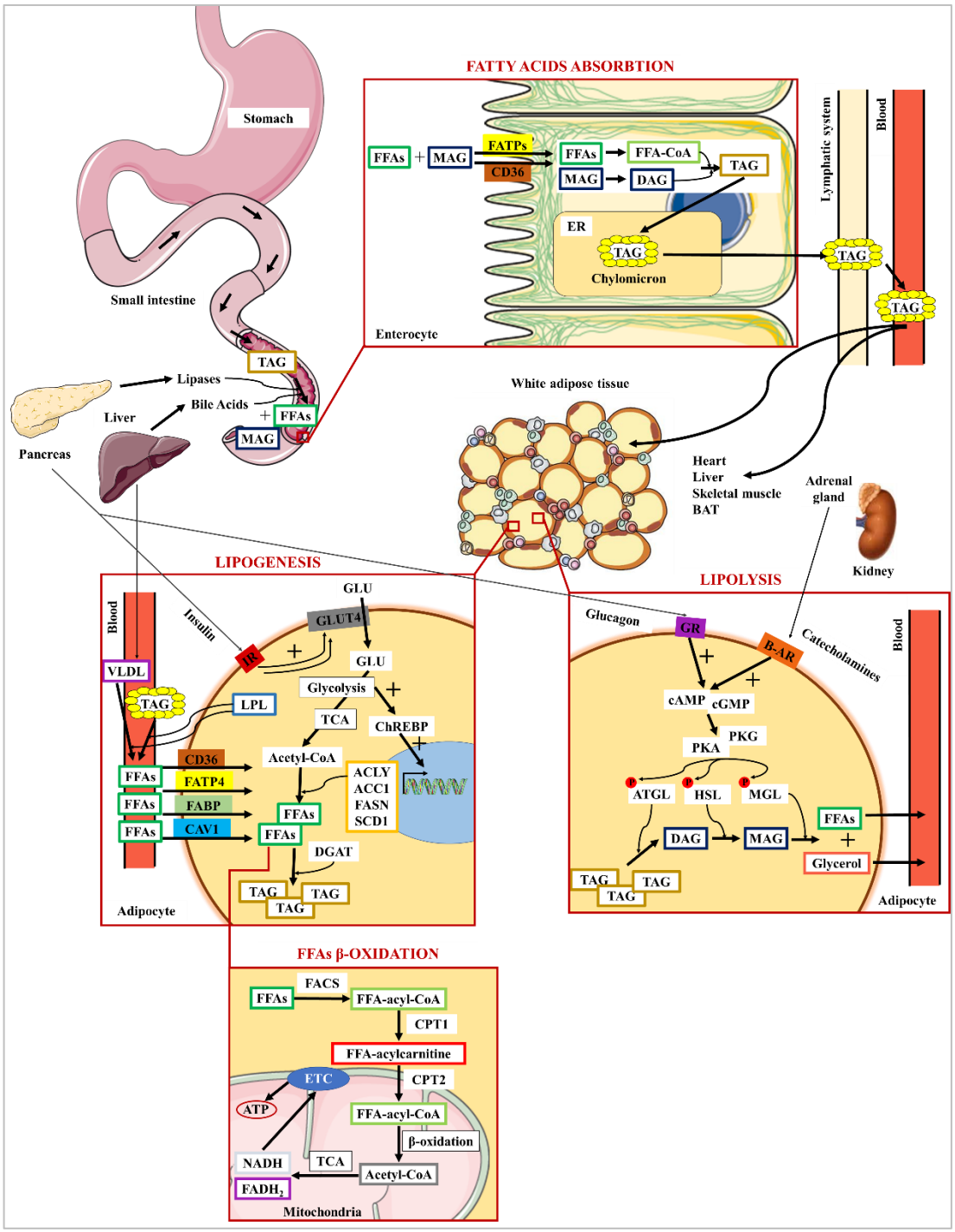


Figure 1.2 White adipose tissue metabolism. *In the small intestine, dietary TAGs are broken down into FFAs and MAG by the action of hepatic bile acids and pancreatic lipases.* ***FAs absorption:*** *FFAs and MAG are transported inside the enterocytes by FATPs and CD36*

transporters. Here, FFAs and MAG are converted into FFAs-CoA and DAG, respectively. Both FFAs-CoA and DAG are re-converted into TAGs and translocated into ER, in which they are assembled into chylomicrons. Chylomicrons are secreted into the lymphatic system and then into the bloodstream, by which they can reach different organs and tissues. ***Lipogenesis:*** *in the blood vessels that irrorate the adipose tissue, both chylomicrons and VLDV (from the liver) are converted into FFAs by LPL, an enzyme produced by the adipocytes. These FFAs are translocated into the adipocyte by CD36, FATP4, FABP, CAV1 transporters. FAAs within the adipocytes can derive from de novo lipogenesis in the presence of carbohydrates. Insulin binds IRs that promote the adipocytes' intake of glucose through GLUT4. In the cell, glucose is converted in acetyl-CoA by glycolysis and TCA cycle. Glycolytic metabolites activate ChREBP, which upregulates the expression of ACLY, ACC1, FASN and SCD1. All these enzymes contribute to the conversion of acetyl-CoA in FFAs. Both dietary- and de novo lipogenesis-derived FFAs can be converted into TAGs by DGAT or they undergo β-oxidation.* ***FFAs β-oxidation:*** *FFAs are conjugated to a CoA group by FACS. Then, CPT1 converts FFA-acyl-CoA into FFA-acylcarnitine. In this form, FFAs can translocate into the mitochondria. Within the mitochondrial inner membrane, CPT2 re-converts FFA-acylcarnitine into FFA-acyl-CoA. This molecule undergoes different biochemical reactions that result in the production of acetyl-CoA. Subsequently, acetyl-CoA units join the TCA cycle and the NADH and FADH2 produced are used by ETC to produce ATP*. ***Lipolysis:*** *it is stimulated by glucagon and catecholamines binding to their receptors, GR and β-AR, respectively. This enhances the production of cAMP and cGMP, which activate PKA and PKG. In turn, the kinases promote the phosphorylation and, thus, the activation of ATGL, which converts TAG into DAG, HSL which converts DAG into MAG, and MGL which converts MAG into FFAs and glycerol.*

<u>Abbreviations (in order of appearance)</u>: TAG, triacylglycerol; FFA, free fatty acid; MAG, monoacylglycerol; FA, fatty acid; FATPs, fatty acid transporters; CD36, cluster of differentiation 36; CoA, coenzyme A; DAG, diacylglycerol; ER, endoplasmic reticulum; BAT, brown adipose tissue; VLDL, very low-density lipoprotein; LPL, lipoprotein lipase; FABP, plasma membrane fatty acid binding protein; CAV1, caveolin-1; GLUT4, glucose transporter 4; ChREBP, carbohydrate response element-binding protein; ACLY, ATP-citrate lyase; ACC1, acetyl-CoA carboxylases 1; FASN, fatty acid synthase; SCD1, stearoyl-CoA desaturase-1; DGAT, diacylglycerol acyltransferase; FACS, fatty acyl-CoA synthase; CPT1 and 2, carnitine palmitoyltransferase 1 and 2; NADH, nicotinamide adenine dinucleotide; $FADH_2$, flavin adenine dinucleotide; ETC, electron transport chain; ATP, adenosine triphosphate; GR, glucagon receptor; β-AR, beta-adrenergic receptor; cAMP, adenosine 3',5'-cyclic monophosphate; cGMP, guanosine 3,5-cyclic monophosphate; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; ATGL, adipose TAG lipase; HSL, hormone-sensitive lipase; MGL, monoglyceride lipase.

1.3 Obesity: a metabolic and endocrine dysregulation of energy balance

Obesity is a chronic, relapsing, progressive disease. It is characterized by genetic or diet-induced dysfunctions in several tissues, including the adipose tissue, the gut and the hypothalamus (Halsted 1999, Williams 2012) (**Fig. 1.3**). As there hasn't been a substantial evolutionary pressure to change the human gene pool in the last century alongside the gradual rise in prevalence of obesity, there is a growing recognition that, instead, the change in the dietary habits and how they impact on the existing gene pool, underlie the basis for the obesity problem. This simplistic view is further complicated by the discovery that diet induced changes in the composition of the gut microbiota may also impact on our ability to regulate energy balance. The reason why not all humans develop obesity in the current obesogenic environment appears to be due to the inter-individual variation (polymorphisms) in the existing genes and how they respond to the diet and the microbiota.

1.3.1 Obesity-induced adipose tissue dysfunction and leptin resistance

The physiological and endocrine functions of the adipose tissue are highly compromised during obesity. High fat mass affects centre of gravity, posture and balance of the body, leading to an exaggerated lumbar spine curve, difficulties in movements and asymmetries that predispose to injuries (Maciałczyk-Paprocka et al 2017). High amount of adipose tissue also causes organ compression, which leads to high pressure and, because of an enlargement of adipocytes, bringing about hypoxia. For these reasons, the adipose tissue in obese individuals is more susceptible to inflammation and insulin resistance (Ye 2009) (**Fig. 1.3**). Once the adipose tissue becomes saturated with fat, the excess fat gets deposited ectopically in other tissues including the liver, heart and skeletal muscle. Ectopic fat deposition exacerbates the disease by increasing the risk of hepatic steatosis and by decreasing muscular oxygen metabolism, followed by loss of muscular strength (Snel et al 2012). In addition,

during obesity, BAT activity and mass decrease (Seale & Lazar 2009). It has been shown that a short-term HFD exposure is sufficient to induce BAT whitening (i.e., BAT transition in a WAT-like phenotype), which implies a reduction of mitochondrial oxidation and dynamics, together with an increase of inflammation (Kotzbeck et al 2018, Kuipers et al 2019) (**Fig. 1.3**). In light of this, a rational approach to combating obesity can be through the restoration of BAT activity (Seale & Lazar 2009).

1.3.1.1 White adipose tissue lipogenesis dysregulation

In a situation of excess energy, the morphology, size and organization of adipocytes change. Higher fat intake, as expected, causes a higher TAG accumulation within the adipocytes, which become hypertrophic. In turn, they secrete more paracrine factors that induce the transit from premature adipocytes to mature adipocytes, causing an increase of the adipose mass and, thus, an adipose tissue remodelling (Choe et al 2016, Pellegrinelli et al 2016). This situation over long-term can lead to ectopic fat deposits in other organs, exacerbating metabolic syndrome. It was demonstrated that during HFD feeding, the FFA uptake into the adipocytes from the circulation increases, together with a dysregulation of factors involved in lipid uptake, such as FABP and FATP1 (Berk et al 1999, Wu et al 2006). In parallel, an upregulation of DGAT enzyme was observed (Tinahones et al 2010). With the abnormal increase of TAG storage within the adipocytes during HFD feeding, it is not surprising that *de novo* lipogenesis is, in turn, affected. Indeed, obesity is characterized by an inhibition of *de novo* lipogenesis, mainly through a downregulation of ACC. This phenomena is even more evident if obesity is accompanied by insulin-resistance (Marcelino et al 2013, Tinahones et al 2010).

1.3.1.2 White adipose tissue lipolysis and β-oxidation dysregulation

HFD and obesity lead to an imbalance in lipogenesis and, also, in lipolysis rate. In rodents, HFD feeding showed a decrease in the lipolysis within the adipose tissue (Tepperman et al 1986). The same scenario was observed in obese individuals (Arner et al 2011). In contrast, an increase in the lipolytic rate of the subcutaneous abdominal adipose tissue has been shown in obese humans consuming HFD (Howe et al 2011).

This excess of FFAs released from the adipose tissue was linked with ectopic fat deposition and insurgence of insulin-resistance and non-alcoholic fatty liver diseases (NAFLD) (Samuel & Shulman 2012). Accordingly, targeting lipolysis with compounds able to inhibit enzymes involved in this metabolic process, such as ATGL, can ameliorate HFD-induced complications (i.e., insulin-resistance and NAFLD) (Schweiger et al 2017). However, genetic deletion of ATGL, HSL and MGL lead to different physiological outcomes, making the role of lipolysis in the pathogenesis of obesity still unclear. For instance, a lack of ATGL and HSL caused an exacerbation of obesity compliance (Haemmerle et al 2006, Haemmerle et al 2002b). Conversely, lack of MGL attenuated HFD-induced insulin-resistance (Taschler et al 2011). In light of this, further investigations are required to fully understand the role of lipolysis during different phases and degrees of metabolic disorders.

On the other hand, it is well established that WAT β-oxidation is enhanced during obesity (Serra et al 2013). An increase in β-oxidation rate during HFD feeding was also observed in skeletal muscle and the heart. This is due to an upregulation of FATP, CD36 and PPARγ gene expression (Cameron-Smith et al 2003, Sikder et al 2018). An enhancement of β-oxidation causes electron transport chain overactivation, which results in a surplus of ATP and reactive oxygen species (ROS). The oxidative stress induced, due to ROS increase, leads to mitochondrial disfunction and inflammation. In general, HFD-induced derangements lead to metabolic syndrome exacerbation, mainly causing insulin-resistance (Lee et al 2015, Serra et al 2013).

1.3.1.3 Leptin resistance

In addition to an adipose tissue dysfunction, a deregulation of hormone production also occurs during obesity. One of the most important hormones in the pathogenesis of obesity is leptin (**Fig. 1.3**). In humans and rodents without mutations in *Ob* gene, the concentration of circulating leptin correlates with body mass index (BMI): higher BMI corresponds to a higher circulating leptin (Maffei et al 1995). A chronic high leptin level in the circulation might results in leptin resistance phenomena, which leads to a further aggravation/exacerbation of obesity and other metabolic complications. Myers and colleagues defined leptin resistance as “the failure of endogenous or exogenous leptin to promote anticipated salutary metabolic outcomes in states of over-

nutrition or obesity, although the hormone's inability to promote desired responses in specific situations results from multiple molecular, neural, behavioural, and environmental mechanisms" (Myers et al 2012). Leptin resistance can have different causes other than hyperleptinemia such as chronic inflammation, ER stress and defective autophagia within the hypothalamus. Many mechanisms seem to be involved in the development of leptin resistance. Among those, the most important are: decrease of leptin transport across the BBB, impairment in LepRb signalling cascade components (which lead to a downregulation or, in general, a desensitization of LepRb) and deficiency or impairment downstream signalling components including melanocortin system (which orchestrate the hypothalamic neural circuit in charge of the control of energy balance) (Zhou & Rui 2013).

Many studies have been carried out, both in humans and rodents, with the aim to assess the efficacy of leptin injection for treating obesity in individuals with leptin deficiency. The results are contrasting: in some studies no amelioration in hyperphagia and body weight loss in *Ob/Ob* mice was observed upon leptin administration and, in others, there are evidences of energy balance and body weight improvement in mice and humans (Cummings et al 2011, Dardeno et al 2010, Hamann et al 1997, Heymsfield et al 1999, Mistry et al 1997). Thus, it is still unclear if leptin can be employed for obesity therapy.

A very recent study carried out using several mutant murine models and anti-leptin antibodies showed that a partial reduction in circulating leptin levels can restore hypothalamic leptin sensitivity with a long-term reduction in weight gain, with further improvement of insulin sensitivity. Moreover, it was confirmed that hyperleptinemia is one of the main driving forces for metabolic disorders (Zhao et al 2019). This can be another starting point for the development of new therapeutic strategies in the treatment of obesity and associated metabolic disorders.

1.3.2 Obesity-induced gastrointestinal dysfunction

1.3.2.1 Gut hormone dysfunction

Part of the comorbidities that come from obesity are due to a dysregulation of the hormones produced by the GIT, in particular those involved in the control of energy balance, glucose homeostasis and appetite (**Fig. 1.3**). Circulating levels of PYY were found to be inversely correlated with markers of adiposity. In obese individuals, both the basal fasting levels of PYY_{3-36} and its postprandial response after meals were significantly lower in obese subjects relative to lean ones. This led to a reduction of satiety (Karra et al 2009). Chronic PYY administration in rodents, as well as transgenic mice that overexpressed PYY, decreased body weight and caused resistance to HFD-induced obesity (Boey et al 2008, Chelikani et al 2007, Vrang et al 2006).

Similar to PYY, CCK satiety-signalling and sensitivity to lipids is disrupted during HFD feeding. In these animals, a significant downregulation of CCK receptors was observed in a specific zone of the vagus nerve called the nodose ganglia (Covasa et al 2000, Duca et al 2013). Furthermore, HFD interferes with the capacity of CCK to induce gastric emptying (Covasa & Ritter 2000). Unlike for PYY, therapeutic approaches targeting CCK signalling were not successful and there were no advances in clinical practice (Kim et al 2011).

Regarding GLP-1, how it changes during HFD feeding is still unclear since different studies are in disagreement with each other. In human subjects, it was reported that postprandial GLP-1 was lower in obese/overweight individuals compared to normal weight individuals (Adam & Westerterp-Plantenga 2005, Verdich et al 2001). Conversely, in another study, postprandial GLP-1 was higher in obese versus lean subjects (Bowen et al 2006a). Despite this discrepancy, liraglutide, a GLP-1 receptor agonist has been tested in different clinical studies that demonstrated its efficacy in the short-term reduction of body weight by 10% and in the improvement of insulin secretion (Mehta et al 2017).

Unexpectedly, ghrelin was found to be lower in obese humans and rodents (Gomez et al 2012, Tschöp et al 2001). In mice fed with HFD, a lower expression of *Ghsr* was observed in both the hypothalamus and vagus nerve nodose ganglion, together with higher inflammation in the same sites. This is a possible cause of ghrelin signalling disruption during HFD-induced obesity (Naznin et al 2015). During diet-induced weight loss, ghrelin resistance is reversed and its circulating levels are higher than during a normal situation. This possibly suggests that the brain senses a reduced food availability or the rapid loss of body weight (negative energy balance) and responds accordingly by enhancing the ghrelin system to prevent further weight loss. Ghrelin may take months to return to a normal level after weight loss (Zigman et al 2016).

1.3.2.2 Nutrient absorptive defects within the gut

Diets enriched in fat affect intestinal fat absorption (**Fig. 1.3**). During HFD consumption in rats, an elongation of the intestinal villi was observed, which caused an increase of the intestinal absorptive surface (Thomson et al 1986). In humans, the gene expression of fatty acids transporters, such as FATP4 and CD36, were upregulated and, additionally, the expression of genes involved in re-conversion of FFAs into TAG within the enterocytes were dysregulated in the presence of HFD (Tremblay et al 2013). Moreover, HFD causes the upregulation of factors involved in chylomicrons synthesis and release (Hernández Vallejo et al 2009).

In other studies, it has been observed that obesity causes an elevated abundance of dietary fat availability in the enterocytes, which interfere with nutrient transporter expression (Uchida et al 2011, Zhou et al 2018). Accordingly, HFD exposure in rodents decreases the abundance of sodium glucose transporter protein (sodium-dependent glucose transporter, SGLT1), cluster of differentiation 36 (fatty acids importer, CD36), peptide transporter 1 (PEPT1), Niemann-Pick C1-Like 1 (sterol transporter, NPC1L1) and glucose transporter 2 (GLUT2) (Betters & Yu 2010, Cifarelli & Abumrad 2018, Losacco et al 2018, Roder et al 2014, Wang et al 2017). At the gene expression level, it was shown that genes encoding CD36 and other lipid transporters are overexpressed during HFD feeding, creating a discrepancy between protein and gene expression level (Primeaux et al 2013, Torelli Hijo et al 2019). The

mechanism that regulates these changes and the explanation of the gene expression-protein disconnection is still to be elucidated.
Moreover, many studies carried out in obese patients showed that these individuals are deficient of several micronutrients (D, B12 vitamins, minerals and proteins) (Daniel et al 2015, Lespessailles & Toumi 2017, Via 2012). In conclusion, obesity status and long-term exposure to fat-enriched foods lead to an alteration of nutrient transporters density, as well as a malabsorption of micronutrients, which are contributors to the progress of the disease.

1.3.3 Obesity-induced inflammation

Another consequence that comes from a long-term HFD exposure is a systemic chronic low-grade inflammation. The organs that are more likely to be inflamed are the hypothalamus within the CNS, together with the liver, intestine, adipose tissue and skeletal muscle in the periphery (**Fig. 1.3**). The excess of adipose tissue during obesity status causes the infiltration of activated immune cells which, once in the tissue, overproduce proinflammatory cytokines that are able to attract other immune cells, enhancing the inflammatory response. This status, which often proceeds silently, can cause the development of other chronic diseases that threaten the health and longevity of the individuals, exacerbating obesity condition (Duan et al 2018).
In this thesis, the focus is mainly on intestinal and adipose tissue inflammation.

1.3.3.1 Adipose tissue inflammation

Other than adipocytes, adipose tissue contains stromal and vascular cells, such as inflammatory cells, vascular endothelial cells and fibroblasts (Murawska-Cialowicz 2017). In obesity, the main cause of increased adipose mass is adipocyte enlargement. In this situation, the extracellular space between adipocytes, where the other resident cell types and vasculature are present, decreases, thus provoking hypoxia. Chronic hypoxia causes cell death and fibrosis, together with an activation of the resident immune cells. These cells increase the production of cytokines, also considered as adipokines (paragraph 1.1.4) (Lee et al 2014). In particular, in HFD-induced obese

rodents, an increase in IL6, TNFα and MCP-1 was observed (Morin et al 1997, Poret et al 2018). To aggravate the situation, macrophages infiltration within the tissue was observed in both rodents and humans, which contributes to the humoral mediators and cytokine production (Hotamisligil 2006). Obesity-related macrophage infiltration has been shown several times through adipose tissue histological staining (immunohistochemistry), flow cytometry and by CD68 gene expression. CD68 is a protein highly expressed by the macrophages (Morgan-Bathke et al 2017, Todoric et al 2006). The recruitment of macrophages in the adipose tissue is abolished when IL6-trans-signalling is blocked in mice, confirming the chemotactic properties of IL6. However, IL6 blockage did not prevent the onset of inflammation-induced insulin resistance (Kraakman et al 2015). Despite increased MCP-1 in obesity, disruption of MCP-1 signaling did not confer resistance to diet-induced obesity (DIO) in mice or reduce adipose tissue macrophage infiltration (Chen et al 2005). In contrast, Kanda *et al* showed that MCP-1 homozygous KO mice extensively reduced insulin resistance, hepatic steatosis, and macrophage accumulation in adipose tissue induced by HFD (Kanda et al 2006). These findings might suggest that MCP-1 is an important contributor to HFD-induced macrophage infiltration but is not the only one involved. Thus, a strategy to efficiently block HFD-induced macrophages infiltration within the adipose tissue might be to simultaneously disrupt the signaling of different cytokines and chemokines.

1.3.3.2 Intestinal inflammation

The GIT is the part of the anatomy that is most exposed to dietary components, and it is not surprising that diets enriched in fat have a direct effect on this site. A prolonged exposure to HFD has been shown to impact on the inflammatory status of the intestine. More specifically, HFD causes a decrease in differentiation of goblet cells, which are particular cells located on the surface of the respiratory and intestinal epithelium (Gulhane et al 2016). Their main function is to secrete mucus that contain mucin, anti-microbial proteins, cytokines and chemokines, which protect the intestinal barrier from luminal microbes. In addition, goblet cells have the ability to deliver luminal substances to antigen-presenting cells (APCs) present in the lamina propria of the epithelium and, in this manner, they contribute to the modulation of the immune

response (Knoop & Newberry 2018). Additionally, a significantly higher expression of cytokines such as interleukin 1 beta (IL1β), TNFα and interleukin 17 alpha (IL17α) was observed within the intestinal epithelium after both, short- and long-term HFD feeding (Ding et al 2010, Gulhane et al 2016). High expression level of TNFα in the intestine has been associated with weight gain and increase of glucose and insulin in the bloodstream (Ding et al 2010).

1.3.4 Obesity-induced insulin resistance

A chronic inflammation status, created by a long-term energy disequilibrium, facilitates the development of insulin resistance. The overactivation of inflammatory pathways within the adipose tissue causes a defect in TAG storage and stimulates FFA release. This FFAs excess contributes to insulin resistance within the liver and the muscle (Guilherme et al 2008). Additionally, inflammation can cause the development of insulin resistance through other mechanisms, particularly in the adipose tissue and in the liver (**Fig. 1.3**). Obesity-induced low-grade inflammation is able to inhibit IR and its signalling pathway, as well as the activity of peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor, the main role of which is to regulate the transcription of genes involved in glucose homeostasis, fatty acids biosynthesis and fat storage (Gayet et al 2007, Kubota et al 2017). However, during obesity, insulin resistance can be triggered by inflammation-independent mechanisms. The excess of lipids causes an overactivation of fatty acid β-oxidation within the mitochondria which, in turn, leads to extra energy availability (ATP). This energy surplus activates a negative feedback that aims to reduce ATP levels by inhibiting insulin-induced glucose uptake (Ye 2013). Finally, insulin resistance can come from an imbalance between insulin production and insulin clearance that results in hyperinsulinemia, even in fasting conditions. This imbalance is facilitated by leptin dysfunction (Corkey 2012).

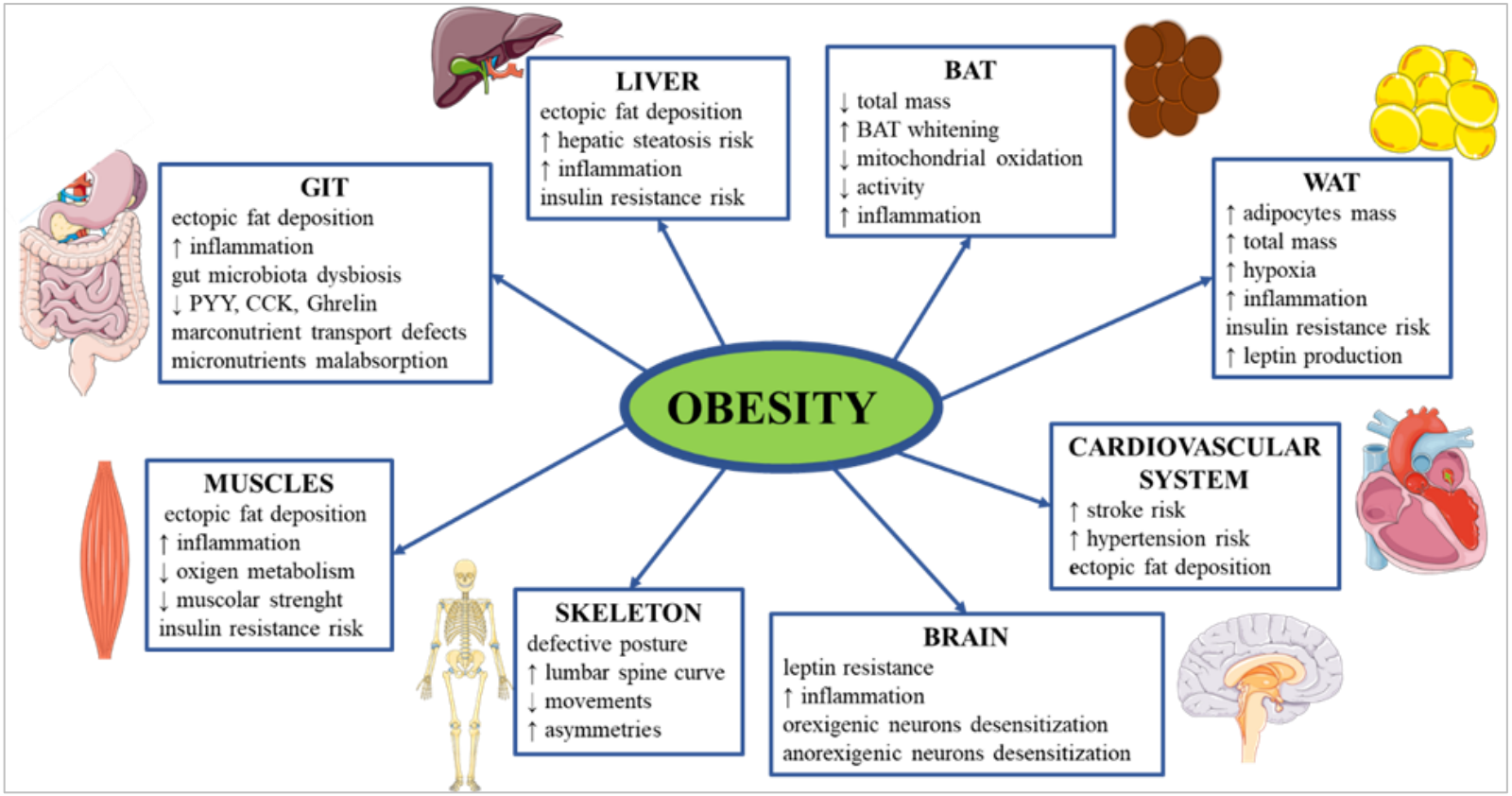


Figure 1.3 Obesity-induced multi-organ dysfunctions. *Obesity-induced liver, BAT, WAT, cardiovascular system, brain, skeleton, muscles, and GIT dysfunctions. Abbreviations: BAT, brown adipose tissue; WAT, white adipose tissue; GIT, gastrointestinal tract; PYY, peptide YY; CCK, cholecystokinin; ↑, increase; ↓, decrease.*

1.3.5 Obesity-induced hypothalamic energy balance-related signaling defects

Obesity or HFD exposure results in hypothalamic inflammation. Both in humans and in rodents, a diet enriched in fats increases the expression of proinflammatory cytokines within the hypothalamus, with IL6, TNFα and nuclear factor kB (NF-kB) considered to be most important (Thaler et al 2012). A chronic hypothalamic inflammation is one of the main causes of leptin and insulin resistance (Milanski et al 2009). This is partially due to a dysregulation of AgRP neuron signalling which, additionally, causes hyperphagia and impairment of POMC neurons to sense glucose sensing capacity (Parton et al 2007, Tsaousidou et al 2014). In addition, HFD induces the expression of SOCS3, which has an inhibitory activity of LepRb. This causes a drop in responsiveness of anorexigenic neurons following leptin and insulin stimulation (Ueki et al 2004). An impairment in Ca^{++} balance, important for mitochondrial functionality, has been observed in POMC neurons during HFD feeding. This leads to POMC neuronal desensitisation (Paeger et al 2017). Recently, it has been shown that a short-term HFD exposure causes a dramatic change in the hypothalamic proteome, which indicates an increase in cellular stress and derangements in synaptic plasticity and mitochondrial dysfunction (McLean et al 2019).

In conclusion, obesity causes derangements of hypothalamic energy balance control system through inflammation and orexigenic and anorexigenic neuronal desensitization.

1.4 Gut microbiota: characteristics, functions and role in the pathogenesis of obesity

Over the last 20 years, the scientific community has investigated the composition and the function of the gut microbiota in a greater depth than ever before. The trillions of microbes that inhabit our gut, together with its genetic repertoire (Qin et al 2010b, Tierney et al 2019), has been recently proposed to represent an organ in recognition of the wide variety of essential functions that it performs. The composition of the gut microbiota is characterized by a wide inter-individual variation, although there are a conserved set of metabolic functions that are shared between individuals known as the "core gut microbiome" (Turnbaugh et al 2009). This suggests that the overall functionality of the gut microbiota is of more importance to the host than its composition. Modulation of the host immune system, protection against opportunistic pathogens, metabolism of some xenobiotic and drugs and regulation of the harvest, storage and expenditure of energy obtained from the diet are among the most important functions encoded by the core gut microbiome (Jandhyala et al 2015). These functions are essential for the correct functionality of the gut. For this reason, it is not surprising that a chronic perturbation to the composition and function of the normal gut microbiota have been frequently associated with the pathogenesis of several gastrointestinal diseases such as obesity and inflammatory bowel disorders (Delzenne et al 2019, Harsch & Konturek 2018, Nishida et al 2018). In addition, in the last few years, it has been established that there exists a communication link between the gut microbiota and the CNS through neural, endocrine and immune pathways important for brain function and behaviour (Cryan & Dinan 2012b). Therefore, new treatments that focus on the selective modulation of the gut microbiota have been considered recently as a means to address metabolism-related diseases and mental disorders.

Bacterial colonization of the GIT occurs immediately after birth, but it is only after 2 years of age that this becomes more stable, remaining relatively consistent until the elderly phase of life. The stable adult gut microbiota is primarily comprised of bacterial species that are members of the Firmicutes and Bacteroidetes phyla, with the remaining species being predominantly members of the Actinobacteria,

Verrucomicrobia, Proteobacteria, Fusobacteria and/or Cyanobacteria phyla (Qin et al 2010a). The factors that most considerably influence gut microbiota composition throughout life include the method of delivery at birth, genetic makeup of the host, diet, antibiotic usage and ageing (Wen & Duffy 2017). Among these factors, diet is particularly important. Depending on the kind of diet consumed (i.e., quantity and type of carbohydrates, fats, proteins and fibres), the composition and functionality of the gut microbiota can be strongly affected. For example, diet-induced changes in the colonic gut microbiota responsible for the fermentation of carbohydrates and proteins results in the altered production of short-chain fatty acids (SCFAs) under anaerobic conditions (paragraph 1.4.2.1). The SCFAs, of which butyrate, propionate and acetate are the major components produced by the gut microbiota, can influence host metabolism by acting as an energy source, by influencing hosts' energy intake (Morrison & Preston 2016, Ríos-Covián et al 2016) or by exerting anti-inflammatory effects on the host (Li et al 2018, van der Beek et al 2017). Thus, modulation of the gut microbiota by diet may provide a way to alter the host metabolism and, in turn, improve the metabolic deregulation seen in people with metabolic diseases. This, however, requires a more detailed understanding of the role of the gut microbiota in metabolic diseases and its interaction with the diet.

1.4.1 How to analyse the microbiome? 16S rRNA-based and shotgun metagenomics: a brief description

The emergence of the human microbiome (defined in **Table T1.2**) as a hot topic in science has been facilitated by the development of new methods that allow the detailed analysis thereof. For this purpose, the use of classical microbiological methods to classify and study the morphology and biochemistry of the microorganisms, such as Gram stain and culture-dependent techniques with different media, was not practical as many of the microbes within the gut cannot be cultured. Thus, the scientific community started to adopt culture-independent techniques, which involve in the extraction of DNA from the whole microbial community within a sample (referred to metagenomic DNA) and the determination of its composition and diversity and/or

microbial functional potential through high throughput DNA sequencing and subsequent bioinformatic analysis (Morgan & Huttenhower 2012). This relied on the use of the two most commonly used techniques, i.e., 16S rRNA-based and shotgun metagenomics (defined in **Table T1.2**). These approaches have been applied to the analysis of multiple microbial communities, such as those in human saliva, skin, lung and stool. In this thesis, I will focus on faecal samples only. During this process, detailed protocols describing how to collect samples for microbiota analysis have been developed in order to avoid contamination and ensure reliability (Kumar et al 2014, Ogai et al 2018, Wang et al 2018b).

Table T1.2 Gut microbiota-related terminology and definitions *(Behjati & Tarpey 2013, Koboldt et al 2013, Marchesi & Ravel 2015, Thomas et al 2012, Whittaker 1960, Willis 2019).*

	Definition
Metagenome/Metagenomics	Metagenome is the collection of genomes and genes present in a sample. Metagenomics is the analysis of the genomes contained in a sample.
Microbiome	Entire habitat, including all the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses) and their genomes, present in a sample.
α-diversity	The number (richness) and the distribution (evenness) of taxa measured within a population (sample).
β-diversity	The amount of variation of taxa measured between populations (samples).
Operational taxonomic units (OTUs)	Cluster of sequences similar at the sequence level beyond some threshold. Thresholds of 95% and 97% of similarity are used in place of genus and species, respectively.
Next generation sequencing (NGS)	Different modern DNA and RNA sequencing technologies. They are more efficient than older techniques (such as Sanger method), thus causing a revolution in genetics and molecular biology fields. NGSs adopt a high-throughput sequencing approach.
Taxonomic rank	Domain (Domains) Kingdom (Kingdoms) Phylum (Phyla) Class (Classes) Order (Orders) Family (Families) Genus (Genera) Species (Species)

1.4.1.1 16S rRNA metagenomics

Bacterial genomes generally consist of a single circular DNA molecule, typically under 10Mb in length, with smaller stretches of DNA contained on smaller circular plasmids (Bobay & Ochman 2017). Bacterial genomes contain marker genes, which allow one to distinguish different bacterial members within a population. Among them, the most ubiquitous and significant one is the small 16 ribosomal RNA subunit gene (16S rRNA gene). This particular gene, which is approximately 1500 bp long, is characterized by 9 separate hypervariable regions, called V1-V9 (Van de Peer et al 1996). For 16S rRNA-based microbiota analysis, the hypervariable regions V1-V5 are most frequently used. Once bacterial DNA is extracted from samples and quantified, it is possible to create a 16S rRNA metagenomic library. This involves the completion of two PCR steps, required to amplify a region of the 16S rRNA using two highly conserved binding sites at either end of the variable region of interest and apply a sample-specific bar code at the extremity of each amplicon (Nurul et al 2019) (**Fig. 1.4**). The 16S rRNA library is sequenced using different sequencing platforms, such as, Illumina MiSeq, Ion Torrent PGM, PacBio Sequel or Oxford Nanopore MinION. The sequencing platform choice depends on the sequencing depth, read length and number of samples required and the error rates that can be tolerate (Allali et al 2017). Subsequently, the reads are analysed using bioinformatic approaches that utilise different databases and tools, which are not detailed in this thesis. The outputs that result from the bioinformatic analysis are: taxonomical classification assignment (down to genus and OTU level; species level assignment is generally unreliable), α- and β- diversity at all the taxonomic levels and an inference of bacterial function profile (only an estimation through tools such as PiCrust) (Jovel et al 2016) (**Table T1.3**). Definitions of OTU, α- and β- diversity and taxonomic rank are detailed in **table T1.2**.

Table T1.3 16S rRNA-based metagenomics vs shotgun metagenomics *(Callahan et al 2016, Jovel et al 2016, Langille et al 2013).*

	16S rRNA metagenomics	Shotgun metagenomics
Costs	Low	High
Taxonomic resolution	OTUs, no detection of species or strains	Species and strains detected
Detection power	Only bacteria are detected	Bacteria, viruses, plasmids and fungi are detected
Functional profiling	No. Only an estimation of the microbial functions is applicable	Detailed functional profile and novel genes recognition
Lowest DNA input allowed	≤ 5 ng/μL	≤ 0,2 ng/μL
Computational power	More consistent and reliable tools available.	Tools are very variable among each other.
False positive	Low	High

1.4.1.2 Shotgun metagenomics

The shotgun metagenomic approach does not target a specific gene within the bacterial genome but, instead, the whole genetic information contained in each sample is considered. The whole metagenome present within the microbial community is fragmented into small pieces of different length (through tagmentation or other approaches) (Quince et al 2017a). In contrast to 16S rRNA sequencing, this includes DNA from phages, viruses, bacteria, archaea, fungi and other eukaryotes genomes. Every fragment is bar coded and sequenced in an independent manner (**Fig. 1.4**). The reads are analysed using bioinformatic approaches that utilise different databases and tools packages, which are not detailed in this thesis (Morgan & Huttenhower 2012). In general, all the reads that result from the sequencing are aligned with DNA sequence databases. Consensus sequences will be informative with respect to the taxonomic profile and the functions encoded in the genome (Quince et al 2017b, Sharpton 2014). The outputs that result from the bioinformatic analysis are several and more complex compared to 16S rRNA-based approach. It is possible to detect taxonomical classification assignment (down to species and strain levels), α- and β- diversity at all the taxonomic levels and reliable data describing the functions (such as of metabolic and biochemical potential) characteristic of a sample. In addition, is possible to

recognise and annotate new genes (Ekblom & Wolf 2014, Morgan & Huttenhower 2012) (**Table T1.3**).

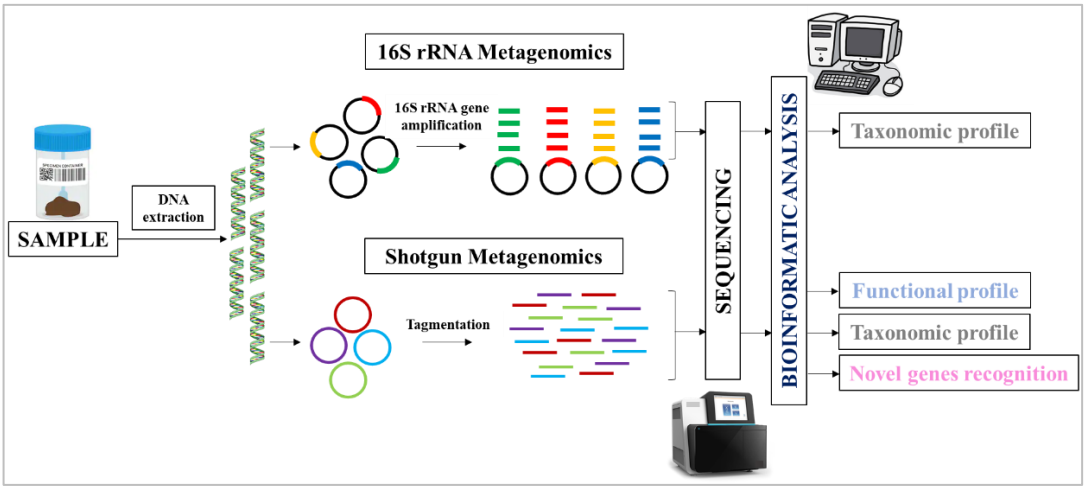


Figure 1.4 16S rRNA-based and shotgun metagenomics: methodology overview. *DNA is extracted from a sample, such as stool. For the 16S rRNA-based approach, a region of the 16S rRNA is amplified using two highly conserved binding sites at either end of the variable region of interest and applying a sample-specific bar code at the extremity of each amplicon. For the shotgun approach, the whole metagenome of the microbial community is fragmented into small pieces of different length (tagmentation), which are bar coded in an independent manner. For both approaches, the resulting libraries are sequenced and analysed with different bioinformatic tools. The outputs from the bioinformatic analysis will be a taxonomic profile for the 16S rRNA-based approach and a taxonomic and functional profile, as well as novel gene recognition, for the shotgun approach.*

1.4.2 Gut microbiota metabolites and their influence on the host

The microbial community within the gut contributes to the regulation of the host metabolism through the production of metabolites. This crosstalk between microbiota and host is important for good health and good metabolic homeostasis. Different microbial taxa are associated with the production of a specific metabolites. For this reason, is not surprising that an alteration of the gut microbiota causes, not only an imbalance in the proportions of the microbes, but also in the metabolites produced by them. This can result in the development of health disorders (Canfora et al 2019b, Lavelle & Sokol 2020). In this thesis, an overview of some important metabolites, produced by gut microorganisms through fermentation of fibers and metabolism of

amino acids, carbohydrates, nucleotides and lipids, is provided (Oliphant & Allen-Vercoe 2019).

1.4.2.1 Short-chain fatty acids

The fermentation of non-digestible fibres occurs in the colon, with the main products being SCFAs. Butyrate, acetate and propionate represent 95% of the total amount of SCFAs (Morrison & Preston 2016) (**Fig. 1.5**). Firmicutes, in particular *Faecalibacterium prausnitzii* and *Clostridium spp.* of the family *Ruminococcaceae*, and *Eubacterium rectale* and *Roseburia spp*. of the family *Lachnospiraceae*, are among the main SCFAs producing-bacteria within the human gut (Louis & Flint 2009, Louis & Flint 2017). Most of SCFAs are absorbed by the host, and just 5-10% are excreted in the faeces (Cummings et al 1987). In the past few years, several studies observed that SCFAs can influence the host metabolism by acting on different organs such as the liver, adipose tissue, brain and muscle. SCFAs interact with these different body regions by binding G protein-coupled receptor 41 and 43 (GPR41 and 43), also known as free fatty acid receptor 3 and 2, respectively (FFAR3 and 2) (Layden et al 2013). *In vitro* and in rodents, it has been demonstrated that SCFAs stimulate the production of anorexigenic hormones GLP-1 and PYY within the intestine and leptin in the adipose tissue (Al-Lahham et al 2010, Larraufie et al 2018, Psichas et al 2015, Tolhurst et al 2012, Xiong et al 2004). In addition, an injection of acetate leads to a reduction in appetite through a modulation of anorexigenic hypothalamic neuropeptides (Frost et al 2014). Accordingly, SCFAs are actively involved in gut-brain axis networking (van de Wouw et al 2018). Moreover, SCFAs have a role in attenuating body weight gain by increasing energy expenditure through promoting lipid oxidation in the WAT and thermogenesis in the BAT (Reynés et al 2019, Sukkar et al 2019). Additionally, SCFAs are involved in the development of immune system, increasing the number of myeloid precursors post-haematopoiesis, and participating in the amelioration of allergic reactions (Khosravi et al 2014, Trompette et al 2014). This evidence is consistent with a positive role for SCFAs against inflammatory and metabolic diseases such as obesity and diabetes and the importance of adopting beneficial dietary habits, including the consumption of large amounts of fibres.

1.4.2.2 Neurotransmitters

An alteration of the gut microbiota is associated with behavioural disturbances and neurodegenerative diseases. This is partially due to the role of the microbes in the production of neurotransmitters, such as gamma-aminobutyric acid (GABA), serotonin (5-hydroxytryptamine; 5-HT) and catecholamines (adrenalin, nordrenalin, dopamine) (**Fig. 1.5**). GABA comes from glutamic acid metabolism and is produced by glutamic acid decarboxylase (GAD). It acts in the CNS, modulating pain, anxiety, depression and sleep, as well as in the ENS, regulating intestinal function and motility (Hyland & Cryan 2010, Ting Wong et al 2003). *Bacteroides*, *Parabacteroides*, *Escherichia*, *Lactobacillus* and *Bifidobacterium* are among the GABA producing genera in the gut (Strandwitz et al 2019, Yunes et al 2019).

Serotonin is a molecule that acts both as a hormone and neurotransmitter and covers an important role in sleep regulation, anxiety, happiness, cognition and mood control (Jouvet 1999). It is thus not surprising that a decreased serotonin level is associated with mood disorders, such as anxiety and depression. In addition, serotonin plays a role in the gut through the modulation of gastrointestinal motility and is involved in the pathophysiology of irritable bowel syndrome (IBS) (Duval et al 2000). The conversion of tryptophan to serotonin requires two essential enzymes: the first, tryptophan hydroxylase, hydroxylates the tryptophan to produce 5-hydroxiltriptophan (5-HTP) and the second, L-aromatic amino acid decarboxylase, decarboxylates 5-HTP to generate 5-HT. The GIT is responsible for 90% of total 5-HT production, which occurs in ECs and gut bacteria. *Escherichia*, *Streptococcus*, *Enterococcus*, *Akkermansia*, *Alistipes* and *Roseburia* have been identified as potential serotonin-producing taxa (Cryan & Dinan 2012a, Valles-Colomer et al 2019). Just 5% of total serotonin synthesis occurs in the CNS. Notably, germ-free (GF) mice have been shown to have a higher tryptophan/serotonin ratio in plasma compared to conventional mice (Jenkins et al 2016, Mohammad-Zadeh et al 2008). When bacteria that are able to metabolize tryptophan are added to the GF gut, levels of circulating tryptophan drop and levels of serotonin rise. At the same time, serotonin levels in the hippocampus increase and there is a reduction in anxiety-like behaviour (Clarke et al 2013). In a recent study, depression-like behaviour was further linked with the gut microbiota

using GF mice and specific pathogen-free (SPF) mice that underwent an acute tryptophan depletion procedure (Lukić et al 2019). These studies clearly indicate the importance of tryptophan intake and the effect that its conversion to serotonin, mediated by the gut microbiota, has on mood and behaviour.

Dopamine is a neurotransmitter that controls reward-motivated behaviour, in particular affection, emotions, working memory, attention, motivation, reward, locomotor activity and neuroendocrine regulation (Baik 2013). This catecholamine is synthetized from phenylalanine and tyrosine and is the precursor of adrenalin and noradrenalin. The species within the gut involved in dopamine production are still under investigation. *E. coli, E. coli K12* and *Bacillus* spp. are able to produce dopamine in *vitro*, together with *Proteus vulgari, Serratia marcescens* and *Staphylococcus aureus* (Clarke et al 2014a, Strandwitz 2018). In *vivo*, GF mice showed a lower levels of β-glucuronidase activity and, consequently, of free catecholamines both in the caecum and in other tissues and those levels are restored followed by colonization by either a faecal microbiota from SPF mice or a mix of *Clostridium* species. However, it is unclear if *Clostridium spp.* directly produce catecholamines or if this is an indirect effect (Asano et al 2012).

1.4.2.3 Vitamins

Gut bacteria provide the host with a range of vitamins important for the regulation of host metabolism. The most abundant vitamins produced by the intestinal bacteria are vitamin K and Bs, which include thiamine (B1), riboflavin (B2), niacin (B3), pantothenate (B5), pyridoxine (B6), biotin (B7), folate (B9) and cobalamin (B12) (Hill 1997) (**Fig. 1.5**). Vitamin K is important for blood coagulation due to its role as a coenzyme in the reaction that is crucial for the production of clotting factors (Merli & Fink 2008). Notably, GF mice showed a lower prothrombin levels and high risk of haemorrhage (Gustafsson et al 1962). Among the main vitamin K producers bacteria there are *E. coli*, *Mycobacterium phlei* and *Bacillus subtilis* (Meganathan 2001, Walther & Chollet 2017, Walther et al 2013). It has been recently discovered that, within the human gut, at least 256 common human gut bacteria (based on their genome annotations alone) are involved in the synthesis of different kinds of B vitamins and

actively exchange B vitamins among each other. This evidence suggests that human gut microbiota has co-evolved relationships that are specific to the gut environment (Magnusdottir et al 2015). B vitamins produced by gut bacteria are directly absorbed in the colon, unlike food-derived B vitamins, which are absorbed in the small intestine (Said 2011). Those gut-derived vitamins have been shown to regulate important metabolism pathways within the host, among which the most important is immunoregulation (Hosomi & Kunisawa 2017).

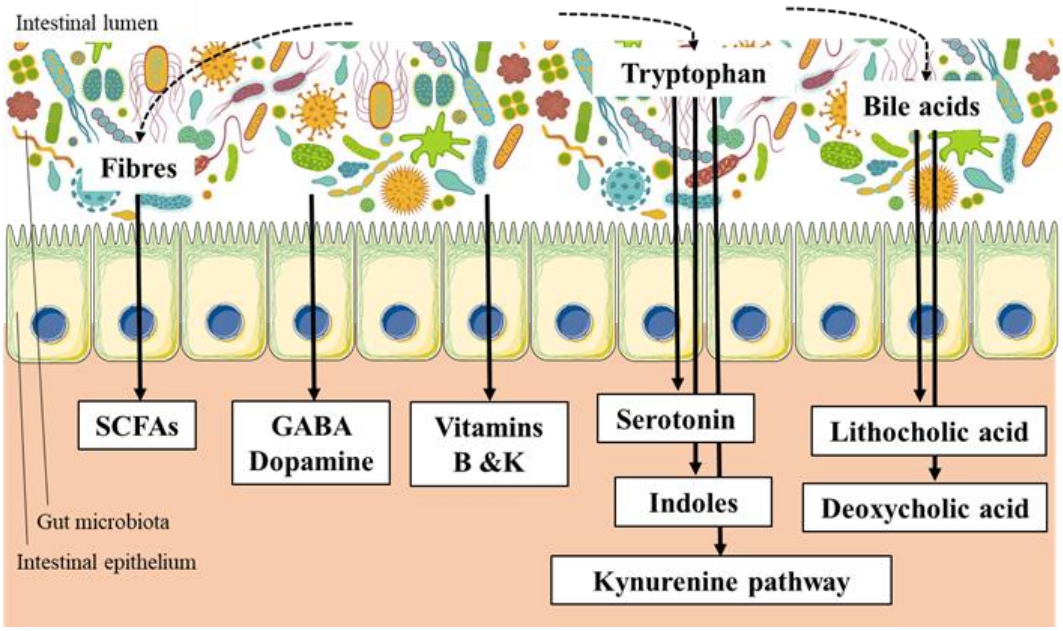


Figure 1.5 Metabolites produced by the gut microbiota. *Gut microbiota produces SCFAs (from the fermentation of dietary fibers), serotonin, indoles and kynurenine pathway-related metabolites (from dietary tryptophan), and lithocholic acid and deoxycholic acid (from the metabolism of bile acids present in the intestinal lumen). In addition, gut microbiota can synthetize neurotransmitters, such as GABA and dopamine, as well as vitamins B and K. Abbreviations: SCFA, short-chain fatty acid; GABA, gamma-aminobutyric acid.*

1.4.2.4 Bile acids

Bile acids derive from cholesterol metabolism and are synthesised within hepatocytes. Once conjugated with taurine or glycine, they are secreted into the duodenum, where they participate in fat digestion. Subsequently, almost 95% of the bile acids are reabsorbed in the distal ileum and return to the liver through the portal vein. The remaining 5% of bile acids are excreted within faeces (Chiang 2013). The microbes within the gut can modify bile acids. The microbial enzyme bile salt hydrolase (BSH)

dissociates taurine and glycine from the rest of the molecule. The taxa in which BSH activity has been observed include *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, *Clostridium* and *Enterococcus* (Jia et al 2019). A second step in the bile acids metabolism occurs after BSH action. It consists in the production of deoxycholic acid (DCA) from cholic acid (CA) and lithocholic acid (LCA) from chenodeoxycholic acid (CDCA) (Ridlon et al 2006) (**Fig. 1.5**). The enzymes involved in these transformations are produced by anaerobic bacteria (Ridlon & Hylemon 2012). There exists an important interplay between the quantity and quality of bile acids and the composition and function of the gut microbiota (Golubeva et al 2017, Molinero et al 2019).

1.4.2.5 Indole and kynurenine derivates

Beside serotonin, tryptophan is a precursor of other classes of metabolites produced by intestinal bacteria. Two important such metabolites for bacteria-host crosstalk are indole and kynurenine derivates (**Fig. 1.5**). There is evidence that indole-derivate metabolites have positive effects on the host, particularly during bacterial infection, intestinal health and neurological, metabolic and inflammatory diseases (Beaumont et al 2018, Wikoff et al 2009). Since indole from gut bacteria leads to an increase in GLP-1 levels in the small intestine and a diet high in sugar drastically decreases indole biosynthesis, indole metabolites can be beneficial to ameliorate metabolic diseases such as diabetes and obesity (Chimerel et al 2014, Isaacs et al 1994). It was shown that bacterial indoles are involved in the control of body weight and adiposity during HFD feeding. In particular, indole-3-carboxilic acid was demonstrated to be a key modulator of the expression of micro RNA miR-181 within the adipocytes. miR-181 directly regulates a network of genes controlling adipose tissue function and development, energy expenditure and insulin signalling (Virtue et al 2019). In obese humans, a reduction in plasma indoles has been observed (Cussotto et al 2020). However, after gastric bypass surgery, microbiota-derived indoles increase (Jennis et al 2018). In addition, indole-derivates, such as 7-hydroxyindole, have the capacity to improve intestinal permeability and integrity by increasing tight junction resistance and reducing the level of inflammatory markers in the colon. This is relevant for the amelioration of gut-related diseases such as cancer and IBS (Bansal et al 2010, Shimada et al 2013). Indole-3-propionic acid is a very interesting indole-derivate

metabolite. This compound exerts a variety of beneficial effects because of its potent anti-oxidant properties and its capacity to inhibit the formation of β-amyloid fibrils in neurons (Chyan et al 1999, Poeggeler et al 1999).

Tryptophan is also the precursor of kynurenine, from which other metabolites are synthetized (i.e., quinolinic acid, kynurenic acid and xanthurenic acid), all belonging to the "kynurenine pathway". Gut bacteria have an impact on kynurenine-derived metabolism and this activity is influenced by the inflammatory status. For instance, the kynurenine pathway is upregulated by pro-inflammatory cytokines, such as interferon γ (INFγ) (Campbell et al 2014). Moreover, kynurenine metabolites contribute to gut-brain axis signalling (Kennedy et al 2017). Quinolinic acid was observed as a NMDA receptor agonist within the CNS and an increase or an injection of this specific metabolites causes neurodegeneration in mice (Lugo-Huitron et al 2013). Kynurenine metabolites have also been linked with metabolic diseases. Accordingly, kynurenic acid controls energy metabolism by signalling to different cell types through the binding with its receptor, i.e., GPR35. GPR35 is expressed by adipocytes, immune cells, muscle fibres and enterocytes and it was recently considered as a good therapeutic target for obesity and diabetes treatment (Dadvar et al 2018). During obesity, kynurenine-derived metabolites are significantly higher in the plasma. This observation has been linked with increased inflammation (Favennec et al 2015).

1.4.3 The microbiota-gut-brain axis

In the last few years, the existence of a gut-brain axis has been established, defined as a bidirectional crosstalk between the CNS and the gut. These anatomic parts are able to communicate *via* afferent and efferent routes. It has been shown that the intestinal microbial population can strongly influence the gut-brain axis structure and function (Cryan et al 2019). Indeed, the intestinal microbial population exploits the gut-brain link routes to, in turn, communicate with the brain.

The vagus nerve and the bloodstream are considered the most important gut-brain axis communication routes. In paragraph 1.1.3, it was already noted that important appetite control-related hormones produced by the GIT (i.e., ghrelin, CCK, GLP-1) exert their regulatory function within the hypothalamus via both the vagus nerve and blood. In addition, as described in paragraph 1.4.2, intestinal bacteria produce a large number of metabolites in response to different physiological conditions, such as diet. Some of these metabolites, such as SCFA, neurotransmitters and tryptophan-derivate metabolites, take advantage of afferent routes to cross the BBB and act in specific regions of the brain (Cryan & Dinan 2012b, De Vadder et al 2014, Nicholson et al 2012). The gut microbiota is also involved in the maturation of intestinal epithelial cells as well as in the maturation of the immune system (Edelman & Kasper 2008, Okumura & Takeda 2017). This relates not just to immune system within the intestine, but also systemically and in the CNS, suggesting a gut-brain interaction that is immune-system dependent (Fung 2020).

In parallel, the brain is able to communicate with the gut through efferent signaling. The most important is the hypothalamic-pituitary gland-adrenal (HPA) axis. This specific signaling pathway is responsible for the production of corticosterone in rodents and cortisol in humans, which are defined as “stress hormones”. The activity of the HPA axis has been shown to impact on the immune system both in the periphery and in the gut, together with consequences on gut physiology mediated by the ENS (Bhatia & Tandon 2005, Savastano et al 1994). Gut microbes are able to influence the HPA axis activation, making the HPA axis-gut communication bidirectional (Frankiensztajn et al 2020).

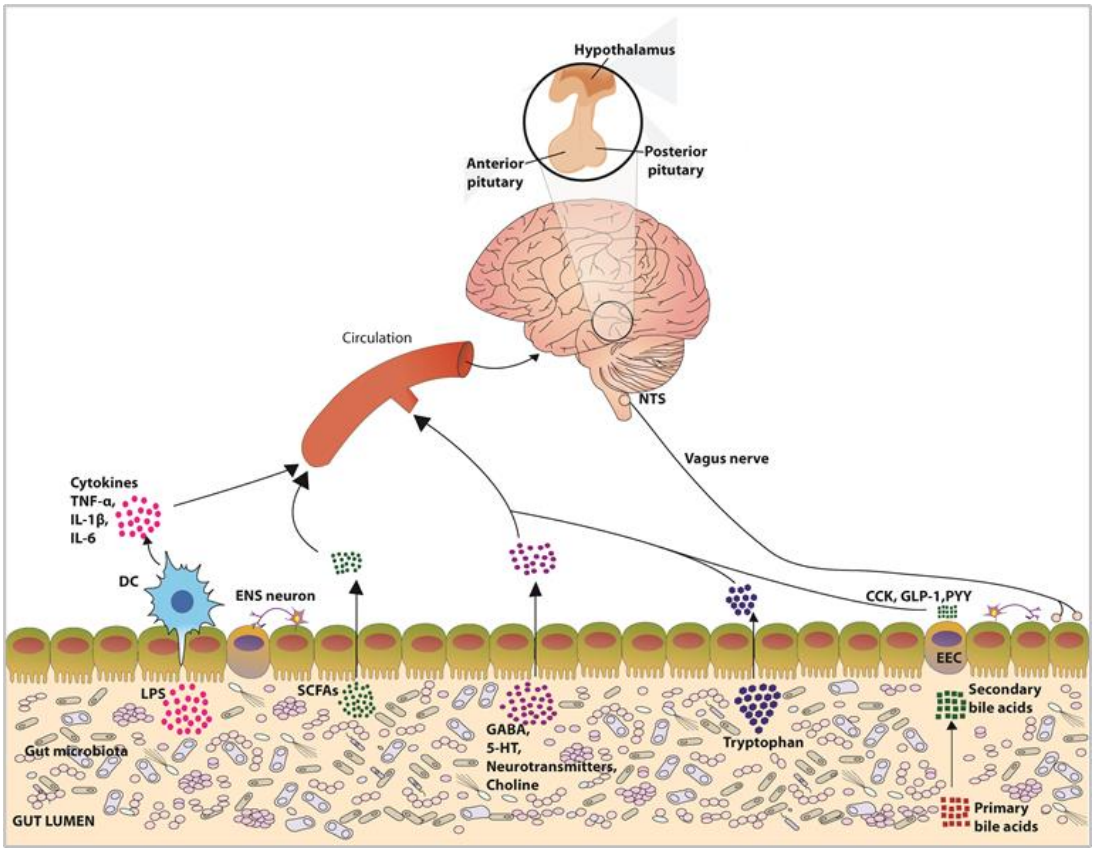


Figure 1.6 The microbiota-gut-brain axis. *These routes include the vagus nerve, production of SCFAs, immune activation with production of immune mediators, production of neurotransmitters and tryptophan. The gut microbiota is also able to convert primary bile acids into secondary bile acids, which activate receptors on EECs and stimulate the secretion of gut peptides. Neuroactive compounds produced by gut microbiota enter the circulation and reach the brain, subsequently affecting neuroendocrine function. Abbreviations: 5-HT serotonin, CCK cholecystokinin, DC dendritic cell, EEC enteroendocrine cell, ENS enteric nervous system, GABA ɣ-aminobutyric acid, GLP-1 glucagon-like peptide-1, IL interleukin, LPS lipopolysaccharide, NTS nucleus tractus solitarii, PYY peptide YY, SCFAs short chain fatty acids. Figure adapted from (Cussotto et al 2018).*

1.4.4 The role of gut microbiota in obesity

An alteration in the gut microbiota composition, with a concurrent reduction in overall diversity, has been suggested to be among the changes associated with obesity status (Turnbaugh et al 2008, Turnbaugh & Gordon 2009). The two most dominant phyla in the human gut are the Bacteriodetes and the Firmicutes. In studies conducted both in obese mice (Ley et al 2005a, Turnbaugh et al 2006b) and in humans (Ley 2006), the proportion of the phylum Firmicutes was observed to increase with decreases in the Bacteroidetes in the obese condition compared to their normal weight counterparts. These studies gave rise to the idea that an increased Firmicutes:Bacteroidetes (F/B) ratio could be used as an obesity biomarker. However, other findings contradict this notion (Schwiertz et al 2010). Thus, the precise alteration in the microbial composition that contributes to obesity remains the subject of debate. Despite this doubt, several important studies demonstrate an important role for the enteric microbiota in the pathogenesis of obesity.

Backhed *et al* showed that GF mice have 40% less total body fat than conventional mice, although they ingest 29% more calories than their conventionally raised littermates (Backhed et al 2004). GF mice also gain less weight than conventionally raised mice and they were protected against diet-induced glucose intolerance and the development of insulin resistance (Piya et al 2013). In addition, faecal microbiota transplanted from conventionally raised mice to GF mice triggered a 57% increase in the amount of body fat and a dramatic increase in hepatic triglyceride levels and insulin resistance without modifying the amount of food consumed (Backhed et al 2004). The expression of host genes involved in energy homeostasis, lipid metabolism and mitochondrial metabolism in different parts of the gut, as well as in the liver and adipose tissues, is markedly different in GF mice and conventionally raised mice (Larsson et al 2012). These data highlight the role played by the gut microbiota in energy harvest and storage in host tissues.

Recent studies have further defined the potential bacterial candidates for the above effects. Fei *et al* have demonstrated that the opportunistic pathogen, *Enterobacter cloacae* B29, isolated from the gut of an obese human, causes obesity and insulin resistance in GF mice (Fei & Zhao 2013). In another study, using gnotobiotic mice fed

a HFD or low-fat diet (LFD), the authors assigned a role for *Clostridium ramosum* in the upregulation of glucose and fat transporters in the intestine, as well as increased body fat deposition (Woting. A et al 2014). In addition, *Staphylococcus aureus*, *Halomonas* and *Sphingomonas* have been associated with an obese phenotype (Kalliomaki et al 2015), accompanied by low *Bifidobacterium* numbers when compared to lean individuals. On the other hand, other bacterial members in the gut can have a protective role against obesity and adiposity both in rodents and in humans. Among them, *Akkermansia muciniphila*, a mucin-degrading bacteria, is present in significantly lower levels in obese *Ob/Ob* mice (i.e., mice with a mutation in the gene encoding leptin). The oral administration of *A. muciniphila* reverses HFD associated metabolic disorders including fat mass gain and insulin resistance in these mice (Everard. A et al 2013). Other commensal enteric bacteria such as *Enterococcus faecalis* FK-23, *Lactobacillus gasseri* SBT2055 and the family *Christensenellaceae* have been shown to exert anti-obesity and anti-adiposity effects (Kadooka et al 2010, Motonaga. C et al 2009). *Christensenellaceae*, a bacterial family present in lean individuals, has been shown to promote a lean host phenotype and has an impact on the diversity of the bacterial community when transplanted to mice (Goodrich et al 2014). Considering these results together, a targeted modulation of specific gut microbiota components may prove a viable alternate target in the treatment of obesity, that can be adjusted in order to resemble as much as possible a healthy configuration.

1.4.5 Strategies of gut microbiota manipulation

To investigate and to understand the role of the gut microbiota in different circumstances, for example during diet intervention, drug administration or disease progression, several methods are commonly employed in *in vivo* studies. All those methods aim to disturb or manipulate the gut microbiota in order to assess the consequent amelioration or worsening of the original phenotype in the animal model.

1.4.5.1 Prebiotics

Prebiotics are defined as selectively fermented non-digestible food ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefits upon the host health (Bindels et al 2015, Gibson et al 2010). Prebiotics include different kinds of food components such as fructans (inulin), galacto-oligosaccharides, starch and glucose-derivate oligosaccharides and non-carbohydrate oligosaccharides (Davani-Davari et al 2019). The main products of prebiotics fermentation/degradation by the gut microbiome are SCFAs and other molecules used by other intestinal microbial population (cross-feeding) (Belenguer et al 2006). Several studies in rodents and humans have shown that different kind of prebiotics have beneficial effects on gastrointestinal diseases, immunity and on the nervous system through the gut-brain axis (Davani-Davari et al 2019). Moreover, specific prebiotics consumption, for example inulin, showed several beneficial effects against metabolic syndrome-related comorbidities (Hiel et al 2018, Rodriguez et al 2020).

1.4.5.2 Probiotics

Probiotics are defined as live microorganism that, when they are present in adequate amounts, confer a health benefit to the host (Hill et al 2014). The majority of commercially used probiotics belong to the genera *Lactobacillus* and *Bifidobacterium*, with *L. acidophilus*, *L. casei*, *L. plantarum*, *L. rhamnosus, L. reuteri*, *L. brevis*, *L. johnsonii*, *L. plantarum, B. infantis*, *B. adolescentis*, *B. animalis B. lactis*, *B. longum*, and *B. bifidum* among the most important species (Fijan 2014). Probiotics can help to maintain a balanced proportion of bacteria that are important for a correct gut microbiota functionality (Oelschlaeger 2010). In addition, they counteract the action of pathogenic bacteria within the gut by impeding their growth (Fijan 2016). For these reasons, a probiotic therapy is recommended after antibiotic treatment. In addition, in recent years, some probiotics have demonstrated positive effects with respect to different GIT-related diseases, metabolic syndrome, immunity, oral health, hypercholesterolemia and the gut-brain axis modulation (Markowiak & Śliżewska

2017, Salazar et al 2019). Moreover, a prebiotic-probiotic mix (i.e., synbiotic) have also been employed in order to improve gut health (Markowiak & Śliżewska 2017).

1.4.5.3 Antibiotic treatment

Antibiotics are antibacterial molecules. They are classified as bactericidal, which directly kill the bacteria, or bacteriostatic, which inhibit bacterial growth (Nemeth et al 2014). Different antibiotics target and inhibit/interfere with a specific function essential for the bacterial cell, such as DNA replication, RNA synthesis, protein synthesis and cell wall biosynthesis (Kohanski et al 2010). Moreover, antibiotics can be broad-spectrum, which act on different bacterial taxa, and narrow-spectrum, which are able to target just specific taxa. Broad-spectrum antibiotic usage can have the generally undesirable effect of also impacting on non-pathogenic bacteria (Melander et al 2018). However, for scientific research, when compared to GF mice, antibiotic treatment offers a more rapid, cheap and manageable method to study the depletion of the gut microbiota. The main advantages in the usage of antibiotics versus GF mice are (Kennedy et al 2018):

- possibility to deplete the gut microbiota in any mouse genotype or condition;
- possibility to deplete the gut microbiota in mice that were normally colonized since birth. Accordingly, mice that start an antibiotic treatment would have normal metabolism and neurodevelopment since birth. In GF mice, many metabolic, immunological, physiological and neurodevelopmental parameters are impaired since birth.

A single antibiotic or an antibiotic cocktail, usually broad-spectrum, are given to the rodents dissolved in drinking water or placed directly into the GIT using oral gavage (Kennedy et al 2018). However, this gut microbiota depletion method has some disadvantages:

- 100% of the bacteria are not killed by antibiotic treatment and antibiotic resistant pathogens and non-bacteria, e.g. fungi, can flourish;

- the antibiotic treatment is not always localized within the intestine, but many antibiotics have a systemic effect, thus, they can influence other organs and eukaryotic cells. This leads to antibiotic-induced side effects, such as problems in the immunomodulation, metabolism and digestion (Blaser 2016, Langdon et al 2016).

1.4.5.4 Faecal microbiota transplantation

Faecal microbiota transplantation (FMT) involves the administration in the upper part of the GIT, or the placement directly in the colon, of faecal material from a healthy donor to a recipient with an altered gut microbiome (Kim & Gluck 2019). This practice is increasingly being used in the clinic to treat patient with *Clostridium difficile* infection, a pathogenic bacterium that cause gastrointestinal disturbances such as diarrhoea, fever and general sickness. *C. difficile* infection often happens after antibiotic usage (Ofosu 2016). FMT is also employed in rodent studies. To assess if the gut microbiota is implicated in the development or amelioration of certain conditions, FMT can be carried out from a donor rodent with a certain disease to a recipient healthy rodent, or vice versa (Backhed et al 2004, Ellekilde et al 2014, Lai et al 2018). Moreover, through FMT, it is possible to create so-called humanized rodents, by placing stool from a human donor in a rodent recipient. Despite the important discoveries carried out in rodents in understanding the crosstalk between the gut microbiota and the host, it is still difficult to translate these findings to humans, which have important differences compared to mice and rats. The use of humanized rodents provides one means to try to reduce these differences (Hugenholtz & de Vos 2018, Nguyen et al 2015).

1.5 Intestinal integrity and permeability: an overview

The intestinal epithelium is an important barrier. Beyond its role as a physical protective barrier, one of the most important roles of the single layer of epithelial cells that clothe the intestinal tract is in nutrient and fluid absorption, together with bidirectional electrolyte flux. Moreover, the intestinal surface is enriched in cells from the innate and adaptive immune system such as B, T and DCs, macrophages as well as mucosal lymphocytes. They ensure intestinal protection from pathogens (Kagnoff 2014). The enterocytes physically communicate and adhere to each other through different kinds of tight junction proteins (TJ). The most abundant in the intestinal epithelium are claudins, TJ-associated MARVEL proteins (TAMP, such as occludins and tricellulin), junctional-adhesion molecules (JAMs) and cytosolic plaques TJ proteins constituted by *Zonula occlundes* (ZO) proteins (Ivanov 2012). The quantity and, consequently, the functions of the TJs is controlled at different levels (transcriptional and post-transcriptional) by different factors, such as cytokines, growth factors, the gut microbiota, pathogens, and nutrients. This directly impacts on intestinal permeability. Moreover, intestinal permeability is affected by different pathological condition, such as GIT diseases and metabolic syndrome, as well as during psychological stress, oxidative stress and ageing (Maeda et al 2010, Saunders et al 2002, Suzuki 2013, Thevaranjan et al 2017).

1.5.1 Role of high-fat diet and gut microbiota in gut permeability modulation

A growing body of evidence indicates that one of the side effects of obesity is an increase in gut permeability. Both genetically obese *Ob/Ob* and *Db/Db* mouse models showed an alteration in TJ assembly that leads to a disruption in intestinal barrier function (Brun et al 2007). Accordingly, HFD feeding causes disfunction at the intestinal barrier level through a decrease in abundance of occludin, claudin-1, claudin-3, and JAM-1 (Suzuki & Hara 2010). Gut permeability disruption in genetically obese mice or caused by HFD is associated with higher levels of circulating

cytokines such as IL1β, IL6 and TNFα (Al-Sadi et al 2009, Rohr et al 2019). It has been shown that HFD-induced gut permeability and altered plasma inflammatory markers are related to a gut microbiota alteration during HFD (Cani et al 2008, Cani et al 2009). Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria that starts an inflammatory reaction through toll like receptor 4 (TLR4) interaction. HFD induces higher LPS levels in the plasma and a mild antibiotic treatment with ampicillin and neomycin abolished this phenotype, confirming the key importance of the gut microbiota in this phenomenon (Cani et al 2008). Moreover, this suggests that the composition of the gut microbiome influences intestinal permeability. Indeed, the gut microbiota is known to help the maturation of intestinal epithelial cells and it is not surprising that GF mice have a "leaky gut" due to TJ dysfunction. However, it has been shown that colonization of GF animals with a human microbiota results in a structural and functional intestinal barrier improvement, together with a reduced risk of intestinal injury (Hayes et al 2018). In line with this, a depletion of the gut microbiota with antibiotics induces TJs disruption and increase in gut permeability *in vitro* and in *vivo* (Feng et al 2019, Spiller 2018). FMT, probiotics and prebiotics, are among the approaches available to ameliorate intestinal permeability linked to a gut microbiota alteration (Yoon & Yoon 2018). Among probiotics, several species of *Saccharomyces*, *Lactobacillus* and *Bifidobacterium*, together with specific *E. coli* strains have been found to be efficient in addressing intestinal barrier disruption (Rao & Samak 2013, Terciolo et al 2019).

1.5.2 Use of Ussing chamber as means to measure gut permeability

In rodent studies, Ussing chamber provide a means to easily and reliably measure the permeability of some molecules and ions across the intestinal barrier (Thomson et al 2019, Ussing & Zerahn 1951). Here, only the experimental procedure to measure molecules permeability is described. Primarily, an intestinal region is selected. Usually the permeability measurement is applied to the ileum or colon. Approximately one-centimetre length of the intestinal zone selected is longitudinally cut and placed as a sheet between two parts of the apparatus, which together create a chamber (**Fig. 1.7**).

In this way, serosal (physiologically in contact with blood vessels) and mucosal (physically in contact with the intestinal lumen) parts of the intestinal portion are each facing one half of the chamber. Both halves of the chamber are filled with a carbonate sugar-enriched solution and a fluorescent macromolecule, 4KDa FITC-dextran, is added to the mucosal side. The chamber is attached to a gas source that maintains the flow of the solution within the chamber and, in the back part, the chamber is water jacketed to ensure a constant temperature of 37°C. Sampling is carried out at different timepoints, over 2 or 3 hours, in the chamber that faces the serosal side of the intestinal tissue. Subsequently, FITC-dextran abundance is detected by measuring its absorbance with a spectrophotometer at 485nm excitation / 535nm emission wavelengths. The timepoint zero (T0) absorbance of each tissue is subtracted from the following timepoints of the same tissue. The output is commonly a curve that describes the serosal concentration of FITC over time. Alternatively, it is also possible to calculate the total FITC flux that penetrates from the mucosal to the serosal side of the tissue surface in the total observation time.

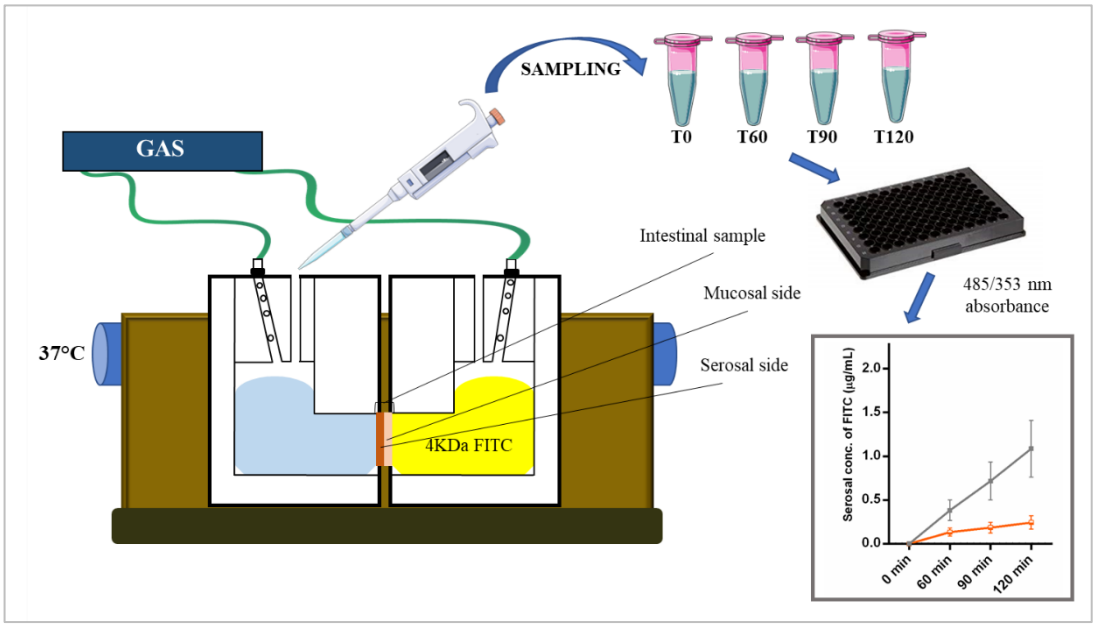


Figure 1.7 Measurement of macromolecules permeability using Ussing chamber apparatus. *An intestinal region is mounted in the Ussing chamber and FITC-dextran is added to the mucosal chamber. Samples are collected from the serosal chamber at different timepoints, and levels of FITC-dextran are quantified by measuring its absorbance with a spectrophotometer at 485nm excitation / 535nm emission wavelengths.*

1.6 Bovine whey protein and its action on energy balance, gut microbiota and mood

Whey is the milk serum that has been separated from the milk during cheese manufacture. Specifically, whey is formed by the action of chymosin after casein curd formation. Whey is composed of vitamins, lactose, minerals, fat (in small amounts) and soluble proteins (Zadow 1994). In bovine milk, 80% of protein is in the form of casein, with whey protein (WP) accounting for the remaining 20%, which includes β-lactoglobulin (LAG), α-lactalbumin (LAB), bovine serum albumin (BSA) and a small amount of, lactoferrin (Lf), lactoperoxidase (Lp), immunoglobulin (Ig), glycomacropeptide (GMP) and lysozyme (Ly) (Morr & Ha 1993) (**Fig. 1.8**). However, the composition varies between species. For instance, human milk has more WP than casein (60% and 40%, respectively) (Zhu & Dingess 2019). The specific composition of WP also varies but, regardless, all are considered to represent high-quality protein sources because of their high content of essential amino acids. This includes high concentrations of branched-chain amino acids (BCAAs), such as leucine, isoleucine and valine, as well as tryptophan, which are all important for muscle growth and repair (Hulmi et al 2010, Millward et al 2008). Indeed, WP are well known for improving muscle building and health (Davies et al 2018).

Moreover, WP and its components have been linked with other beneficial effects on health, such as lowering adiposity, lipid metabolism and cholesterol modulation, anti-hypertensions, immunomodulation, wound healing, antioxidant, anticarcinogenic and glucose homeostasis (McAllan et al 2012, Pal & Radavelli-Bagatini 2013, Yalçin 2006) (**Fig. 1.9**).

WP The commercial sources of WP supplementation of diets exist in the form of whey protein concentrate (WPC), in which the amount of proteins varies between 50% and 75%, or whey protein isolate (WPI), which contains a minimum of 90% protein (Morr & Ha 1993).

Unlike casein, WP does not coagulate in the stomach. Instead, the components can rapidly enter the jejunum and be digested, releasing amino acids (Pal & Ellis 2010a). Thus, the absorption of digested WP components is faster than for caseins. A brief description of the most important WP is provided.

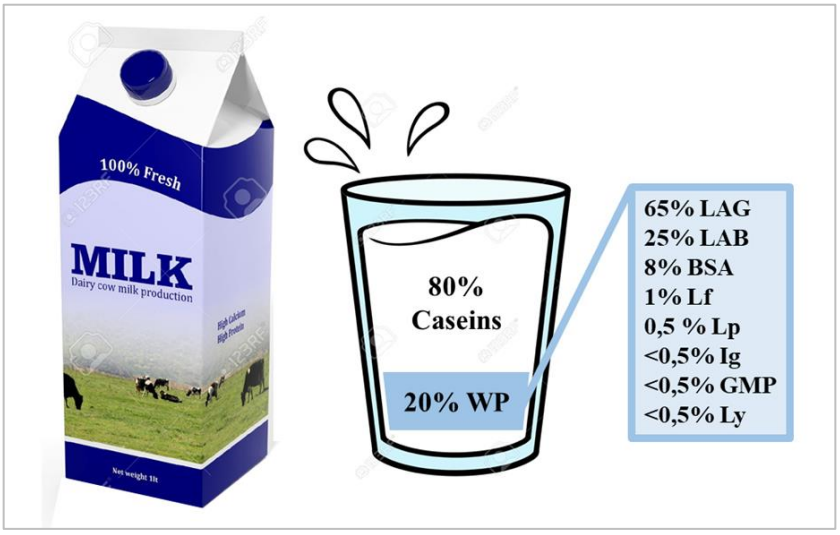


Figure 1.8 Bovine milk proteins: casein and whey. *Bovine milk contains two kinds of proteins: casein (80% of the total) and whey protein (WP, 20% of the total). WP, in turn, includes different components at different percentages. Abbreviations: LAG, β-lactoglobulin; LAB, α-lactalbumin; BSA, bovine serum albumin; Lf, lactoferrin; Lp, lactoperoxidase; Ig, immunoglobulin; GMP, glycomacropeptide; Ly, lysozyme.*

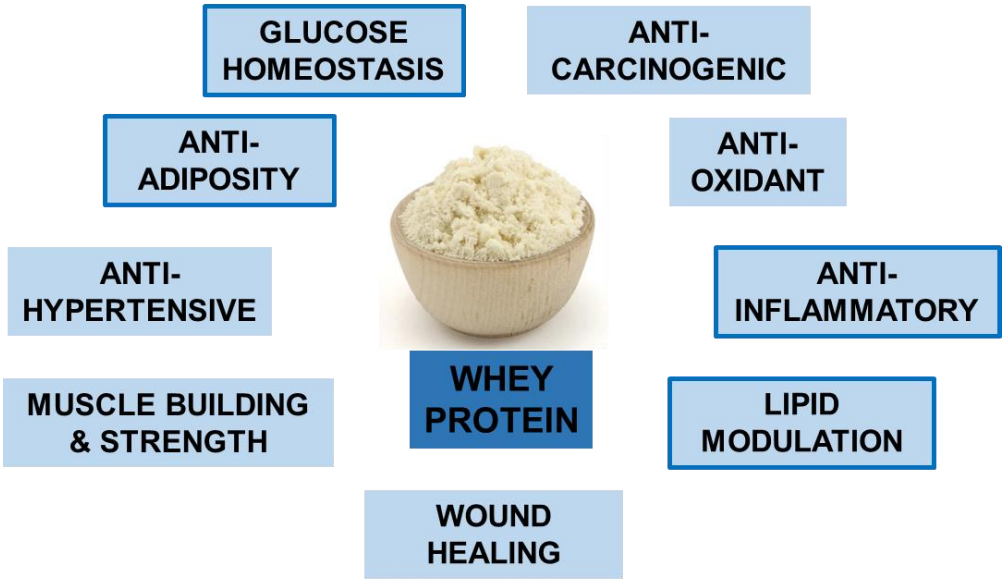


Figure 1.9 Beneficial effects of whey protein. *Among these, this thesis focuses on anti-adiposity, lipid modulation, glucose homeostasis and anti-inflammatory outcomes.*

1.6.1 Whey protein components

1.6.1.1 β-lactoglobulin

LAG accounts for ~ 60-65% of the total WP content and ~ 12% of total bovine milk proteins. LAG consists of 162 amino acids residues in its primary structure and it has a globular structure, which is stable against the acids and enzymes present in the stomach. The molecular weight of the purified cattle LAG was measured as 17.44 KDa (Aich et al 2015). While LAG is the most abundant protein in bovine whey, it is absent from both human and rodent milk. Indeed, LAG is regarded as one the primary allergen in cow's milk and its removal from whey has been suggested for the production of hypoallergenic food products such as milk formula (Chiancone; E 1991). In addition, to providing a rich source of both essential amino acids and BCAAs, LAG has also been shown (in preruminant calves) to play an important role in the intestinal uptake of small hydrophobic ligands such as retinol, TAGs and LCFAs (Kushibiki et al 2001). Recent *in vitro* experiments using hybridoma cell models, demonstrated that LAG can influence human immunity and promote cell proliferation (Tai et al 2016). LAG is not heavily affected by gastric digestion *in vivo* and apparently remains mostly intact after its transition through the stomach (Ebeler et al 1990). For this reason, LAG has been proposed as a vehicle for the delivery of prebiotic compounds, viable probiotic bacteria and pH-sensitive drugs to the colon (Teng et al 2015).

1.6.1.2 α-lactalbumin

Bovine LAB, which consists of 123 amino acid residues, is the second most abundant protein found in bovine whey (~20-25% of the total). It is a small Ca^{++} binding protein composed of a singular globular domain which is essential for the molecule stability and rigidity. Its ability to strongly bind Ca^{++} further enhances the stability of the folded state (Permyakov & Berliner 2000a). This specific WP is also able to bind other ions such as Mg^{++}, Mn^{++}, Na^{+}, K^{+} and Zn^{++} and can interact with membranes, proteins, peptides and low molecular weight substrates (Permyakov & Berliner 2000b). There is evidence that LAB contributes to a reduced risk of incidence of some cancer types

through several mechanisms such as the anti-proliferative effects, as demonstrated in assays with colon adenocarcinoma cell lines (Sternhagen & Allen 2001). Indeed, BAMLET, which is a complex of bovine LAB and oleic acid, was shown to have the ability to kill tumour cells that are high resistant to apoptosis (Rammer et al 2010). Although when examined against several Gram-positive and Gram-negative bacteria, native bovine LAB did not exhibit any bactericidal activity (Pellegrini et al 1999). Pihlanto-Leppälä *et al* demonstrated that LAB reduced the metabolic activity of *E. coli* JM103 by 79% when pre-hydrolysed with pepsin or trypsin (Pihlanto-Leppala et al 1999). Furthermore, Brück *et al* showed that a combination of LAB and GMP inhibited the association between CaCo-2 cells and the GIT pathogens enteropathogenic *E. coli* (EPEC), *Salmonella typhimurium* and *Shigella flexneri*. This suggests that the supplementation of milk with both LAB and GMP might be effective in the prevention of some GIT infections (Bruck et al 2006). In the GIT, LAB is able to exert other biological functions, such as an ability to protect against gastric mucosal injuries. This was demonstrated using two acute ulcer models in rats (Matsumoto et al 2001). A recent review by Layman *et al* provides more information relating to LAB characteristics and the potential application of this WP for human health (Layman et al 2018a).

1.6.1.3 Bovine serum albumin

Unlike other WP, BSA is not synthesised in the mammary gland but, instead, joins the milk through its leakage from the bloodstream. For this reason, it may be considered a whey associated protein. BSA, which contains 583 amino acid residues, is involved in the maintenance of colloid osmotic pressure and acts as a carrier protein for FFAs, catecholamines and other hormones (Danon & Sapira 1972, Hankins 2006). In addition, BSA is also able to bind different kinds of drugs. More specifically, recent studies have explored the interaction between BSA and nitrofuran antibiotics, cephalexin, caffeic acid, the anticancer drug neratinib and the flavonoid compound astilbin (Hamishehkar et al 2016, Liu et al 2018, Precupas et al 2017, Wani et al 2018). Moreover, because of its chemical and physical properties, synthetic BSA nanoparticles that are able to efficiently carry and release drugs, such as salicylic acid, have been developed *in vitro* (Bronze-Uhle et al 2017). While known to be a good

source of essential amino acids, knowledge about the therapeutic potential of BSA is still limited. One notable property associated with BSA is its ability to inhibit tumour growth in a MCF-7 human breast cancer cell line (Laursen et al 1990).

1.6.1.4 Lactoferrin

Lf is a 80 KDa single chain iron-binding protein. It is a member of the transferrin protein family and accounts for ~1% of the total proteins in bovine whey. Lf is produced by mucosal epithelial cells in several mammalian species and is present in mucosal secretions such as tears, saliva and GIT fluids (Pierce et al 2009, van der Strate et al 2001). Of all the proteins contained within bovine whey, Lf is the most widely studied. Lf is a multifunctional protein whose properties include anti-bacterial activity and stimulation of beneficial intestinal bacteria (discussed in paragraph 1.6.4), as well as other functional properties such as anti-viral, immunomodulatory, anti-cancer and anti-inflammatory activities (García-Montoya et al 2012, Jenssen & Hancock 2009). Briefly, the antimicrobial activity of Lf has been studied both *in vitro* and *in vivo*. Lf is effective against both Gram-positive and Gram-negative bacteria, exerting both bacteriostatic and bactericidal activity. Lf comprises two homologous lobes, each of which can bind one ferric ion (Fe^{2+} or Fe^{3+}) (Baker et al 2003). Lf, therefore, exerts bacteriostatic activity through the sequestration of iron away from bacterial pathogens, thus limiting the use of this vital nutrient and down-regulating the expression of virulence factors (Parrow et al 2013). Lf also invokes bactericidal effects on pathogenic bacteria through direct interaction with bacterial surface proteins (Ellison et al 1988, Miyazawa et al 1991). For all these reasons, Lf may now be thought of as a nutraceutical protein and its use in nutritional and pharmaceutical application has been encouraged.

1.6.1.5 Lactoperoxidase

A member of the mammalian peroxidases family, the 78 KDa enzyme Lp accounts for 0.25-0.50% of the total protein content of whey and is found in biological secretions such as tears, saliva and milk. It is a heme-containing chain glycoprotein and binds a covalently linked heme prosthetic group, a derivative of protoporphyrin IX, in its

catalytic centre (Sharma et al 2013). One of the most important enzymes in plain milk, Lp plays an important role in its preservation and has several commercial applications such as food and crop preservation and protection of the oral cavity against bacterial infection (Boots & Floris 2006, Reiter & Harnulv 1984, Seifu et al 2005). As well described by Köksal *et al*, antibacterial activity relies on the presence of both the thiocyanate anion (SCN^-; which is present in significant levels in natural secretions such as saliva and milk) and hydrogen peroxide (H_2O_2) and is based upon the ability of Lp to catalyse the oxidation of SCN^- (Köksal et al 2016). This Lp-catalysed reaction results in the formation of short-life intermediary compounds, primarily hypothiocyante ($OSCN^-$) and hypothiocyanous acid (HOSCN), which are thought to invoke anti-bacterial activity *via* the inhibition of the essential metabolic pathways such as glycolysis by the oxidation of sulfhydryl groups of bacterial proteins in both Gram-positive and Gram-negative bacteria (Bafort et al 2014, Tenovuo 2002). The formation of intermediary compounds from the Lp-catalysed oxidation of SCN^- is pH dependant and may result in the formation of other bactericidal compounds such as cyanosulphuros acid (HO_2SCN) and cyanosulphuric acid (HO_3SCN). The Lp anti-bacterial system plays an important role in the maintenance of oral health, where the $Lp/SCN^-/ H_2O_2$ system was shown by Welk *et al* to have bactericidal activity against both *Streptococcus mutans* and *S. sanguinis*, which are associated with both dental caries and periodontal disease (Ge et al 2008, Welk et al 2011).

1.6.2 Casein: a brief overview

Bovine milk, the most popular animal milk consumed by humans, together with goat milk, contains four caseins: α_{S1}, α_{S2}, β and κ (Farrell et al 2004). Also present is γ-casein, which is a product of degradation of β-casein (Andrews 1983). Caseins provide one of the best example of intrinsically or natively disordered (or natively unfolded) proteins, with very little secondary and tertiary structures, despite being functional (Thorn et al 2015). In raw milk, caseins are the most abundant proteins and they are responsible for the white colour of the milk. In addition, caseins can assemble into micelles, which are spherical colloidal structures that include all four casein species. These entities are stabilized by physiochemical properties, which allow them to bind calcium. For this reason, the key biological functions of caseins are to carry calcium and phosphate and to form a clot in the stomach for efficient digestion (Huppertz et al 2018).

These secretory calcium-binding phosphoproteins possess high heat stability: only temperatures above 120˚C make caseins insoluble (Tamime 2007). Their content of non-polar amino acids amounts to 32 -42%, making these proteins high hydrophobic. However, the association with calcium and phosphates allow them to be soluble in aqueous solvents (Fox et al 2015).

Once ingested, caseins coagulate in the acidic environment of the stomach, which delays its gastric emptying. When they reach the stomach, the proteolytic enzymes attack the external part of the micelles first, and then the hydrophobic subunits inside, which are insoluble. For this reason, caseins take a relatively long time to be digested, inducing a slow postprandial increase in plasma amino acids; this ensures a dietary amino acid availability that lasts for few hours (Boirie et al 1997).

Caseins are essential for human growth and development and they contribute to functions related to nutrient and vitamins uptake (Hoffman & Falvo 2004). Many *in vivo* and *in vitro* studies have attributed anticarcinogenic, antibacterial activity and hypocholesterolemic effects to caseins (Davoodi et al 2016).

1.6.3 Whey protein effects on satiety, body weight, energy balance and inflammation

Acquiring a better understanding of the fundamental mechanisms by which WP interventions affect mechanisms regulating energy balance would provide the basis for the development of enhanced health promoting food products for the consumer. The following provides an overview of what is known about the effects of dietary WP (both individual and collective proteins in the form of WPI or WPC) and the outstanding knowledge deficit in terms of developing these proteins or their bioactive components as food ingredients for the affected individuals.

1.6.3.1 Whole and individual whey protein effects on satiety control

WP can influence energy balance through appetite control *via* their satiety properties (**Table T1.4**). While it is generally accepted that dietary protein induces a greater satiety effect than other macronutrients such as carbohydrates or fats (Tome et al 2009), data from several studies have suggested that WP is more potent satiety inducer than other protein sources. A study by Hall *et al* reported that the intake of WP (48g) delayed the desire to consume a subsequent meal by 180 minutes when compared to casein in lean individuals (Hall et al 2003). In another human study it was shown that the consumption of a diet of protein, carbohydrate and fat, which provided 10%, 55% and 35% energy, respectively, reduced hunger more effectively when WP were included in the diet rather than casein or soy protein (Veldhorst et al 2009a). In a further study in lean subjects, Pal and Ellis reported significantly reduced mean energy intake and appetite during a subsequent buffet meal (4h later) in individuals who had consumed a blended drink containing WP, compared to those which had consumed a blended drink containing the same amount (50g) of either egg, tuna or turkey protein (Pal & Ellis 2010a). Later on, they showed that WP ingestion in the morning time has a stronger satiety effect and increased fullness just before lunch time (approximately 4 hours later) compared to casein in overweight and obese humans (Pal et al 2014). It has been suggested that WP provides a greater satiety effect than other proteins due to

its higher content of BCAAs and, in particular, leucine (Sharp et al 2019). Leucine, which enters the brain more rapidly than any other amino acid (Yudkoff et al 2005), has been shown through intracerebroventricular injection to play an important role in appetite suppression, which suggests that WP invokes a central effect on energy intake (Morrison et al 2007). It is also possible that these effects are mediated by the high tryptophan content in whey. Tryptophan is a precursor of serotonin, an important appetite regulator (Sharma & Sharma 2012), thus the high tryptophan content of LAB may, in turn, increase satiety through an increase in serotonin production. Regardless, a study by Veldhost *et al* demonstrated that a breakfast diet where LAB provided either 10% or 25% energy reduced lunchtime energy intake when compared to a breakfast diet deriving protein energy from casein, soy or whey (Veldhorst et al 2009b). This is consistent with the observations of Hursel *et al* who also reported that a LAB-enriched diet had a greater effect on appetite suppression than a whey diet (Hursel et al 2010). These results suggest a use for LAB in appetite control. Something to keep in mind is the extreme importance of amino acid quality in energy balance regulation. It has recently been shown that a long-term ingestion of an imbalanced quantity of amino acids, in particular BCAAs, leads to a dysfunction of appetite regulation and, consequently, to hyperphagia (Solon-Biet et al 2019).

Another mechanism by which WP (or whey peptides resulting from gastric digestion) may influence energy intake is by the stimulation of hormones described in paragraph 1.1.3, which are important regulators of energy intake. In support of this, it was shown that dietary WP intake (50g) decreased appetite in both lean and overweight individuals and this change was coupled with increased plasma levels of CCK and GLP-1 and reduced levels of ghrelin when compared to glucose intake (Bowen et al 2006a, Bowen et al 2006b). The results of this study are in line with those of other studies in both humans and animal models (Hall et al 2003, Zhou et al 2011). Normal-weight women that consumed a drink enriched in WP containing naturally present GMP showed an increase in plasmatic levels of CCK, GLP-1 and PYY at different timepoints after the drink administration, relative to women having a maltodextrin-supplemented drink (Chungchunlam et al 2015). In obese male rats consuming a diet enriched in WP (WPI, LAB or Lf), an increase in plasma levels of PYY was noticed and, consequently, a stronger satiety effect compared to the control group (Zapata et

al 2018). These results suggest that WP or its associated bioactive peptides can reduce energy intake by the activation or suppression of important appetite regulating hormones within the gut.

All together, these results suggest that, in lean individuals/animals, WP supplementation enhances satiety. However, some studies have suggested that the satiety effect of WP may be reduced in obese individuals/animals despite WP-induced reduction in body weight gain. In a study using diet-induced obese (DIO) rats, a similar energy intake pattern was observed in mice, which had whey supplemented drinking water (100g/L) compared with those provided with un-supplemented drinking water, despite the former group having significantly lower body weight (Eller & Reimer 2010). This trend has also been shown in humans where supplementation reduced subsequent energy intake in normal weight but not obese individuals (Bellissimo et al 2008). A few studies have demonstrated that, in addition to influencing the concentration of circulating satiety hormones, the presence of WPI had an impact on gene expression level of neuropeptides within the hypothalamic cells. It was shown that mice fed a LFD with a high or low sucrose dose that contained WPI as the main protein source showed a trend towards a decrease and increase in the genes encoding POMC and NPY, respectively, compared to the CAS counterparts (Nilaweera et al 2017). In contrast, in Wistar rats, a phyto-oestrogen free (PF) diet given with WPC supplementation showed an increase in the hypothalamic expression of POMC, together with a decrease in energy intake relative to rats fed a PF diet without any supplementation (Andreoli et al 2016).

Results from these studies suggest that the beneficial effects of WP on appetite suppression may be more significant and potent in lean than obese individuals. Based on this observation, it is possible that the presence of HFD might interfere with the satiety properties exerted by WP.

Table T1.4 In vivo studies reporting whey protein (WP) effects on energy intake, satiety and satiety hormones and neuropeptides. *Abbreviations: LAB, α-lactalbumin; BSA, bovine serum albumin; Lf, lactoferrin; Lp, lactoperoxidase; GMP, glycomacropeptide; HFD, high-fat diet; LFD, low-fat diet; HSD, high-sucrose diet; LSD, low-sucrose diet; CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; PYY, peptide YY; PP, pancreatic polypeptide; POMC, proopiomelanocortin; NPY, neuropeptide Y;* IL3, *interleukin 3; INFγ, interferon gamma, Foxp3, forkhead box protein 3.*

In vivo **Experimental approach**	**WP in the diet**	**Dietary intervention**	**Dietary intervention length**	**Details**	**References**
Humans (lean)	WP (48g)	Buffet meal *ad libitum*		↓ Appetite ↑ Plasma CCK and GLP-1	Hall et al, 2003
Humans (healthy)	WP (10% energy)	Breakfast		↓ Hunger ↑ Plasma insulin and GLP-1	Veldhorst et al, 2009a
Humans (lean and overweight)	WP (50g)	Buffet meal		↓ Appetite ↑ Plasma insulin, CCK and GLP-1 ↓ Plasma ghrelin	Pal & Ellis 2010; Bowen et al, 2006a; Bowen et al, 2006b
Humans (overweight and obese)	WP (27g)		12 weeks	↑ Satiety and fullness before lunch	Pal et al, 2014
Humans (normal weight)	GMP-enriched WP (45g)			↑ Plasma insulin, CCK, GLP-1, PYY and PP	Chungchunlam et al, 2015
Humans (healthy)	LAB (10% and 25% energy)	Breakfast		↓ Appetite	Veldhorst et al, 2009b
Humans (healthy)	LAB-enriched WP	Breakfast		↓ Hunger	Hursel et al, 2010
Wistar rats (males)	WPI, LAB or Lf (15% energy) WPC (g/Kg)	phyto-oestrogen free diet	10 weeks	↓ Energy intake ↑ POMC hypothalamic expression ↓ Fasting plasma insulin	Andreoli et al, 2016

Obese-prone rats (males)	WP, LAB or Lf (15% energy)	HFD (40% fat)	7 days	↓ Energy intake ↑ Plasma PYY ↑ PYY colonic expression	Zapata et al, 2018
C57BL/6 mice (males)	WPI (20% energy)	LFD (10% fat)	15 weeks	↓ Cumulative energy intake ↓ PYY expression in the small intestine	McAllan et al, 2015
C57BL/6 mice (males)	WPI (20% energy)	LSD (35% sucrose) HSD (7% sucrose)	17 weeks	↑ Energy intake ↓ POMC hypothalamic expression ↑ NPY hypothalamic expression	Nilaweera et al, 2017
Obesity Prone CD (OP-CD) rats (males)	LAB (15% energy) Or Lf (15% energy)	HFD (40% fat)	56 days	Transient hypophagia (LAB) Prologed hypophagia (Lf)	Zapata et al, 2017
C57BL/6 mice (males)	BSA (20% energy)	HFD (45% fat)	12 weeks	↑ Energy intake	McManus et al, 2015b
BALB/cByJ Jcl mice (female)	Lp supp. (2.5g/Kg)	Standard diet	1 or 3 hours	Change in the expression of genes related to metabolism and immunity	Wakabayashi et al, 2007
Colitis model of CBA/J mice (female)	Lp supp. (62.5g/mouse/day)	Standard diet	3 or 9 days	↑IL10 and regulatory T cell (Treg) marker, Foxp3 colonic expression ↓INFγ colonic expression	Shin et al, 2009

1.6.3.2 Whole whey protein effects on body weight, energy balance and inflammation

With the current worldwide obesity epidemic, the anti-obesity properties associated with WP has attracted much attention in the recent years. Several studies have highlighted the anti-obesity effects of WP or, more specifically, its ability to reduce HFD associated increased body weight and fat mass accumulation (**Table T1.5**). Also casein, compared to other non-dairy proteins, is more efficient in preventing weight gain and accretion of adipose mass. In a recent study conducted in C57BL/LJ mice, Liisberg *et al* investigated the effect of a high-fat diet/high-protein diet using casein, soy, cod filets, chicken, beef, pork as protein sources. Casein was revealed to be the most efficient of these protein sources in obesity prevention and in lowering fat mass (Liisberg et al 2016). A decrease in body fat accretion and changes in the urinary excretion of phase II metabolites in the presence of hydrolysed casein were also demonstrated in obesity prone mice (Clausen et al 2015). However, in humans it was recently shown that casein consumption before sleep did not affect fat or glucose metabolism, RER, or suppress appetite in hyperinsulemic obese men, relative to the placebo control (Kinsey et al 2016). Notably, WP was not tested in this study.

The reason why WP, and not casein, was selected in this thesis as a better candidate in the treatment of metabolic diseases is because this protein is more effective than casein in influencing body weight and energy balance. Pezhesky and colleagues recently demonstrate that WP and casein have a differential effect on energy balance, gut hormones and glucose metabolism in rats. A diet enriched in both CAS and WP caused a reduction in body weight, adiposity and food intake and an improvement of glucose tolerance but, in all cases, the effect of WP was more robust compared to CAS, without showing changes in energy expenditure (Pezeshki et al 2015). Another study showed that both WP and casein ameliorated energy balance, indices of renal inflammation and damage, and prevented morbidity associated with stroke in spontaneously hypertensive stroke-prone rats fed with HFD supplemented with 1% salt in drinking water for 12 weeks. However, only WP decreased fat mass and blood pressure (Singh et al 2016). In humans, WPI supplementation improves body composition by increasing lean mass in women without affecting changes in fat mass. The body weight

composition improvement was more evident if coupled with energy restriction (Bergia et al 2018). Several studies from our group aimed to extend the understanding of WP and energy balance regulations. The effect of WPI supplementations was investigated in the presence of a low/standard-fat diet (LFD; 10% fat by calories), high-fat diet (HFD; 45% fat by calories), low-sucrose diet (7% sucrose by calories) high-sucrose diet (HSD; 35% sucrose by calories) in male C57BL/J6 mice. Mice fed a LFD supplemented with 20% of WPI for 15 weeks showed a decrease in weight gain, cumulative energy intake and dark-phase VO_2 compared with CAS-fed mice. In addition, a reduction in stomach and small intestine size was observed in mice that ingested WPI-supplemented diet, which had an additional decrease in the small intestinal expression of genes encoding PYY, FATP4 and IR compared to mice fed with LFD-CAS. Plasma leptin, insulin, TAG and GLP-1 remain unchanged between the two groups (McAllan et al 2015). In the same mouse strain, but in females, in which WPI was supplemented in drinking water, a decreased in hepatic lipid droplets and an increase in basal metabolic rates, respiratory quotient, glucose tolerance and insulin sensitivity were noticed (Shertzer et al 2011). Mice fed with HFD containing 20% WPI for 8 weeks gained less weight and had an increase in the lean mass compared to their CAS counterparts (McAllan et al 2013). Interestingly, the change in energy intake could not account for the decrease in body weight gain, suggesting another mechanism was at play. A higher percentage of lean mass was observed also when the same diet was given for 21 weeks. Increasing the WPI portion in the HFD up to 40% (and proportionally reducing the carbohydrate content) caused a drastic drop in body weight gain, with observed values very similar for the low-fat diet control group. In addition, WPI reduced HFD-induced plasma leptin and liver TAGs (McAllan et al 2014). Again, in this study also, at moderate WPI content (20%), energy intake was not different to CAS-fed controls, but there was a trend towards a decrease in fat mass in the former group. Finally, in our recent study, we did observe differences in body weight gain but no changes in energy expenditure between mice fed with high-sucrose diet (HSD)-WPI and HSD-CAS. However, the HSD-WPI showed a reduction in RER, adipose tissue weight and ileal gene expression of the amino acid transporter SLC6a19, glucose transporter 2 (GLUT2) and FATP4. In this study, it was noticed that the mice that were ingesting WPI, both in HSD and LSD, increased energy intake

compared to the CAS groups and yet they reduced weight gain (Nilaweera et al 2017), further strengthening the idea that there is a, so far unidentified mechanism through which whey affects energy balance that does not rely on energy intake.

Pilvi *et al* have suggested several mechanisms by which WPI may reduce symptoms of obesity in a series of studies in C57BL/J6 mice consuming HFD (60% fat by calories). Initially, mice receiving HFD containing 18% WPI and 1.8% of calcium carbonate ($CaCO_3$, which has anti-obesity properties) showed a significant decrease in body weight and fat coupled with significantly high levels of fat excretion when compared to mice in receipt of a similar diet where CAS was the protein source and which had a lower percentage of $CaCO_3$ (0.4%). It was suggested that the reduction in weight gain and fat excretion results from the high leucine content of whey modulating insulin signalling, thus directing energy towards muscle biosynthesis rather than fat storage in adipose tissue (Pilvi et al 2007). In a follow-up study employing the same diet groups for the same 21-week study period, the authors observed the same inhibition of fat mass accumulation within the whey consuming cohort combined with a significant up-regulation of leptin and β-adrenergic receptor genes, leading the authors to suggest that WPI attenuates symptoms via improved leptin sensitivity and increased lipolysis (Pilvi et al 2008). Recently, WPI demonstrated a positive effect on cardiovascular derangements. A chronic 10% or 20% WPI supplementation (18 weeks) improved high-fat/high-cholesterol diet-induced atherosclerosis in atherosclerosis-prone apolipoprotein E-deficient (ApoE null) mice. In particular, in the presence of WP, reductions in aorta lesions and cholesterol accumulation within the liver were observed along with an increase in serum high-density lipoprotein cholesterol levels. Moreover, serum IL6, a pro-inflammatory cytokine, and IL10, an anti-inflammatory cytokine, were decreased and increased, respectively, by WP supplementation (Zhang et al 2018).

In summary, the above findings suggest that dietary WP can affect energy balance without relying on energy intake. The mechanisms for this effect are still to be elucidated.

1.6.3.3 Specific whey protein effects on body weight, energy balance and inflammation

All the aforementioned studies assessed the effect of whole WP components administered together. However, it is important to explore which specific effects can be attributed to WP components (**Table T1.5**). In a study from Pilvi *et al*, mice previously fed with HFD were placed on a low-calorie weight loss diet containing different WP components (LAB, LAG, WPI and Lf) for 50 days before being returned to *ad libitum* feeding. Mice which consumed the LAB diet showed a significant loss of fat mass during the calorie restricted period and a subsequent reduction in visceral fat gain during the weight recovery period compared to all other dietary groups (Pilvi et al 2009). In another study, Gao *et al* initially provided mice with HFD (60% fat by calories) and then, after 8 weeks, introduced hydrolysed LAB (LABH) in the diet for further 12 weeks. The presence of LABH in the HFD caused a decrease in the weight gain, blood glucose, serum insulin and downregulated the inflammation-related genes and the gene encoding insulin receptor substrate (*Irs-1*) in the adipose tissue. In addition, TNFα, IL6 and MCP-1 expression was downregulated within the adipose tissue in the presence of LABH. This suggests that LABH ameliorates adipose insulin resistance and inflammation in HFD-fed C57BL/6J mice (Gao et al 2018). Recently, in another study, LABH administration was shown to reduce inflammation and inhibit the destruction of the gut barriers through an upregulation of some TJs expression in the colon in HFD-induced endotoxemia and systematic inflammation in mice (Li et al 2019a). In rats, Zapata *et al*, remarked on the importance of protein quantity and quality in energy balance improvement. DIO rats were randomized to isocaloric diets containing WPI, LAB, Lf or pair-fed to Lf. Both LAB and Lf led to a decrease in calories intake, body weight, plasma leptin and plasma insulin in the animals. Specifically, rats with Lf-supplemented diet decreased the energy expenditure and rats supplemented with LAB increased plasma PYY (Zapata et al 2017b). Recently, a study in pigs was performed, in which Caesarean-delivered preterm pigs were fed with bovine milk or bovine milk containing WPC enriched in LAB. The presence of WPC enriched in LAB improved organ growth and immunity parameters (Nielsen et al 2020). A study conducted in humans reported that LAB had the same efficacy in reduction of body weight, fat mass and in the preservation of fat-free mass as similar

daily intakes of milk proteins during six months of calorie restriction (Soenen et al 2011).

A few studies have been focusing also on the role of bovine Lf in energy balance. A 12-week Lf administration to HFD-induced obese mice decreased body weight gain, blood cholesterol and glucose concentration, and alleviated liver steatosis. Moreover, the HFD-Lf group showed a decrease in leptin and plasminogen activator inhibitor (PAI-I) expression in the adipose tissue, which could be attributed to a decreased LPS translocation and gut inflammation (Sun et al 2016). Conversely, we have shown that the body weight HFD-Lf-fed mice did not differ from their CAS counterparts. However, the Lf group had lower levels of leptin and corticosterone in the blood (McManus et al 2015a).

There is evidence that BSA may also beneficially influence fat mass accumulation as noted in our study by McManus *et al* (McManus et al 2015b). In this study, C57BL/6 mice fed with HFD, where BSA was the dominant dietary protein source, for 13 weeks had significantly reduced subcutaneous fat mass, plasma leptin and plasma corticosterone relative to animals that consumed a HFD where CAS was the dominant dietary protein source. Notably, BSA-fed mice had elevated energy intake but reduced body weight.

There are no evidences for an impact by LAG or Lp on body weight and energy balance, but they have an influence on immunity. As already mentioned in paragraph 1.6.1.1, LAG plays a role in enhancing the immune response and its enzymatic hydrolysis produces a higher yield of short bioactive peptides with potential antioxidant and anti-inflammatory effects (Bamdad et al 2017, Tai et al 2016). Similarly, in BALB/c mice, it was demonstrated that the oral administration of Lp causes a change in expression of several genes involved in metabolism, apoptosis and immunity (in particular the gene that encode the immunity-regulator FKBP5) in small intestinal epithelial cells (Wakabayashi et al 2007). Lp administration also ameliorates colitis by upregulating colonic anti-inflammatory cytokines and maintaining peripheral regulatory T cells (Shin et al 2009). Taking into account the link between

metabolic diseases, such as obesity, and increase in chronic low-grade inflammation (Saltiel & Olefsky 2017), it is not odd to think that LAG and Lp could have a positive effect on these diseases and have a co-adjuvant effect for other specific WPs within WPI.

All of these findings suggest that WP modulate energy balance, lipid metabolism and body composition, even if it is still a challenge to precisely assign each WP component a specific role or mechanism of modulation. It is important to highlight that the activity of WP can vary according to quantity of protein intake, kind and quantity of micro (i.e., $CaCO_3$) and macronutrients (i.e., fat, sucrose) present in the diet, the hydrolysation status and model used in the study (mice, rat or humans).

Table T1.5 In vivo studies reporting whey protein (WP) effects on energy expenditure, body weight, metabolism regulation and inflammation. *Abbreviations: LAB, α-lactalbumin; BSA, bovine serum albumin; Lf, lactoferrin; WPC, whey protein concentrate; DIO, diet-induced obesity; HFD, high-fat diet; LFD, low-fat diet; HSD, high-sucrose diet; LSD, low-sucrose diet; βAR, beta adrenergic receptor; TAG, triacylglycerol; VO2, oxygen consumption rate; FATP4, fatty acid transporter 4; IR, insulin receptor; RER, respiratory exchange ratio; SLC6a19, Sodium-dependent neutral amino acid transporter B(0)AT1 gene; GLUT2, glucose transporter 2; IL6, IL10, interleukin 6 and 10; IRS1, insulin receptor substrate 1; TNFα, tumour necrosis factor alpha, MCP-1, monocyte chemoattractant protein 1; TJ, tight junction; LPS, lipopolysaccharide; PAI-I, plasminogen activator inhibitor-1.*

***In vivo* Experimental approach**	**WP in the diet**	**Dietary intervention**	**Dietary intervention length**	**Details**	**References**
Humans (healthy women)	WP supp.			↑ Lean mass (especially during energy restriction)	Bergia et al, 2018 *sistematic review
Obesity Prone CD (OP-CD) rats (males)	WP (14% energy)	HFD (33% fat)	5 weeks	↓ Body weight ↓ Adiposity ↑ Glucose tolerance	Pezeshki et al, 2015
Spontaneously hypertensive stroke-prone rats (males)	WP (40% energy)	HFD (33% fat)	12 weeks	↓ Body weight ↓ Adiposity ↑ Energy expenditure ↓ Renal inflammation ↓ Blood pressure	Singh et al, 2016

C57BL/6 mice (males)	WPI (18% energy) +1.8% CaCO3	HFD (60% fat)	21 weeks	↓ Body weight ↓ Adiposity ↑ Fat excretion ↑ Leptin and βAR fat expression	Pilvi et al, 2007; Pilvi et al, 2008
C57BL/6 mice (females)	water + 100 g/L of Natural Pure WPI (90% WPI)	HFD (40% fat)	11 weeks	↓ Hepatic lipid droplets ↑ Energy expenditure ↑ Glucose tolerance	Shertzer et al, 2011
C57BL/6 mice (males)	WPI (20% energy)	HFD (45% fat)	8 weeks	↓ Weight gain ↑ Lean mass	McAllan et al, 2013
C57BL/6 mice (males)	WPI (20%, 30% or 40% energy)	HFD (45% fat)	21 weeks	↓ Weight gain ↑ Lean mass ↓ Plasma leptin ↓ Hepatic TAGs	McAllan et al, 2014
C57BL/6 mice (males)	WPI (20% energy)	LFD (10% fat)	15 weeks	↓ Weight gain ↓ Dark phase VO2 ↓ stomach and small intestine size ↓ FATP4 and IR expression in the small intestine	McAllan et al, 2015
C57BL/6 mice (males)	WPI (20% energy)	LSD (35% sucrose) HSD (7% sucrose)	17 weeks	↓ Weight gain ↓ Adiposity ↓ RER ↓ SLC6a19, GLUT2, FATP4 ileal expression	Nilaweera et al, 2017
ApoE−/− mice (males)	WPC suppl. (10% or 20% energy)	High fat/High-cholesterol diet	18 weeks	↓ Hepatic cholesterol ↓ Serum IL6 ↑ Serum IL10	Zhang et al, 2018
Obesity Prone CD (OP-CD) rats (males)	LAB (15% energy) Or Lf (15% energy)	HFD (40% fat)	56 days	↑ Energy expenditure ↓ Weight gain ↓ Adiposity gain ↓ Plasma leptin and insulin	Zapata et al, 2017

				↑ Glucose clearance ↑ Hepatic fatty acids β-oxidation ↓ Hepatic lipogenesis	
DIO C57BL/6 mice (males)	LAB (18% energy)	Calories restriction (CR) diet + *ad libitum*	50 days + 50 days	↓ Adiposity during CR ↓ Visceral fat gain during *ad libitum*	Pilvi et al, 2009
DIO C57BL/6 mice (males)	Hydrolysed LAB supp. (100, 200 or 400 mg/Kg)	HFD (60% fat)	12 weeks	↓ Weight gain ↓ Blood glucose ↓ IRS1 fat expression ↓ TNFα, IL6 and MCP-1 fat, colonic expression and in the serum ↑ Colonic TJs expression ↓ Serum and faecal LPS	Gao et al, 2018; Li et al, 2019.
Caesarean-delivered preterm pigs	LAB-enriched WPC supp. (6.3 g/L total LAB)	Unpastorized bovine milk	19 days	↑ Organ growth ↑ Immunity parameters	Nielsen et al, 2020
C57BL/6 mice (males)	Lf (20% energy)	HFD (45% fat)	13 weeks	↓ Plasma leptin	McManus et al, 2015a
DIO C57BL/6 mice (males)	Lf supp. (100 mg/Kg)	HFD (40% fat)	12 weeks	↓ Weight gain ↓ Blood glucose and cholesterol ↓ Hepatic steatosis ↓ Leptin and PAI-I fat expression	Sun et al, 2016
C57BL/6 mice (males)	BSA (20% energy)	HFD (45% fat)	13 weeks	↓ Adiposity ↓ Plasma leptin	McManus et al, 2015b

1.6.4 Whole and individual whey protein effects on the gut microbiota

The composition of the gut microbiota undergoes dramatic changes throughout the life cycle of the host. Protein components of whey appear to play important roles in the development of the infant gut microbiota by stimulating beneficial bacteria species such as members of the genera *Bifidobacterium* and *Lactobacillus* and providing protection against opportunistic pathogens, including pathogenic *E. coli* strains (Hascoet et al 2011, Mastromarino et al 2014, Subramanian et al 2014). However, the impact of dietary WP intake on the stable adult gut microbiota is currently poorly understood. Some promising results have been reported in the few studies examining the effect of dietary protein on the gut microbiota. Firstly, when compared to other dietary protein sources such as red and white meat, soy and casein, WP is shown to exert significantly less colonocyte DNA damage in rats (Toden et al 2007a, Toden et al 2007b). This result suggests that bacterial fermentation of WP does not result in a large array of the putrefactive metabolites traditionally associated with high dietary protein intake, and therefore WP represent potential novel candidates for beneficial gut microbiota modulation. Sprong *et al* reported that the consumption of a diet containing WP increased faecal *Lactobacillus* and *Bifidobacterium* counts in Wistar rats, when compared to those consuming the same diet but where the protein energy source was CAS (Sprong et al 2010). Similar results were reported by our group where, using 16S rRNA sequencing, we demonstrated significantly higher *Lactobacillus* and *Bifidobacterium* populations with a concurrent significant decrease in *Clostridium* populations in C57BL/6 mice consuming HFD-WPI compared to mice consuming a HFD-CAS (McAllan et al 2014). However, in a similar study, Tranberg *et al* did not observe any difference in the composition of the gut microbiota between two groups of C57BL/6 mice consuming HFD where the protein source was either casein or whey. Although, in the latter case, microbiota analysis was determined by less detailed, denaturant gradient gel electrophoresis (DGGE)-based, approach (Tranberg et al 2013). In mice fed with WP-standard diet but with different percentages of sucrose, the proportion of Firmicutes was consistently lower, compared to the CAS counterparts, at different timepoints (Nilaweera et al 2017). Notably, another study

showed that, in comparison to a standard diet, an enteral whey-peptide based murine diet significantly increased concentration of cecal SCFAs (Tomoda et al 2015). While results from this study indicate that the microbial fermentation of WP or associated bioactive peptides promotes gut health through increased SCFA production, the authors did not examine the composition of the gut microbiota or the SCFA producing bacteria species (Tomoda et al 2015). *In vitro* gastrointestinal digestion following faecal batch culture fermentations (mimicking colonic fermentation), using faeces from normal-weight and obese donors, has been the basis of another study. Through this, the authors showed how a diet containing WP can selectively stimulate the growth of *Bifidobacterium* and *Lactobacillus*, enhancing SCFA production and contributing to improved intestinal health and reducing obesity (Sanchez-Moya et al 2017).

There have been few human studies, focusing on assessing the effect of dietary WP coupled with exercise on gut microbiota composition. Clarke *et al* observed a significantly higher microbial diversity, which is a biomarker of good health, associated with the gut of professional rugby players relative to controls. The higher diversity was positively correlated with protein intake with, notably, whey protein supplements represented a significant proportion of the athletes' total protein consumption (Clarke et al 2014b). The relationship between exercise and WP consumption was recently investigated also in healthy but sedentary adults that underwent short-term exercise and/or WP supplementation of their diets. During the exercise period the participants either consumed or refrained from consuming daily WP. The changes in gut microbial composition and function were modest following increases of physical activity but, in the whey protein-supplemented group, a significant change in the gut virome was evident. In this study, no differences were found in bacteria and archaea diversity following whey protein consumption (Cronin et al 2018). While data from these initial studies suggest a potential use for WP in the beneficial modulation of the gut microbiota, more studies using specific component proteins of whey are required to fully elucidate their effects on enteric bacterial population.

As mentioned in paragraph 1.6.1.4., bovine Lf is a growth promoter of several bacteria that colonize our gut, such as *Bifidobacterium spp.* (Oda et al 2014). *In vitro*, both apo-Lf (iron free) and holo-Lf (iron saturated) exhibited bifidogenic activity with respect to several species of *Bifidobacterium* (i.e., *B. infantis, B. breve, B. bifidum, B. longum*) (Morshedur Rahman et al 2010, Saito et al 1996). Multiple *in vivo* studies have also established that Lf exerts bifidogenic effects. Hentges *et al* reported gnotobiotic mice associated with a human infant gut microbiota consuming a milk fortified with bovine Lf had increased counts of *Bifidobacterium* compared with mice consuming un-supplemented formula (Hentges et al 1992). Furthermore, recombinant human Lf (rhLf) was shown to modulate the intestinal microbiota of piglets and increase ileal populations of *Bifidobacterium spp*. and *Lactobacillus spp* .(Hu et al 2012). Bovine Lf has also been shown to exert a positive effect on the gut microbiota also in HFD-induced obese mice, increasing the *Bifidobacterium spp.* and modestly inhibiting the growth of Enterobacteriales (Sun et al 2016). These combined results suggest that the ingestion of Lf positively modulate the gut microbiota through exerting a probable prebiotic effect.

While at the moment there is no data relating the specific effects of LAG, BSA and Lp on the modulation of the gut microbiota during normal or HFD, a few studies have focused on LAB. ln HFD-fed mice supplemented with LAB hydrolysates (LABH), there was an increase in the relative abundance of *Lachnospiraceae* and *Blautia* (Li et al 2019a). In addition, Cesarean-delivered preterm pigs fed with milk containing LAB-enriched WPC showed an increase in *Clostridiaceae*, *Streptococcus*, *Enterobacteriaceaea* and *Strepromyces* within the gut microbiota, together with changes is some bacterial metabolites, such as acetic acid and butanoic acid (Nielsen et al 2020).

In summary, in many cases, supplementation of WP in a normal diet or in a HFD have been show to positively modulate the gut microbiota by increasing the growth of beneficial taxa, such as *Bifidobacterium spp.* and *Lactobacillus spp.*. Based on results to date, the most important players would seem to be LAB and Lf.

1.6.5 Whole and individual whey protein effects on mood and behavioural control

As mentioned in paragraph 1.6.3.1, bovine WP is rich in BCAAs and tryptophan. We have previously noted that the essential amino acid tryptophan is a precursor of serotonin. WP contains a higher percentage of tryptophan than other protein sources. Furthemore, LAB is known to have the highest proportion of tryptophan (approx. 6%) compared to other WP components (Madureira et al 2007). Due to its particularly high tryptophan contents, diets enriched in LAB were thought to have beneficial effects on mood and cognition. Several studies have been done in light of this hypothesis. Markus *et al* carried out three such studies. Initially, they recruited both highly stressed, vulnerable and relatively-stressed invulnerable subjects, which have been exposed to experimental stress after diet enriched in LAB or CAS intake. The supplementation with LAB resulted in an increase in plasma tryptophan levels in both groups and, through profile of mood states, measurement of skin conductance and peripheral pulse frequency, a reduction in depressive-like behavior and stress were recorded among stress-vulnerable subjects. These findings were coupled with a decrease in salivary cortisol, the "stress-hormone", and an increase in plasma prolactin, which is an indication of stress reduction, and brain serotonin activity (Markus et al 2000). Again in stress-vulnerable subjects, the authors found increased ratio of plasma tryptophan, coupled with large neutral amino acids, and improved cognitive performance during a dietary intervention that included WP enriched in LAB (Markus et al 2002). A few years later, the same research group carried out another human study that included healthy subjects with or without sleep complaints. They demonstrated that LAB-enriched diet intake in the evening caused, as expected, a dramatic increase in tryptophan in the blood before bedtime. In the morning, in both groups but especially in subjects with sleep complaints, subjects that had LAB-enriched diet were feeling "less sleepy" and their behavioral performance, together with brain sustained-attention processes, were improved (Markus et al 2005). Despite these encouraging findings, a human study carried out on unmedicated recovered depressed subjects showed that, although a diet enriched in LAB led to the expected rises in circulating tryptophan, only minimal effects were found on mood and cortisol response to experimental stress,

which is not sufficient to prevent a stress-induced mood deterioration in this specific category of subject (Merens et al 2005). However, a dietary LAB supplementation improved abstract visual memory in unmedicated recovered depressed subjects, suggesting a possible utilization of this WP component to mediate age- or decline-related memory decline (Booij et al 2006). Notably, in these last two studies, the authors pointed out that one of the limitations of the studies is the effective exposure to LAB-enriched diet, discussing that the long-term effect of LAB intake should be investigated. In addition to the beneficial effects exerted by dietary LAB on stress, sleep control and memory, a study conducted in rats attributed an anxiolytic-like effect to LAB. Notably, Wistar rats were fed with LAB or CAS-enriched diets acutely (30 minutes meal) or chronically (3 and 6 days). Animals fed with LAB-supplemented diet significantly increased the amount of serotonin produced by the hypothalamus, coupled with an increase in the time spent on the open arms of the elevated plus maze and increased number of visits to the center of the open field, indicating a less anxious status. Furthermore, a single LAB meal decreased sucrose consumption, reflecting an appetitive and/or rewarding action of this WP component (Orosco et al 2004). Further details on LAB applications in human nutrition are detailed elsewhere (Layman et al 2018b).

WP-derived peptides have also been investigated with respect to a potential role in mood and behavior. In a recent study, healthy humans received either whey peptide rich in tryptophan-tyrosine-related peptides (produce by enzymatic digestion of WP), or placebo supplements for 12 weeks. Notably, the whey peptide had been previously shown to have a positive effect on cognitive and memory performance in mice (Ano et al 2018). In agreement with the study conducted in rodents, this specific whey-derivate peptide improved some cognitive functions in people with a high level of subjective fatigue. This study is in accordance with previous epidemiological and preclinical findings suggested that intake of whey peptide in daily life might experience benefit with respect to cognitive function (Kita et al 2018).

In conclusion, WPs and, in particular LAB, thanks to their amino acid composition containing a high percentage of BCCAs and tryptophan, have a positive effect in ameliorating anxiety and stress, improving cognition and feeling refreshed after sleep.

The mechanism through which a protein source rich in tryptophan could modulate the cortisol levels and, thus, mood can be explained by two different scenarios:

- a higher tryptophan availability results in a higher serotonin production. It has been shown that serotonin plays a role in the functionality of the HPA axis and, in turn, influences the production of cortisol. Moreover, a hyperproduction of cortisol have been shown to decrease the availability of tryptophan, increasing the risk of development of anxiety and depression (Fuchs & Flügge 2003, López et al 1998, Nuller & Ostroumova 1980).
- The gut microbiota uses tryptophan to produce serotonin, indoles and kynurenine pathway-related metabolites. These metabolites, in turn, affect different functions in the host, including mental health. It could be, thus, there might be a link between tryptophan-derived gut metabolites and the neuropsychiatric disorders (Agus et al 2018).

1.7 Aims and Objectives

Obesity has become one of the most prevalent health issues over the last decades, due to modern eating habits and an increase of sedentary lifestyle. Obesity is a multifactorial condition that comes from a long-term imbalance between energy intake and energy expenditure (paragraph 1.3). Despite the adoption of several therapies (i.e., pharmacotherapies, surgery and dietary therapies) for the prevention or amelioration of this metabolic disease, scientific effort is still required to expand therapeutic options (Ruban et al 2019). In this thesis, we aim to investigate the impact of a dietary strategy focused on bovine whey protein (WP) (paragraph 1.6). Our general goal was to better understand WP actions on adiposity, metabolism, energy balance, gut functionality, and gut microbiota in the presence of high-fat or low-fat diets. Further, we sought to provide mechanistic insight into the various WP-related effects (**Fig. 1.10**).

1.7.1 Aim 1: Does alpha-lactalbumin influence energy balance, nutrient absorption, fat metabolism and gut microbiota during high-fat diet feeding?

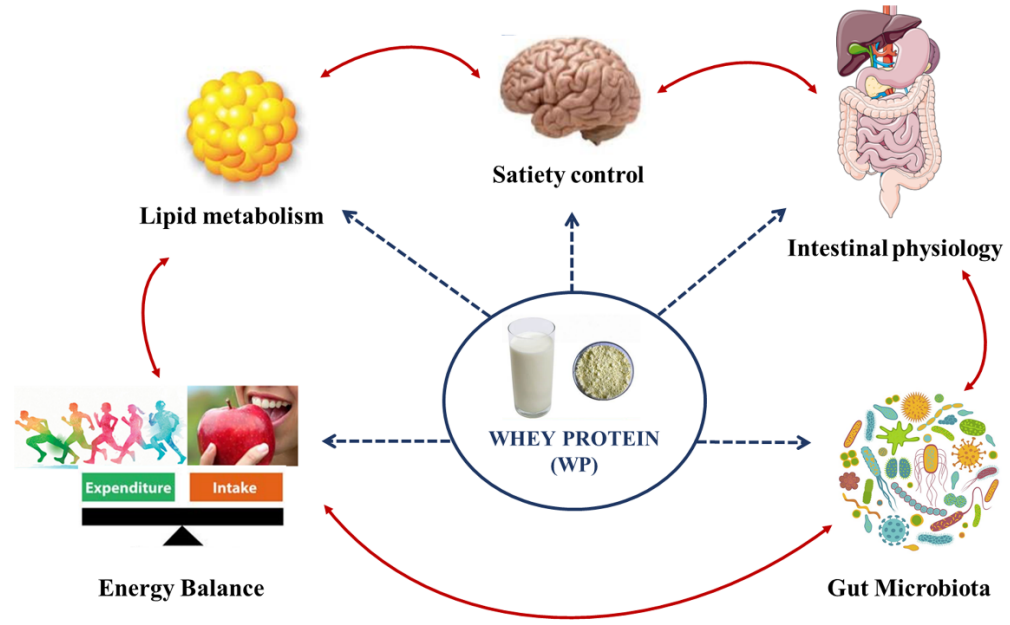
Bovine whey is composed by different protein types, present in different proportions. Previous studies in our group explored the effect of two WP components, BSA and Lf, on energy balance and metabolism (McManus et al 2015a, McManus et al 2015b). These studies had contrasting outcomes, confirming the fact that different WP components have different characteristics and influence differently energy balance and metabolism (paragraph 1.6.1). In this study, we sought to examine if the specific WP, LAB, countered the effects of a high-fat diet on gut-hypothalamic-adipose control of energy balance.

1.7.2 Aim 2: Does a high-fat diet containing whey protein differentially affect energy balance, nutrient absorption, fat metabolism and gut microbiota at different ages?

Individuals' gut microbiota and metabolism are influenced by diet throughout life (Conlon & Bird 2014, Drewnowski & Shultz 2001, St-Onge & Gallagher 2010). Adolescence is a particular period of development, marked by physical maturation, where dietary habits can have a health impact late in life (Das et al 2017, Joung et al 2012). Thus, we sought to assess if an HFD containing WPI given at two different ages, before the adulthood period, would differentially affect energy balance, adipose tissue metabolism, intestinal nutrient absorption and gut microbiota configuration. In addition, we wanted to assess if WPI given before and during high-fat diet exposure would prevent or ameliorate HFD-induced metabolic syndrome.

1.7.3 Aim 3: Does the gut microbiota mediate the effects of whey protein on adiposity, gut physiology and lipid metabolism during high-fat diet feeding?

The aforementioned studies (described in Aim 1 and Aim 2) and paragraph 1.6.4 showed that the gut microbiota configuration is affected by individual and whole WP supplementation within HFD. Notably, WPI had a positive effect on HFD-induced dysfunctions, such as body weight gain, adiposity, and metabolism-related changes in addition to gut microbiota changes. In light of these observations, we investigated if WPI-related changes in body weight and adiposity during were HFD mediated by the gut microbiota. To assess this, the microbiota of HFD-WPI-fed mice was depleted by treating with an antibiotic cocktail for 5 and 10 weeks. During this study, we had the opportunity to extend the knowledge of the effect of WP on inflammation, gut permeability, and metabolites within the caecum in the presence of HFD.



Aims of the thesis

Aim 1	Aim 2	Aim 3
LAB	**WPI, age and duration**	**WPI and gut microbiota depletion**
Effect of HFD-LAB on: - energy balance - nutrient absorption - lipid metabolism - gut microbiota.	Effect of HFD-WPI on: - energy balance - nutrient absorption - lipid metabolism - gut microbiota at different ages.	To investigate if the gut microbiota mediates the effect of HFD-WPI on: - adiposity - gut physiology - lipid metabolism.

Figure 1.10 Diagram representing the general aim of the thesis. *Top: the general aim of the thesis is to better understand WP action on adiposity, metabolism, energy balance, satiety control, intestinal physiology, and gut microbiota. In addition, this thesis aims to find a link among WP-related changes. Bottom: three aims of the thesis. Abbreviations: HFD, high-fat diet; LAB, α-lactalbumin; WPI, whey protein isolate.*

Chapter 2

Dietary Alpha-lactalbumin Alters Energy Balance, Gut Microbiota Composition and Intestinal Nutrient Transporter Expression in High-Fat Diet-Fed Mice

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Abstract

Rationale Recently there has been a considerable rise in the frequency of metabolic diseases, such as obesity, due to changes in lifestyle and resultant imbalances between energy intake and expenditure. Whey protein is considered as potentially important component of a dietary solution to the obesity problem. However, the roles of individual whey proteins in energy balance remain poorly understood.

Objectives This study investigated the effects of a high-fat diet (HFD) containing alpha-lactalbumin (LAB), a specific whey protein, or the non-whey protein casein (CAS), on energy balance, nutrient transporters expression, and enteric microbial populations. C57BL/6J mice (n = 8) were given a HFD containing either 20% CAS or LAB as protein sources or a low-fat diet (LFD) containing CAS for 10 weeks.

Results HFD-LAB fed mice showed a significant increase in cumulative energy intake ($P=0.043$), without differences in body weight, energy expenditure, locomotor activity, respiratory exchange ratio or subcutaneous and epididymal adipose tissue weight. HFD-LAB intake led to a decrease in the expression of glucose transporter *glut2* in the ileum ($P=0.05$) and in the fatty acid transporter *cd36* ($P<0.001$) in both ileum and jejunum. This suggests a reduction of absorption efficiency within the small intestine in the HFD-LAB group. DNA from faecal samples was used for 16S rRNA-based assessment of intestinal microbiota populations; the genera *Lactobacillus*, *Parabacteroides* and *Bifidobacterium* were present in significantly higher proportions in the HFD-LAB group.

Conclusions These data indicate a possible functional relationship between gut microbiota, intestinal nutrient transporters and energy balance, with no impact on weight gain.

Introduction

Obesity has emerged as one of the most prevalent global health problems over the last 30 years because of its association with comorbidities such as chronic inflammations, type II diabetes, cardiovascular disorders and certain types of cancer (O'Neill & O'Driscoll 2015). Whilst the aetiology of obesity is multifactorial, there is a growing recognition that high energy density diets (containing high levels of fat and sugar) are major contributors to long-term imbalance between energy intake and energy expenditure (Hu et al), with comparable effects seen in rodents including mice such as the C57BL/6J strain. Indeed, energy dense diets increase intestinal energy absorption and fat storage in adipose tissue (Galgani & Ravussin 2008).

Additionally, these diets impair the hypothalamic regulation of hormones involved in the control of energy balance (Wang et al 2012). Thus, one approach to reducing weight gain or causing weight loss would be to devise interventions that affect the cross-talk between the gut, adipose tissue and the hypothalamic mechanisms regulating energy balance.

Nutrient digestion and absorption provides the necessary energy for the survival of living organisms and the gastrointestinal (GI) tract has evolved to optimise these processes (Goodman 2010). In particular, nutrient absorption takes place in the duodenum, jejunum and ileum, through paracellular movements or by uptake through specific nutrient transporters, which are able to transport fats (i.e., Fatty Acid Transporter, FATP4; Cluster Differentiation 36, CD36), sugars (i.e., Glucose Transporter 2, GLUT2; Sodium-Glucose transporter Protein 1, SGLT1) and amino acids (i.e., L-type Amino acid Transporter 4, LAT4; neural amino acid transporter 1, B^0AT1) into the blood stream (Ferraris & Vinnakota 1995).

The complexity of nutrient absorption is further highlighted by the network of diverse intrinsic and extrinsic factors regulating this process. With regard to intrinsic factors, it is notable that deficiency of the anorexigenic hormone leptin in mice (Ferraris & Vinnakota 1995) increases energy ingestion leading to a greater adiposity and weight gain, coupled with associated hypothalamic-neuropeptide changes and chronic intestinal inflammation (Monteiro-Sepulveda et al 2015). Similarly, targeted deletion

of the gene encoding GLUT2, a key intestinal glucose transporter, also increases energy intake (Schmitt et al 2017).

Of the extrinsic factors, the gut microbiota plays a key role in harvesting energy from ingested food and providing it for host metabolism (Cani 2013, Clarke et al 2012, Torres-Fuentes et al 2017, van de Wouw et al 2017). Hence, germ-free mice are protected against obesity despite consuming more calories than control mice, but this phenotype is reverted after faecal microbiota transplantation from conventionally raised mice (Backhed et al 2004). Using gnotobiotic mice fed a high-fat or low-fat diet (HFD and LFD, respectively), a role has been suggested for *Clostridium ramosum* in the upregulation of body fat deposition promoting factors (Woting. A et al 2014). These data, coupled with the observed impact of gut microbiota on brain formation, including hypothalamic micro-structures (JM. Fernandez-Real JM et al 2015), provide novel avenues for modulating the activity of the intestine, adipose tissue and hypothalamus, involving microorganisms.

Alpha-lactalbumin (LAB) is a globular protein well known as a source of peptides having beneficial properties such as antioxidant bioactivity and immune modulation ability (Mburu et al 2010). It constitutes around 25% of the bovine milk whey protein (Mburu et al 2010). Whey can be extracted during the manufacture of cheese and comprises also other protein types, varying in abundances, including β-lactoglobulin (~ 65%), bovine serum albumin (BSA, ~8%), lactoperoxidase (0.25-0.5%), lactoferrin (Lf, ~1%) as well as other minor proteins such as immunoglobulins (<1%) (Morr & Ha 1993). Recently, whole whey protein has been considered as a potential dietary solution to obesity in light of the discovery that this protein, as an isolate (WPI) or concentrate (WPC), acutely increases the production of hormones that are involved in satiety in both humans and rodents (Chungchunlam et al 2015, Zhou et al 2011). Moreover, intake of whey protein in humans reduces fat absorption (Stanstrup et al 2014). This effect might be attributed to an altered composition of the gut microbiota involved in energy harvest, since other studies have shown that dietary whey protein can influence the composition of the gut microbiota (McAllan et al 2014, Nilaweera et al 2017, Sanchez-Moya et al 2017, Sprong et al 2010). Indeed, we showed that WPI reduced the proportion of Firmicutes in the mouse gut microbiome (Nilaweera et al 2017), and this was accompanied by a reduction in the expression of intestinal nutrient

transporters, specifically *glut2* and *fatp4*. In addition, we showed that WPI reduced epididymal adipose tissue (eWAT) weight and overall body weight gain. Consistent with changes in the gut, WPI increased energy intake, which was reflected in the altered hypothalamic gene expression of neuropeptides, namely proopiomelanocortin (*pomc*), neuropeptide Y (*npy*) and ghrelin (Nilaweera et al 2017).

While these data suggest a modulatory effect of WPI on the gut microbiome, hypothalamus and adipose tissue, it was not clear if the changes in energy intake were driven directly from the gut/hypothalamus axis or were secondary to the changes in adiposity. Indeed, previously it was shown that specific whey protein components, such as BSA and Lf, influenced in a different way body weight, energy intake and plasma leptin level (McManus et al 2015a, McManus et al 2015b).

Given that, LAB has been shown to affect the energy balance but the underlying mechanism is still unclear (Gao et al 2018, Zapata et al 2017a), in the current study we sought to further investigate the link established previously between gut-hypothalamic-adipose control of energy balance using LAB as the main protein source. The protein was given in a physiological amount (i.e., 20% calories) during HFD-induced weight gain phase in the C57BL/6J strain of mice, which was used as the model for humans. LAB was introduced early in development (5 weeks of age), given WPI specificity to influence weight gain during this period (Tranberg et al 2013). As the control, we fed mice with LFD or HFD containing the same physiological amount of a non-whey milk protein, casein.

Methods

Experimental Strategy

The *in vivo* experiments were approved by the University College Cork Animal Experimentation Ethics Committee (2011/005) and were licensed under the European Directive 2010/63/EU. Twenty-four C57BL/6J three-week-old male specific pathogen free mice were purchased commercially (Harlan; UK) and were singly housed in each cage with enrichment (Litaspen 8/20 and alpha-dry Plus bedding; LBS-biotech, UK) on a 12 h light/dark cycle with humidity maintained at 45-60% and temperature between 19-22°C. The mice had *ad libitum* access to food and water throughout the study unless otherwise stated. The health of the animals and the environment parameters were checked and recorded daily. No adverse effects were observed by the dietary interventions or procedures detailed below. The mice were provided with a diet containing 10% (low) fat and 20% casein (LFD-CAS; #D12450Bi; Research Diets, USA) (all % values by energy) during the initial 2 week acclimatization period, and then weight matched mice were switched to a 45% (high) fat diet containing either 20% casein (HFD-CAS; #D12451i) or alpha-lactalbumin (HFD-LAB; #D13081701i) (n=8 per group). The controls continued to receive LFD-CAS (n=8). The diets were OpenSource and were made by Research Diets, USA, with LAB sourced from Sigma, USA (diets composition details, **Table 2.TS1**). Body weight as well as food intake were measured weekly. The latter was converted to gross energy intake using the dietary energy density of the diets (HFD=19.79 KJ/g and LFD=16.11 KJ/g; Research Diets, USA).

During weeks 8 and 9, mice were placed in TSE Phenomaster cages (Germany) for 3 days to measure metabolic parameters, specifically, oxygen consumption (mL/h, VO2), carbon dioxide production (mL/h, VCO2), respiratory exchange ratio (VCO2/VO2, RER) and locomotor activity (X, Y and Z planes). The data were collected in the final 24h of the housing period as detailed previously (McAllan et al 2013).

At week 10, mice were fasted for 10-12h commencing at 22.00 in the dark phase, then anaesthetized (100mg/Kg Ketamine and 10 mg/Kg Xylazine) and blood samples were

collected. Mice were sacrificed by cervical dislocation and tissues were collected and samples were stored at -80˚C for subsequent analysis. The length of the small intestine and the weight of all the tissues were recorded on fresh tissues before snap freezing them.

DNA Sequencing

Faecal samples were collected directly from the colon, homogenized and processed using mechanical and chemical lysis. The 16S rRNA gene (V3-V4 region) was amplified with Universal primers (PCR1 Forward and Reverse primer as to the Illumina 16S Metagenomic Sequencing Protocol) that facilitated sequencing on the Illumina MiSeq platform (2x250bp paired-end reads; V3 sequencing chemistry). The fastq files were filtered on the basis of quality (removal of low quality nucleotides at the 3' end) and length (removal of sequences with less than 200nt) with prinseq (Schmieder & Edwards 2011), and paired reads with a minimum overlap of 20 bp were joined using Fastq-join (Aronesty 2013). Sequences were clustered (97% identity) to obtain Operative Taxonomic Units (OTUs) using closed-reference usearch v7.0 algorithm (Edgar 2010) and chimeric OTUs were removed through use of the GOLD database. The taxonomic assignment of OTUs was obtained using the Ribosomal database project (RDP) (Cole et al 2014). Alpha- and beta-diversity was determined using QIIME (Caporaso et al 2010), and additional analyses were performed with the R package phyloseq (McMurdie & Holmes 2013).

To identify chow-associated microbes, total DNA was extracted from ~ 10 g of chow containing 20% fat and 20% casein (LFD-CAS; #D17052702; Research Diets, USA), and ~ 10 g of chow containing 40% fat and 20% casein (HFD-CAS; #D17052705; Research Diets, USA) using the Gene All Extragene SoilSV kit (GeneAll). The Qubit high-sensitivity DNA assay (Bio-Sciences, Dublin, Ireland) was used for accurate quantification of the total DNA. Whole-metagenome shotgun libraries preparation was performed following the Nextera XT DNA library preparation guide from Illumina (Clooney et al 2016) and sequencing was performed using the Illumina NextSeq 500 with a v2 NextSeq 500/550 high-output reagent kit (300 cycles).

The raw shotgun metagenomic sequences were filtered on the basis of quality (removal of low quality nucleotides at the 3' end and application of sliding window trimming,

cutting once the average quality within the window falls below a treshold quality of 20 bp) and length (removal of sequences of less than 200nt) with prinseq (Schmieder & Edwards 2011). The filtered sequences were then converted to bam files using SAMtools (Li et al 2009), and duplicate reads were subsequently removed using Picard Tools (https://github.com/broadinstitute/picard). The quality of the sequences was tested using SAMtools in combination with Picard Tools, removing the low-quality sequences. Taxonomic analysis was performed using Kaiju (Menzel et al 2016).

Gene expression

The small intestine samples, stored at -80˚C, were initially immersed in a RNAlater-Ice frozen tissue transition solution and stored at -20˚C according to the instructions of the manufacturer (Ambion). This step allows untangling of the intestine while preserving the integrity of the RNA so that tissue samples, corresponding to the ileum (1 cm from the distal end of the small intestine) and jejunum (the central part of the small intestine), could be cut. Total RNA was extracted from the intestinal samples and hypothalamic blocks using RNeasy Minikit and QIAshredder columns (Qiagen), and from epididymal adipose tissue (eWAT) and subcutaneous adipose tissue (sWAT) using QIAzol Lysis Reagent (Qiagen). The extracted RNAs were treated with DNase (Qiagen, Ireland). Complementary DNA was synthetized from 600ng total RNA using SuperscriptTM II Reverse Transcriptase kit (Life Technologies, Ireland), and subjected to Real-Time PCR (Roche, Ireland) using SYBR Green Select Master Mix (Roche, UK) as detailed before (McAllan et al 2015). The gene expression was calculated using 2-ΔΔCp and normalized against the reference gene β-actin (intestine and adipose tissue) and YWHAZ (hypothalamus). The sequence of the primers can be found elsewhere (McAllan et al 2013, McAllan et al 2015, McManus et al 2015b).

Plasma leptin, glucose and triglyceride levels

Plasma leptin and glucose levels were determined using Mouse Leptin ELISA kit (Crystal Chem, USA) and Mouse Glucose Assay (Crystal Chem, USA) respectively.

Triglyceride (TAG) level in the plasma was measured using Triglyceride Quantification Assay Kit (abcam, UK).

Statistical analysis

For power calculation, the coefficient of variation was measured using data (body weight gain) from a previous study (McAllan et al 2015) and determined to be 8.8%. For a power of 80% and a significance level of 5%, this allows detection of a difference of 14% with a sample size of 8 mice per treatment. Body weight and energy intake differences over 10 weeks were analysed by a two-way repeated-measures ANOVA with Bonferroni's *post hoc* pairwise comparisons. Statistical analysis of gene expression, cumulative energy intake, intestine weight/intestine length, plasma leptin and triacylglycerol data were performed using one-way ANOVA followed by pairwise comparison using Bonferroni's *post hoc* test. Non-parametric data were compared by Kruskal-Wallis ANOVA followed by Mann-Whitney *U* test. Data were expressed as mean ± SEM and significance was set at $P<0.05$ using SPSS software version 24 (IBM Corp). VO_2 and heat production was analysed by ANCOVA (SAS software version 9.3), with total body weight being used as the covariant.

Gut microbiota data were statistically analysed by Adonis and Anosim for beta-diversity analysis. ANOVA was used to calculate significance for alpha-diversity-related analyses (statistical significance established at $P<0.05$). Statistical differences across multiple samples were determined by Kruskal-Wallis and False discovery rate (FDR, qvalue) control based on the Benjamini-Hochberg procedure, used to correct for multiple testing with the R statistical package (https://www.r-project.org/). Test for association across all groups of samples were performed through correlation analysis. This was based on Spearman's rank correlation test by the R function of cor.test, which aimed to examine similar expression association profiles between groups of samples (https://www.r-project.org/package=corrplot) and adjustment of the Pvalue by the Benjamini-Hochberg method. Statistical significance was established at $P<0.05$ (*), with other ranges, i.e., $P<0.01$ (**) and $P<0.001$ (***), also being noted.

Results

LAB increased cumulative energy intake but not weight gain

Mice fed HFD-LAB and HFD-CAS had similar body trajectories (**Fig. 2.1A**) but differed from the LFD-CAS group in the time taken to increase weight. Notably, the HFD-CAS group had a significantly higher weight than LFD-CAS by week 3 (P=0.033) while the weight of the HFD-LAB group did not significantly exceed that of the LFD-CAS group until week 6 (P=0.045) ($F_{(8,160)}$=440.2, P<0.001 for the effect of the Time, $F_{(2,20)}$=5.5, P<0.05 for the effect of the Diet, $F_{(16,160)}$=11.6, P<0.001 for the effect of the Time x Diet interaction) (**Fig. 2.1A**). Despite having similar body weight trajectories, the energy intake of HFD-LAB showed a trend towards an increase at week 2 (P=0.053) (413±9 KJ in HFD-LAB, 383±8 KJ in HFD-CAS and 293± 8 KJ in LFD-CAS) and it was higher in HFD-LAB compared to HFD-CAS at week 4 (P=0.037) ($F_{(7,140)}$=7.2, P<0.001 for the effect of the Time, $F_{(2,20)}$=81.7 , P<0.001 for the effect of the Diet, $F_{(14,140)}$=1.3 , P>0.05 for the effect of the Time x Diet interaction) (**Fig. 2.1B**). The cumulative energy intake of HFD-LAB was significantly increased by week 10 relative to HFD-CAS (P=0.043) (**Fig. 2.1C**). The difference in energy intake was reflected in the hypothalamic expression of neuropeptides. Notably, at week 10, the expression of the anorexigenic neuropeptide *pomc* was reduced relative to the LFD-CAS control (P=0.043 compared to HFD-CAS and P=0.001 compared to HFD-LAB), without showing significant difference between the two HFD groups. (**Fig. 2.1D**). While the gene expression for the orexigenic neuropeptides ghrelin and *npy* remained unchanged, the gene expression of growth hormone secretagogue receptor (*ghsr*) was reduced in both HFD groups compared to LFD-CAS (P=0.012 relative to HFD-CAS and P=0.001 relative to HFD-LAB) (**Fig. 2.1D**). Similar to *pomc* expression, the expression of *ghsr* between HFD-CAS and HFD-LAB groups did not change. There was no effect due to diet on the expression of the fatty acid synthase (*fasn*). The mismatch between energy intake and weight gain was unrelated to energy expenditure as both HFD-CAS and HFD-LAB groups had similar 24h VO_2 levels (97 ±3 in HFD-LAB, 95±3 in HFD-CAS and 101±3 in LFD-CAS; all values in mL/h) and heat production (2.1±0.05 in HFD-LAB, 2.0±0.05 in HFD-CAS and 2.2±0.06 in LFD-CAS; all values in KJ/h), where both parameters were measured during week 8 and 9

period. There were no differences between groups in terms of 24h locomotor activity (303±28 in HFD-LAB, 293±29 in HFD-CAS and 365±37 in LFD-CAS). While the HFD-CAS and the HFD-LAB groups had similar RER, the corresponding values were lower compared to LFD-CAS (both with P<0.001), consistent with increased fat consumption by former groups (0.816±0.014 in HFD-LAB and 0.809±0.014 in HFD-CAS versus 0.916±0.008 LFD-CAS).

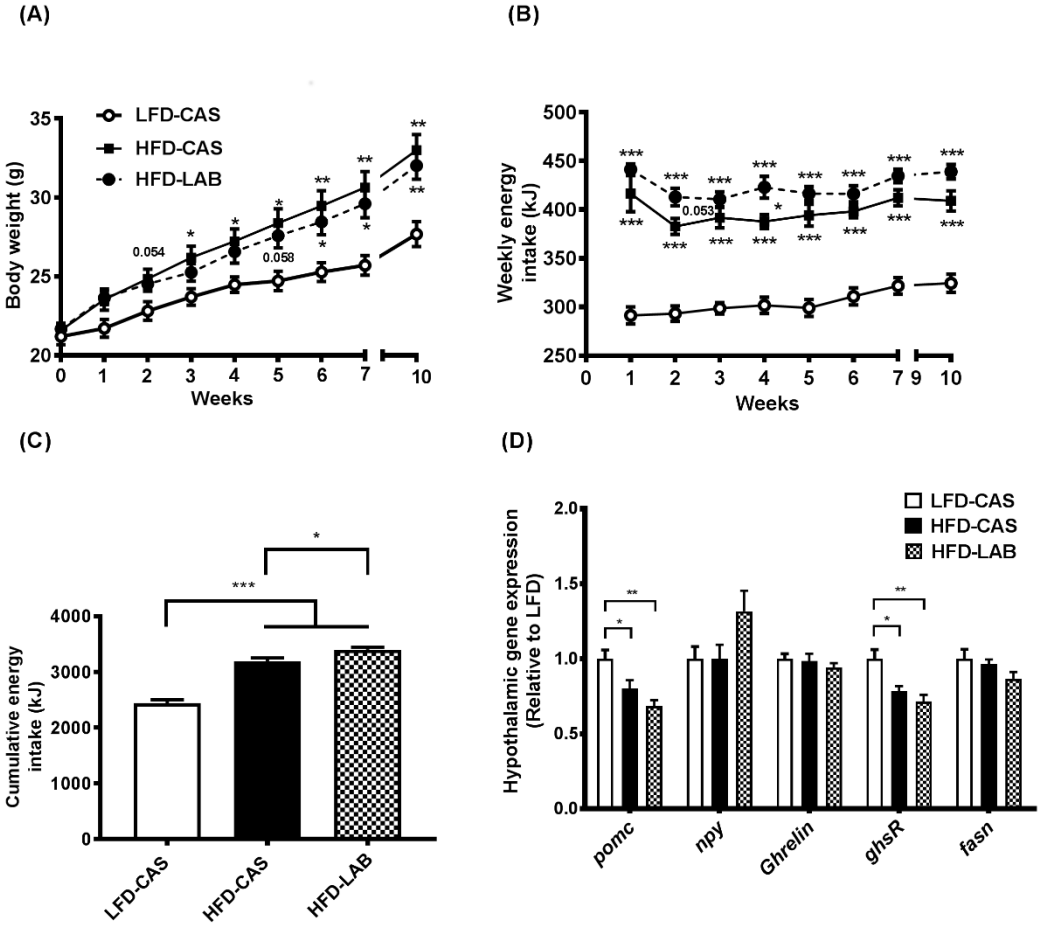


Figure 2.1 HFD-LAB has an effect on energy intake without impacting body weight gain. Impact of HFD-CAS, HFD-LAB and LFD-CAS on body weight **(A)** and energy intake **(B)** trajectories during 10 weeks experiment. Also shown the cumulative energy intake **(C)** after 10 weeks. In the hypothalamus were detected the level of expression of some genes involved in energy balance control **(D)** after 10 weeks of HFD-CAS, HFD-LAB or LFD-CAS intake relative to the control group LFD-CAS. Groups showing * are significant (* P<0.05 or ** P<0.01 or *** P<0.001; detailed statistical analysis in "supplementary statistic"). Abbreviation; POMC; proopiomelanocortin, NPY; neuropeptide Y, GHSR; growth hormone secretagogue receptor, FASN; fatty acid synthase.

LAB decreases the intestinal expression of glucose and fatty acid transporters

To find an explanation for the apparent energy loss in the HFD-LAB group, the impact on gastro-intestinal size and related gene expression of several nutrient transporters were measured. The intestinal length was unaffected by the diet (33.2±0.61cm in HFD-LAB, 32.4±0.74 cm in HFD-CAS and 32.9±0.38 cm in LFD-CAS). While the intestinal weight expressed relative to the length and body weight was similar between HFD-CAS and HFD-LAB groups, this was significantly lower compared to LFD-CAS controls (P=0.039 relative to HFD-CAS and P=0.019 relative to HFD-LAB) (**Fig. 2.2A**). The data from ANCOVA analysis showed that there was not significant effect of diet on intestinal weight/length when body weight was used as a covariant (0.024±0.001g in HFD-LAB, 0.025±0.001g in HFD-CAS and 0.026±0.001g in LFD-CAS).

In the ileum, we observed a significant decrease in the expression of the sodium-glucose transporter protein (*slgt1*) in both HFD groups compared to the LFD-CAS (P=0.026 relative to HFD-CAS and P=0.029 relative to HFD-LAB), but *glut2* gene expression was specifically reduced in the HFD-LAB compared to LFD-CAS (P=0.01) and it showed a trend towards a decrease, at the significance threshold, compared to HFD-CAS (P=0.053) (**Fig. 2.2B**). While *fatp4* gene expression was unaffected in the ileum, the cluster of differentiation 36 (*cd36*; an integral membrane protein responsible for importing fatty acids into the cell) was reduced in the HFD-LAB compared to the LFD-CAS (P=0.001) and HFD-CAS (P<0.001) (**Fig. 2.2B**). The expression of genes encoding the L-amino acid transporter, *lat4*, and the neutral amino acid transporter *slc6a19* was not significantly affected (**Fig**. **2.2B**). The same gene expression was measured in the jejunum. Similar to the ileum data, *cd36* gene expression in the jejunum was reduced in the HFD-LAB group compared to the other two groups (P=0.001 relative to LFD-CAS and P<0.001 relative to HFD-CAS), whereas *glut2* expression did not change across the groups (**Fig. 2.2C**). In addition, in the jejunum, the level of expression of *fatp4* was significantly higher in the HFD-LAB group compared to the LFD-CAS control group (P<0.001) and the expression of *lat4* showed a trend towards an increase in the HFD-LAB fed mice relative to HFD-CAS fed mice (P=0.061) (**Fig. 2.2C**).

In light of the results obtained for *glut2* and *cd36* gene expression, we measured the levels of glucose and triglyceride in the plasma. We observed that the glucose in the plasma of HFD-LAB fed mice (311±23 mg/dL) was not significantly different to HFD-CAS (293±8 mg/dL) but was lower compared to LFD-CAS fed mice (364±8 mg/dL, P=0.018). The level of triglyceride in the HFD-LAB (37±4 mg/dL), HFD-CAS (35±6 mg/dL) and LFD-CAS (36±5 mg/dL) groups was not significantly different.

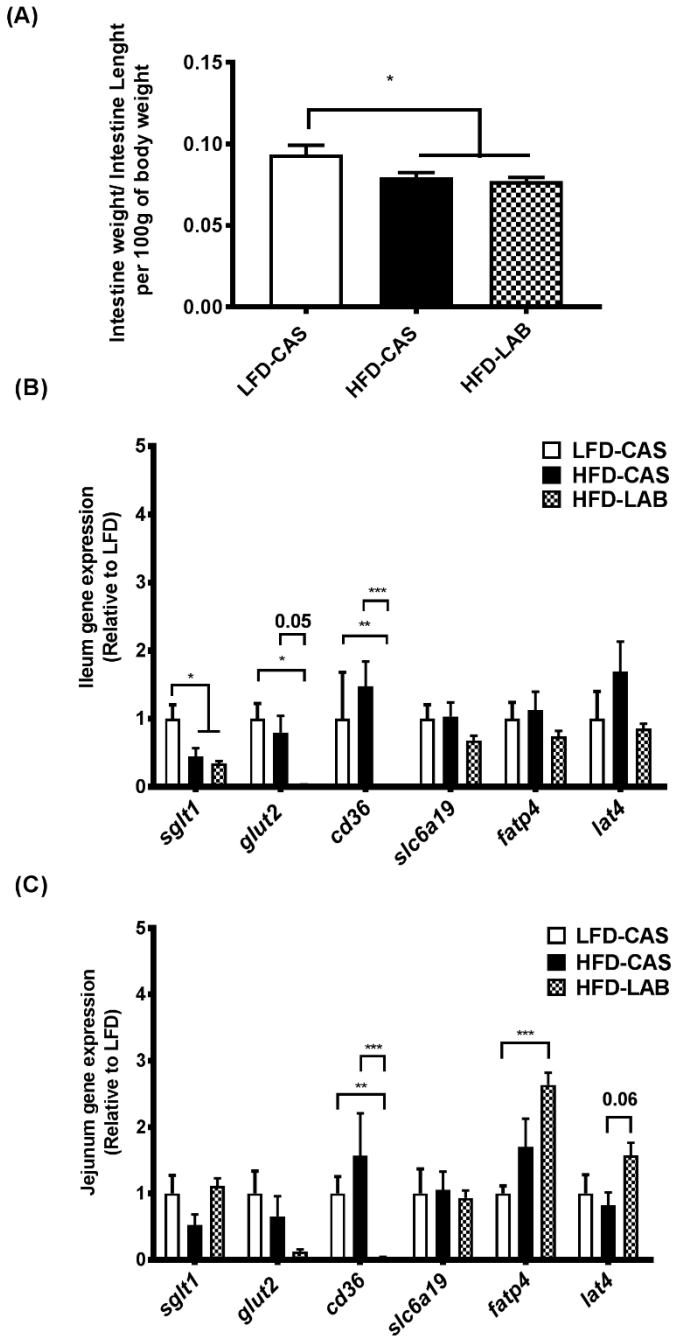


Figure 2.2 LAB influences the level of nutrient transporter expression within the small intestine. The data show the effect of LAB on intestine weight normalized by intestine length per 100g of body weight **(A)**. The level of gene expression in the ileum **(B)** and in the jejunum **(C)** after 10 weeks of intake of LFD, HFD-CAS or LAB groups relative to the control LFD-CAS group has been reported. Groups showing * are significant (* P<0.05 or ** P<0.01 or *** P<0.001; detailed statistical analysis in "supplementary statistic"). Abbreviation; SGLT1; sodium-glucose transporter 1, GLUT2; glucose transporter 2, CD36; cluster of differentiation 36, SLCa19; methionine transporter, FATP4; fatty acid transporter 4, LAT4; L type amino acid transporter 4.

LAB does not affect leptin gene expression and plasma hormone availability

Several tissues were harvested from the mice and their weights were recorded (**Fig. 2.3A**). The weights of both the subcutaneous adipose tissue (sWAT) and the eWAT were greater in both the HFD groups compared to the LFD-CAS control group (sWAT: P=0.002 relative to HFD-CAS and P=0.056 relative to HFD-LAB; eWAT: P=0.001 relative to HFD-CAS and P<0.001 relative to HFD-LAB) but there were no differences in the adipose tissues weights between HFD-CAS and HFD-LAB (**Fig. 2.3A**). Next, the expression of several genes involved in the catabolism and anabolism of fatty acids in the eWAT and sWAT was measured. In the current study, the expression in the eWAT of genes involved in fatty acid catabolism (carnitine palmoitoyltransferase I, *cpt1*; uncoupling protein, *ucp*; hormone-sensitive lipase, *hsl*; beta-3 adrenergic receptor *β-3ar*) and anabolism (acetylCoA carboxylase 1, *acc1*; fatty acid synthase, *fasn*; lipoprotein lipase, *lpl*) between HFD-LAB and HFD-CAS fed animals did not differ (**Fig. 2.3B-C**). There was also no change in the expression of genes involved in fatty acids transport, such as *cd36, fatp1* and *glut4* (**Fig. 2.3C**). However, consistent with the increased adiposity of the high fat fed groups, *acc1* gene expression was down regulated in HFD-CAS (P=0.008) and HFD-LAB (P=0.002) compared to LFD-CAS (**Fig. 2.3C**). In addition, the inflammatory marker, cluster of differentiation 68 (*cd68*) expression showed a trend towards an increase in the HFD-CAS group compared to the LFD-CAS (P=0.057), but for the HFD-LAB group, the increase was not significant relative to LFD-CAS (**Fig. 2.3C**). The unchanged adipose tissues weights, coupled with gene expression data in the eWAT, suggests an alternative mechanism of energy loss in the HFD-LAB group. We also measured leptin (*ob*) gene expression in the eWAT to assess if there is a specific effect of LAB in leptin regulation, similar to that attributed to the whey protein Lf (McManus et al 2015a).

Consistent with the gain in body weight and adipose tissue weight, the expression of the *ob* gene in the HFD-CAS group increased compared to the control LFD-CAS (P<0.001) and, in the HFD-LAB group, it showed a trend towards an increase relative to the LFD control (P=0.059), without showing any differences between the two HFD groups (P=0.08) (**Fig. 2.3C**).

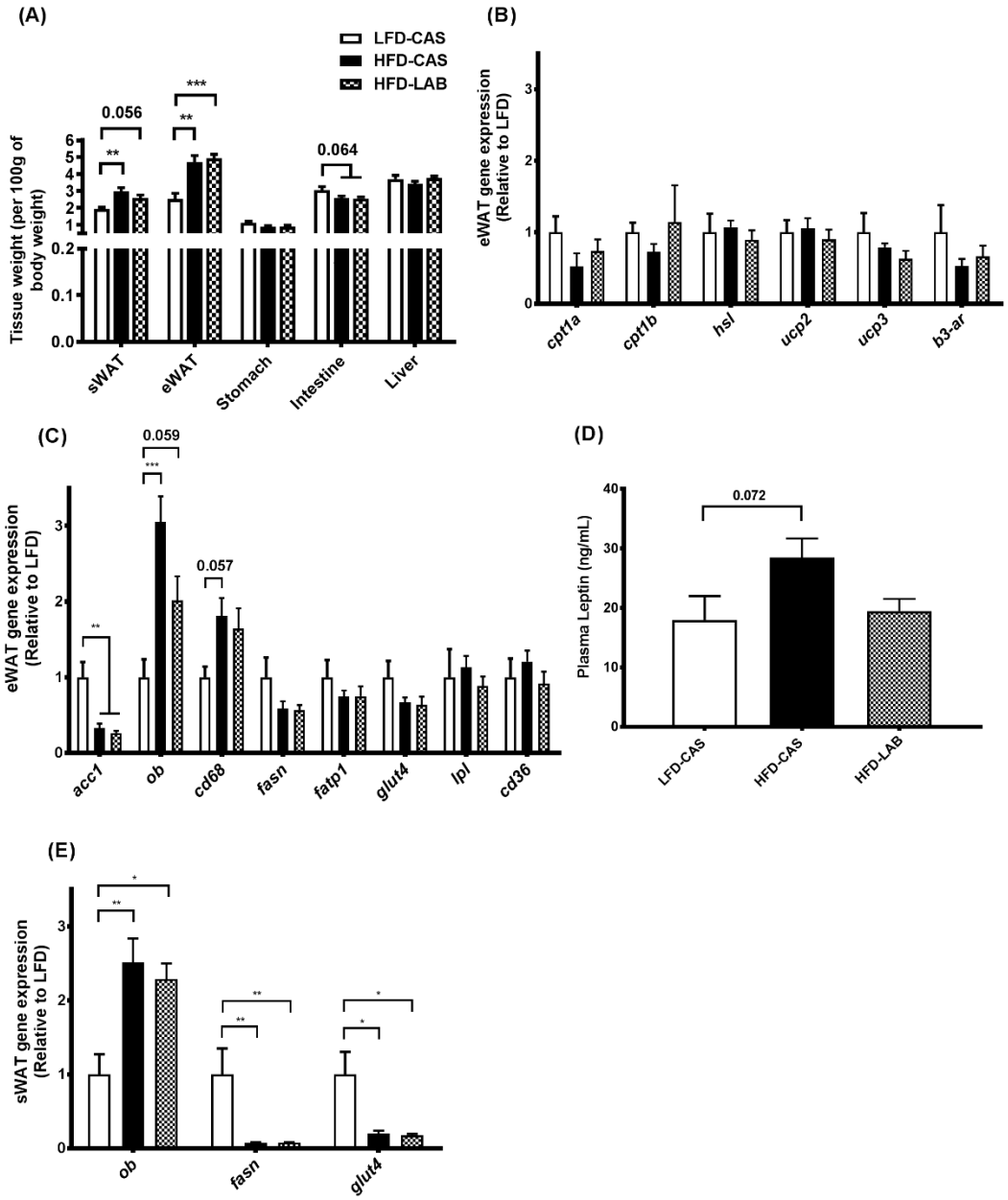


Figure 2.3 Effect of LAB on tissue weights and on the expression of genes involved in the catabolism and anabolism of adipose tissue. The data show the tissue weights normalized by 100g of body weight **(A)**. Expression of catabolic **(B)** and anabolic **(C)** genes in the epididymal adipose tissue (eWAT) was investigate after 10 weeks of intake of LFD, HFD-CAS or WPI groups relative to the control LFD-CAS group. Abbreviation; CPT1a and b; carnitine palmitoyltransferase I, HSL; hormone-sensitive lipase, UCP2 and 3; uncouplin protein, β3-AR; beta-3 adrenergic receptor, ACC; acetyl-CoA carboxylase, OB; leptin gene, CD68; cluster of differentiation 68, FASN; fatty acid synthase, FATP1; fatty acid transporter 1, GLUT4; glucose transporter 4, LPL; lipoprotein lipase, CD36; cluster of differentiation 36. Again after 10 weeks, it was measured the level of plasma leptin **(D)** in the three groups of study. In the subcutaneous adipose tissue (sWAT), the expression of the genes *ob*, *fasn* and *glut4* **(E)** has been reported. Groups showing * are significant (* P<0.05 or ** P<0.01 or *** P<0.001; detailed statistical analysis in "supplementary statistic").

Similarly, no differences in *ob* expression were detected in the sWAT between the HFD groups (**Fig. 2.3E**), and the expression of *fasn* and *glut4* genes were decreased compared with the LFD-CAS group (*fasn*: P=0.001 relative to HFD-CAS and P=0.001 relative to HFD-LAB; *glut4*: P=0.021 relative to HFD-CAS and P=0.035 relative to HFD-LAB), with no difference between HFD-CAS and HFD-LAB groups (**Fig. 2.3E**).

The *ob* gene expression in eWAT and sWAT was reflected in the plasma availability of leptin, as the presence of LAB in the HFD did not influenced the plasma hormone level, relative to HFD-CAS (Fig. **2.3D**).

Beta-diversity and gut bacterial composition differs due to LAB intake.

After quality filtration and length trimming an average of 182,494 high-quality 16S rRNA sequences per sample (i.e., faecal pellets collected from the colon) were obtained across two groups, i.e., HFD-CAS (n=8) and HFD-LAB (n=8). Analysis of this data revealed that, while alpha-diversity did not differ significantly across groups, a significant difference in beta-diversity (P=0.002) between the microbiota of the HFD-CAS and HFD-LAB animals was apparent, as represented by distinct clustering upon Principal Coordinates Analysis (PCoA) of all 16S rRNA reads (clustered at 97% similarity) (**Fig. 2.4A**).

Differences in beta-diversity were reflected by taxonomic differences between the HFD-CAS and HFD-LAB groups. At the phylum level, a lower Firmicutes/Bacteroidetes (ratio F/B) was apparent in the HFD-CAS samples (ratio F/B=1.23) relative to the HFD-LAB equivalents (ratio F/B=1.42), though significance was not achieved (**Fig. 2.4B**). At the family level, two families differed significantly in a diet-associated manner. These were *Lactobacillaceae* (higher abundance in HFD-LAB samples) and *Streptococcaceae* (higher abundance in HFD-CAS samples) (**Table 2.T1**).

At the genus level, statistically significant differences were apparent in the abundance of *Parabacteroides*, *Bifidobacterium*, *Parvibacter* and *Lactobacillus*, all of which were present in higher proportions in the HFD-LAB sample groups, as well as

Lactococcus, *Roseburia* (P=0.021), *Phascolarctobacterium* (P=0.018) and *Turicibacter*, present in higher proportions in the HFD-CAS group (**Table 2.T1**), were noted.

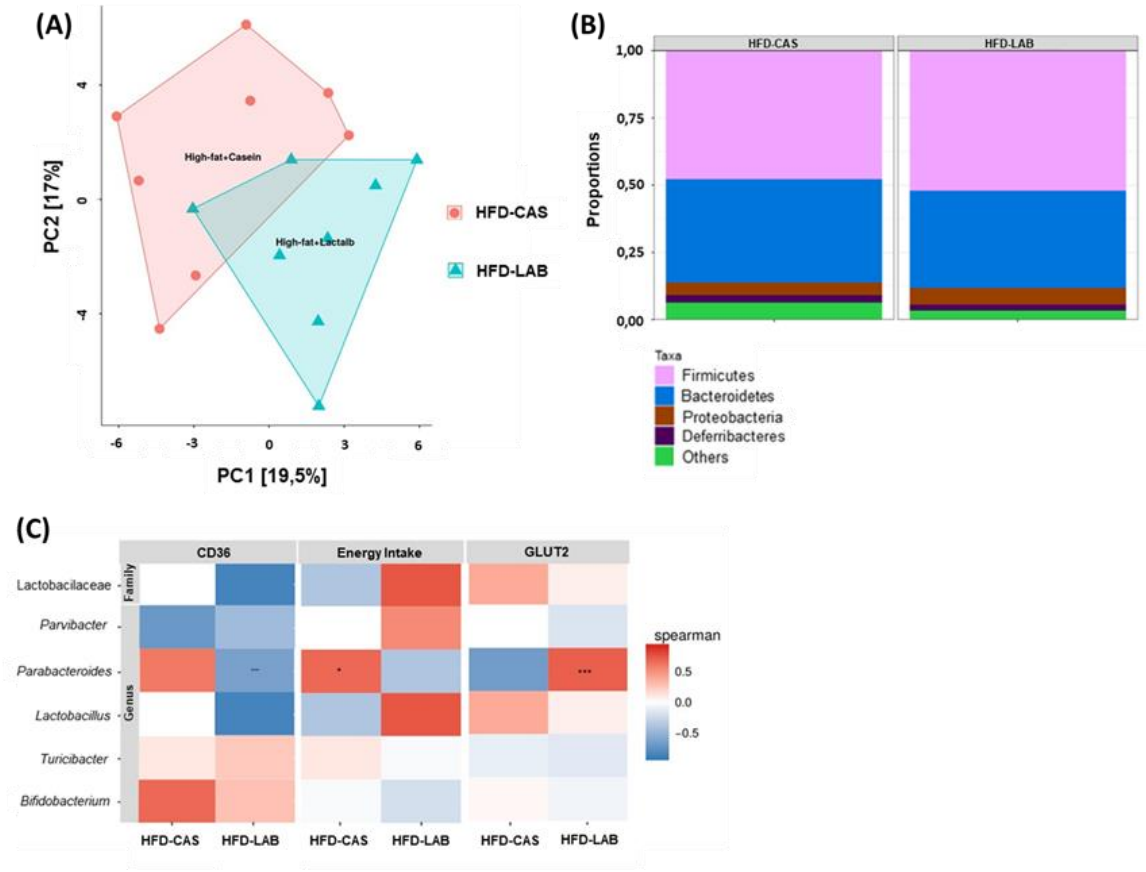


Figure 2.4 Impact of LAB on the composition of the gut microbiota. Beta-diversity of the two groups (HFD-CAS: red, HFD-LAB: light blue) calculated using Principal Coordinate Analysis (PCoA) **(A)**. Bar chart representing the phylum-level proportions in faecal samples of mice fed with HFD-CAS and HFD-LAB using Kruskal Wallis method **(B)**. Heatmap illustrating Spearman's rank correlations between the abundance of family and genus-level gut microbes and energy intake, *glut2* expression and *cd36* expression in both HFD-CAS and HFD-LAB groups. The pValue adjustment was done using Benjamini-Hochberg Method **(C)**. Groups showing * are significant (* P<0.05 or ** P<0.005 or *** P<0.001).

To assess the possibility that the presence of particular taxa within the chow containing CAS could be responsible for the associated increased proportion of *Streptococcaeae* in the gut, analysis of the microbiota of the CAS-containing chow was performed. This revealed that the family *Streptococcaceae* (99.6% and 99.8% in LFD-CAS and HFD-CAS chow, respectively) and the associated genus, *Lactococcus* (99.6% and 99.8% in LFD-CAS and HFD-CAS chow, respectively), were dominant (**Table 2.TS2**).

Correlation and independent analyses of expression association profiles were conducted between *cd36* and *glut2* expression levels, as well as energy intake, against bacterial composition at the genus level. Dairy taxa potentially sourced from the food substrate were not considered. After adjustment for multiple testing (BH-corrected), it was noted that energy intake positively correlated within the group of HFD-CAS samples with *Parabacteroides*. Within the HFD-LAB group, *Parabacteroides* negatively correlated with *cd36* expression. *Glut2* expression was positively correlated with *Parabacteroides* in HFD-LAB samples (**Fig. 2.4C**).

Family	**HFD-LAB vs HFD-CAS**	**Relative Abundance**
Streptococcaceae	P=0.029 ↑ HFD-CAS	0.082895775
Lactobacillaceae	P=0.015 ↑ HFD-LAB	1.18133309
Genus		
Lactococcus	P=0.001 ↑ HFD-CAS	0.077810661
Turicibacter	P=0.024 ↑ HFD-CAS	0.301261529
Streptococcus	P=0.001 ↑ HFD-LAB	0.148479572
Parvibacter	P=0.039 ↑ HFD-LAB	0.012092907
Lactobacillus	P=0.011 ↑ HFD-LAB	1.180503082
Parabacteroides	P=0.026 ↑ HFD-LAB	0.045986668
Bifidobacterium	P=0.05 ↑ HFD-LAB	0.159330093

Table 2.T1 Relative abundance (%) of genera and families in which their abundance is significantly higher either in the HFD-CAS group or in the HFD-LAB group. Statistical differences across multiple samples were estimated by the bioinformatics methods "False discovery rate" control based on the "Benjamini-Hochberg" procedure.

Discussion

In this study, we investigated the effects of a specific dietary whey protein (LAB) on intestinal-adipose-hypothalamic control of energy balance. We show that supplementation of LAB in a HFD reduced *cd36* and *glut2* gene expression in the small intestine and was associated with changes in the composition of the gut microbiota. These changes were accompanied by increased cumulative energy ingestion. This, coupled with the fact that targeted deletion of the *glut2* gene increases energy intake (Schmitt et al 2017), suggests a potential link between gut microbiota, intestinal nutrient transporters and energy intake in response to LAB supplementation. These effects were unrelated to adiposity, as HFD-LAB-fed mice had similar weight gain, adipose tissue weight (both eWAT and sWAT) and plasma TAG.

Previous studies have shown that diets rich in whey protein cause short-term satiety effects in mice and in humans (Lam et al 2009, MacKenzie-Shalders et al 2015, Yu et al 2009). Long-term effects on energy balance, however, do not involve the production of satiety hormones (Zhou et al 2011). On a background of HFD intake, which has been shown to reduce the brain sensitivity to satiety hormones (Westerterp 2006), the increased energy intake in mice fed with HFD-CAS compared to LFD and even higher (cumulative) energy intake in HFD-LAB is likely to involve another mechanism of regulation within the adipose tissue-gut-brain connection.

Hypothalamic neuropeptide expression provided an insight into the regulatory effect of LAB on energy intake. Notably, the expression of the anorexigenic neuropeptide *pomc* gene expression was significantly lower in the HFD-CAS group in line with the higher energy intake compared to LFD-CAS, similar to that shown in previous studies (McAllan et al 2013, McManus et al 2015a). In the HFD-LAB group, while *pomc* expression remained low, the expression of the orexigenic neuropeptide, *npy*, did not change across the groups. We performed a *t* test analysis on *npy* gene expression data between HFD-CAS and HFD-LAB and we found a trend towards an increase (not significant) in the presence of LAB. This suggests that an increase in sample size might bring about a significant difference in hypothalamic *npy* expression. Since NPY has an orexigenic action, an increase in this neuropeptide would be in agreement with the

increased energy intake observed during LAB consumption. Significant effects on energy intake and on hypothalamic *pomc* and *npy* expression were previously found in mice fed with WPI compared to casein in a LFD (Nilaweera et al 2017). In addition, neither the expression of *npy* nor the cumulative energy intake changed in mice fed a HFD containing lactoferrin compared to casein (McManus et al 2015a). These data suggest that the whey protein LAB have bioactivity that influences energy intake and that is not mediated by the modulation of hypothalamic neuropeptide genes investigated in this study.

In the current study, while the HFD-CAS group increased weight rapidly, reaching a significant increase relative to LFD values by week 3, it was a further 3 weeks before the mice fed HFD-LAB diet significantly increased body weight relative to LFD fed animals. The finding that the higher cumulative energy intake was not reflected in the increased body weight in the HFD-LAB group suggests a mechanism must exist to explain the missing energy from the supply. This is consistent with a rat study, which showed that HFD intake with LAB did not change energy intake but decreased adiposity (Zapata et al 2017a). The difference in energy intake between this study and our current study could be related to the fact that the rat study included both egg albumin and LAB as the protein sources in the same diet, whereas we used LAB as the only protein source. This apparent energy loss could be related to lipid oxidation, as a study showed that pre-exercise LAB-enriched whey protein meal preserved lipid oxidation alongside rapid delivery of amino acids to tissues for use during exercise, decreasing the adiposity in rats (Bouthegourd et al 2002). However, we did not observe this effect in the sedentary mice used in our current study, as RER was similar between HFD-LAB and HFD-CAS fed mice and adipose-specific gene expression linked to lipid catabolism also did not change. This, coupled with the similar sWAT and eWAT weights between HFD-LAB and HFD-CAS groups, suggests that the energy loss in the sedentary mice fed LAB occurs through another mechanism.

It has been shown previously that WPI reduces fat absorption in humans and mice (Frestedt et al 2008b, Pilvi et al 2007, Tranberg et al 2013), and we showed that this effect could be related to decreased ileal *fatp4* gene expression (Nilaweera et al 2017). In addition, we also showed a time course reduction of *glut2* gene expression in the

same region with 17 weeks of WPI intake and this effect on intestinal gene expression was independent of sucrose content in the diet (Nilaweera et al 2017). In the present study, by feeding a constituent protein of WPI, i.e., LAB, to mice for a shorter time period (10 weeks) and by analyzing their intestinal gene expression, we now observed a reduction in the expression of *sglt* in both HFD groups relative to LFD groups. This can be a reflection of higher carbohydrate content in the LFD compared to the two HFDs (**Table 2.TS1**). In addition, we show a reduction of *glut2* expression in the ileum exposed to HFD, specifically with LAB. The similar effects of LAB and WPI on intestinal *glut2* expression in the ileum suggests that LAB may contributes to the bioactivity in WPI influencing *glut2* expression and this bioactivity, whatever it may be, appears to be effective independent of sucrose or fat content in the diet (Nilaweera et al 2017). It was shown that the recruitment of apical GLUT2 in the small intestine is detrimental for health, i.e., it leads to an excess of glucose uptake with increased obesity and type 2 diabetes risk (Kellett et al 2008). Our study suggests a potential effect of a diet enriched in LAB in the control of intestinal glucose transporter-related transcripts, that could influence glucose absorption. In another *in vitro* study, using human intestinal Caco-2 cells, it was shown that polyphenols can also lead to a decrease in the abundance of apical GLUT2 (Alzaid et al 2013).

In the current study, we also observed a reduction in *cd36* gene expression within both ileum and jejunum. Thus, in combination, these data further support the hypothesis the whey protein LAB seems to affect different components of the mechanism involved in the absorption of glucose and fatty acids within the small intestine, at least at the gene expression level. This would explain, at least in part, the loss of energy that occurs in mice fed LAB. Further analysis aimed to look at the protein level and energy content in the faeces need to be done to confirm this hypothesis. Notably, differences in the level of *cd36* and *glut2* in the jejunum during LAB intake are in agreement with the fact that the modality and the degree of absorption can change along the intestinal tract. This is due to a different density of nutrient transporters across the three major regions of the small intestine (Spiller 1994). Based on the effect of LAB in a HFD on expression of nutrient transporters in the intestinal epithelium and associated energy loss, we speculate that mice compensate by increasing energy intake. In fact, in this

study we observed that the levels of glucose and triacylglycerol in the plasma did not show significant changes between HFD groups.

Furthermore, the HFD groups consumed more energy than LFD. Since there were no differences in the energy expenditure, the energy surplus has been stored in the adipose tissue. Thus, the lower expression of *acc* and *fasn* in the adipose tissue, presumably reflects the reduced necessity to produce fat endogenously when there is an external supply of excess energy. A similar reduction in *fasn* gene expression has been noted in a previous study from our group (McAllan et al 2014). On the contrary, other studies show that HFD intake increases *fasn* and *acc* in the adipose tissue (de Oliveira Santana et al 2016, Jiang et al 2005, Morgan et al 2008). The reasons for these discrepancies are unclear, but we speculate that contributing factors can include different genetic background, age, diet (composition and duration) and different housing environment.

Leptin, a hormone encoded by the *ob* gene, controls food intake and body weight through an interaction with specific receptors within the hypothalamus (Havel 2007). In obese humans and in mice fed an obesogenic diet the adipose tissue mass increases, enhancing significantly the level of plasma leptin and leading to metabolic dysfunctions (Myers et al 2010). In addition to the effect on intestinal nutrient transporters and energy intake, WPI in a LFD also reduced eWAT weight and plasma leptin, raising the possibility that the reduced adipose tissue and associated signals, including reduced leptin, could have stimulated the energy intake in mice. However, feeding mice with a HFD containing 20% of WPI for 8 weeks did not have an effect on plasma leptin levels, compared to mice fed a HFD containing CAS (McAllan et al 2013). On the contrary, HFD containing Lf significantly reduced plasma leptin levels compared to the HFD-CAS controls (McManus et al 2015a). This is another demonstration of the distinct bioactivities that WPI-associated whey protein components can have. In this study, mice fed a HFD containing LAB as the sole protein source showed no significant decrease in plasma leptin, which was reflected in no change in eWAT and sWAT *ob* gene expression in the HFD-LAB group, compared to the HFD-CAS group. However, a *t*-test analysis on plasma leptin data between HFD-CAS and HFD-LAB showed a significant decrease in plasma leptin

during LAB consumption. Thus, an increased sample size might be important to show a significant effect of LAB on leptin availability.

We also demonstrated that a HFD containing LAB has a strong influence on the composition of the gut microbiota. Beta-diversity data highlighted differences in the clustering of samples from the HFD-LAB group compared to the HFD-CAS group. Corresponding taxonomic analyses were also completed. In particular, we observed a significantly higher proportion of *Parabacteroides* in the HFD-LAB group, which is notable in that this genus showed a significantly positive correlation with the expression of nutrient transporters. Notably other studies have suggested that *Parabacteroides* protects the gut from inflammation and there have been reports of the apparent absence of this genus in the digestive tract of people affected by Inflammatory Bowel Diseases (Noor et al 2010, Zitomersky et al 2013). Moreover, *Parabacteroides* has been associated with anti-obesity effects. An increase in *Parabacteroides goldsteinii* was linked with the anti-obesity properties of a prebiotic polysaccharide isolated from the fungus *Hirsutella sinensis* (Wu et al 2019). A way through which *Parabacteroides* triggers its anti-obesity effects is by increasing the production of succinate and secondary bile acids (Wang et al 2019). These metabolites have been shown to be involved in central control of energy balance and glucose homeostasis (de Vadder & Mithieux 2018, Ma & Patti 2014).

In this study, we also observed that the genera *Bifidobacterium* and *Lactobacillus* are significantly more abundant in the HFD-LAB group. In several human studies, an association between lower levels of *Bifidobacterium* and obesity was noted (Kalliomaki et al 2008, Million et al 2012b, Schwiertz et al 2010). In addition, higher levels of *Bifidobacterium* have been linked with an increase in SCFA production, an improvement of gut mucosal barrier and lower intestinal LPS levels (Pinzone et al 2012, Schwiertz et al 2010). Furthermore, specific strains of *Bifidobacterium* and *Lactobacillus* spp. have been shown to exhibit anti-obesity effects (Torres-Fuentes et al 2015). It is worth highlighting the importance of species and strain level differences as, for example, human and animal studies have highlighted an association between different *Lactobacillus* species with weight gain or weight protection (Drissi et al 2014, Million et al 2012a). For this reason, more accurate in-depth metagenomic

analyses, such as shotgun analysis, should be considered in the future to investigate the *Lactobacillus* species that populate the gut of HFD-LAB fed mice.

A number of bacterial taxa typically associated with dairy products were also found to be altered in the gut. A recent human feeding study demonstrated that many bacteriophages and *Streptococcus* and *Lactococcus* genera, that were significantly altered in individuals whose diet was supplemented with whey protein, were present in high proportions within the whey protein supplement (Cronin et al 2018), and thus presumably originated from the food source. The family *Streptococcaceae* and the associated genus, *Lactococcus,* are significantly more abundant in the HFD-CAS group compared to the HFD-LAB group. This is consistent with our previous study that showed that *Streptococcaceae* proportion increased significantly in the CAS-fed mice compared to WPI-fed mice (Nilaweera et al 2017). However, this phenomenon is likely a reflection of the high proportion of *Streptococcaceae* and *Lactococcus* reads detected after analysis of both LFD-CAS and HFD-CAS chows.

The changes in the gut microbiota in the presence of LAB, coupled with the reduced intestinal expression of genes for nutrient transporters, support and provide a potential scenario for the energy mismatch in the HFD-LAB group compared to the control group. However, the specific mechanism that connects these changes in intestinal transporters gene expression, gut microbiota and energy intake in the presence of LAB remains to be elucidated. Further studies are needed to assess if these effects are seen with different genetic background, sex, housing environment (group versus single housing) and dietary fat content and duration. In conclusion, this present study has demonstrated for what is to our knowledge the first time, how a HFD containing a specific whey protein, i.e., LAB, specifically affects the metabolism, microbiota and nutrient absorption regulation, without preventing weight gain and adipose tissue mass accumulation in mice.

Supplemental Material

Supplemental Tables

Table 2.TS1 Composition of the experimental diets: high-fat diet containing casein (HFD-CAS), high-fat diet containing alpha-lactalbumin (HFD-LAB) and low-fat diet containing casein (LFD-CAS).

	HFD-CAS		HFD-LAB		LFD-CAS	
Ingredients	**gm**	**Kcal**	**gm**	**Kcal**	**gm**	**Kcal**
Casein	200	800	0	0	200	800
α-Lactalbumin	0	0	200	800	0	0
Glycomacropeptide	0	0	0	0	0	0
L-Cysteine	3	12	3	12	3	12
Corn Starch	72,8	291	72,8	291	315	1260
Maltodextrin 10	100	400	100	400	35	140
Sucrose	172,8	691	172,8	691	350	1400
Cellulose, BW200	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225
*Lard**	177,5	1598	177,5	1598	20	180
Mineral Mix S10026	10	0	10	0	10	0
DiCalcium Phosphate	13	0	13	0	13	0
Calcium Carbonate	5,5	0	5,5	0	5,5	0
Potassium Citrate, 1 H2O	16,5	0	16,5	0	16,5	0
Vitamin Mix V10001	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0
FD&C Yellow Dye #5	0	0	0,05	0	0,05	0
FD&C Red Dye #40	0,05	0	0	0	0	0
Total	858,15	4057	858,15	4057	1055,05	4057

*Typical analysis of cholesterol in lard = 0.95 mg/gram.
Cholesterol (mg)/4057 Kcal=19
Cholesterol (mg)/Kg=18

Table 2.TS2 Relative abundance (%) of families and genera in the LFD-CAS (20% fat, 20% CAS) and HFD-CAS (45% fat, 20% CAS) chows, using shotgun analysis approach. The taxonomic abundance analysis was performed using Kaiju.

Family	**Chow LFD-CAS**	**Chow HFD-CAS**
Streptococcaceae	99.56243	99.78502
Lactobacillaceae	0.43757	0.21498
Genus		
Lactococcus	99.56243	99.76647
Streptococcus	0	0.01855
Lactobacillus	0.43757	0.21498

Supplemental Statistic

Statistical output related to **Figure 1** (**HFD-LAB has an effect on energy intake and hypothalamic gene expression without impacting body weight gain)**

Body weight $F_{(8,160)}$=440.2, P<0.001 for the effect of the Time, $F_{(2,20)}$=5.5, P<0.05 for the effect of the Diet, $F_{(16,160)}$=11.6, P<0.001 for the effect of the Time x Diet interaction **(A)**. Energy Intake $F_{(7,140)}$=7.2, P<0.001 for the effect of the Time, $F_{(2,20)}$=81.7, P<0.001 for the effect of the Diet, $F_{(14,140)}$=1.3, P>0.05 for the effect of the Time x Diet interaction **(B)**. Cumulative energy intake one-way ANOVA, $F_{(2,22)}$=81.7, P<0.001 followed by Bonferroni's *post-hoc* **(C)**. Hypothalamic gene expression: one-way ANOVA, *pomc* $F_{(2,22)}$=9.2, P<0.01; *npy* $F_{(2,22)}$=2.8, P=0.079; *ghsR* $F_{(2,22)}$=9.8, P<0.01; all followed by Bonferroni's *post-hoc* except *npy* **(D)**.

Statistical output related to **Figure 2 (LAB influences the level of nutrient transporter expression within the small intestine)**

Intestine weight/ Intestine Length per 100g of body weight one-way ANOVA, $F_{(2,20)}$=5.5, P<0.05 followed by Bonferroni's *post-hoc* **(A)**. Ileum gene expression: Kruskal-Wallis test for *sglt1* (P=0.035) and *cd36* (P=0.000). Mann-Whitney *U* test for *sglt1*: **1 VS 2** $U_{(15)}$= 9, P=0.029; **1 VS 3** $U_{(14)}$= 7, P=0.026. Mann-Whitney *U* test for *cd36*: **1 VS 3** $U_{(14)}$=0, P=0.001; **2 VS 3** $U_{(15)}$= 0, P=0.000. One-way ANOVA, *glut2* $F_{(2,19)}$=6.4, P<0.01 **(B)**. Jejunum gene expression: Kruskal-Wallis test for *cd36* (P=0.001) and *fatp4* (P=0.016). Mann-Whitney *U* test for *cd36*: **1 VS 3** $U_{(14)}$=0, P=0.001; **2 VS 3** $U_{(15)}$= 0, P=0.000. Mann-Whitney *U* test for *fatp4*: **1 VS 3** $U_{(15)}$=0, P=0.000. One-way ANOVA, *lat4* $F_{(2,20)}$=3.6, P<0.05 followed by Bonferroni's *post-hoc* **(C)**.

Statistical output related to **Figure 3 (Effect of LAB on tissue weights and on the expression of genes involved in the catabolism and anabolism of adipose tissue)**

Tissue weights normalized by 100g of body weight: one-way ANOVA, sWAT $F_{(2,22)}$=8.3, P<0.01; stomach $F_{(2,22)}$=3.4, P=0.051; Intestine $F_{(2,20)}$=4, P<0.05; eWAT $F_{(2,21)}$=12.7, P<0.001; all followed by Bonferroni's *post-hoc* **(A)**. eWAT gene expression (anabolism): Kruskal-Wallis test for *acc* (P=0.007). Mann-Whitney *U* test for *acc*: **1 VS 2** $U_{(15)}$= 4, P=0.008; **2 VS 3** $U_{(15)}$= 1, P=0.002. One-way ANOVA, *ob* $F_{(2,21)}$=12.6, P<0.001; *cd68* $F_{(2,21)}$=3.5, P<0.05; both followed by Bonferroni's *post-hoc* **(C)**. Plasma leptin: one-way ANOVA, $F_{(2,22)}$=3.3, P=0.056 **(D)**. sWAT gene expression: one-way ANOVA, *ob* $F_{(2,21)}$=8.2, P<0.01 followed by Bonferroni's *post-hoc*. Kruskal-Wallis test for *fasn* (P=0.001) and *glut4* (P=0.032). Mann-Whitney *U* test for *fasn*: **1 VS 2** $U_{(15)}$= 1, P=0.001; **1 VS 3** $U_{(14)}$= 0, P=0.001. Mann-Whitney *U* test for *glut4*: **1 VS 2** $U_{(15)}$= 8, P=0.021; **1 VS 3** $U_{(13)}$= 6, P=0.035.

Chapter 3

Age- and Duration-Dependent Effects of Whey Protein on High-Fat Diet-induced Changes in Body Weight, Lipid Metabolism and Gut Microbiota in Mice

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Abstract

Rationale Bovine whey protein has been demonstrated to exert a positive effect on energy balance, lipid metabolism and nutrient absorption. Additionally, it affects gut microbiota configuration. Thus, whey protein is considered as good dietary candidate to prevent or ameliorate metabolic diseases, such as obesity. However, the relationship that links energy balance, metabolism and intestinal microbial population mediated by whey protein intake remains poorly understood.

Objectives In this study we investigated the beneficial effects attributed to whey protein in the context of high-fat diet (HFD) in mice at two different ages, with short or longer durations of whey protein supplementation.
Here, a 5-week dietary intervention with HFD in combination with either whey protein isolate (WPI) or the control non-whey milk protein casein (CAS) was performed using 5-week or 10-week-old C57BL/6J mice. Notably, the younger mice had no prior history of ingestion of WPI, while older mice did.

Results 5-week-old HFD-WPI-fed mice showed a decrease in weight gain and changes in the expression of genes within the epididymal white adipose tissue including those encoding leptin, inflammatory marker CD68, fasting-induced adipose factor FIAF and enzymes involved in fatty acids catabolism, relative to HFD-CAS-fed mice. Differences in β-diversity and higher proportions of *Lactobacillus murinus*, and related functions, were evident within the gut microbiota of HFD-WPI mice. However, none of these changes were observed in mice that started the HFD dietary intervention at 10-weeks-old, with an extended period of WPI supplementation.

Conclusions These results suggest that the effect of whey protein on mouse body weight, adipose tissue and intestinal parameters depends on diet duration and stage of life during which the diet is provided. In some instances, WPI influences gut microbiota composition and functional potential, which might orchestrate observed metabolic and physiological modifications.

Introduction

Bovine whey protein is a milk protein present in the liquid that results from cheese production, called whey. Whey protein includes subcategories of proteins present in different quantities, such as α-lactalbumin (~25%), β-lactoglobulin (~65%), bovine serum albumin (~8 %), lactoperoxidase (0·25–0·5 %), lactoferrin (~1%) and minor proteins such as immunogloblulins (<1%) (Morr & Ha 1993). Because of the specific amino acid composition including branched chain amino acids, intake of these proteins has been shown to bring about several health benefits. Notably, a diet supplemented with whey protein has been found to modulate appetite, and ileal expression and plasma levels of satiety hormones such as insulin, ghrelin, cholecystokinin and glucagon-like peptide 1 (Chungchunlam et al 2015, McAllan et al 2012, Pal et al 2014). Additionally, in several studies in which whey protein constituted the main protein source of a high-fat diet (HFD), this protein ameliorated the impairment of glucose tolerance, and reduced the increase in body weight and adiposity compared to other dietary proteins, such as casein (CAS, a non-whey milk protein which is the main component of cheese), soy, meat and fish proteins (Madsen et al 2017, McAllan et al 2013, Pilvi et al 2007). Whey protein also influences lipid metabolism in adipocytes, including plasma release of triacylglycerol (TAG), and reduces intestinal fat absorption (McAllan et al 2013, Pal et al 2010, Stanstrup et al 2014). For these reasons, whey protein has been chosen as a good candidate for dietary interventions aiming to prevent or ameliorate metabolic diseases such as obesity.

Obesity is a complex and multifactorial disease characterised by an excess of adiposity due to an imbalance between energy intake and energy expenditure (De Lorenzo et al 2016). According to the World Health Organization, in 2016, 1.9 billion adults worldwide were overweight or obese of which 650 million had obesity. It is well known that metabolic disorders are linked with other metabolic diseases, such as cardiovascular diseases, diabetes, adipose tissue dysfunction, as well as a dysbiosis of the intestinal microbial population (Carding et al 2015). The gut microbiota plays an important role in the modulation of the host metabolism and in energy harvesting through fermentation of fibres and proteins from food that were not digested and

absorbed within the small intestine. Some studies have shown that low-fat diet (i.e., LFD) and HFD containing whey protein isolate (WPI) have similar impacts on the gut microbiota, increasing the abundance of the families *Bifidobacteriaceae* and *Lactobacillaceae*, and *Bifidobacterium spp.* and *Lactobacillus spp*.. However, these studies were performed with *in vitro* gastrointestinal digestion models or 16S rRNA compositional metagenomic approaches and, thus, there are no studies describing in detail which species and which microbial functions are influenced by the presence of WPI in the diet (McAllan et al 2014, Sanchez-Moya et al 2017, Sprong et al 2010).

Other factors that considerably shape the gut microbiota in humans in early stage of life of the individual are modality of delivery and genetics, which influence the microbial colonization in the perinatal and early-postnatal period. The microbiota evolves and becomes more similar to an adult microbial configuration by the end of 3-5 years of life. From this stage, the gut microbiota becomes relatively stable throughout life, but factors such as diet, antibiotic treatment, lifestyle and bacterial infections can strongly affect and deviate the gut microbiota stability. In turn, this can have an impact on host metabolism and host physiology (O'Toole & Jeffery 2015, Rodriguez et al 2015). In contrast to the human case, the link between age and microbiota in rodents is still poorly understood. A recent study carried out in rodents, particularly in healthy Sprague Dawley rats, revealed a significant change in the composition of their microbiota from 3 weeks to 2 years of age, with consequences on the microbial fermentative capacity and thus, host metabolism regulation (Flemer et al 2017). Notably, we have shown that WPI intake (20% energy) starting from early adolescence and continuing for 8 week period reduces weight gain and adiposity (McAllan et al 2013), but extending intake into 21 weeks (and hence as mice get older), there is an impairment of the effects of WPI (McAllan et al 2014). These data suggested a potential interplay between duration of WPI and HFD intake on parameters related to energy balance that appear to be sensitive to the animal's age. Untangling the relative importance of each for energy balance regulation is difficult to achieve because duration of the diet intake and age go together, instead one can focus on how different dietary component interplay with age by fixing one dietary component and changing another, and then assess the influence on energy balance. This would provide the basis for development of better interventions for prevention of

weight gain and development of obesity in individuals at risk as they grow from young to older age.

In the present work we aimed to investigate the relationship between WPI-supplemented diet duration, age, microbiome and host energy balance, using 5-week and 10-week-old C57BL/6J mice. To assess these interactions, we fixed HFD to a limited period (5 weeks) in young and older mice. We have previously shown that feeding high (30% and 40% by energy) WPI as part of a 45% energy HFD to adolescent mice was shown to reduce weight gain, with animals attaining a lean phenotype but where the microbial composition was distinct to LFD fed mice (McAllan et al 2014). This led to our first hypothesis that WPI intake as part of LFD during adolescence would bring about the microbial and endocrine changes necessary to impede weight gain resulting from a subsequent switch to HFD in older mice but whilst continuing to ingest WPI. Because microbial changes in the gut occurs early life, and it was not clear at what stage in adolescence the change in microbial composition occurs in relation to WPI intake, we also assessed the impact of WPI as part of a HFD much earlier on in adolescence without pre-intervention with LFD containing WPI. To this end, we focus mainly on the effects of a HFD containing a standard amount of WPI (20% energy) on body weight, energy intake, plasma hormones, metabolites and genes encoding nutrient transporters, lipid metabolism enzymes and hormones in the ileum and in the adipose tissue in mice at two different stages of life (i.e., adolescence, from week 4 to week 8-9 of life, and early adulthood, from week 9 to 17-18 of life) (Brust et al 2015). In addition, using a high-throughput shotgun metagenomic approach, we performed a gut microbiota analysis on faecal samples. Unlike the 16S rRNA gene sequencing approach, shotgun metagenomics allows a more detailed view of the microbial composition and genes and metabolic pathways related to the microbial community (Quince et al 2017a). Specifically, we performed a first experiment with 5 week-diet duration (i.e., 5w) in which 5-week-old mice were fed a HFD, containing WPI (HFD-WPI) or the control CAS (HFD-CAS) as the protein source. In parallel, we performed a second experiment with 10 week-diet duration (i.e., 10w), in which 5-week-old mice were fed LFD-CAS or LFD-WPI for 5 weeks and, when they were 10-weeks old, their diet was switched to HFD-CAS or HFD-WPI for a further 5 weeks. The control groups were maintained on LFD-CAS

or WPI for the entirety of the experiment (**Fig. 3.1a**). The above experimental design allowed us to fix the HFD feeding duration to 5 weeks in both experiments in younger and older mice, and focus on the potential interplay of the effects of WPI duration and age (shorter duration in younger mice in experiment 1 versus longer duration as mice age in experiment 2).

We predicted that: (i) giving WPI in LFD during a pre-HFD period (i.e., second experiment 10w) would prevent/ameliorate the metabolic syndrome outcomes that would occur after switching to HFD and extending the WPI supplementation; (ii) energy balance, adipose tissue metabolism, gut microbiota and nutrient absorption would exhibit differences due to the dietary intervention carried out at two different stages of life (i.e., both experiments, 5w and 10w).

Methods

Experimental Strategy

The *in vivo* experiments were approved by the University College Cork Animal Experimentation Ethics Committee (2015/007) and were licenced under the European Directive 2010/63/EU. Eighty C57BL/6J 3-week-old male mice were purchased commercially (Harlan; UK) and were housed 4 per cage on a 12 h light/dark cycle. The mice had ad libitum access to food and water throughout the study unless otherwise stated. During the initial 2 weeks of acclimatisation period, mice were provided with a diet containing 10% low fat diet and 20 % casein (LFD-CAS; #D12450B; Research Diets, USA; % by values of energy). Subsequently, weight matched mice received different diets varying only in protein quality or fat content (Research Diets, USA): (1) LFD-CAS, (2) 10% fat and 20 % whey protein isolate (LFD-WPI; #D1208/601), (3) 45% fat and 20% casein (HFD-CAS; #D12451) and (4) 45% fat and 20% whey protein isolate (HFD-WPI; #D11040501). The composition of each diet is detailed in the **Table 3.T1**.

Two experiments were done:

Experiment in which mice started the HFD dietary intervention at 5-week-old (5w): 5-week-old mice were provided HFD-CAS or HFD-WPI (4 cages per group, each with 4 animals) and LFD-CAS or LFD-WPI (controls; 2 cages per group, each with 4 animals) for 5 weeks. After 5 weeks of dietary intervention, only the 10-week-old animals fed with HFD (groups 1 and 2) were euthanized (**Fig. 3.1a**).

Experiment in which mice started the HFD dietary intervention at 10-week-old (10w): 5-week-old mice were provided LFD-CAS or LFD-WPI for 5 weeks and switched to HFD-CAS or HFD-WPI for further 5 weeks (4 cages per group and 4 animals per cage). The controls were the same animals as the experiment 5w, i.e., mice fed with LFD-CAS or LFD-WPI (2 cages per group, each with 4 animals) for 10 weeks. At the end of the dietary intervention, all the 15-week-old animals (groups 3,4,5, and 6) from the four groups (i.e., HFD-CAS, HFD-WPI, LFD-CAS and LFD-WPI) were euthanized (**Fig. 3.1a**).

In both experiments body weight was measured weekly, as well as the food intake per cage and was converted in energy (using the energy density HFD=19.79 KJ/g and

LFD=16.11 KJ/g of the rodent diets; Research Diets, USA). Notably, in the results are reported energy intake data for HFD-fed mice and not for LFD-fed mice because the latter groups had a sample size of 2, thus too small to carry out appropriate statistical analysis. For this reason, we omitted energy intake data for LFD-fed mice.

The faecal samples were collected from individual mice the day before euthanasia and these were stored at -80˚C for subsequent analysis. In both experiments, mice were fasted for 8h commencing at 22.00 in the dark phase, then anaesthetized (100mg/Kg Ketamine and 10 mg/Kg Xylazine). Mice were euthanized by cervical dislocation and blood samples and tissues were collected. Tissues weight and/or length was recorded, and the samples were stored at -80˚C for subsequent analysis.

	Low-fat diet with casein		Low-fat diet with whey protein isolate		High-fat diet with casein		High-fat diet with whey protein isolate	
From Research Diets, USA	**LFD-CAS**		**LFD-WPI**		**HFD-CAS**		**HFD-WPI**	
	#D12450B		#D1208/601		#D12451		#D11040501	
fat % (by values of energy)	10		10		45		45	
CAS or WPI % (by values of energy)	20		20		20		20	
Ingredients	**gm**	**Kcal**	**gm**	**Kcal**	**gm**	**Kcal**	**gm**	**Kcal**
Casein	200	800	0	0	200	800	0	0
Whey Protein	0	0	200	800	0	0	200	800
L-Cysteine	3	12	3	12	3	12	3	12
Corn Starch	315	1260	315	1260	72,8	291	72,8	291
Maltodextrin 10	35	140	35	140	100	400	100	400
Sucrose	350	1400	350	1400	172,8	691	172,8	691
Cellulose, BW200	50	0	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225	25	225
*Lard**	20	180	20	180	177,5	1598	177,5	1598
Mineral Mix S10026	10	0	10	0	10	0	10	0
DiCalcium Phosphate	13	0	13	0	13	0	13	0
Calcium Carbonate	5,5	0	5,5	0	5,5	0	5,5	0
Potassium Citrate, 1 H2O	16,5	0	16,5	0	16,5	0	16,5	0
Vitamin Mix V10001	10	40	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0	2	0
Total	1055	4057	1055	4057	858,1	4057	858,1	4057

Table 3.T1 Composition of the experimental diets.

RNA Extraction and Gene Expression Analysis

Total RNA was extracted from the ileum and liver (approximately 30 mg of tissue per each sample) using RNeasy Minikit and QIAshredder columns (Qiagen), and from epididymal white adipose tissue (eWAT) and subcutaneous white adipose tissue (sWAT) using QIAzol Lysis Reagent (Qiagen). The extracted RNAs were treated with DNase (Qiagen, Ireland). Complementary DNA was synthetized from 600ng total RNA using SuperscriptTM II Reverse Transcriptase kit (Life Technologies, Ireland), and subjected to Real-Time PCR (Roche, Ireland) using SYBR Green Select Master Mix (Roche, UK) as detailed before (McAllan et al 2015). The gene expression was calculated using 2-ΔΔCp and normalised against the reference gene *β-actin* (intestine and adipose tissue) and *Gapdh* (liver), and presented as a ratio versus average of HFD-CAS 5w. The sequence of the primers is detailed in **Table 3.ST1**. RNA extraction and gene expression analysis were carried out in HFD-CAS and HFD-WPI-fed mice in both experiments (i.e., 5w and 10w), but not in the LFD control groups.

Leptin, glucose, insulin and triglyceride levels

Plasma leptin and glucose levels were determined using Mouse Leptin ELISA kit (Crystal Chem, USA) and Mouse Glucose Assay (Crystal Chem, USA) respectively. Mouse Insulin ELISA Kits, Ultra-Sensitive Assay (Crystal Chem, USA) was used to determine the plasma insulin level.

The weights of liver and previously dried caecum content samples were recorded, and the samples were homogenised in 1,5 mL of %NP40/ddH2O solution. After two repeated steps of heating (80-100°C for 3-5 min) and cool down (15 minutes at RT), all the samples were centrifuged at maximum speed. The supernatant, containing the lipids, was collected and diluted 1:10. Triacylglycerol (TAG) level in the plasma, in the liver extract and in the caecum content extract it was measured using Triglyceride Quantification Assay Kit (abcam, UK).

Leptin, glucose, insulin and TAG levels detection was carried out in all the groups, in both experiments.

DNA extraction and library preparation

Genomic DNA from faecal samples collected from HFD groups at the end of experiment 5w and 10w was purified according to the protocol for the standard Qiagen QiaAMP DNA Fast Stool Kit (Qiagen, Germany). Total DNA was accurately quantified with Qubit dsDNA HS Assay kit (Bio-Sciences, Dublin, Ireland).
After DNA extraction, samples were processed for shotgun metagenomic sequencing using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA). After passing quality checks, libraries were prepared with Nextera XT DNA library prep kits (Illumina), the samples were sequenced on the Illumina MiSeq platform (2x250 bp paired-end reads) in the Moorepark Teagasc sequencing facility, following standard Illumina sequencing protocols (Illumina 2013). DNA extraction and library preparation were carried out in HFD-CAS and HFD-WPI-fed mice in both experiments (i.e., 5w and 10w), but not in the LFD control groups.

Bioinformatic analysis (shotgun metagenomic sequencing data)

Metagenomic raw sequence reads were filtered on the basis of quality (removal all bases at the 3' end with an average Phred score < 25 over a sliding window of 10 bp) and length (only reads with a minimum length of 180 bases were considered for further analysis) with PRINSEQ-lite 0.20.4 (Schmieder & Edwards 2011). After filtering, the sequences from the host, in our case the mouse, were eliminated. The mouse genome (*Mus musculus* (Mouse) GRCm38) downloaded from llumina iGenomes (https://support.illumina.com/sequencing/sequencing_software/igenome.htmL) was use as reference to remove host reads using BMTagger (Version 3.101) (Rotmistrovsky & Agarwala 2011). Paired-end reads were joined and filtered for duplicates using Picard tools 2.7.1, specifically FastqToSam and MarkDuplicates tools respectively ("Picard Toolkit." 2019. Broad Institute, GitHub Repository. http://broadinstitute.github.io/picard/). Subsequently, the reads underwent another stage of quality filtering, sub-quality, using a modified version of the script trimBWAstyle.pl that works directly from BAM files (TrimBWAstyle.usingBam.pl.2010;https://github.com/genome/genome/blob/master/lib/perl/Genome/Site/TGI/Hmp/HmpSraProcess/trimBWAstyle.usingBam.pl). This

script was used to delete reads with a Quality threshold value less than Q30. In addition, the reads shorter than 200 bp were also eliminated.

The analysis of the microbial composition was carried out using the MetaPhlAn2 species classifier (Segata et al 2012). Taxa with a relative abundance of <0.01% were categorized as "Other" for each classifier. Subsequently, the functions were assigned with HUMAnN2 tool (Franzosa et al 2018), based on ChocoPhlan and UniRef databases (Suzek et al 2007). The HUMAnN2 gene abundance table was regrouped by a mapping of GO terms for all categories of bacterial metabolism and dividing the functional table into two files (one stratified and one non-stratified). Metagenome assembly was performed using IDBA-UD (Peng et al 2012).

Statistical analysis

Power and coefficient of variation were calculated using data (body weight gain) from a previous study (McAllan et al 2015) and determined to be 8.8%. For a power of 80% and a significance level of 5%, this allows detection of a difference of 14% with a sample size of at least 8 mice per treatment. Body weight, cumulative weight gain and cumulative energy intake differences over both experiments 1 and 2 were analysed by a two-way repeated-measures ANOVA with Bonferroni's *post hoc* pairwise comparisons. Statistical analysis of tissue weight, intestine weight/intestine length, gene expression, plasma leptin, insulin, glucose and TAG, and caecal and hepatic TAG data were performed using two-way ANOVA followed by pairwise comparison using Bonferroni's *post hoc* test. Non-parametric data were compared by Kruskal-Wallis ANOVA followed by Mann-Whitney *U* test. Tissues weight was also evaluated using ANCOVA. Data were expressed as mean ± SEM and significance was set at $P<0.05$ using SPSS software version 24 (IBM Corp). A complete description is detailed in "Supplementary Statistic" and figures **3.S4** and **3.S5**.

Bacterial and archaeal reads were retrieved from the output of MetaPhlAn2 for taxonomic profiling. Functional assignment was performed at the GO gene level. Statistical analyses were carried out with R (R version 3.4). Different R's packages have been used (R Development Core Team 2016). To perform alpha-diversity analyses (Richness, Shannon Index and Simpson Index), as well as the

multidimensional scaling analysis (MDS) based on Bray-Curtis, the statistical package "vegan" version 2.3.0 (Oksanen et al 2015) was used. The Adonis function in "vegan" was used for PERMANOVA (permutational analysis of variance) analysis, and betadisper function for MDS figures. To compare alpha-diversity metrics among groups, ANOVA (aov) test was performed and it was adjusted with the Benjamini-Hochberg method. Statistical differences between multiple samples in taxonomic and functional analyses were estimated by Kruskal-Wallis and False discovery rate (FDR). The Benjamini-Hochberg procedure was used to correct for multiple testing, and differences between two samples were estimated by Mann-Whitney test analysis and corrected with Family-wise error rate. The ggplot2 package (version 3.2.0) was used for data visualization (Wickham 2016).

Statistical significance was established at $P<0.05$ (*), with other ranges, i.e. $P<0.01$ (**) and $P<0.001$ (***), $P<0.0001$ (****), based on the corrected p-values, also being noted.

Results

HFD containing WPI increased energy intake but decreased body weight gain in younger mice

During the first 5 weeks (5w, **Fig. 3.1a**), 5-week-old mice fed HFD-CAS significantly increased body weight starting from week 2 compared to the LFD-CAS (**Fig. 3.1b**). On the contrary, mice fed with HFD-WPI did not differ from their control group (i.e., LFD-WPI) for the duration of the experiment. HFD-WPI-fed mice exhibited significantly lower body weight, from week 3, and cumulative weight gain, from week 1, compared to HFD-CAS-fed mice (**Fig. 3.1b** and **3.1c**). During the experiment, not just HFD-CAS-fed mice, but also HFD-WPI-fed mice, had significantly higher cumulative body weight gain compared to LFD groups at every timepoint (**Fig. 3.1c**). The energy intake did not match the body weight and cumulative weight gain data: the HFD-WPI-fed mice showed an increase in the cumulative energy intake compared to CAS counterparts (week 5 timepoint, HFD-CAS vs HFD-WPI: 400.70 ± 12.27 KJ vs 440.07 ± 6.90 KJ; **Fig. 3.1d**).

In contrast, HFD containing WPI given to 10-week-old mice (10w, **Fig. 3.1a**) produced different outcomes with respect to energy balance compared to HFD-WPI feeding in younger individuals. LFD-fed mice supplemented with WPI or CAS had similar body weight trajectories until week 5, similar to our previous study (**Fig. 3.1b**) (Nilaweera et al 2017). When all the mice were 10-week-old, switching the diet to HF significantly increased body weight and cumulative weight gain of HFD-CAS group from week 7 compared to LFD-CAS control group (**Fig. 3.1e** and **3.1f**). In this instance, the body weight trajectory of HFD-WPI-fed mice was similar to the LFD-WPI-fed mice until week 9 (**Fig. 3.1e**), but their cumulative weight gain was higher starting from week 7 compared to LFD counterparts (**Fig. 3.1f**). Unlike 5w mice, in 10w mice no difference between HFD-CAS and HFD-WPI body weight trajectories and cumulative weight gain were observed ($p>0.05$) (**Fig. 3.1e**).

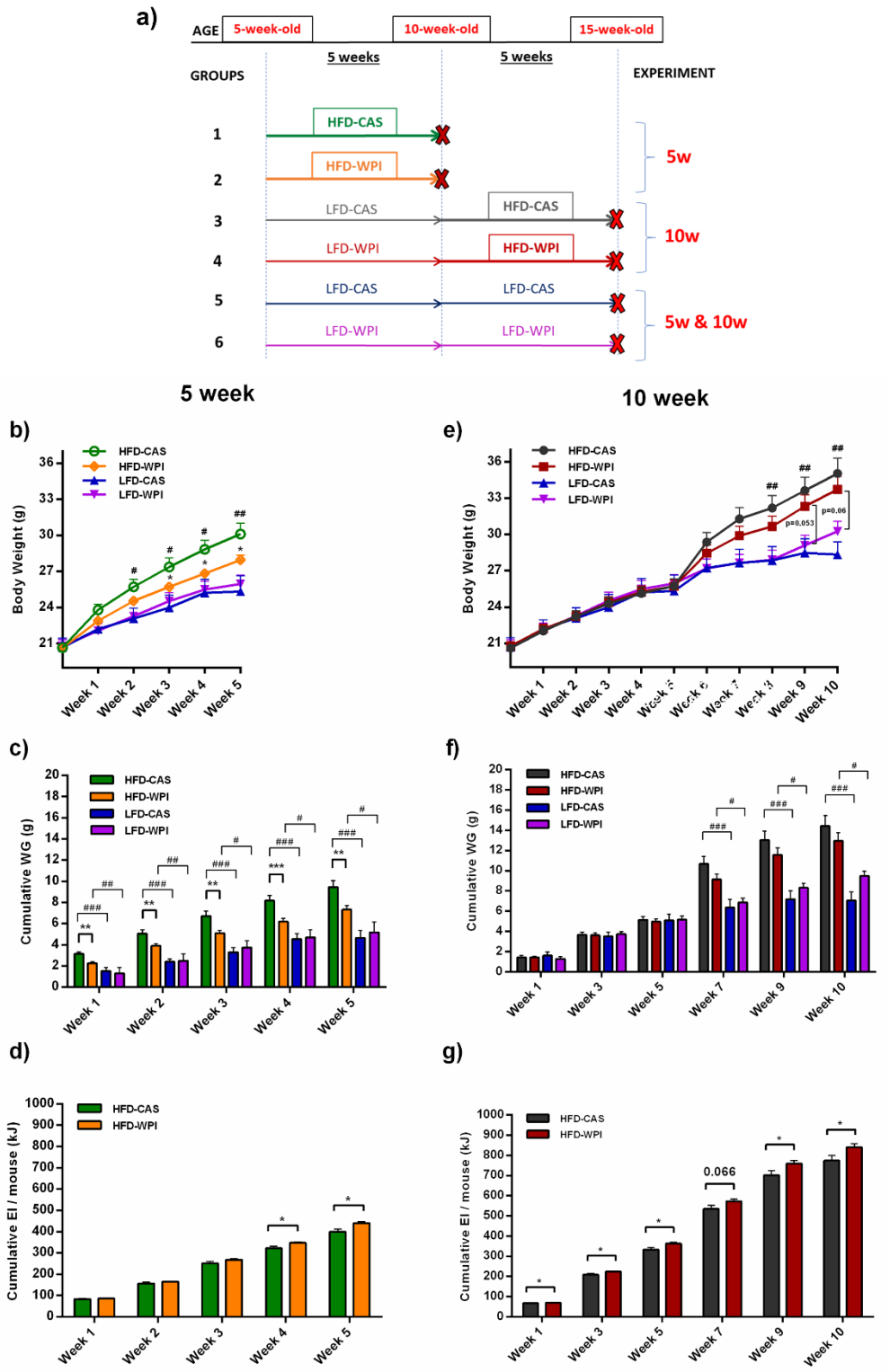


Figure 3.1 Effect of HFD-WPI on body weight and energy intake in 5w vs 10w mice. (a) Mice were divided into 6 groups. 5-week-old mice (5w, groups 1 and 2) were fed a high-fat diet with control casein (HFD-CAS; 45% fat and 20% casein) or whey protein isolate (HFD-WPI; 45% fat and 20% whey protein isolate). As controls for 5w, groups 5 and 6, respectively,

were fed a low-fat diet with casein (LFD-CAS; 10% fat and 20% casein) or whey protein isolate (LFD-WPI; 10% fat and 20% whey protein isolate). Groups 3 and 4 (10w) were initially fed with LFD-CAS and LFD-WPI respectively. After 5 weeks, when they were 10-week-old, the diet of these groups was switched from LF to HF, which lasted for other 5 weeks. The groups 5 and 6 (i.e., LFD-CAS and LFD-WPI) represented the control also for 10w. The present data show **(b)** body weight and **(c)** cumulative weight gain of 5w mice fed with HFD-CAS, HFD-WPI, LFD-CAS and LFD-WPI. In panel **(d)** is indicated cumulative energy intake of 5w mice fed with HFD-CAS and HFD-WPI. Data also show **(e)** body weight and **(f)** cumulative weight gain intake of 10w mice fed with HFD-CAS, HFD-WPI, LFD-CAS and LFD-WPI. In panel **(g)** is indicated cumulative energy of 10w mice fed with HFD-CAS and HFD-WPI. *Statistical analysis*: in figure (b) and (e) groups showing * (for HFD-CAS vs HFD-WPI) and # (HFD-CAS vs LFD-CAS) are significant (*/# $P<0.05$ or **/## $P<0.01$ or ***/### $P<0.001$). In figure (e), the trends refer to HFD-WPI vs LFD-WPI. In figure 1 (c), (d), (f) and (g) groups showing * (for HFD-CAS vs HFD-WPI and LFD-CAS vs LFD-WPI) and # (HFD-CAS vs LFD-CAS and HFD-WPI and LFD-WPI) are significant (*/# $P<0.05$ or **/## $P<0.01$ or ***/### $P<0.001$). A complete statistical description is detailed in Methods and Materials and "Supplementary Statistics".

During 10w, in accordance 5w, the WPI groups fed with HFD showed a significant increase in the cumulative energy intake relative to CAS group (week 10 timepoint, HFD-CAS vs HFD-WPI: 774.85 ± 26.10 KJ vs 841.50 ± 16.68 KJ) (**Fig. 3.1g**). Clearly, this indicates that a loss of energy occurs during WPI supplementation.

To investigate the aforementioned loss of energy, we quantified the level of lipids, more specifically triacyclglycerols, in the caecum, but did not observe any differences between CAS and WPI groups except in the 10w HFD-WPI group, which showed a trend towards a decrease compared to 10w HFD-CAS (**Fig. 3.S1a** and **3.S1b**). In addition, 10w HFD groups showed a significantly higher amount of lipids in the caecum compared to 5w HFD groups (**Fig. 3.S1a**). This analysis did not yield an explanation for the discrepancy of energy between WPI and CAS groups, and the underlying cause remains unclear.

Effect of HFD-WPI on organs and tissues at different ages

Mice that received HFD-WPI at 5-week-old decreased the weight of adipose tissues (i.e., sWAT, eWAT and BAT) and increased liver and stomach weights, compared to the HFD-CAS group (**Fig. 3.2a**). No differences in caecum and small intestine weights were observed between the two groups (**Fig. 3.2a**). In addition, an organ/tissues

weights evaluation using ANCOVA (body weight as covariant) was also performed, which also yielded similar results (see supplementary statistics **Fig. 3.S5b** and **3.S5c**). Details of tissue weight at this timepoint in the LFD-CAS and LFD-WPI have previously been detailed elsewhere (Nilaweera et al 2017).

Prolonged WPI intake as part of LFD (10w mice) had no effect on organs and tissues weights (**Fig. 3.2g**). However, when WPI-fed mice and their controls were exposed to HFD at 10-week-old, they had increased eWAT and sWAT weights compared to LFD-fed mice (**Fig. 3.2g**). While there was no difference observed in eWAT and BAT weight between HFD-CAS and HFD-WPI-fed mice, sWAT weight was significantly lower for 10w mice fed with HFD-WPI than those fed with HFD-CAS (**Fig. 3.2g**). No major changes were observed in stomach, caecum, small intestine and liver across the groups (**Fig. 3.2g**).
The small intestine length (data not shown) and intestinal weight normalized to the length were similar across both 5w and 10w mice (**Fig. 3.2b** and **3.2h**).

From the organs weight data, we noticed that the weight of the liver was higher in mice fed a WPI (even if in 10w mice this difference did not achieve the significance). To investigate the possible reason behind this finding, we measured the level of gene expression of selected enzymes involved in hepatic fatty acids biosynthesis, namely acetyl-CoA carboxylase (*Acc1*), fatty acids synthase (*Fasn*), malonyl CoA-acyl carrier protein transacylase (*Mcat*) and acyl-CoA desaturase 1 (*Scd1*). To carry out this analysis, we solely focused on HFD-fed mice, both 5w and 10w. While no changes between HFD-CAS and HFD-WPI (both 5w and 10w) were observed in the gene expression of *Acc1*, *Fasn* and *Mcat*, the expression level of the gene *Scd1* was lower in both HFD-WPI 5w and 10w compared to the CAS counterparts (**Fig. S2a**). SCD1 takes part in TAG biosynthesis and animals with a deficiency in SCD1 have a lower rate of TAG biosynthesis (Flowers & Ntambi 2008). According to these results, we found that 5w mice showed a lower level of hepatic TAG in the presence of WPI. However, no differences in liver TAG were detected between 10w mice (**Fig. S1c**). This last result disagrees with *Scd1* gene expression data. In addition, hepatic TAG of both 10w mice were similar to HFD-WPI 5w mice and lower than HFD-CAS 5w mice

(**Fig. S1c**). No statistical difference in hepatic TAG has been observed between LFD-CAS and LFD-WPI fed mice (**Fig. S1d**).

In summary, the reason why the liver is heavier during WPI supplementation is not because of a hepatic TAG accumulation. Thus, the underlying cause remains to be elucidated.

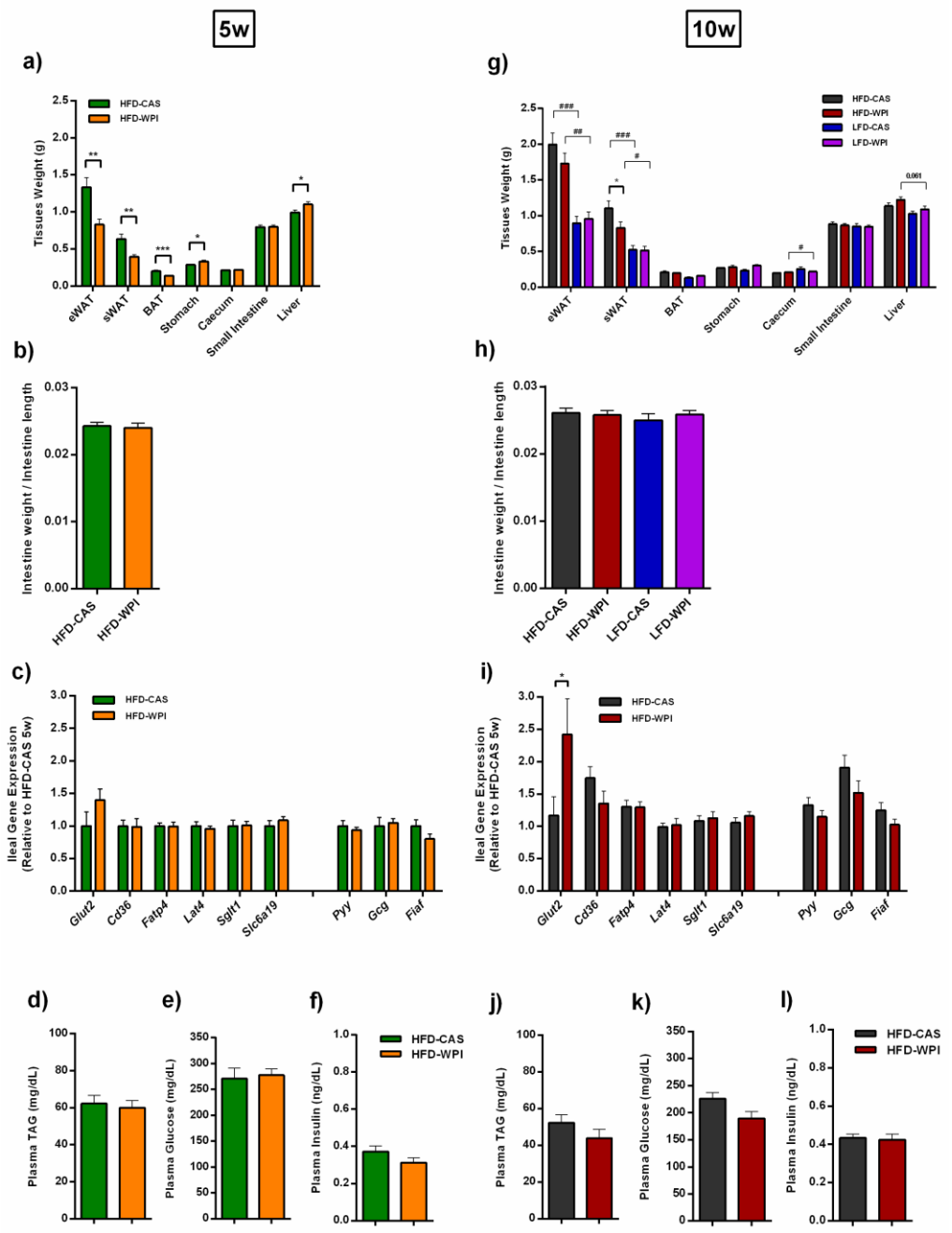


Figure 3.2 HFD-WPI effect on tissues and organs weight, ileal gene expression and plasma metabolites and insulin in 5w vs 10w mice. Data show **(a)** the tissues and organs absolute weight, **(b)** intestine weight normalized by intestine length, **(c)** ileal gene expression

of hormones and nutrient transporters, plasma levels of **(d)** triacylglycerol (TAG), **(e)** glucose and **(f)** insulin of 5w mice fed with HFD-CAS and HFD-WPI. Also shown are **(g)** the tissues and organs absolute weight, **(h)** intestine weight normalized by intestine length, **(i)** ileal gene expression of hormones and nutrient transporters, plasma levels of **(j)** triacylglycerol (TAG), **(k)** glucose and **(l)** insulin of 10w mice fed with HFD-CAS and HFD-WPI. Abbreviation; eWAT; epididymal white adipose tissue, sWAT; subcutaneous white adipose tissue, BAT; brown adipose tissue, GLUT2; glucose transporter 2, CD36; cluster of differentiation 36, FATP4; fatty acid transporter 4, LAT4; L type amino acid transporter 4, SGLT1; sodium-glucose transporter 1, SLCa19; methionine transporter, PYY; peptide YY, GCG; proglucagon, FIAF; fasting-inducing adipose factor. *Statistical analysis*: groups showing * (for HFD-CAS vs HFD-WPI) and # (HFD-CAS vs LFD-CAS and HFD-WPI and LFD-WPI) are significant (*/# $P<0.05$ or **/## $P<0.01$ or ***/### $P<0.001$). A complete statistical description is detailed in Methods and Materials and in **figures 3.S4** and **3.S5**.

HFD-WPI does not influence ileal gene expression, glucose and insulin plasma levels

Considering the differences observed in the body/organs weight and energy intake data across the different groups, we investigated further by measuring the ileal expression level of genes involved in the transport of nutrients and genes encoding for proteins involved in energy balance metabolism. We focused most of our analysis specifically on mice that received HFD-CAS and HFD-WPI at both ages (i.e., **Fig. 3.1a** groups 1,2,3 and 4), as differences in mice fed with LFD-CAS or WPI have been previously investigated (McAllan et al 2013, McAllan et al 2014, McAllan et al 2015).

WPI intake during 5w had no significant impact on the ileal expression of nutrient transporters and satiety hormones in HFD mice (**Fig. 3.2c** and **3.2i**). In contrast, adults fed a HFD-WPI increased glucose transporter 2 (*Glut2*) expression compared to HFD-CAS-fed mice (**Fig. 3.2i**). Furthermore, fatty acids transporter (*Cd36*) expression increased in the 10w HFD-CAS group compared to the 5w HFD-CAS group, without showing any differences between the WPI groups, and the gene encoding fatty acids transporter *Fatp4* was significantly more expressed in both 10w groups, compared to the 5w groups (**Fig. 3.S2b**). No differences were detected in the amino acid transporters (i.e., *Lat4* and *Slc6a19*) and the sodium-glucose transporter *Sglt1* gene expression across all groups (**Fig. 3.2c**, **3.2i** and **3.S2b**). In addition, we observed an increase in the expression of anorexic hormone Peptide Tyrosine Tyrosine (*Pyy*) in 10w HFD-CAS-fed mice relative to 5w HFD-CAS-fed mice, and an increase in *Gcg* (i.e., a gene that codes for the precursor of the satiety hormone GLP-1 in the intestinal

L cells of the ileum) in both 10w HFD groups, compared to 5w HFD groups. The expression of the Fasting-Induced Adipose Factor (*Fiaf*) remained unchanged (**Fig. 3.S2b**).

Although the expression of fatty acids transporters seems to be higher in older mice, no differences were observed in TAG levels in the plasma between 10w HFD-CAS and 5w HFD-CAS-fed mice and between LFD fed mice (**Fig. 3.2d**, **3.2j**, **3.S1e** and **3.S1f**). On the contrary, TAG levels in the plasma were higher in the 5w HFD-WPI group relative to the 10w counterpart (**Fig 3.S1e**).

In addition, no changes were detected between CAS and WPI groups at both ages in glucose and insulin plasma level (**Fig. 3.2e**, **3.2f**, **3.2k** and **3.2l**). However, 10w HFD-WPI-fed mice showed a significant decrease in plasma glucose and an increase in plasma insulin relative to 5w HFD-WPI-fed mice. The plasma glucose levels were also lower in 10w HFD-CAS-fed mice compared to the 5w counterparts, but insulin remained unchanged between these two groups (**Fig. 3.S1i** and **3.S1j**). According to our previous study (McAllan et al 2015), the two LFD groups had a similar plasma level of insulin (**Fig. 3.S1j**); however, in discordance with the study just mentioned, LFD-WPI-fed mice showed lower plasma glucose levels compared to LFD-CAS-fed (**Fig. 3.S1h**).

These results suggest that the stage of life at which the mice start HFD-feeding has an influence on glucose and insulin levels and on the expression of genes involved in fatty acids transporters and genes coding for satiety hormones located in the distal part of the small intestine. In this context, the dietary protein typology did not particularly affect our measurements.

HFD-WPI influences leptin and fat catabolism-related genes in younger mice

A gene expression analysis was also carried out in the eWAT. Interestingly, we observed that *Ob*, *Cd68* (i.e., genes that encode for leptin and for a protein expressed in the monocytes and macrophages, respectively) and *Fiaf* expressions were significantly lower in the 5w HFD-WPI-fed mice relative to the HFD-CAS control, but this difference was no longer observed between the two 10w groups (**Fig. 3.3a**, **3.3e** and **3.S2c**). These findings were in accordance with body weight and cumulative weight gain data. Notably, the levels of plasma leptin data matched the *Ob* gene

expression levels between the 5w groups (**Fig. 3.3b**). In older mice, unlike the *Ob* gene expression data, the WPI groups showed a lower level of leptin compared to CAS groups, although this difference was less pronounced compared to the difference between HFD-CAS and HFD-WPI in younger mice (**Fig. 3.3f**). It is also important to highlight that, both *Ob* gene expression and plasma leptin levels were significantly higher in mice that began HFD-feeding at 10-week-old compared to mice that started the HFD intervention at 5-week-old (**Fig. 3.S2c** and **3.S1k**). No differences in plasma leptin were detected between the LFD groups (**Fig. 3.S1l**).

Analysis of the expression of genes involved in the catabolism within adipose tissue revealed an increase in *Cpt1a* and *Ucp2* and a decrease in *Hsl* and *Lpl* expression in the 5w HFD-WPI-fed mice, relative to HFD-CAS-fed mice (**Fig. 3.3c**). Again, in older age, there were no differences detected between HFD-CAS and HFD-WPI-fed mice (**Fig. 3.3g**). No significant differences were detected in *Ctp1b* and *Ucp3* expression (**Fig. 3.3g** and **3.S2d**).

Conversely, the anabolic gene *Acc1* was expressed more in the HFD-WPI group compared to the HFD-CAS group during 10w, without any changes during 5w (**Fig. 3.3d** and **3.3h**). Only *Fatp1* was more highly expressed in both HFD adolescent groups, compared to the HFD adult groups (**Fig. 3.S2e**). The expression of the anabolic gene *Fasn* and the transporter *Cd36* remained unchanged.

Altogether, the results showed the specific effect of whey protein within a HFD on leptin expression/plasma availability and on the expression of *Cd68*, *Fiaf* and some important genes involved in the catabolism of fatty acids in eWAT. However, these effects were only observed when the animals started the dietary intervention at 5-week-old; the same diet given at 10-week-old did not have the same effects.

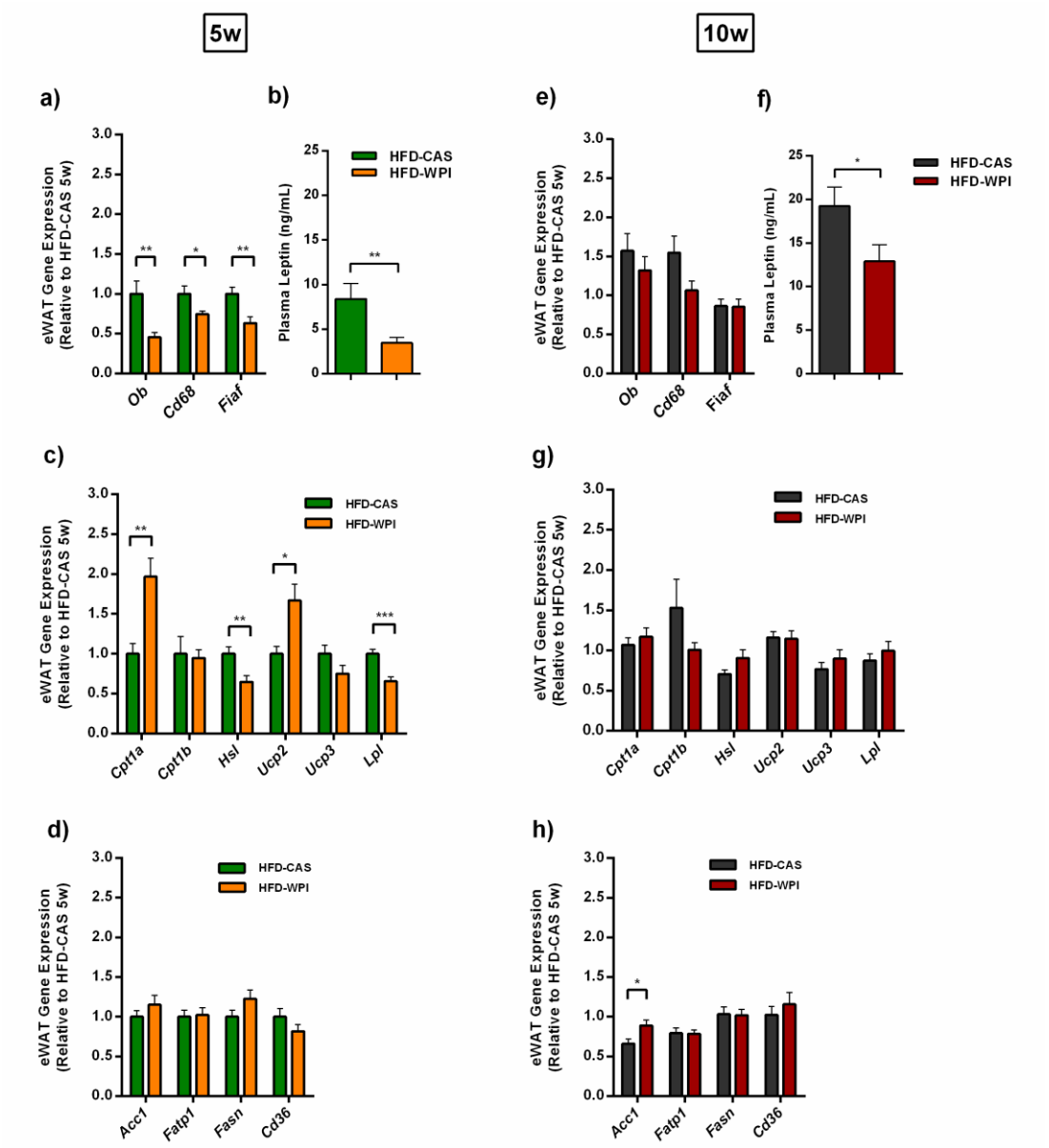


Figure 3.3 Differential effect of HFD-WPI on eWAT gene expression and plasma leptin in 5w compared to 10w mice. Data show **(a)** expression of genes encoding for leptin (*Ob*), inflammation marker CD68 and the FIAF in the epididymal white adipose tissue (eWAT) and **(b)** plasma levels of leptin of 5w mice fed with HFD-CAS and HFD-WPI. Expression of genes encoding for **(c)** catabolic and **(d)** anabolic enzymes in the eWAT of 5w mice fed with HFD-CAS and HFD-WPI were also investigated. Data also show **(e)** expression of genes encoding for leptin (*Ob*), inflammation marker CD68 and the FIAF in the eWAT, **(f)** plasma levels of leptin and expression of genes encoding for **(g)** catabolic and **(h)** anabolic enzymes in the eWAT of 10w mice fed with HFD-CAS and HFD-WPI. Abbreviation; OB; leptin, CD68; cluster of differentiation 68, CPT1a and b; carnitine palmitoyltransferase I, HSL; hormone-sensitive lipase, UCP2 and 3; uncoupling protein, LPL; lipoprotein lipase, ACC; acetyl-CoA carboxylase, FATP1; fatty acid transporter 1, FASN; fatty acid synthase, CD36; cluster of differentiation 36. *Statistical analysis*: groups showing * (for HFD-CAS vs HFD-WPI) are significant (* P<0.05, ** P<0.01, *** P<0.001). A complete statistical description is detailed in Methods and Materials and **figure 3.S4**.

Younger mice fed with HFD-WPI have a higher proportion of *Lactobacillus murinus* within the gut

Faecal DNA was subjected to shotgun metagenomic sequencing. The total number of reads per sample averaged 5,733,118 ± 1,768,632. These reads were used to investigate taxonomical and functional differences across the HFD-fed mice at different ages. At the family and species level, the taxonomical analysis of alpha-diversity indexes (i.e., Simpson and Shannon) did not differ across the groups, although Richness index was higher in 5w HFD-WPI-fed mice compared to their CAS counterpart with no differences between the two 10w groups (**Fig. 3.4a**). The NMDS graph based on the dissimilarity matrix of Bray-Curtis showed clustering related to the type of diet. A clear separation between CAS and WPI groups was observed (**Fig. 3.4b**, **3.4c** and **3.S3b**). At the family level, no significant differences were detected between HFD-CAS and HFD-WPI groups, either in 5w or in 10w (**Fig. 3.4b**), even if the two younger groups showed a more evident separation (R^2=0.14, p=0.066 PERMANOVA pairwise analysis) compared to the older individuals. However, at species level, a significant separation was noted between 5w HFD-CAS and HFD-WPI (R^2=0.147, p=0.036; PERMANOVA pairwise analysis), with no differences between the two 10w counterparts (**Fig. 3.4c**).

A significantly higher proportion of the family *Streptococcaceae* and the corresponding genus and species *Lactococcus* and *Lactococcus lactis* were present in all groups fed with a diet containing CAS as the main protein source (**Fig. 3.S3a** and **3.S3c**). This is in accordance with our previous study, in which we demonstrated that the high abundance of this taxa in mice fed with CAS is due to the presence of *L. lactis* in the diet (Boscaini et al 2019) (Chapter 2). *Bacteroides vulgatus* was also more abundant in both CAS groups (**Fig. 3.4d** and **3.S3a**). Interestingly, *Lactobacillus murinus*, was present in higher proportion in HFD-WPI fed mice compared to their CAS counterparts during 5w, with no difference in the abundance of this species between the 10w groups (**Fig. 3.4d** and **3.S3a**). The relative abundance of *L. murinus* across the groups (represented in a taxa plot and as a percentage) is reported in **figures 3.S3a** and **3.S3e**.

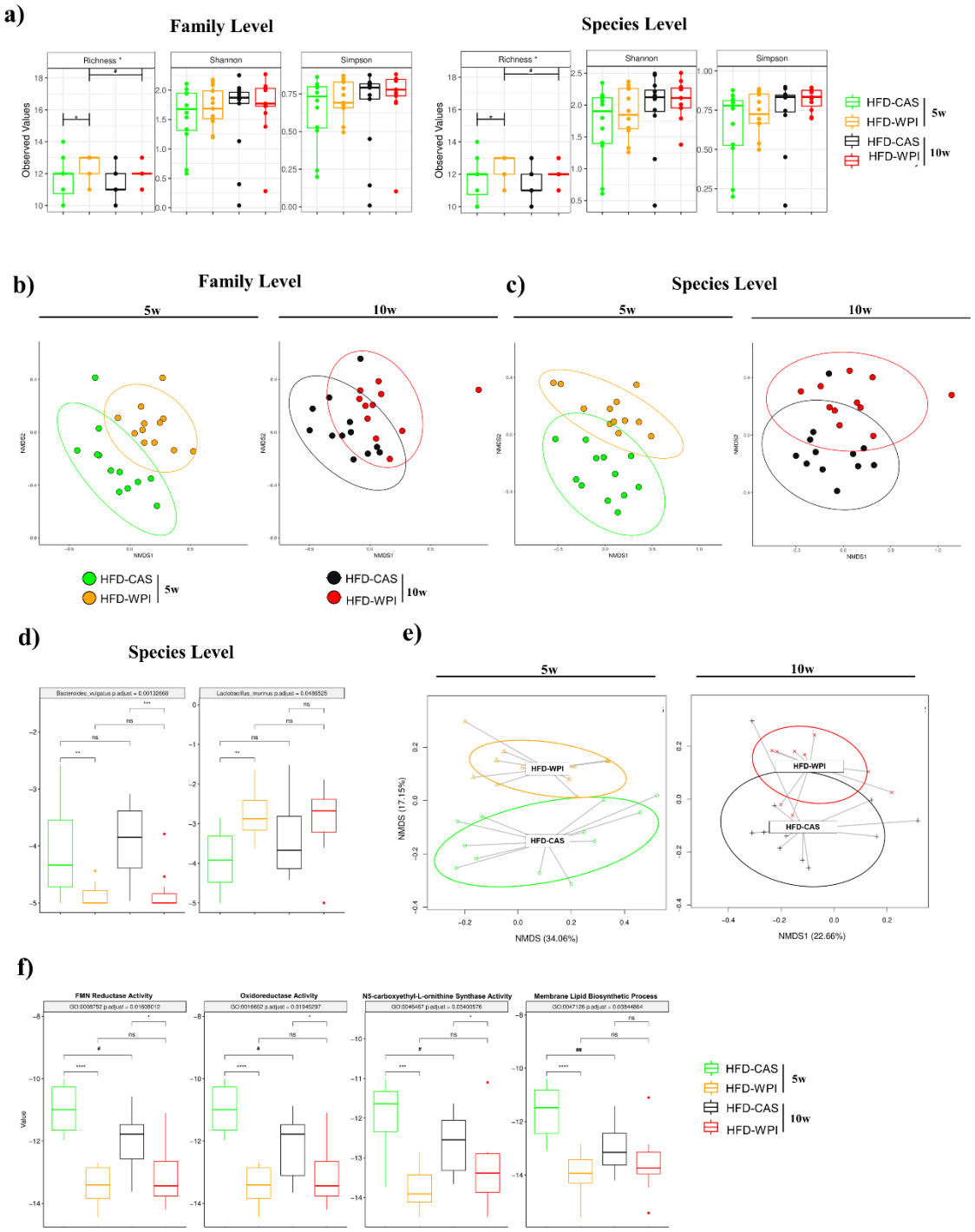


Figure 3.4 Age- and duration-dependent effect of HFD-WPI on the gut microbiota taxonomy and function. Taxonomic **(a)** alpha-diversity at family and species level, measured with richness, Shannon and Simpson indexes, and beta-diversity, calculated using NMSD ordination, both **(b)** at family and **(c)** species level, of HFD-CAS and HFD-WPI-fed mice at both ages (5w and 10w). **(d)** Bar chart representing taxonomic differences at species level across the groups, using Kruskal Wallis method. **(e)** Functional beta-diversity calculated using NMSD ordination, and **(f)** bar chart representing differences in metabolic activities or

processes across the HFD-CAS and HFD-WPI-fed mice at both ages. *Statistical analysis*: groups showing * (for HFD-CAS vs HFD-WPI) and # (5w vs 10w) are significant (*/# $P<0.05$ or **/## $P<0.01$ or ***/### $P<0.001$). A complete statistical description is detailed in Methods and Materials.

In addition to the taxonomical data, functional analyses were carried out. Alpha-diversity analysis performed with HUMAnN2 did not show changes across the groups (data not shown). As for the taxonomic data, a clear separation between CAS and WPI functions in the NMDS graph based on the dissimilarity matrix of Bray-Curtis beta-diversity plot was observed (**Fig. 3.4e** and **3.S3d**). HFD-WPI groups, at both ages, were significantly different compared to their CAS counterparts (5w: HFD-CAS vs HFD-WPI $R^2= 0.145$, $p=0.006$; 10w: HFD-CAS vs HFD-WPI $R^2= 0.118$, $p=0.012$; PERMANOVA pairwise analysis) (**Fig. 3.4e**). In addition, both WPI groups showed decreased metabolic FMN reductase, oxidoreductase and N5-carboxyethil-L-ornithine synthase activities (**Fig. 3.4f**). In contrast the membrane lipid biosynthetic process was decreased in 5w HFD-WPI-fed mice, relative to HFD-CAS-fed mice, with no difference between the 10w mice (**Fig. 3.4f**).

In addition, we analysed in detail *L. murinus* -related functions that were significantly different across the groups. In total, we found 62 significantly different activities and functions across the groups (**Fig. 3.5**) and, among these, 49 functions were significantly higher in HFD-WPI-fed mice compared to HFD-CAS-fed mice during 5w with no differences between the 10w groups. Several processes and functions, attributed to *L. murinus*, were found to be abundant within the younger HFD-WPI group, such as amino acids biosynthesis, sugars metabolism (including glycolysis and fructose, mannose, lactose metabolism), vitamins metabolism (vitamin C catabolism and vitamin K2 biosynthesis), DNA and nucleic acid-related processes (including pentose phosphate pathway) and oxalate metabolism (**Fig. 3.5**). Altogether, these results suggest that the composition and the function of the intestinal bacterial population can be differently influenced based on the stage of life at which the dietary interventions start, together with other metabolic and physiologic outcomes.

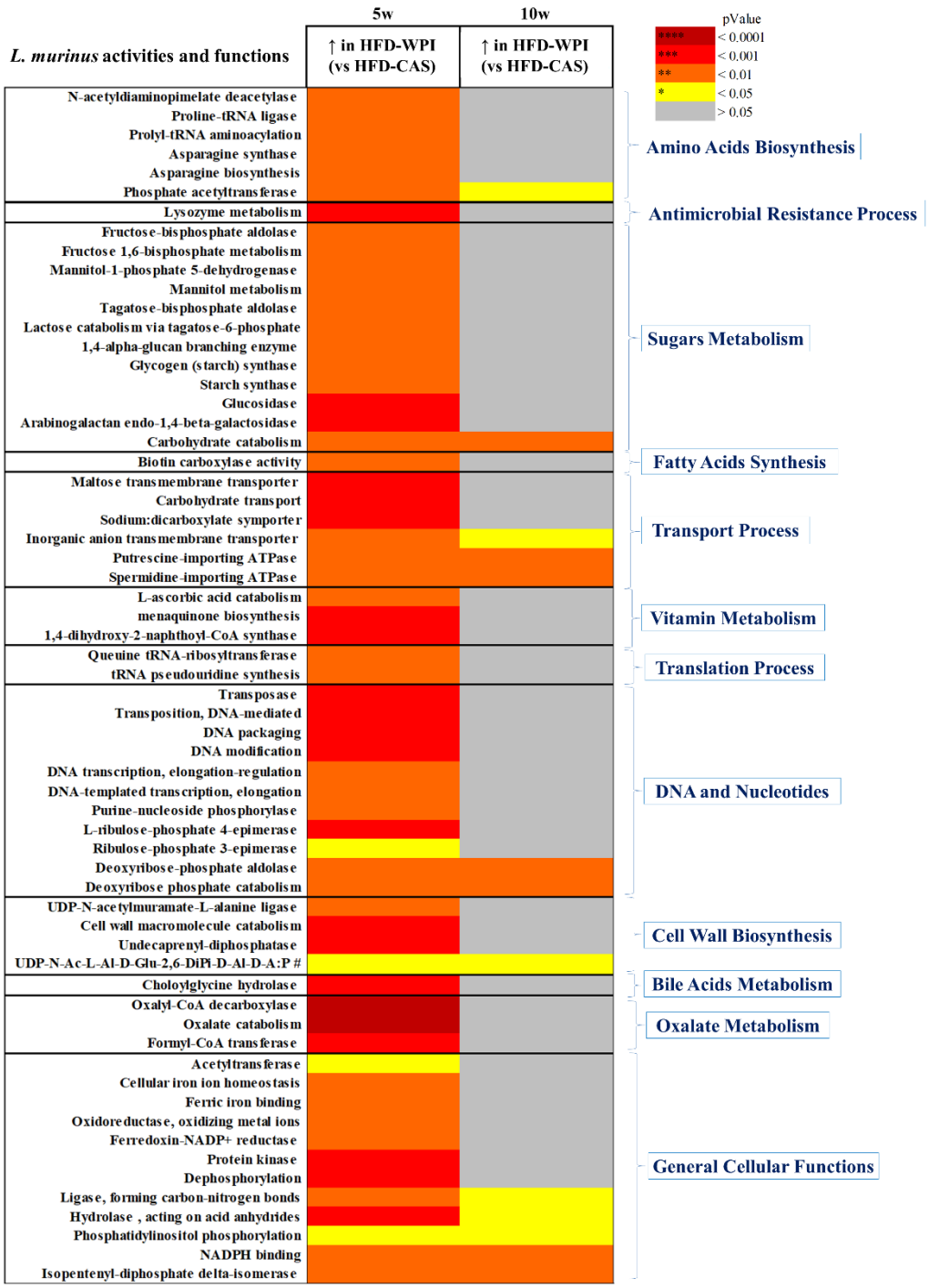


Figure 3.5 Differences in metabolic activities and processes of *Lactobacillus murinus*. Heat map showing significant differences in the increase of metabolic activities and processes abundance belonging to faecal *Lactobacillus murinus* in HFD-WPI, relative to HFD-CAS, in 5w compared to 10w mice. Abbreviation # (in cell wall biosynthesis): UDP-N-Ac-L-Al-D-Glu-2,6-DiPi-D-Al-D-A:P as UDP-N-acetylmuramoyl-L-alanyl-D-glutamyl-meso-2,6-diaminopimelyl-D-alanyl-D-alanine:undecaprenyl-phosphate transferase. *Statistical analysis*: groups showing * (for HFD-CAS vs HFD*-WPI) are significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). A complete statistical description is detailed in Methods and Materials.

Discussion

There is an increasing emphasis on understanding the microbiota function and composition in response to diet (Hughes et al 2019). In particular, its modulation to a healthier configuration to ameliorate or prevent metabolic diseases, such as obesity, is of interest (Torres-Fuentes et al 2015, Torres-Fuentes et al 2017). Here, we have shown that a 5-week-diet intervention with HFD-WPI, compared to HFD-CAS, in mice that started the HFD intervention at 5-week-old, reduced weight gain and adiposity, changed eWAT catabolism and leptin production, with an increase in *Lactobacillus murinus* abundance and related functions within the gut. In contrast, none of these changes was observed in mice that underwent the same diet intervention starting from 10-week-old and an extended period of WPI supplementation. These results suggest that the efficacy of this dietary intervention is age-dependent and, based on age, the interplay with diet duration and the quality of response is different. Thus, this is in accordance with our second hypothesis.

It has previously been shown that the presence of WPI as the protein component of a HFD caused a reduction in weight gain in rodents (McAllan et al 2013, McAllan et al 2014, Pilvi et al 2008). With the present paper, we confirmed a reduction in weight gain and adiposity in mice starting a 5-week HFD-WPI dietary intervention at 5-week-old. When the same dietary intervention was started at 10-week-old, the body weight trajectory and the cumulative weight gain of HFD-WPI-fed mice were not statistically significantly different, compared to their CAS counterparts. It is worth noting that, while total weight gain was not significant, sWAT weight was significantly lower in HFD-WPI compared to their CAS fed counterparts. Notably, WPI administration in LFD at 5-week-old did not prevent an increase in weight gain after the switch to HFD at 10-week-old. This finding goes against our first hypothesis.

In contrast with previous studies in rodents that showed a satiety effect due to WPI-enriched diets as being the mechanism of action responsible for reduced weight gain (Hall et al 2003, Pezeshki et al 2015, Zhou et al 2011), here we found an increase in energy intake, both in HFD and LFD-fed mice, suggesting a loss of energy during WPI

supplementation. Notably, we also found a discrepancy between body weight and energy intake in our previous study, in which we were investigating the metabolic and gut microbial differences in mice fed with HFD containing alpha-lactalbumin, a specific whey protein, compared to CAS counterparts (Boscaini et al 2019) (Chapter 2). To investigate the loss of energy that occurred between WPI and CAS groups, we measured the level of TAG in the caecum. Variation of fat, specifically free fatty acids, cholesterol and TAG, in faeces is an indication of intestinal function and fat absorption changes under certain conditions (Kraus et al 2015, Mataki et al 2007). No differences were detected in the TAG levels between WPI and CAS-fed mice, but we cannot exclude that free fatty acids or cholesterol can be involved in the afore-mentioned loss of energy.

Surprisingly, during organ weight data analysis, we observed that the liver was heavier in the presence of WPI within the diet. Since we did not observe either an accumulation of hepatic TAG or an overexpression of genes involved in fatty acids biosynthesis in the liver of WPI-fed mice, we could not explain the higher weight of the liver in these mice. Perhaps, this phenomenon is due to inflammation or an accumulation of other macromolecule such as glycogen or cholesterol or vitamins (Bhattacharya 2015, Li et al 2016). Future investigation, using histological and transcriptomics approaches, will be required to fully elucidate the effect of WPI on liver weight.

In previous studies from our group it was observed that a LFD enriched in WPI changed the gene expression of some ileal nutrient transporters and satiety-control hormones, such as FATP4, methionine transporter and PYY (McAllan et al 2013, Nilaweera et al 2017). In the present study, we showed that, with the exception of *Glut2* in 10w, WPI provided in presence of HFD decreases the capacity to regulate the expression of ileal nutrient transporters and satiety hormones. Instead, some of the analysed genes (i.e., *Cd36*, *Fatp4*, *Pyy*, *Gcg*) underwent increased expression in 10w relative to 5w mice. In accordance with these data, plasma levels of TAG, glucose and insulin also remained unchanged between WPI and CAS across the groups, but they showed some variations with age. This is in line with other studies testing TAG, glucose and insulin during HFD-WPI administration (McAllan et al 2013, Pilvi et al 2007). The fact that nutrient transporter expression and plasma metabolites are

different between mice at different ages might be because they started the HFD dietary intervention at different stages of life with different duration of WPI intake. Consequently, we suggest that the decreased weight gain and adiposity observed in the presence of WPI-enriched HFD at younger age is not controlled or modulated by a differential nutrient absorption mechanism within the small intestine.

Our data suggest that decreases in weight gain and adiposity in younger mice fed a HFD-WPI might be linked with eWAT catabolism. *Cpt1a* and *Ucp2* are genes coding respectively for a mitochondrial enzyme that catalyses the rate-limiting step of fatty acids β-oxidation and a mitochondrial membrane protein that dissipates metabolic energy with prevention of oxidative stress accumulation (Bäckhed et al 2007, Horvath et al 2003, Pierelli et al 2017, Warfel et al 2017). While these two genes were expressed more in mice fed a HFD-WPI when younger, *Hsl* and *Lpl* genes, together with the gene coding for LPL-fasting induced inhibitor FIAF (also involved in triglyceride metabolism), were expressed less in the same group, compared to 5w mice fed with HFD-CAS. HSL is an intracellular neutral lipase responsible for the hydrolysis of TAG, diacylglycerols, monoacylglycerols and cholesteryl-esters (Haemmerle et al 2006, Shen et al 2011). LPL is an enzyme that hydrolyses TAG and controls the kinetics and transport of the majority lipoproteins in the plasma (Wang & Eckel 2009). This suggests that WPI modulates the expression of key genes involved in adiposity control and that play a role in metabolic syndrome development, thereby accelerating eWAT fatty acids catabolism and ameliorating adiposity and HFD-induced obesity phenotype.

In 5w mice fed with HFD-WPI, the level of expression of the eWAT *Ob* gene, together with the plasma level of the satiety hormone produced by this gene, leptin, were lower compared to the CAS counterparts. Notably, plasma leptin, but not *Ob* expression, was also lower in adult HFD-WPI-fed mice compared to the CAS controls. Also in the presence of LFD, WPI have been shown to reduce plasma levels of leptin (McAllan et al 2014), however we found no differences in circulating leptin between LFD-WPI and LFD-CAS, after 10 weeks of dietary intervention. As is well established for HFD-induced obese mice and obese humans, leptin signalling to the brain is disrupted and, consequently, its level in periphery is higher than in the healthy status (Zhou & Rui

2013). WPI, not only by influencing fatty acids catabolism, but also possibly by lowering both plasma leptin and *Ob* gene expression, positively modulate adiposity in mice and this effect seems to be more powerful in the presence of HFD than LFD. Notably, our data suggest that a reduction in plasma leptin in HFD-WPI-fed mice is directly due to a reduction in *Ob* gene expression, rather than a reduction in adipose mass. Low *Cd68* expression in eWAT, an inflammatory marker, gives a first insight regarding a low inflammatory state within the adipose tissue in the presence of HFD-WPI, which is usually very high in mice and humans with obesity (Stolarczyk 2017).

Aside from eWAT catabolism and leptin production, the gut microbiota composition and function showed changes between 5w HFD-WPI- and HFD-CAS-fed mice, that were no longer observed between 10w HFD groups. Specifically, the most important difference lay at species-level, where the species *Lactobacillus murinus* showed an increased abundance in younger HFD-WPI-fed mice relative to the CAS controls. *L. murinus* can attenuate allergic responses in mice with food allergy-induced dysbiosis within the gut microbiota and its high proportions in the gut are correlated with a lower degree of necrotizing enterocolitis, suggesting a protective role in the intestine (Huang et al 2016, Isani et al 2018). In addition, a recent study showed that there was a high proportion of *L. murinus* in the gut of mice that underwent calorie restriction, which was also linked with anti-inflammatory effects. In the same study, it was also demonstrated that *L. murinus* reduced intestinal permeability and systemic inflammatory markers in old microbiota-colonized gnotobiotic mice (Pan et al 2018).

In the present study, *L. murinus* levels within the gut of younger HFD-WPI-fed mice were linked to changes in the functional profile, such as upregulation of amino acids, fatty acids and cell wall biosynthesis functions, together with functions related to transport, translation, DNA, nucleotides, sugar metabolism and general cellular function. This can be explained by the fact that WPI stimulates the growth of gut *L. murinus*, thus all the bacterial functions related to growth, cell division and survival are upregulated. Additionally, oxalate, bile acid and vitamin metabolism were found to be *L. murinus*-related upregulated functions in the presence of WPI.

Oxalate is a toxic compound found in fruit, vegetables, grains and nuts. Excessive concentration of oxalate can cause problems in the gastrointestinal tract and exceptionally kidney failure. Recently, probiotic bacteria belonging to *Bifidobacterium* and *Lactobacillus* have been studied for their potential to degrade oxalate. A direct correlation between the oral administration of probiotic bacteria and the reduction of urinary oxalate excretion has been observed in rats and humans (Noonan & Savage 1999, Turroni et al 2007).

Gut bacterial vitamin and bile acids metabolism are among the most important examples of gut bacteria-host metabolism interaction. In particular, it was recently shown that HFD causes a serious alteration of the bile acid pool, that may contribute to obesity morbidity and development (Lin et al 2019). Accordingly, in this study, we observed a significant increase in *L. murinus*-related choloylglycine hydrolase activity in younger mice fed with HFD-WPI. Cholylglycine hydrolase activity is related to the bile salt hydrolase enzyme (BSH), which is commonly produced by *Lactobacillus spp.* to promote bile acids biotransformation (Urdaneta & Casadesús 2017). This process, in turn, plays an important role in regulating host lipid and cholesterol, glucose, and energy metabolism, drug metabolism, and the modulation of immune response (Joyce et al 2014, Li & Chiang 2014). Thus, the anti-obesity effect exerted by WPI might be mediated by an increase in *L. murinus* and its bile acids biotransformation activity. In light of this, in the future, will be insightful to perform a detailed analysis on bile acids and gut microbiota upon WPI consumption.

This work builds upon a decade of our research where we have tried to understand the energy balance related effects of WPI, which have led to the finding that this protein can reduce adiposity and body weight without relying on energy intake. Here, we have shown that early nutritional intervention with whey protein alters the composition of the gut microbiota and adiposity, where the effects of the catabolic response in the tissue were evident, suggesting a potential functional relationship between changes in the gut and the adipose tissue, which is not seen in later stage of the mouse life despite prolonged protein intake. It is interesting that preterm babies fed whey protein gain weight but this effect is lost in term babies (Berger et al 1979). More recently, it has been shown that babies aged 28 day fed formula with a higher proportion of whey

protein consume more of the formula but gain less weight (gain per intake) than babies fed low amounts of the protein (Fleddermann et al 2014).

Taken together our findings show that WPI exerts a possible prebiotic effect within the gut thanks to its ability to promote *L. murinus* particularly at a young age. Consequently, we propose that the role of WPI, during HFD administration, in the amelioration of weight gain and regulation of adipose tissue metabolism might be directly linked to modulation of the gut microbiota. Future work is planned, which aims to address this hypothesis.

Supplemental Material

Supplemental Tables

Table 3.S1 Sequences of qPCR primers.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
Acetyl-CoA carboxylase *(Acc1)*	5'-cagtgctatgctgagattgagg-3'	5'-acacagccagggtcaagtg-3'
Fatty acid transporter 1 (*Fatp1)*	5'-ccggtgtggtggctgctcttctc-3'	5'-gctgccatctccccgccataaatg-3'
Fatty acid synthase (*Fasn*)	5'-gctgctgttggaagtcagc-3'	5'-agtgttcgttcctcggagtg-3'
Lipoprotein lipase (*Lpl*)	5'-tgtctaactgccacttcaaccac-3'	5'-gggcacccaactctcatacattc-3'
Cluster of differentiation 36 (*CD36*)	5'-ttgaaaagtctcggacattgag-3'	5'-tcagatccgaacacagcgta-3'
Leptin (*Ob*)	5'-ccccgcaccgctggaagtac-3'	5'-atgtgccctgaaatgcggtatg-3'
Cluster of differentiation 68 *(CD68)*	5'-cacttcgggccatgtttctcttg-3'	5'-aggggctggtaggttgattgtcgtc-3'
Carnitine palmitoyltransferase 1a (*Cpt1a*)	5'-gactccgctcgctcattc-3'	5'-tctgccatcttgagtggtga-3'
Carnitine palmitoyltransferase 1b (*Cpt1b*)	5'-gagtgactggtgggaagaatatg-3'	5'-gctgcttgcacatttgtgtt-3'
Hormone-sensitive lipase (*Hsl*)	5'-ctattcagggacagaggcag-3'	5'-cgatgtggtcttttggggc-3'
Uncoupling protein 2 (*Ucp2*)	5'-ccatttcctgcaccccgatttacttcc-3'	5'-gctgggctggggatgaagatgaag-3'
Uncoupling protein 3 (*Ucp3*)	5'-acaggcccacacggtccagaacc-3'	5'-cccatcaggtcagtgcaaaacagagg-3'
Angioprotein-like 4 (*Fiaf*)	5'-gctcattggcttgactcccaac-3'	5'-aaaagtccactgtgccgctc-3'
Glucose transporter 2 (*Glut2*)	5'-tcctacttggcctatctgctgtgc-3'	5'-tgccctgacttcctcttccaac-3'
Fatty acid transporter 4 (*Fatp4*)	5'-tggcgtttcatccgggtcttcatc-3'	5'-gcaaacagcaggggcaccgtcttc-3'
L-type amino acid transporter 4 (*Lat4*)	5'cccgcttcctgttgttggtgctaac3'	5'ggggcttcttctcaggctttcaag 3'
Sodium-glucose co-transporter 1 (*Sglt1*)	5'-gagccccgcggttactgc-3'	5'-cctgcggctgctcctgtg-3'
System B(0) neutral amino acid transporter 1 (*Slc6a19*)	5'gtgtggcgcttcccctacctatg-3'	5' cctctgaccgatggcaaactcc-3'
Peptide YY (*Pyy*)	5'-ggacgcctaccctgccaaacca-3'	5'-agtgccctcttcttaaaccaaaca-3'
Proglucagon (*Cgc*)	5'-agggacctttaccagtgatgtga-3'	5'-acgagatgttgtgaagatggttgt-3'
Malonyl CoA-acyl carrier protein transacylase (*Mcat*)	5'-cagtgtgggagagtttgctg-3'	5'-ccttcaccgcatacagacc-3'
Stearoyl-CoA desaturase *(Scd1)*	5'-ttccctcctgcaagctctac-3'	5'-cagagcgctggtcatgtagt-3'
Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*)	5'-aagagggatgctgcccttac-3'	5'-ccattttgtctacgggacga-3'
β-actin (*ActB*)	5'-agagggaaatcgtgcgtgac-3'	5'-caatagtgatgacctggccgt-3'

Supplemental Figures

Figure 3.S1 Plasma metabolites and hormones, and TAG in the caecum and in the liver. Data show triacylglycerol (TAG) level in the caecum **(a)** between HFD groups in 5w vs 10w and **(b)** between LFD-CAS and LFD-WPI groups. TAG level in the liver **(c)** between HFD groups in 5w vs 10w and **(d)** between LFD-CAS and LFD-WPI groups. Plasma levels of TAG **(e)** between HFD groups in 5w vs 10w and **(f)** between LFD-CAS and LFD-WPI groups. Plasma levels of glucose **(g)** between HFD groups in 5w vs 10w and **(h)** between LFD-CAS and LFD-WPI groups. Plasma levels of insulin **(i)** between HFD groups in 5w vs 10w and **(j)** between LFD-CAS and LFD-WPI groups. Plasma levels of leptin **(k)** between HFD groups in 5w vs 10w and **(l)** between LFD-CAS and LFD-WPI groups. *Statistical analysis*: groups showing * (for HFD-CAS vs HFD-WPI) and # (HFD-CAS 5w vs HFD-CAS 10w and HFD-WPI 5w vs HFD-WPI 10w) are significant (*/# P<0.05 or **/## P<0.01 or ***/### P<0.001). A complete statistical description is detailed in Methods and Materials and in **figures 3.S4** and **3.S5**.

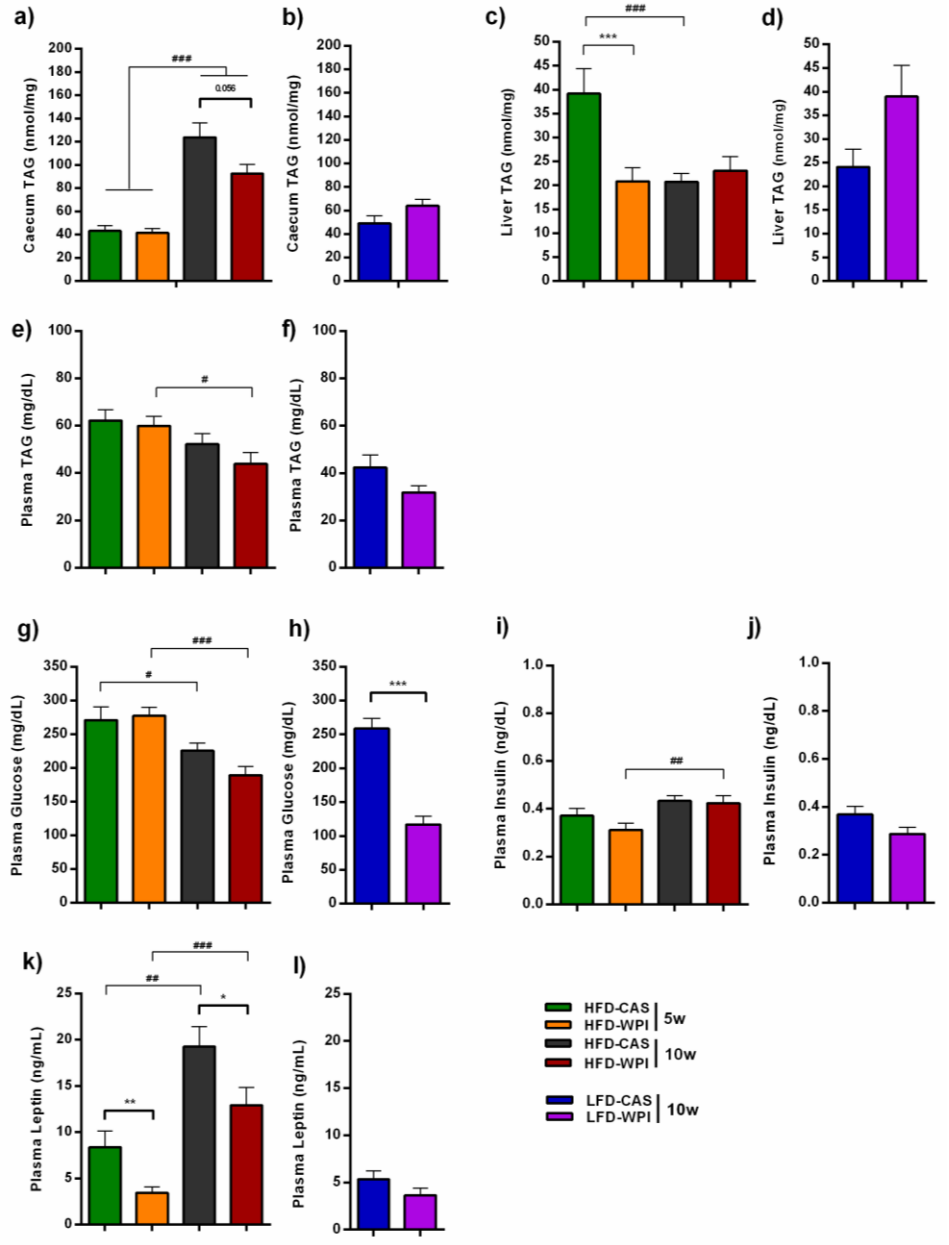
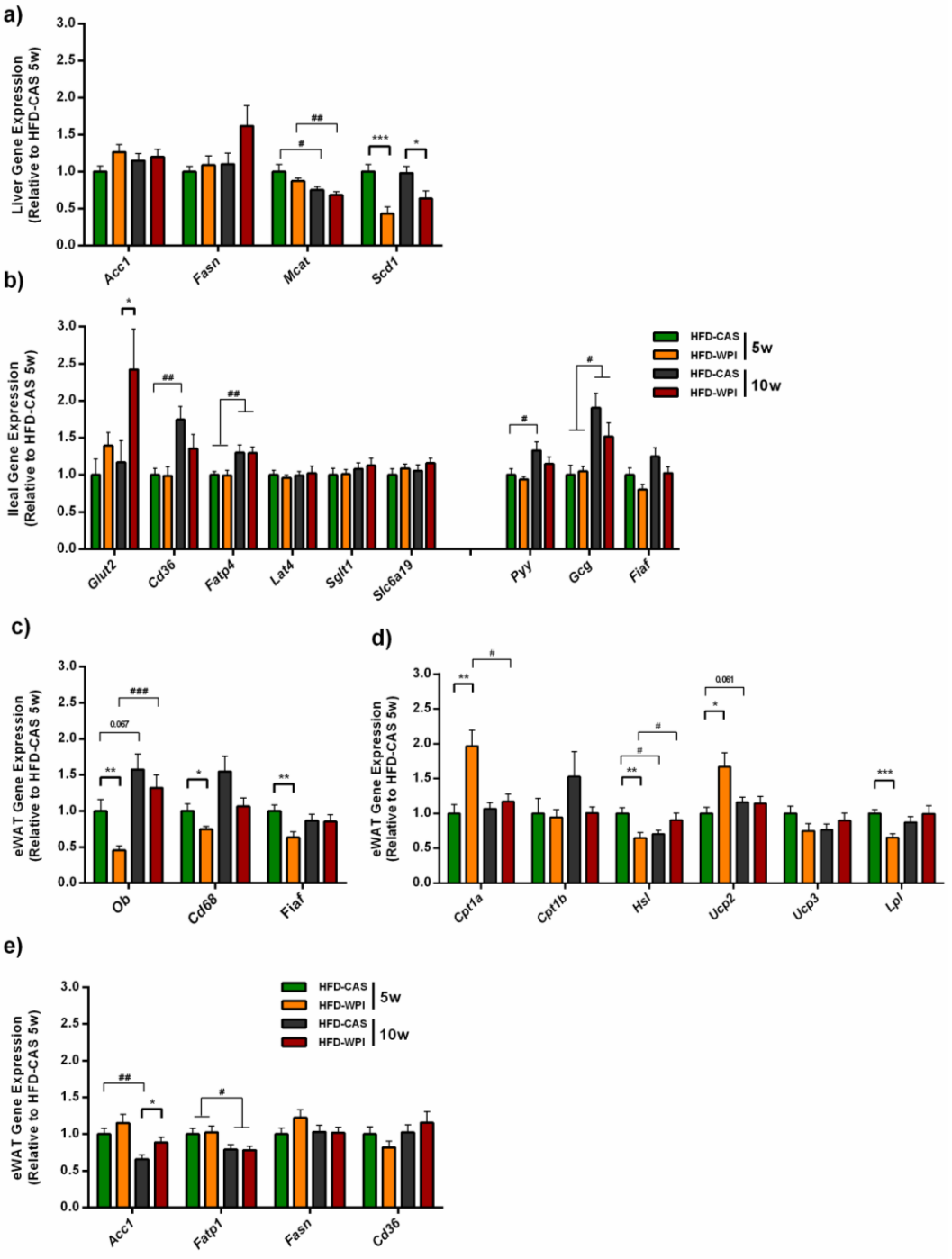


Figure 3.S2 Hepatic, Ileal and eWAT gene expression. Data show **(a)** expression of genes coding for enzymes involved in fatty acids biosynthesis within the liver, **(b)** ileal gene expression of hormones and nutrient transporters, **(c)** expression of genes coding for leptin, inflammation marker CD68 and the FIAF, **(d)** catabolic and **(e)** anabolic enzymes in the eWAT. Abbreviations: ACC1; acetyl-CoA carboxylase 1, FASN; fatty acids synthase; MCAT, malonyl CoA-acyl carrier protein transacylase; SCD1, stearyl-CoA desaturase; other figure descriptions in figures 2 and 3. *Statistical analysis*: groups showing * (for HFD-CAS vs HFD-WPI) and # (HFD-CAS 5w vs HFD-CAS 10w and HFD-WPI 5w vs HFD-WPI 10w). A complete statistical description is detailed in Methods and Materials and in **figure 3.S4**.



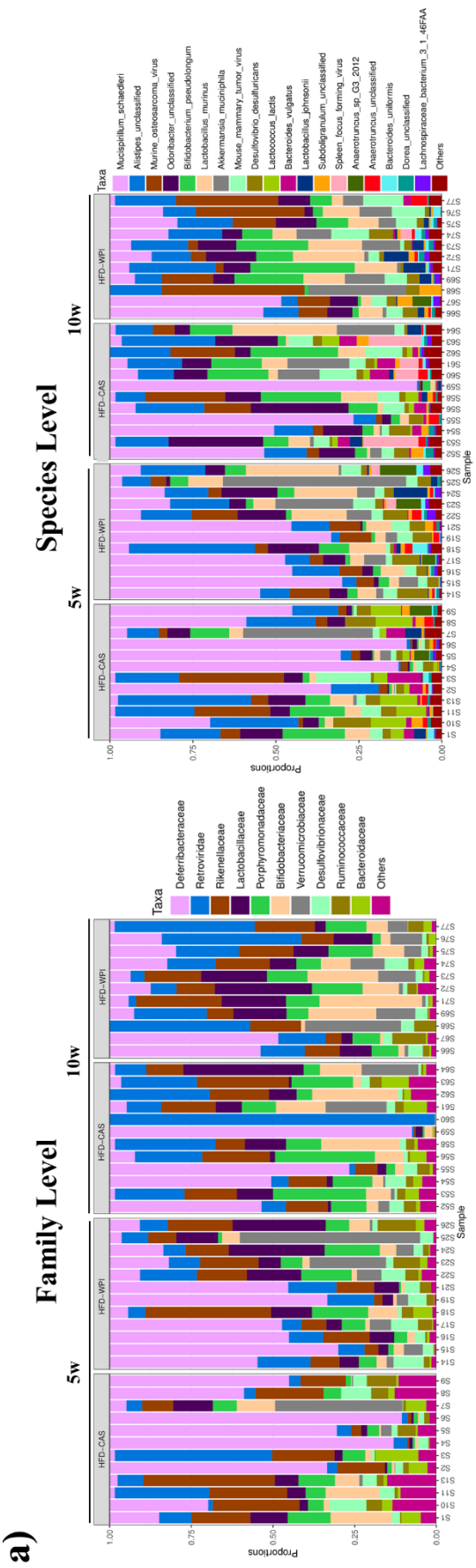
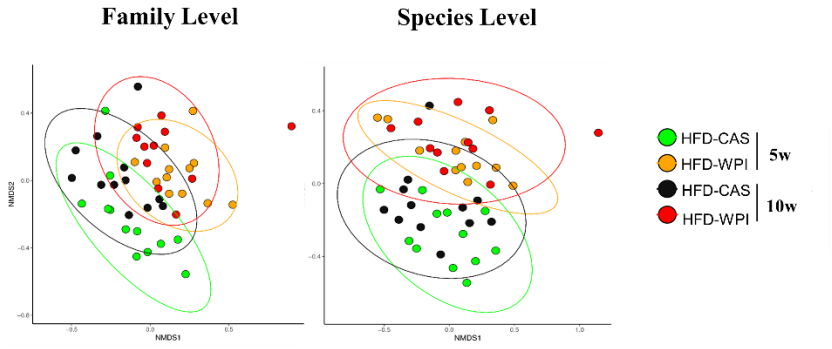
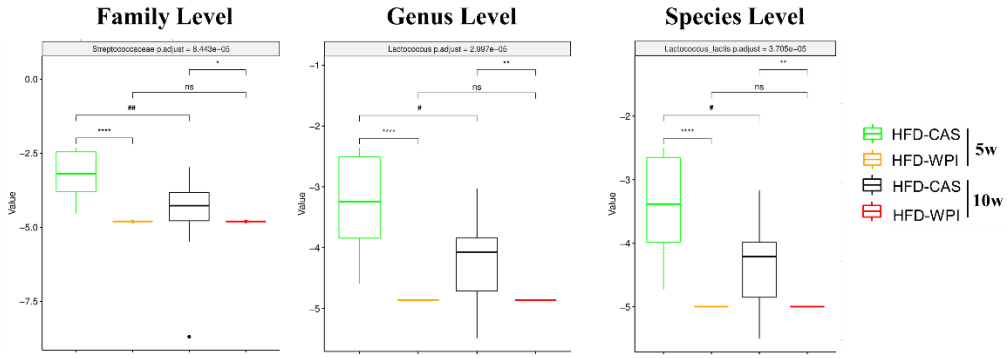


Figure 3.S3 Effect of HFD-WPI in the gut microbiota of 5w vs 10w mice. (a) Taxaplot showing families and species within the faecal samples of 5w and 10w mice fed with HFD-CAS and HFD-WPI. Also reported **(b)** taxonomic beta-diversity, calculated using NMSD ordination, both at family (R^2=0.146, pValue=0.001) and species level (R^2=0.181, pValue=0.001), **(c)** bar charts representing taxonomic differences at family, genus and species level across the HFD groups (5w and 10w), using Kruskal Wallis method. The last graph shows **(d)** functional beta-diversity (R^2=0.174, pValue=0.001) calculated using NMSD ordination, across the HFD groups (5w and 10w). **(e)** Relative abundance (%) of the species *Lactobacillus murinus* across the groups, using shotgun metagenomic analysis approach. *Statistical analysis*: groups showing * (for HFD-CAS vs HFD-WPI) and # (HFD-CAS 5w vs HFD-CAS 10w and HFD-WPI 5w vs HFD-WPI 10w) are significant (*/# P<0.05 or **/## P<0.01 or ***/### P<0.001 or ****/#### p<0.0001). A complete statistical description is detailed in Methods and Materials.

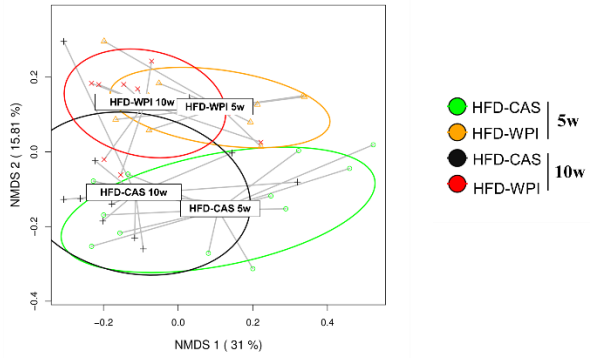
b)



c)



d)



e)

Species	HFD-CAS 5w	HFD-WPI 5w	HFD-CAS 10w	HFD-WPI 10w
Lactobacillus murinus	2,697	6,468	1,744	4,588

Figure 3.S4 Statistical analysis details. Complete statistical description organs and tissues weight data **(a)** in 5w and **(b)(c)** in 10w. Complete statistical description of **(d)(e)** hormones and metabolites data, and gene expression data. Mann Whitney *U* test: 1= HFD-CAS 5w; 2= HFD-WPI 5w; 3 = HFD-CAS 10w, 4 = HFD-WPI 10w.

(a)

Indipendent Sample Test	
Organ/tissue weights (5w)	**(HFD-CAS vs HFD-WPI)**
eWAT	$t_{(30)} = 3.35$, $p < 0.01$
sWAT	$t_{(30)} = 3.3$, $p < 0.01$
BAT	$t_{(30)} = 4.61$, $p < 0.001$
Stomach	$t_{(30)} = -2.24$, $p < 0.05$
Caecum	$t_{(30)} = -0.21$, p=0.84
Small Intestine	$t_{(30)} = -0.15$, p=0.88
Liver	$t_{(30)} = -2.22$, $p < 0.05$
Colon	$t_{(30)} = 1.19$ p=0.24
Small Intestine length	$t_{(30)} = -1.06$, p=0.30
SI weight/length	$t_{(30)} = 0.31$, p=0.76

(b)

ANOVA			
Organ/Tissue (10w)	**Fat content (HFD vs LFD)**	**Protein Type (CAS vs WPI)**	**Interaction**
eWAT	$F_{(1;43)} = 31.29$, $p < 0.001$	$F_{(1;43)} = 0.38$, p=0.54	$F_{(1;43)} = 0.97$, p=0.33
sWAT	$F_{(1;43)} = 18.87$, $p < 0.001$	$F_{(1;43)} = 1.88$, p=0.18	$F_{(1;43)} = 1.64$, p=0.20
Caecum	$F_{(1;43)} = 4.98$, p=0.031	$F_{(1;43)} = 0.69$, p=0.41	$F_{(1;43)} = 2.77$, p=0.10
Small Intestine	$F_{(1;43)} = 0.55$, p=0.46	$F_{(1;43)} = 0.14$, p=0.71	$F_{(1;43)} = 0.03$, p=0.86
Liver	$F_{(1;43)} = 5.87$, p=0.02	$F_{(1;43)} = 2.23$, p=0.14	$F_{(1;43)} = 0.06$, p=0.81
Colon	$F_{(1;43)} = 1.43$, p=0.24	$F_{(1;43)} = 0.34$, p=0.56	$F_{(1;43)} = 2.12$, p=0.15
SI weight/length	$F_{(1;43)} = 0.38$, p=0.54	$F_{(1;43)} = 0.11$, p=0.74	$F_{(1;43)} = 0.51$, p=0.48

(c)

Non parametric test (Kruskal Wallis & Mann Withney)						
Organ/Tissue (10w)	**for the effect of the fat content**	**for the effect of the protein type**	**1 VS 2**	**3 VS 4**	**1 VS 3**	**2 VS 4**
BAT	$\chi^2_{(1)} = 14.14$, $p<0.001$	$\chi^2_{(1)} = 0.25$, p=0.62	NS	NS	$U_{(23)} = 13.5$, p=0.003	$U_{(22)} = 13.5$, p=0.004
Stomach	$\chi^2_{(1)} = 0.03$, p=0.86	$\chi^2_{(1)} = 2.60$, p=0.11	NS	NS	NS	NS

(d)

		ANOVA		
		Fat content (HFD 5w vs HFD 10w)	**Protein Type (CAS vs WPI)**	**Interaction**
Hormones/ Metabolites	**Plasma Glucose**	$F_{(1;60)} = 21.85$, $p < 0.001$	$F_{(1;60)} = 1.06$, p=0.31	$F_{(1;60)} = 2.22$, p=0.14
	Plasma Insulin	$F_{(1;60)} = 9.82$, $p < 0.01$	$F_{(1;60)} = 1.54$, p=0.22	$F_{(1;60)} = 0.79$, p=0.38
	Plasma TAG	$F_{(1;56)} = 8.53$, $p = 0.005$	$F_{(1;56)} = 1.41$, p=0.24	$F_{(1;56)} = 0.46$, p=0.5
	Liver TAG	$F_{(1;56)} = 5.93$, p=0.018	$F_{(1;56)} = 5.75$, p=0.02	$F_{(1;56)} = 9.70$, p=0.003
Ileum	***Cd36***	$F_{(1;56)} = 13.75$, $p < 0.001$	$F_{(1;36)} = 1.81$, p =0.18	$F_{(1;56)} = 1.61$, p =0.21
	Fatp4	$F_{(1;57)} = 15.17$, $p < 0.001$	$F_{(1;57)} = 0.01$, p =0.94	$F_{(1;57)} = 0.01$, p =0.98
	Lat4	$F_{(1;59)} = 0.16$, p =0.69	$F_{(1;59)} = 0.01$, p =0.93	$F_{(1;59)} = 0.24$, p =0.63
	Sglt1	$F_{(1;60)} = 1.39$, p =0.24	$F_{(1;60)} = 0.12$, p =0.73	$F_{(1;60)} = 0.05$, p =0.83
	Pyy	$F_{(1;58)} = 9.18$, $p < 0.01$	$F_{(1;58)} = 1.83$, p =0.18	$F_{(1;58)} = 0.43$, p =0.52
	Fiaf	$F_{(1;60)} = 6.16$, $p < 0.05$	$F_{(1;60)} = 4.86$, $p < 0.05$	$F_{(1;60)} = 0.02$, p =0.90
eWAT	***Fiaf***	$F_{(1;60)} = 0.24$, p =0.623	$F_{(1;60)} = 4.60$, $p < 0.05$	$F_{(1;60)} = 4.12$, $p < 0.05$
	Hsl	$F_{(1;58)} = 0.05$, p =0.82	$F_{(1;58)} = 0.79$, p =0.38	$F_{(1;58)} = 11.05$, $p < 0.01$
	Ucp3	$F_{(1;60)} = 0.17$, p =0.68	$F_{(1;60)} = 0.36$, p =0.55	$F_{(1;60)} = 3.48$, p =0.067
	Fatp1	$F_{(1;59)} = 9.20$, $p < 0.01$	$F_{(1;59)} = 0.01$, p =0.93	$F_{(1;59)} = 0.05$, p =0.82
	Fasn	$F_{(1;58)} = 0.92$, p =0.34	$F_{(1;38)} = 1.31$, p =0.26	$F_{(1;58)} = 1.64$, p =0.21
	Cd36	$F_{(1;58)} = 2.47$, p =0.12	$F_{(1;58)} = 0.04$, p =0.83	$F_{(1;58)} = 1.85$, p =0.18
Liver	***Scd1***	$F_{(1;49)} = 0.97$, p =0.33	$F_{(1;49)} = 22.46$, $p < 0.001$	$F_{(1;49)} = 2.45$, p =0.25

(e)

		Non parametric test (Kruskal Wallis & Mann Withney)					
		for the effect of the fat content	**for the effect of the protein type**	**1 VS 2**	**3 VS 4**	**1 VS 3**	**2 VS 4**
Hormones/ Metabolites	**Plasma Leptin**	$\chi^2_{(1)} = 20.59$, $p<0.001$	$\chi^2_{(1)} = 6.14$, $p<0.05$	$U_{(32)} = 59.00$, p=0.008	$U_{(32)} = 74.00$, p=0.043	$U_{(32)} = 46.00$, p=0.001	$U_{(32)} = 30.00$, $p<0.001$
	Caecum Content TAG	$\chi^2_{(1)} = 3 6.69$, $p<0.001$	$\chi^2_{(1)} = 0.45$, p=0.51	NS	$U_{(29)} = 60.00$, p=0.056	$U_{(28)} = 5.00$, $p<0.001$	$U_{(32)} = 15.00$, $p<0.001$
Ileum	***Glut2***	$\chi^2_{(1)} = 0.46$, p=0.49	$\chi^2_{(1)} = 6.318$, $p<0.05$	NS	$U_{(30)} = 64.00$, p=0.045	NS	NS
	Slc6a19	$\chi^2_{(1)} = 0.85$, p=0.36	$\chi^2_{(1)} = 6.16$, $p<0.05$	NS	$U_{(31)} = 65.00$, p=0.03	NS	NS
	Gcg	$\chi^2_{(1)} = 15.55$, $p<0.001$	$\chi^2_{(1)} = 0.89$, p=0.35	NS	NS	$U_{(30)} = 31.5$, $p<0.001$	$U_{(30)} = 68.00$, p=0.041
eWAT	***Ob***	$\chi^2_{(1)} = 14.24$, $p<0.001$	$\chi^2_{(1)} = 4.25$, $p<0.05$	$U_{(32)} = 53.00$, p=0.004	NS	$U_{(32)} = 79.00$, p=0.067	$U_{(32)} = 33.00$, $p<0.001$
	Cd68	$\chi^2_{(1)} = 5.92$, $p<0.05$	$\chi^2_{(1)} = 5.054$, $p<0.05$	$U_{(31)} = 70.00$, p=0.049	NS	NS	NS
	Cpt1a	$\chi^2_{(1)} = 0.67$, p=0.41	$\chi^2_{(1)} = 8.92$, $p<0.01$	$U_{(31)} = 38.00$, p=0.001	NS	NS	$U_{(32)} = 68.00$, p=0.023
	Cpt1b	$\chi^2_{(1)} = 2.28$, p=0.13	$\chi^2_{(1)} = 0.24$, p=0.62	NS	NS	NS	NS
	Ucp2	$\chi^2_{(1)} = 0.01$, p=0.91	$\chi^2_{(1)} = 2.77$, p=0.056	$U_{(31)} = 63.50$, p=0.024	NS	$U_{(31)} = 72.50$, p=0.060	NS
	Acc1	$\chi^2_{(1)} = 9.61$, $p<0.01$	$\chi^2_{(1)} = 3.39$, p=0.07	NS	$U_{(32)} = 69.00$, p=0.026	$U_{(32)} = 47.00$, p=0.002	NS
	Lpl	$\chi^2_{(1)} = 1.05$, p=0.31	$\chi^2_{(1)} = 1.68$, p=0.19	$U_{(29)} = 26.00$, $p<0.001$	NS	NS	NS
Liver	***Acc1***	$\chi^2_{(1)} = 0.22$, p=0.65	$\chi^2_{(1)} = 2.06$, p=0.15	NS	NS	NS	NS
	Fasn	$\chi^2_{(1)} = 0.85$, p=0.36	$\chi^2_{(1)} = 0.83$, p=0.36	NS	NS	NS	NS
	Mcat	$\chi^2_{(1)} = 12.29$, $p<0.001$	$\chi^2_{(1)} = 0.042$, p=0.36	NS	NS	$U_{(31)} = 64.50$, p=0.027	$U_{(31)} = 48.50$, p=0.004

Figure 3.S5 Hormones and metabolites statistics, and tissue/organs weight ANCOVA. Complete statistical description of **(a)** hormones and metabolites data in LFD-fed mice and organs and tissues weight ANCOVA **(c)** in 5w and **(d)** in 10w.

(a)

Indipendent Sample Test	
Hormones/ Metabolites	(LFD-CAS vs LFD-WPI)
Plasma Glucose	$t_{(13)} = 7.47$, $p < 0.001$
Plasma TAG	$t_{(13)} = 1.84$, $p = 0.09$
Plasma Insulin	$t_{(13)} = 1.88$, $p = 0.08$
Plasma Leptin	$t_{(13)} = 1.49$, $p = 0.16$
Liver TAG	$t_{(12)} = -1.96$, $p = 0.08$
Caecum TAG	$t_{(13)} = -1.74$, $p = 0.11$

(b)

	ANCOVA	
	Organ/Tissue (5w)	Protein Type (CAS vs WPI)
Covariant: Body Weight	eWAT	$F_{(1;29)} = 6.61$, $p=0.016$
	sWAT	$F_{(1;29)} = 5.57$, $p=0.025$
	BAT	$F_{(1;29)} = 14.10$, $p=0.001$
	Stomach	$F_{(1;29)} = 6.53$, $p=0.016$
	Caecum	$F_{(1;29)} = 0.18$, $p=0.67$
	Small Intestine	$F_{(1;29)} = 1.32$, $p=0.26$
	Liver	$F_{(1;29)} = 14.87$, $p=0.001$
	Colon	$F_{(1;29)} = 0.36$, $p=0.55$

(c)

	ANCOVA			
	Organ/Tissue (10w)	Fat Content (HFD vs LFD)	Protein Type (CAS vs WPI)	Interaction
Covariant: Body Weight	eWAT	$F_{(1;42)} = 19.35$, $p<0.001$	$F_{(1;42)} = 1.18$, $p=0.28$	$F_{(1;42)} = 0.58$, $p=0.45$
	sWAT	$F_{(1;42)} = 2.78$, $p=0.10$	$F_{(1;42)} = 5.52$, $p=0.024$	$F_{(1;42)} = 1.56$, $p=0.22$
	BAT	$F_{(1;41)} = 5.72$, $p=0.021$	$F_{(1;41)} = 0.24$, $p=0.63$	$F_{(1;41)} = 1.57$, $p=0.22$
	Stomach	$F_{(1;41)} = 0.46$, $p=0.50$	$F_{(1;41)} = 4.85$, $p=0.033$	$F_{(1;41)} = 2.54$, $p=0.12$
	Caecum	$F_{(1;42)} = 3.84$, $p=0.057$	$F_{(1;42)} = 0.67$, $p=0.42$	$F_{(1;42)} = 2.71$, $p=0.11$
	Small Intestine	$F_{(1;42)} = 4.19$, $p=0.047$	$F_{(1;42)} = 0.09$, $p=0.76$	$F_{(1;42)} = 0.16$, $p=0.69$
	Liver	$F_{(1;42)} = 0.33$, $p=0.57$	$F_{(1;42)} = 5.57$, $p=0.023$	$F_{(1;42)} = 1.22$, $p=0.28$
	Colon	$F_{(1;42)} = 1.36$, $p=0.25$	$F_{(1;42)} = 0.32$, $p=0.57$	$F_{(1;42)} = 2.15$, $p=0.15$

Supplemental Statistics

The outliers were calculated and excluded performing Grubbs' test (significance level = 0.05).

Repeated Measurement

Body weight in experiment 5w (0-5 weeks): $F_{(5;215)} = 436.71$, $p < 0.001$ for the effect of the time, $F_{(1;43)} = 6.14$, $p < 0.05$ for the effect of the diet fat content, $F_{(1;43)} = 2.38$, $p > 0.05$ for the effect of the protein type, $F_{(5;215)} = 21.43$, $p < 0.001$ for the effect of the time x diet fat content interaction, $F_{(5;215)} = 2.54$, $p > 0.05$ for the effect of the time x diet protein type interaction, $F_{(5;215)} = 3.12$, $p < 0.05$ for the effect of the time x diet fat content x diet protein type interaction.

Body weight in experiment 10w (0-10 weeks): $F_{(10;430)} = 339.36$, $p < 0.001$ for the effect of the time, $F_{(1;43)} = 3.21$, $p > 0.05$ for the effect of the diet fat content, $F_{(1;43)} = 0.15$, $p > 0.05$ for the effect of the protein type, $F_{(10;430)} = 30.83$, $p < 0.001$ for the effect of the time x diet fat content interaction, $F_{(10;430)} = 0.53$, $p > 0.05$ for the effect of the time x diet protein type interaction, $F_{(10;430)} = 3.11$, $0.07 < p > 0.05$ for the effect of the time x diet fat content x diet protein type interaction.

Cumulative Weight Gain experiment 5w (0-5 weeks): $F_{(4;172)} = 340.66$, $p < 0.001$ for the effect of the time, $F_{(1;43)} = 33.29$, $p < 0.001$ for the effect of the diet fat content, $F_{(1;43)} = 4.13$, $p < 0.05$ for the effect of the protein type, $F_{(4;172)} = 12.55$, $p < 0.001$ for the effect of the time x diet fat content interaction, $F_{(4;172)} = 1.36$, $p > 0.05$ for the effect of the time x diet protein type interaction, $F_{(4;172)} = 2.55$ $p > 0.05$ for the effect of the time x diet fat content x diet protein type interaction.

Cumulative Weight Gain experiment 10w (0-10 weeks): $F_{(9;396)} = 285.36$, $p < 0.001$ for the effect of the time, $F_{(1;44)} = 16.91$, $p < 0.001$ for the effect of the diet fat content, $F_{(1;44)} = 0.026$, $p > 0.05$ for the effect of the protein type, $F_{(9;396)} = 37.85$, $p < 0.001$ for the effect of the time x diet fat content interaction, $F_{(9;396)} = 0.66$, $p > 0.05$ for the effect of the time x diet protein type interaction, $F_{(9;396)} = 4.61$ $p < 0.05$ for the effect of the time x diet fat content x diet protein type interaction.

Cumulative Energy Intake/mouse experiment 5w (0-5 weeks): $F_{(9;72)} = 6615.09$, $p < 0.001$ for the effect of the time, $F_{(1;8)} = 8.09$, $p < 0.05$ for the effect of the protein type, $F_{(9;72)} = 20.71$, $p < 0.01$ for the effect of the time x diet protein type interaction.

Cumulative Energy Intake/mouse experiment 10w (0-10 weeks): $F_{(4;32)} = 3575.00$, $p < 0.001$ for the effect of the time, $F_{(1;8)} = 11.36$, $p < 0.05$ for the effect of the protein type, $F_{(4;32)} = 8.70$, $p < 0.05$ for the effect of the time x diet protein type interaction.

Chapter 4

Depletion of the Gut Microbiota Differentially Affects the Impact of Whey Protein on High-fat Diet-induced Obesity and Intestinal Permeability

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Abstract

Rationale Whey protein isolate (WPI) is considered a dietary solution to obesity. However, the exact mechanism of WPI action is still poorly understood but is probably connected to its beneficial effect on energy balance, adiposity and metabolism. More recently its ability to modulate the gut microbiota has received increasing attention.

Objectives Here, we used a microbiota depletion, by antibiotic cocktail (ABX) administration, to investigate if the gut microbiota mediates the physiological and metabolic changes observed during high-fat diet (HFD)-WPI consumption. To facilitate this, C57BL/6 mice received a HFD containing WPI (HFD-WPI) or the control non-whey milk protein casein (HFD-CAS) for 5 weeks or 10 weeks.

Results HFD-fed mice supplemented with WPI showed reduced body weight gain, adiposity, *Ob* gene expression level in the epididymal adipose tissue (eWAT) and plasma leptin relative to HFD-CAS-fed mice, after 5- or 10-weeks intervention both with or without ABX treatment. Following 10-weeks intervention, ABX and WPI had an additive effect in lowering adiposity and leptin availability. HFD-WPI-fed mice showed a decrease in the expression of genes encoding pro-inflammatory markers (MCP-1, TNFα and CD68) within the ileum and eWAT, compared to HFD-CAS-fed mice, which did not show alterations following microbiota depletion. Additionally, WPI supplementation decreased HFD-induced intestinal permeability disruption in the distal ileum; an effect that was reversed by chronic ABX treatment.

Conclusions WPI reverses the effects of HFD on metabolic and physiological functions through mainly microbiota-independent mechanisms. In addition, we demonstrate a protective effect of WPI on HFD-induced inflammation and ileal permeability disruption, with the latter being reversed by gut microbiota depletion.

Introduction

Obesity is characterized by a long-term disruption of energy balance, which results in an excessive accumulation of fat mass. There are several health issues that arise in obesity, such as cardiovascular diseases, metabolic and endocrine dysfunction of adipose tissue, alterations of gastrointestinal physiology, dysregulation of hypothalamic satiety-related circuits and a state of chronic low-grade inflammation that can lead to insulin resistance (Cercato & Fonseca 2019, Gregor & Hotamisligil 2011, Longo et al 2019, Teixeira et al 2012, Timper & Brüning 2017). In addition, the diversity, composition and function of the gut microbiota is altered in obese subjects, as well as the abundance of several metabolites produced by the intestinal microbial population, which are important for maintaining good health of the host (Canfora et al 2019a, Sun et al 2018). Specifically, microbial metabolites can communicate with different organs, such as adipose tissue, liver and brain, regulating important metabolic and behavioural functions (Torres-Fuentes et al 2017, Wang et al 2018a). Moreover, HFD-fed mice are characterized by increased intestinal permeability, followed by an increase in plasma endotoxemia (i.e., increase in plasma levels of lipopolysaccharide, LPS) and expression of pro-inflammatory cytokines (Araújo et al 2017, Cani et al 2007). This causes an exacerbation of the pathogenesis of HFD-induced obesity.
The obesity problem is continuously growing in our modern society; thus, a considerable scientific effort is required to find strategies to prevent/ameliorate/treat this condition. Currently, pharmacotherapy, surgery and dietary therapies are the most widely used approaches (Ruban et al 2019).

Bovine whey protein has recently been identified as a dietary candidate for the improvement of obesity-related physiological changes, because of its distinct properties. This protein is present in the liquified compartment (i.e., whey) that can be separated after casein curd formation during cheese production. Whey protein contains different protein components, such as β-lactoglobulin, α-lactalbumin, bovine serum albumin, lactoferrin, lactoperoxidase and glycomacropeptide (Morr & Ha 1993). In several human and rodent studies, whey protein has been shown to attenuate appetite as well as modulate insulin and some gastrointestinal hormones production, such as

cholecystokinin (CCK), peptide YY (PYY) and ghrelin, compared to casein (Hall et al 2003, McAllan et al 2015, Pal & Ellis 2010a, Veldhorst et al 2009a). These findings were observed in lean individuals and mice fed with a low-fat diet (LFD). In the presence of a high-fat diet (HFD), the effect of whey protein on satiety seems to be less evident. However, compared to casein, whey protein supplementation results in a decrease in body weight gain, adiposity and leptin levels, together with an increase in fatty acid β-oxidation and glucose tolerance (Bergia et al 2018, Boscaini et al 2020, McAllan et al 2014, Pilvi et al 2007, Pilvi et al 2008, Shertzer et al 2011) (see Chapter 3).

In rodents and *in vitro*, we and others have shown that whey protein isolate (WPI) significantly alters the composition of the gut microbiota (Boscaini et al 2020, McAllan et al 2014, Sanchez-Moya et al 2017, Sprong et al 2010) (see Chapter 3). In the presence of HFD, these effects are accompanied by a decrease in body weight and adiposity, leptin availability and a change in the expression of genes involved in adipose tissue metabolism (Boscaini et al 2020) (see Chapter 3). These results suggest a possible link between the gut microbiota and metabolic and physiological modifications.

Moreover, it was shown that the milk protein CAS ameliorated intestinal barrier function on diabetes-prone rats and bovine milk has been associated with a decreased gut permeability and improved inflammation and microbial dysbiosis in HFD-induced obese mice (Boudry et al 2017, Visser et al 2010). However, the effect of WPI on gut permeability during HFD-induced obesity is currently not fully understood.

In light of these findings, we hypothesized that, in the presence of HFD, WPI may exert beneficial effects on body weight gain, adiposity and metabolic-related changes through the modulation of the gut microbiota. To test this, we depleted the gut microbiota using an antibiotic cocktail (ABX) and examined the impact on measures of body weight, immunometabolism, gut permeability, gut microbiota composition and caecal metabolomics.

Methods

Experimental Design

Seventy-four three-week-old C57BL/6J male specific pathogen free mice were purchased commercially (Envigo; UK) and were housed 3 per cage on a 12 h light/dark cycle with humidity maintained at 45-60% and temperature between 19-21°C. Mice had *ad libitum* access to food and water throughout the study. During the initial 2 weeks of acclimatization, mice were provided with a diet containing 10% (low) fat and 20% casein (LFD-CAS; #D12450Bi) (all % values by energy). Then, weight-matched cages were assigned to one of the 8 experimental groups (**Fig. 4.1**). Because of the importance of the timing on the ability of WPI to exert its anti-obesity effects (Boscaini et al 2020) (see Chapter 3), we sought to compare some of the readouts during different intervention windows. Mice were provided with a 45% high-fat diet containing 20% casein (HFD-CAS; #D12451i) and with a 45% high-fat diet containing 20% whey protein isolate (HFD-WPI; #D11040501) for 5 weeks (short-term intervention) or 10 weeks (long-term intervention). The diets were OpenSource and were made by Research Diets (diets composition details, **Table 4.T1**). Since the start of the dietary interventions, groups 2, 4, 6 and 8 were given a cocktail of the following antibiotics (ABX) in drinking water: ampicillin (1 g/L), neomycin (0.5 g/L) and vancomycin (0.35 g/L) (Cani et al 2008, Rabot et al 2016, Thackray et al 2018). Mice were carefully monitored for signs of dehydration upon ABX administration and drinking water consumption was recorded every 2-3 days (data not shown). All ABX were obtained from Discovery Fine Chemicals and given for 5 or 10 weeks, as indicated in **figure 4.1**.

The *in vivo* experiments were approved by the University College Cork Animal Experimentation Ethics Committee (2015/007) and were licensed under the European Directive 2010/63/EU.

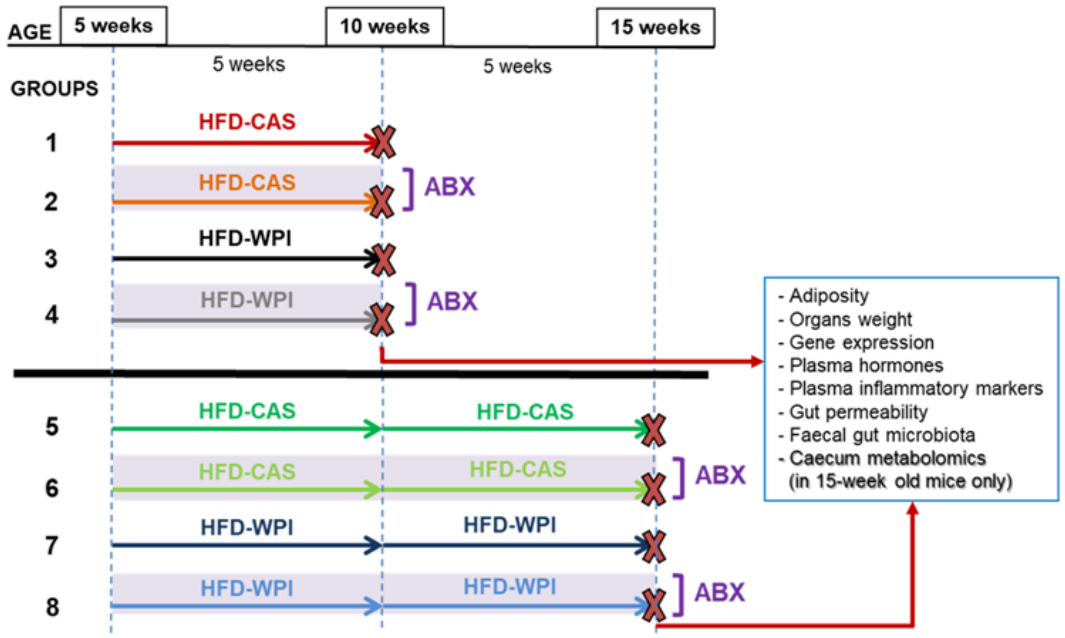


Figure 4.1 Experimental design. 5-week-old mice were fed a high-fat diet with control casein (HFD-CAS; 45% fat and 20% casein) or whey protein isolate (HFD-WPI; 45% fat and 20% whey protein isolate) for 5 (until they are 10-week old; groups 1-4) or 10 weeks (until they are 15-week old, groups 5-8). Groups 2, 4, 6 and 8 were provided with an antibiotic cocktail (ABX; ampicillin (1 g/L), neomycin (0.5 g/L) and vancomycin (0.35 g/L)) in drinking water for 5 or 10 weeks. The other groups (i.e., 1, 3, 5 and 7), which did not undergo ABX treatment, were considered as controls. At the end of the experiment, both at 5 and 10 weeks timepoints, several measurements were carried out. Number of mice: group 1=9, group 2=9, group 3=9, group 4=9, group 5=9, group 6=12, group 7=8, group 8=9.

	LFD-CAS		HFD-CAS		HFD-WPI	
Ingredients	**gm**	**Kcal**	**gm**	**Kcal**	**gm**	**Kcal**
Casein	200	800	200	800	0	0
Whey Protein	0	0	0	0	200	800
L-Cysteine	3	12	3	12	3	12
Corn Starch	315	1260	72,8	291	72,8	291
Maltodextrin 10	35	140	100	400	100	400
Sucrose	350	1400	172,8	691	172,8	691
Cellulose, BW200	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225
*Lard**	20	180	177,5	1598	177,5	1598
Mineral Mix S10026	10	0	10	0	10	0
DiCalcium Phosphate	13	0	13	0	13	0
Calcium Carbonate	5,5	0	5,5	0	5,5	0
Potassium Citrate, 1 H2O	16,5	0	16,5	0	16,5	0
Vitamin Mix V10001	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0
Total	1055	4057	858,1	4057	858,1	4057

Table 4.T1 Composition of the experimental diets.

Body weight and food intake measures

Body weight, as well as food intake, were measured weekly. Unexpectedly, the texture of the diets was soft and crumbly, which skewed the energy intake data. In addition, HFD-WPI diet had a softer consistency than HFD-CAS diet. Metabolic cages allowing precise measurement of food consumption were not available for this study. We, thus, decided to omit these data from our results since we could not guarantee data accuracy.

Post-mortem tissue collection

Following 5 (groups 1-4) and 10 weeks (groups 5-8) of dietary/ABX interventions, mice were euthanized for tissue collection. The day before euthanasia, faecal samples were collected and stored at -80˚C. Then, mice were fasted for 12h commencing at 19.00 in the dark phase. Mice were euthanized by decapitation and organs and adipose tissues i.e., epididymal white adipose tissue (eWAT), subcutaneous white adipose tissue (sWAT), brown adipose tissue (BAT), retroperitoneal adipose tissue (rAT) and mesenteric adipose tissue (mAT) were collected, snap frozen in liquid nitrogen or dry ice and stored at -80˚C for subsequent analysis. Cervical blood samples were collected in tubes with EDTA and centrifuged for 10 minutes at 3000 xg; the supernatant (plasma) was stored at -80˚C. The length of the gut (i.e., whole small intestine and colon) and the weight of all tissues/organs collected were recorded on fresh tissues before snap freezing. The weight of adipose tissues and organs are reported as absolute values.

***Ex vivo* intestinal permeability**

Freshly isolated distal ileum and colon segments (length= 1-2 cm) were emptied of luminal contents with PBS and placed in Krebs solution (1.2 mM NaH_2PO_4, 117 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 25mM $NaHCO_3$, 11 mM $CaCl_2$ and 10 mM glucose). The samples were mounted into the Ussing chamber apparatus (Harvard Apparatus, exposed tissue area of 0.12 cm^2) as previously described (Golubeva et al 2017). To assess intestinal epithelial permeability to macromolecules, 4 KDa FITC-dextran (Sigma-Aldrich) was added to the luminal chamber at a final concentration of

2.5 mg/mL; 200-µL samples were collected from the serosal chamber after 1 h and every 30 min for the following 2 h. The volume in the serosal chamber was replenished with fresh Krebs. To avoid the potential impact of Na^+/glucose co-transporter on paracellular permeability in distal ileum samples (Turner 2000), glucose in the luminal chamber was replaced with 10 mM mannitol. FITC absorbance was measured at 485-nm excitation/535-nm emission wavelength through fluorometric analysis using a multi-mode plate reader (Victor 3, Perkin Elmer). FITC flux at each timepoint was then calculated as an increment in fluorescence intensity vs baseline fluorescence in the serosal compartment and presented in ng/mL. Total flux was presented as $\mu g/h/cm^2$.

RNA extraction and gene expression analysis

Total RNA was extracted from distal ileum (using RNeasy Minikit and QIAshredder columns (Qiagen)) and eWAT and hypothalamic tissues (RNeasy Minikit and QIAzol Lysis Reagent (Qiagen)), according to manufacturer's recommendations. Extracted RNA was treated with RNAse-free DNAse (Qiagen) for complete DNA removal and quantified with Nanodrop (ThermoFisher Scientific). Complementary DNA was synthetized from 600 ng total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and subjected to Real-Time PCR (Roche) using SYBR Green Select Master Mix (Roche) as detailed before (McAllan et al 2015). Relative gene expression values were calculated using 2-ΔΔCt equation, normalized against the reference gene *Actb* and presented as a ratio versus pooled average of all the experimental groups. The sequences of the primers are detailed in **Table 4.TS1**.

Leptin, insulin and inflammatory markers levels in plasma

Plasma leptin and insulin were measured using Mouse Metabolic Kit, Multi-Spot Assay System (Meso Scale Discovery) following the instructions provided within the kit. Plasma MCP-1, TNF-α and IL6 were measured using U-PLEX Biomarker Group 1 (ms) Assay, SECTOR Multiplex Assay (Meso Scale Discovery). The plates from both assays were analysed using the MSD Quickplex instrument (Meso Scale Discovery).

Caecum Metabolomics

Caecum metabolomics was performed in experimental groups that underwent 10 weeks of dietary and ABX intervention (35 samples in total). Caecal water was prepared by homogenising caecal content (approx. 100 mg) with 400 µL of sterile water for 5 min using a bead beater. Samples were centrifuged at 16,000 xg for 30 min, after which supernatants were filtered through 0.22 µm column filters (Costar). Two separate mass spectrometry methods were used to measure metabolites in each sample. 1) A broad profiling of caecal extracts was performed using the LC-MS method. The analysis was carried out using a UPLC system (Vanquish, Thermo Fisher Scientific) coupled with a high-resolution quadrupole-orbitrap mass spectrometer (Q Exactive™ HF Hybrid Quadrupole-Orbitrap, Thermo Fisher Scientific). An electrospray ionization interface was used as ionization source. Analysis was performed in negative and positive ionization mode. A QC sample was analysed in MS/MS mode for identification of compounds. The UPLC was performed using a slightly modified version of the protocol described by Catalin et al (https://www.waters.com/waters/library.htm?locale=en_US&lid=134636355). Data were processed using Compound Discoverer 3.0 (ThermoFisher Scientific).
2) Gas chromatography–mass spectrometry (GC-MS) was used to analyse short and long chain fatty acids (SCFA, LCFA) in caecal extracts. Samples were acidified with hydrochloric acid (for SCFA) or derivatized using methyl chloroformate (for LCFA). The raw GC-MS data was processed by software developed by MS-Omics and collaborators (PARAFAC2 model).

Faecal bacteria quantification

Faecal samples were weighted, homogenised and processed using mechanical (TissueLyser, Qiagen) and chemical lysis. Genomic DNA was extracted purified according to the protocol for the standard QIAmp PowerFaecal Pro DNA Kit (Qiagen). Total DNA was quantified with Qubit dsDNA HS Assay kit (Bio-Sciences). Subsequently, 16S rDNA gene of standards and samples was amplified and quantified using Real-Time PCR. In particular, to create the standards, 2µL of extracted bacterial DNA from each HFD-CAS and HFD-WPI non-treated sample that underwent 5-week

intervention were pooled. The pool represented the standard n=1, 100% of bacteria, concentration (1:1). Starting from standard 1, 4 more standards were creating by 1:10 serial dilution in water. The last standard (n=6) contained water only (0% of bacteria) (total number of standards=6).

Standards and DNA samples from all 8 experimental groups were subjected to Real-Time PCR (Roche) using SYBR Green Select Master Mix (Roche) as detailed before (McAllan et al 2015) to amplify the 16S rDNA region (primer forward - 5' ACTCCTACGGGAGGCAGCAGT 3'- and primer reverse - 5' ATTACCGCGGCTGCTGGC 3'-). Ct values for standards and samples were obtained.

A standard curve was generated by plotting the Ct values for the standards versus bacterial % of each standard. A lineal regression of the standard curve was performed to obtain an estimation of 16S rDNA copy numbers of each standard.

16S rDNA copy numbers for faecal samples were calculated by interpolating Ct values in the standard curve and corrected considering the dilution factor of the sample. Then, 16S rDNA copy numbers per mg of faeces (16S copies / mg faeces) were calculated. At the end, the % of 16S copies/mg of faeces were calculated versus pooled average of HFD-CAS copies/mg of faeces. Notably, HFD-CAS 5w contained the highest concentration of 16S copies, thus we considered it as 100%.

DNA extraction, library preparation and 16S metagenomic sequencing

Faecal samples from all experimental groups were homogenised and processed using mechanical (TissueLyser, Qiagen) and chemical lysis. Genomic DNA was extracted purified according to the protocol for the standard QIAmp PowerFaecal Pro DNA Kit (Qiagen). Total DNA was quantified with Qubit dsDNA HS Assay kit (Bio-Sciences). After DNA extraction, the 16S rRNA gene (V3-V4 region) was amplified with universal primers (PCR1 forward and reverse primer as to the Illumina 16S Metagenomic Protocol). Subsequently, amplicons from each sample were pooled in equimolar amounts and sequenced with Illumina Miseq platform (2 × 250 bp pairedend reads; V3 sequencing chemistry; Illumina). Negative controls were used for the Operative Taxonomic Unit (OTUs) table clean up with the objective of removing

contaminant sequences from the samples, as explained below. Sequencing run variable was taken into consideration in the multivariate models explaining alpha- and beta-diversity for controlling possible confounding effect of differential sequencing depth among runs.

Bioinformatics analysis

The Illumina reads were filtered on the basis of quality (removal of low quality nucleotides at the 3' end, and remove the 20 nt windows with a low average quality) and length (removal of sequences with less than 200 pb) with PRINSEQ-Lite v0.20.4 (Schmieder & Edwards 2011), and the paired-end reads with a minimum overlap of 20 bp were joined using Fastq-Join (Aronesty 2013). A second filtering step with PRINSEQ-Lite was performed at this point, ensuring a mean quality score of the reads of Q25 and a length range between 400-540 bp, approximately the length of the 16S rRNA amplicon. In addition, the sequences of dereplicates and unique sequences and chimeras were eliminated using GOLD database (https://gold.jgi.doe.gov) through the closed-reference Usearch v8.0 algorithm (Edgar 2010). The resulting sequences were clustered with 97% identity to obtain OTUs using UPARSE-OTU algorithm with Usearch v8.0 algorithm (Edgar 2010). The taxonomic assignment of these OTUs was obtained against the Ribosomal Database Project (Cole et al 2007). Negative controls were evaluated to identify contaminants. OTUs present both in samples and negative controls were removed.

Statistical Analysis

Data were analysed in SPSS software version 24 (IBM Corp.). All datasets were checked for the normality with Shapiro-Wilk test and homogeneity of variance with Levene's test. Outliers were removed following Grubbs' test (significance level = 0.05).

Changes in body weight gain and FITC permeability overtime were analysed by a two-way repeated-measures ANOVA (diet and ABX as independent factors and time as a repeated-measure factor) followed by Bonferroni's *post hoc* pairwise comparisons at each timepoint. Tissue and organ weights, intestinal length, gene expression, plasma

leptin, insulin, inflammatory markers and lipopolysaccharide binding protein levels, and total FITC flux data were compared with two-way analysis of variance (ANOVA) followed by pairwise comparison using Bonferroni's *post hoc* test. Non-parametric data were compared with Kruskal-Wallis test followed by Mann-Whitney *U* test. Caecum metabolomics pairwise comparisons were performed with independent Student's *t* test and expressed as Log2 ratios. Benjamin Hochberg (BH) procedure with false discovery rate (FDR) set at 0.05 was used to correct p values for multiple comparisons. $p<0.05$ was deemed significant in all cases. Data are expressed as mean + SEM.

A complete description of statistical analysis is detailed in "Supplementary Statistic" and **figures 4.S5**, **4.S6** and **4.S7**.

All statistical analyses of the gut microbiota data were performed with R version 3.6.0. Normality of the data was evaluated with Shapiro-Wilk test. Microbiota and study variables were included in the estimation of alpha-diversity richness (Shannon, Simpson and total Richness indexes) by the Vegan and Phyloseq R packages (McMurdie & Holmes 2013). Therefore, potential differences in richness of factors included in the study were estimated by repeated measures ANOVA. Statistically significant differences in beta-diversity were assessed by PERmutational Multivariate Analyses Of Variance (PERMANOVA) using a Bray-Curtis dissimilarity measure. Specific differences between groups were assessed by *post hoc* comparisons with Adonis pairwise comparisons. Principal Coordinates Analysis (PCoA) plots based on Bray-Curtis dissimilarity measure were used to visualize beta-diversity plot (Bray & Curtis 1957). Differences in taxa abundance for experimental groups were analysed by non-parametric Kruskal-Wallis test and Pairwise Wilcoxon Rank Sum tests for multiple comparisons and Benjamin-Hochberg *P*-value correction with a threshold of 0.05.

Results

HFD-fed mice treated with antibiotics further reduced body weight gain and fat depots upon WPI supplementation

Two experimental cohorts were included in this study: short-term (5 weeks) and long-term (10 weeks) administration of WPI and ABX cocktail in HFD-fed mice.
In both cohorts, the analysis of body weight gain revealed a significant effect of WPI and ABX administration on weight gain in HFD-fed mice (protein type, $F_{(1;32)} = 5.25$, p=0.029; ABX treatment, $F_{(1;32)} = 11.44$, p=0.002 in 5 weeks cohort and protein type, $F_{(1;34)} = 6.44$, p=0.016; ABX treatment, $F_{(1;34)} = 4.54$, p=0.04 in 10 weeks cohort). HFD-WPI, HFD-CAS+ABX and HFD-WPI+ABX mice showed a marked reduction of body weight gain compared to HFD-CAS group. These effects were significant as early as 1 week following the commencement of treatments (**Fig. 4.2a-b**), and the difference between HFD-CAS and other groups gradually increased throughout the intervention (**Fig. 4.2a-b**). Interestingly, in the long-term administration cohort, HFD-WPI+ABX group demonstrated the slowest trajectory of weight gain. At 9 and 10 weeks of intervention, HFD-WPI+ABX had a significantly lower body weight gain compared to HFD-CAS+ABX counterparts (p=0.048 for 9 weeks and p=0.027 for 10 weeks).

In agreement with body weight gain data, WPI and ABX administration substantially decreased the accumulation of adipose tissue in all major fat depots. The absolute weights of eWAT, sWAT, rAT and mAT were significantly lower in HFD-WPI and HFD-CAS+ABX groups compared with HFD-CAS group (**Fig. 4.2c-d).** The only exemption was BAT, which was marginally reduced by ABX in HFD-CAS diet only following 5 weeks of treatment, and by WPI following 10 weeks of intervention (**Fig. 4.2c-d**). In the long-term administration cohort, HFD-WPI+ABX group showed a further reduction in adipose tissue weights compared to HFD-WPI counterparts, demonstrating a robust anti-obesity effect across all fat depots (**Fig. 4.2d**). Furthermore, HFD-WPI+ABX group displayed the lowest fat depots gain, which was significantly lower compared to HFD-CAS+ABX group (**Fig. 4.2d**). These data

suggest an additive effect of WPI and ABX co-administration against adiposity associated with HFD consumption.

With regard to organ metrics, ABX administration for 5 weeks increased caecum weight (both full and empty), and decreased spleen, stomach and liver weights in both CAS and WPI groups. In addition, the presence of WPI in the diet caused an increase in liver weight, as well as in small intestine length (**Fig. 4.S1a** and **4.S1c**).
Following long-term administration of ABX, only caecum weight was significantly increased by ABX treatment with no major changes in other organs. Notably, an increase in caecum weight following administration of ABX is a hallmark of successful microbiota depletion in rodents.
Similar to 5-week intervention cohort, HFD-WPI and HFD-WPI+ABX mice showed an increase in liver weight relative to CAS counterparts (**Fig**. **4.S1b**). No major differences were detected in small intestine and colon lengths (**Fig. 4.1Sd**).

Since we were unable to collect reliable energy intake data, we analysed the expression of genes encoding neuropeptides involved in satiety control in the hypothalamus.
These included anorexigenic peptides (proopiomelanocortin, POMC and cocaine and amphetamine regulated transcript, CARTPT), orexigenic peptide neuropeptide Y (NPY), as well as glucocorticoid receptor and corticotropin-releasing hormone (GR and CRH). Overall, no robust effects in gene expression were observed in either WPI or ABX treated groups in comparison to HFD-CAS-fed mice and the fold change in expression between the groups was small across most of the significant differences detected (**Table 4.TS2**).

In summary, we have shown that both a WPI consumption and microbiota depletion with ABX equally ameliorate HFD-induced weight gain and adiposity during either 5- or 10-week intervention. Following long-term 10-week administration, ABX and WPI presence showed an additive effect against adiposity. We next looked at a selected range of pathophysiological markers associated with obesity in order to understand the putative pathways affected by WPI and ABX.

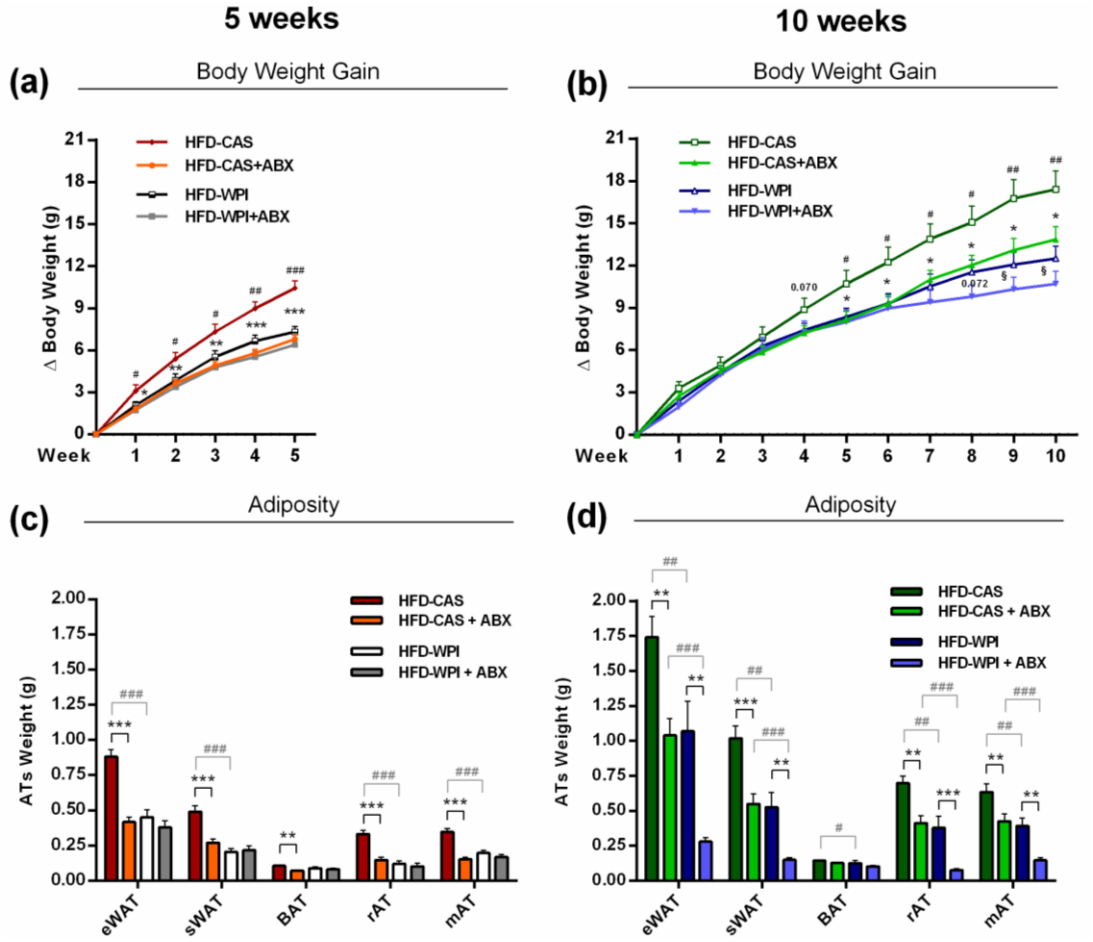


Figure 4.2 Body weight gain and adiposity. The present data show body weight gain **(a)** at 5 and **(b)** 10 weeks timepoints and different kinds of absolute adipose tissue weight **(c)** at 5 and **(d)** 10 weeks timepoints of mice fed with HFD-CAS and HFD-WPI, both controls and ABX-treated. Abbreviation; eWAT; epididymal white adipose tissue, sWAT; subcutaneous white adipose tissue, BAT; brown adipose tissue, rAT; retroperitoneal adipose tissue, mAT; mesenteric adipose tissue. *Statistical analysis*: **(a, b)** groups showing * (for HFD-CAS vs HFD-CAS+ABX), # (HFD-CAS vs HFD-WPI) and § (for HFD-CAS+ABX vs HFD-WPI+ABX) are significant. **(c, d)** groups showing * (for HFD-CAS vs HFD-CAS+ABX and HFD-WPI vs HFD-WPI+ABX) and # (for HFD-CAS vs HFD-WPI and HFD-CAS+ABX vs HFD-WPI+ABX) are significant (*/#/§ $P<0.05$ or **/##/§§ $P<0.01$ or ***/###/§§§ $P<0.001$). A complete statistical description is detailed in Methods and Materials, "Supplementary Statistics" and **figures 4.S5** and **4.S7**.

HFD-fed mice treated with antibiotics further reduced plasma leptin availability production upon WPI supplementation

It is well known that HFD-induced obesity leads to a dysregulation in the production of energy balance- and metabolism-related hormones (Sidhu et al 2000, Ye 2013). In this context, we first measured plasma levels and eWAT gene expression of leptin. Leptin plasma levels agreed with *Ob* (i.e., gene that encodes for leptin) gene expression, and both were matching body weight and adiposity data. At 5 weeks, leptin gene expression and plasma levels were significantly lower in HFD-WPI compared to the HFD-CAS group, with no differences between HFD-WPI and the ABX-treated groups (**Fig. 4.3a** and **4.3e**).

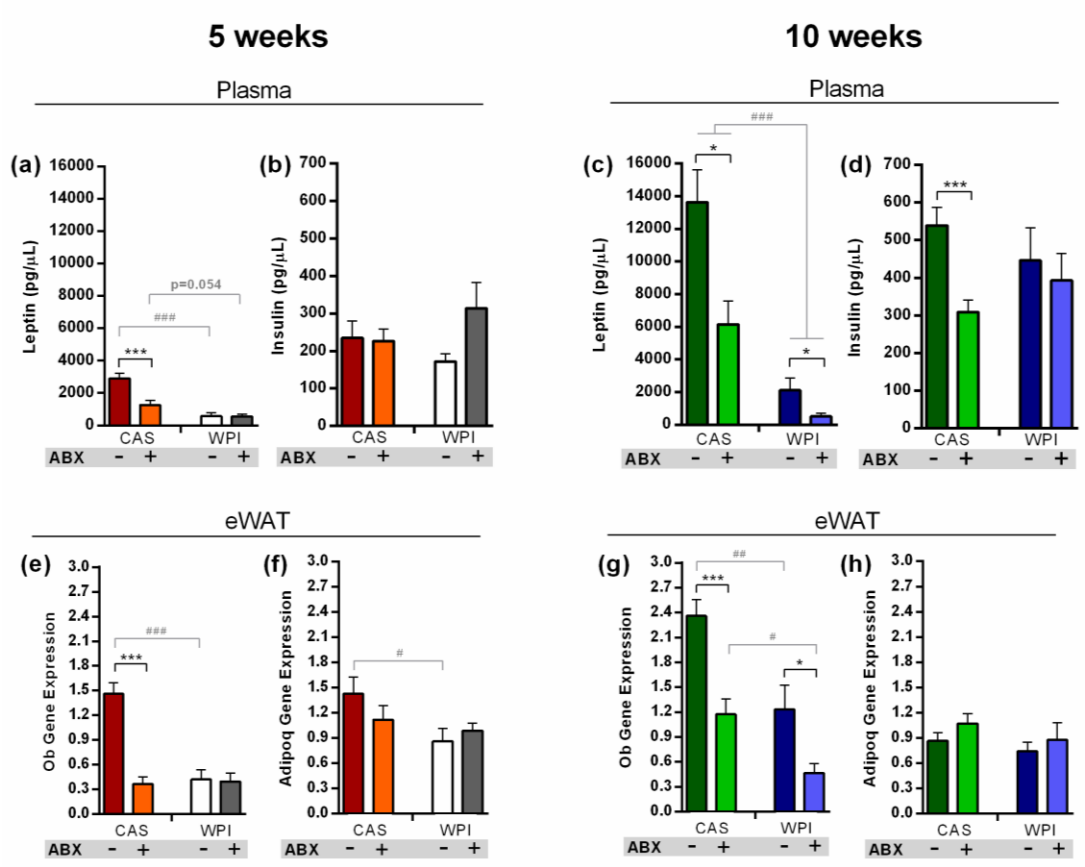


Figure 4.3 Adipokines, insulin and leptin. Data show plasma levels of **(a)** leptin and **(b)** insulin at 5 weeks timepoint and plasma levels of **(c)** leptin and **(d)** insulin at 10 weeks timepoint. Data also show **(e)** Ob and **(f)** Adipoq expression within the eWAT at 5 weeks timepoint and **(g)** Ob and **(h)** Adipoq expression within the eWAT at 10 weeks timepoint. All the data were measured in mice fed with HFD-CAS and HFD-WPI, both controls and ABX-treated. Abbreviation; eWAT; epididymal white adipose tissue, Ob; leptin, Adipoq; adiponectin. Groups showing * (for HFD-CAS vs HFD-CAS+ABX and HFD-WPI vs HFD-

WPI+ABX) and # (for HFD-CAS vs HFD-WPI and HFD-CAS+ABX vs HFD-WPI+ABX) are significant (*/# P<0.05 or **/## P<0.01 or ***/### P<0.001). A complete statistical description is detailed in Methods and Materials and **figures 4.S6** and **4.S7**.

Similarly, 10 weeks, both HFD-WPI and HFD-CAS+ABX groups showed lower leptin gene expression and plasma levels compared to the HFD-CAS group. In addition, leptin levels in the HFD-WPI+ABX group showed a further significant reduction compared to HFD-WPI and HFD-CAS+ABX groups (**Fig. 4.3c** and **4.3g**). This suggests an additive effect of ABX and WPI towards reducing leptin levels in HFD-associated obesity.

In obesity, while leptin production increases, the level of adiponectin decreases. The leptin/adiponectin ratio is considered as a biomarker of WAT dysfunction (Frühbeck et al 2019). Therefore, we assessed the encoding *Adipoq* gene expression levels in the eWAT. Apart from a reduction in the HFD-WPI group compared to HFD-CAS group at 5 weeks, no changes in *Adipoq* expression was observed across the groups (Fig. **4.3f** and **4.3h**).

To further investigate the impact of WPI and gut microbiota depletion on leptin signalling, we measured the expression of a gene that encodes for hypothalamic suppressor of cytokine signalling 3 (i.e., *Socs3*). Socs3 is a protein involved in leptin signalling within the hypothalamus and is overexpressed under HFD feeding. It acts by binding leptin receptor and inhibiting downstream the signalling cascade. Rodents with *Socs3* deletion in proopiomelanocortin (POMC) neurons are resistant to diet-induced obesity and have lower levels of circulating leptin (Bian et al 2013, McEwen et al 2016, Mori et al 2004). On the other hand, Socs3 upregulation promotes hypothalamic leptin resistance (Reed et al 2010). Here we observed that ABX-treated groups, both at 5 and at 10 weeks, had a lower hypothalamic *Socs3* expression relative to their non-treated counterparts (**Table 4.TS2**). This is a first indication that, in the presence of HFD, gut microbiota depletion, and not protein quality within the diet, may have a stronger impact on leptin signalling in the brain.

Plasma levels of fasting insulin were similar across all groups at both timepoints, except in HFD-CAS+ABX group at 10 weeks, which showed a lower insulin level compared to HFD-CAS group (**Fig. 4.3b** and **4.3d**).

In summary, we have shown that WPI and ABX administration had both ameliorated an increase in leptin production induced by HFD consumption, without impacting on insulin and adiponectin levels. Similar to body weight and adiposity results, ABX and WPI showed an additive effect towards reducing leptin production in 10 week-administration cohort. In addition, gut microbiota depletion, and not WPI supplementation, seems to have a stronger impact on leptin signalling responsiveness within the hypothalamus.

Whey protein and antibiotics administration decreased the gene expression levels of pro-inflammatory cytokines

Obesity is also characterized by chronic low-grade inflammation, which, in turn, exacerbates the metabolic syndrome dysfunctions (Ellulu et al 2017). Here we measured the plasma levels of selected pro-inflammatory markers along with their gene expression levels in different tissues.

At 5 weeks, plasma levels of monocyte chemoattractant protein 1 (MCP-1) did not show major differences across groups, except a decrease in the HFD-WPI+ABX group compared to HFD-CAS+ABX group (**Fig. 4.4a**). Plasma levels of tumour necrosis factor α (TNFα) was lower both in HFD-CAS+ABX group and HFD-WPI group relative to HFD-CAS mice (**Fig. 4.4b**).

At 10 weeks, plasma MCP-1 was substantially decreased in HFD-WPI group compared to HFD-CAS group; intriguingly, the effect of WPI was no longer seen under ABX co-administration (**Fig. 4.4c**). No differences in plasma TNFα were detected across the groups (**Fig. 4.4d**).

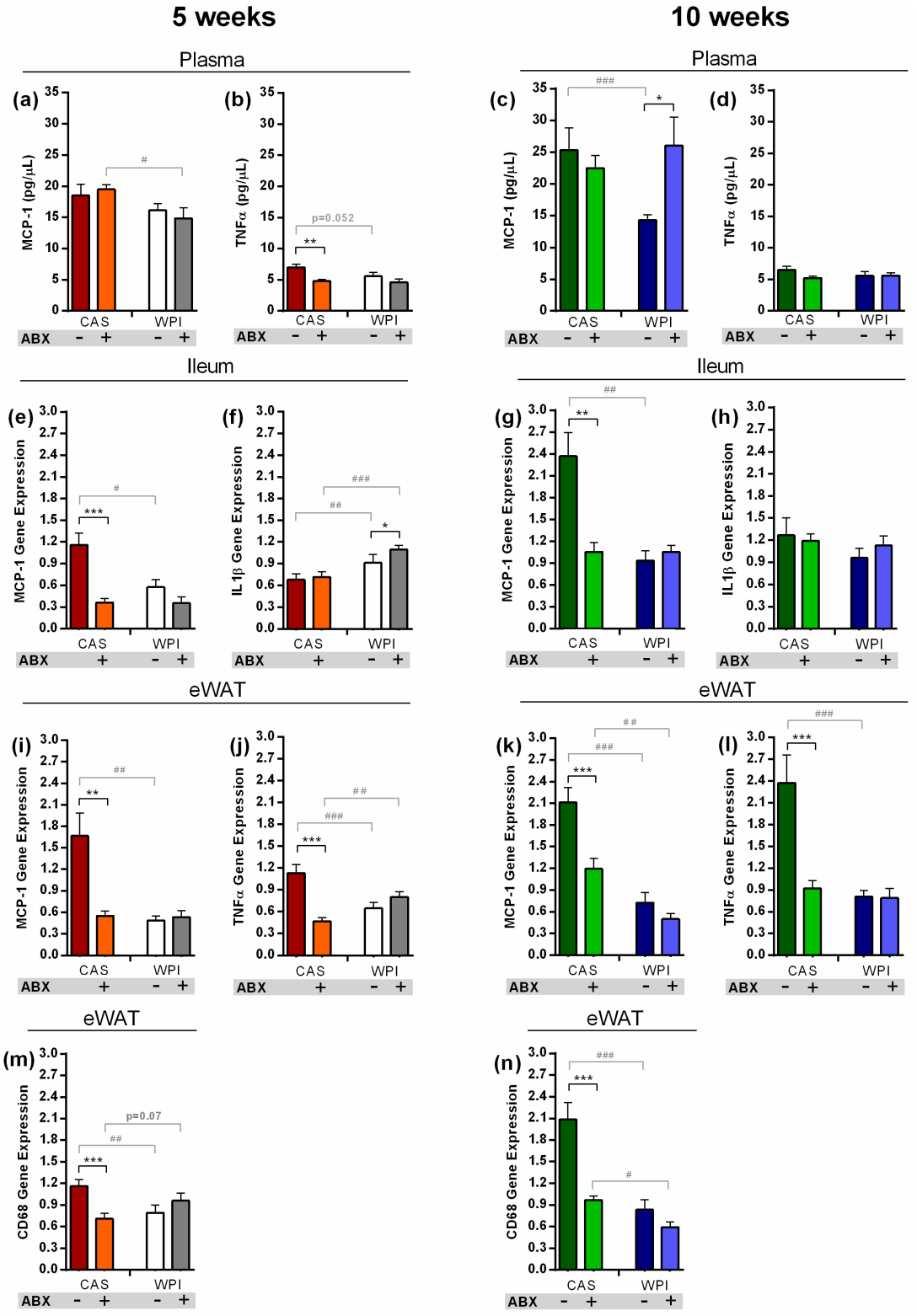


Figure 4.4 Pro-inflammatory cytokines levels. Data show plasma levels of **(a)** MCP1 and **(b)** TNFα at 5 weeks timepoint and plasma levels of **(c)** MCP1 and **(d)** TNFα at 10 weeks timepoint. Data also show **(e)** Mcp1 and **(f)** Il1β ileal expression at 5 weeks timepoint and **(g)** Mcp1 and **(h)** Il1β ileal expression at 10 weeks timepoint. In addition, it is reported **(i)** Mcp1 and **(j)** Tnfα expression within the eWAT at 5 weeks timepoint and **(k)** Mcp1 and **(l)** Tnfα

expression within the eWAT at 10 weeks timepoint. The final graph shows the expression of Cd68 within the eWAT at (m) 5 and (n) 10 weeks timepoint. All the data were measured in mice fed with HFD-CAS and HFD-WPI, both controls and ABX-treated. Abbreviation; eWAT; epididymal white adipose tissue, MCP1; monocyte chemoattractant protein 1, TNFα; tumor necrosis factor alpha, IL1β; interleukin 1 beta, CD68; cluster of differentiation 68. Groups showing * (for HFD-CAS vs HFD-CAS+ABX and HFD-WPI vs HFD-WPI+ABX) and # (for HFD-CAS vs HFD-WPI and HFD-CAS+ABX vs HFD-WPI+ABX) are significant (*/# P<0.05 or **/## P<0.01 or ***/### P<0.001). A complete statistical description is detailed in Methods and Materials and **figures 4.S6** and **4.S7**.

The analysis of cytokine gene expression in the distal ileum showed that *Mcp-1* expression levels were markedly reduced by WPI and ABX administration, both in 5-week and 10-week experimental cohorts (**Fig. 4.4e** and **4.4g**). No differences between two ABX-treated groups were observed. In contrast, interleukin 1β (*Il1β*) gene expression was higher in WPI-fed mice compared to CAS-fed mice at 5 weeks, without showing differences across groups after long-term intervention (**Fig. 4.4f** and **4.4h**).

In the eWAT, the changes in *Mcp-1* expression showed a similar pattern as in the ileum both at 5 and 10 weeks. HFD-WPI and HFD-CAS+ABX groups displayed lower levels of *Mcp-1* gene expression. Furthermore, at 10 weeks *Mcp-1* was less expressed in HFD-WPI+ABX group relative to the CAS counterpart (**Fig. 4.4i** and **4.4k**). At 5 weeks, *Tnfα* and cluster of differentiation 68 (*Cd68*) gene expression levels were lower in HFD-CAS+ABX and HFD-WPI groups compared HFD-CAS group and was higher in HFD-WPI+ABX mice compared to CAS counterparts (**Fig. 4.4j** and **4.4m**). At 10 weeks, both genes were less expressed in HFD-CAS+ABX and HFD-WPI groups compared HFD-CAS group. *Cd68* expression level was lower in HFD-WPI+ABX mice compared to HFD-CAS+ABX mice (**Fig. 4.4l** and **4.4n**).

In conclusion, these data suggest that microbiota depletion attenuated HFD-induced inflammation within the ileum and eWAT, but not in the plasma. The presence of WPI had a protective effect towards HFD-induced inflammation in the ileum and eWAT as well as in systemic circulation.

Gut microbiota depletion reversed the protective effect of WPI on HFD-induced ileal permeability alteration

To investigate if the lower inflammation during ABX and WPI intervention could be related to intestinal barrier function, we measured the *ex vivo* permeability of ileal and colonic epithelium to macromolecules (4 KDa FITC) using Ussing chambers (Chelakkot et al 2018).

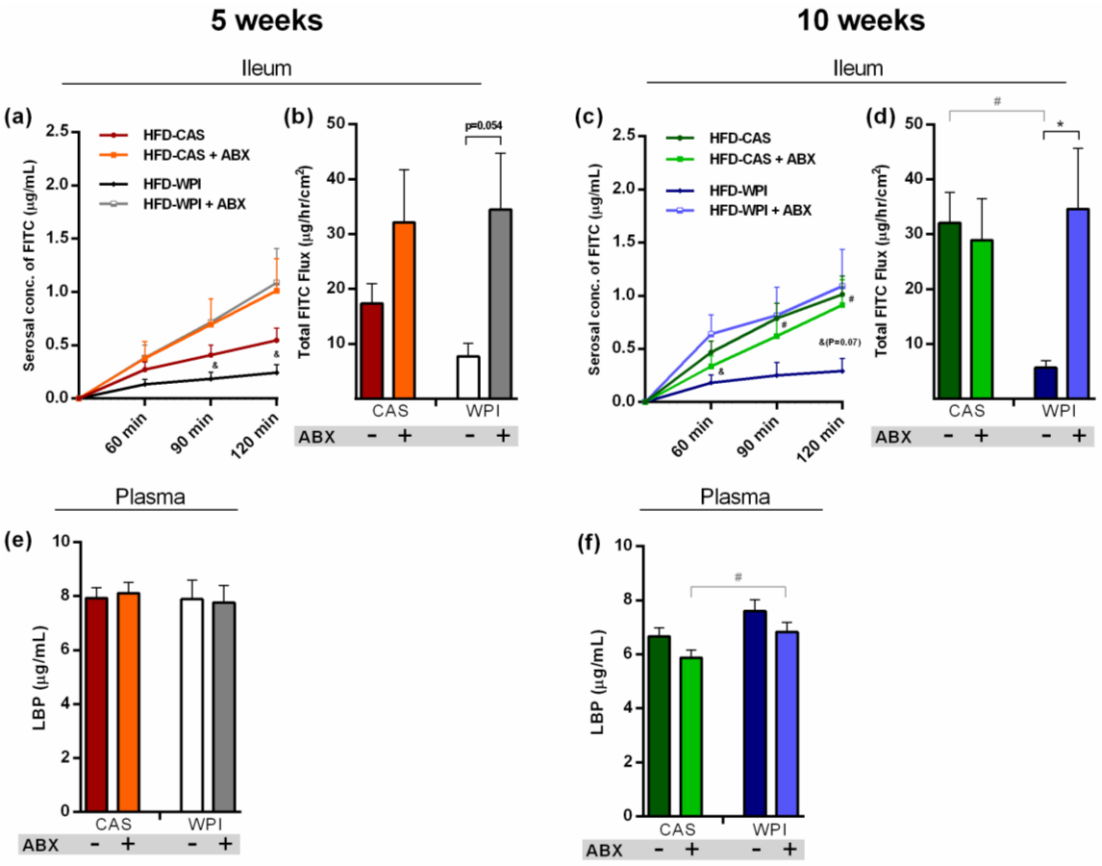


Figure 4.5: Intestinal permeability. Data show **(a)** ileal FITC paracellular permeability at 60, 90 and 120 min and **(b)** the total FITC flux that passed through the ileal epithelium over 2 hours, at 5 weeks timepoint. Data also show **(c)** ileal FITC paracellular permeability at 60, 90 and 120 min and **(d)** the total FITC flux that passed through the ileal epithelium over 2 hours, at 10 weeks timepoint. The final graph shows the plasma level of LBP **(e)** 5 and **(f)** 10 weeks timepoint. All the data were measured in mice fed with HFD-CAS and HFD-WPI, both controls and ABX-treated. Abbreviation; FITC; fluorescein isothiocyanate, LBP; lipopolysaccharide binding protein. Statistical analysis: (a, c) groups showing # (HFD-CAS vs HFD-WPI) and & (for HFD-WPI vs HFD-WPI+ABX) are significant. (b, d, e, f) groups showing * (for HFD-CAS vs HFD-CAS+ABX and HFD-WPI vs HFD-WPI+ABX) and # (for HFD-CAS vs HFD-WPI and HFD-CAS+ABX vs HFD-WPI+ABX) are significant (*/#/& P<0.05 or **/##/&& P<0.01 or ***/###/&&& P<0.001). A complete statistical description is detailed in Methods and Materials, "Supplementary Statistics" and **figures 4.S6** and **4.S7**.

In the distal ileum, ABX administration damaged the integrity of the barrier function. At 5 weeks, both ABX-treated groups showed a strong trend towards increased FITC flux compared to non-treated counterparts (pairwise post-hoc comparisons not significant due to high between-animal variability in ABX groups) (**Fig. 4.5a**, **4.5b**, **4.S6** and **4.S7**).
At 10 weeks, the increase in ileal permeability in HFD-WPI+ABX group compared to HFD-WPI group was even more dramatic. In addition, FITC flux in HFD-CAS group was much higher than HFD-WPI group and was similar to HFD-CAS+ABX and HFD-WPI+ABX groups (**Fig. 4.5c** and **4.5d**).

In the colon, the FITC flux was lower in HFD-CAS+ABX group compared to HFD-CAS group at 5 weeks timepoint, and in HFD-WPI+ABX group relative to HFD-WPI group at 10 weeks timepoint (**Fig. 4.S2** a and **4.S2d**). No effect of WPI compared to CAS in HFD and HFD-ABX mice was observed at both timepoints.

Disruption of gut permeability can cause an increase of lipopolysaccharide (LPS) in the bloodstream. LPS is produced by Gram-negative bacteria and can further boost a pro-inflammatory response (Fuke et al 2019). We could not measure plasma LPS since a sterile environment for blood collection as unavailable. Instead, we measured the plasma levels of lipopolysaccharide binding protein (LBP), which is also considered as a marker of 'leaky' gut under various pathological conditions (Vreugdenhil et al 2000). However, despite the robust changes in small intestinal FITC flux, no prominent changes in plasma levels of LBP were detected across the groups (Fig. **4.5e** and **4.5f**).

No robust effects in the ileal gene expression of tight junctions (i.e., Tjp1: tight junction protein 1, F11: junctional adhesion molecule A, Cldn1: claudin 1, Ocln: occludin) were observed in either WPI or ABX treated groups in comparison to HFD-CAS-fed mice and the fold change in expression between the groups was mainly small across most of the significant differences detected (**Table 4.TS3**).

In conclusion, both HFD and ABX treatment had a negative impact on the epithelial barrier function in the small intestine, causing an increased macromolecular permeability in distal ileum. Here, for the first time, we showed a protective role exerted by WPI supplementation on intestinal barrier, effectively preventing the HFD-induced increase in ileal permeability. This effect was reversed by ABX administration, suggesting that WPI acts through the gut microbiota to exert a protective role on HFD-induced ileal permeability alteration.

WPI effect on the gut microbiota composition in ABX-treated and non-treated mice

Next, using a 16S rRNA-based metagenomic approach, we analysed the composition of faecal gut microbiota to investigate the impact of 5- and 10-week ABX treatment and WPI intervention on taxonomic changes across the groups.

After filtration and trimming, the reads per sample averaged 109095.5±16343.35. As expected, the alpha-diversity measured with three different indexes (i.e., Richness, Shannon and Simpson) was dramatically reduced in ABX-treated mice compared to non-treated mice after both 5 and 10 weeks of intervention (**Fig. 4.6a** and **4.6b**). The successful depletion of microbiota by the selected ABX cocktail was further confirmed by a robust decrease in the bacterial DNA load in the faeces of ABX-treated mice (calculated as a number of 16S copies per mg of faecal matter, **Fig. 4.S2e**). As for the diet effect, Richness and Simpson indexes showed a decrease and increase, respectively, in alpha-diversity of HFD-WPI group relative to HFD-CAS counterparts, with no changes in Shannon index at 5 weeks (**Fig. 4.6a**). At 10 weeks, Richness and Shannon indexes showed a decrease in alpha-diversity of HFD-WPI group compared to HFD-CAS group, with no difference in Simpson index (**Fig. 4.6b**). Notably, across all of the indexes at both timepoints (except Simpson at 10 weeks), the alpha-diversity of HFD-WPI+ABX group was consistently higher than HFD-CAS+ABX counterparts (**Fig. 4.6a** and **4.6b**). This was observed while the bacterial 16S gene quantification in HFD-WPI+ABX group was much lower than in HFD-CAS+ABX group (0.01% vs 8% at 5 weeks and <0.001% vs 7% at 10 weeks) (**Fig. 4.S2e**).

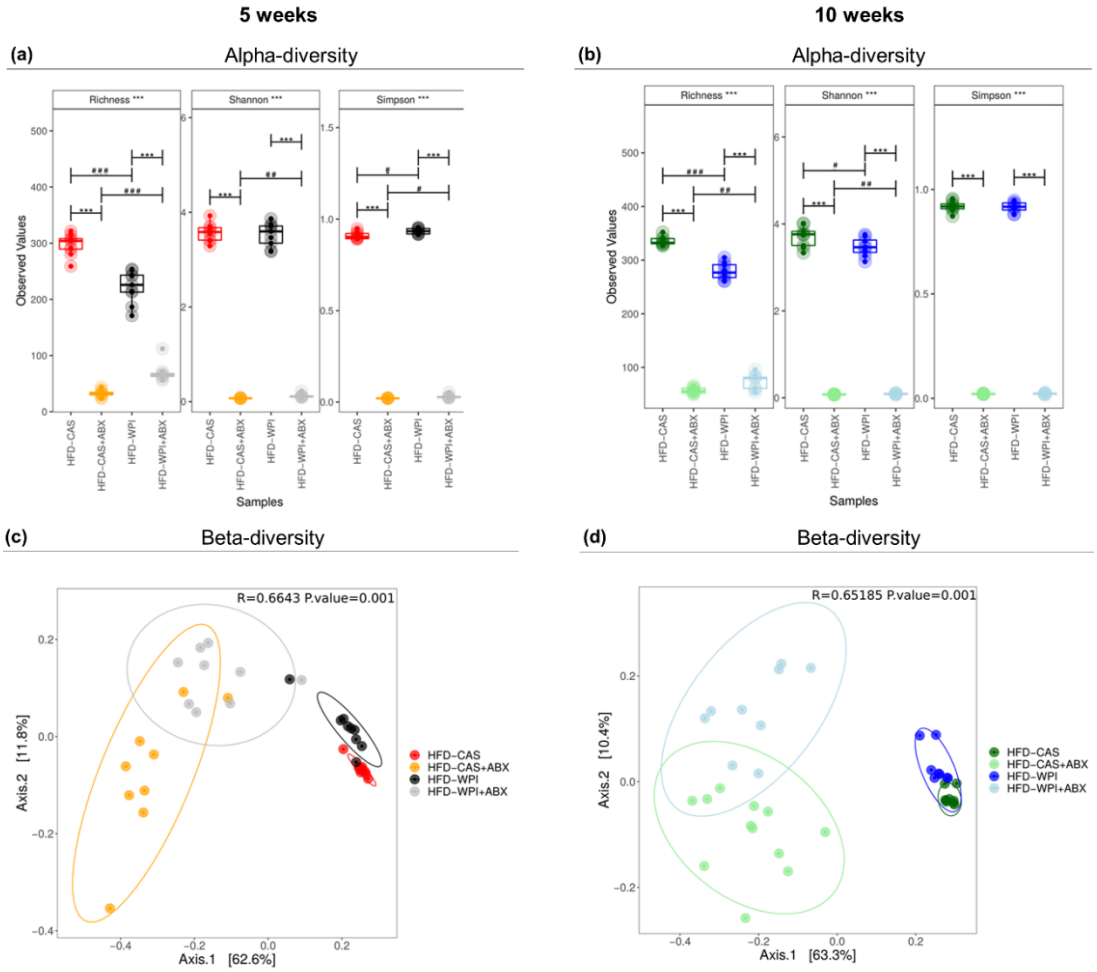
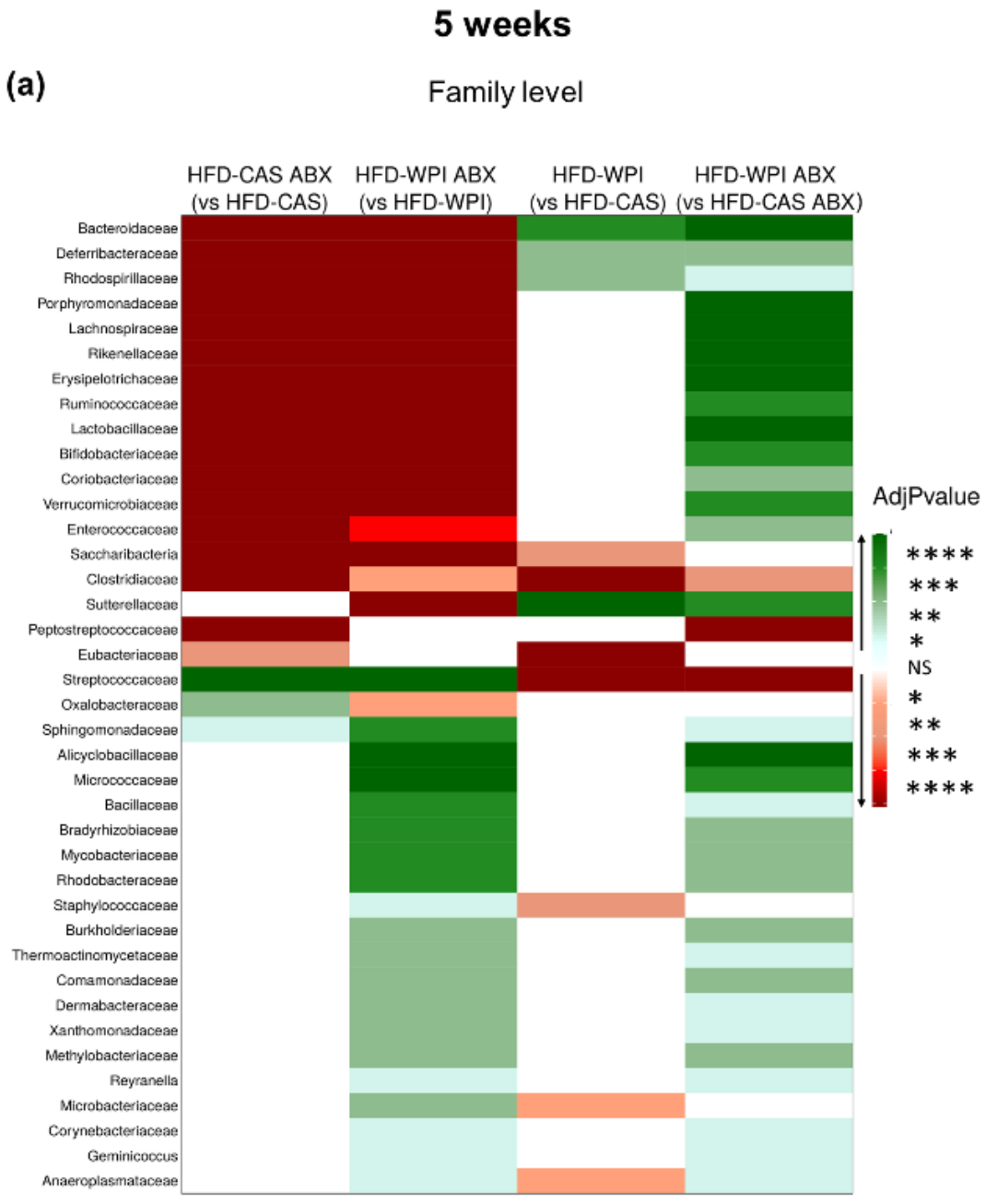


Figure 4.6: Gut microbiota analysis: alpha- and beta-diversity. Taxonomic alpha-diversity, measured with richness, Shannon and Simpson indexes at **(a)** 5 and **(b)** 10 weeks timepoints. Beta-diversity of the all groups, calculated using PCoA ordination at **(c)** 5 and **(d)** 10 weeks timepoints. All the data were measured in mice fed with HFD-CAS and HFD-WPI, both controls and ABX-treated. Groups showing * (for control vs ABX-treated) and # (CAS vs WPI) are significant (*/# P<0.05 or **/## P<0.01 or ***/### P<0.001 or ****/#### P<0.0001). A complete statistical description is detailed in Methods and Materials.

Principal component analysis (PCoA) plots showed a clear beta-diversity separation of ABX-treated compared to non-treated mice, at both timepoints (5 weeks: HFD-CAS vs HFD-CAS+ABX, R^2=0.69, p=0.006; HFD-WPI vs HFD-WPI+ABX, R^2=0.57, p=0.006; 10 weeks: HFD-CAS vs HFD-CAS+ABX, R^2=0.71, p=0.006; HFD-WPI vs HFD-WPI+ABX, R^2=0.59, p=0.006; PERMANOVA pairwise comparisons) (**Fig. 4.6c** and **4.6d**). In addition, at both timepoints, HFD-WPI+ABX showed a significant separation from HFD-CAS+ABX (5 weeks: HFD-WPI+ABX vs HFD-CAS+ABX, R^2=0.26, p=0.012; 10 weeks: HFD-WPI+ABX vs HFD-CAS+ABX, R^2=0.24, p=0.006; PERMANOVA pairwise comparisons).

A significant separation was observed also between non-treated HFD-WPI mice and HFD-CAS mice, both after 5 and 10 weeks of dietary intervention (5 weeks: HFD-WPI vs HFD-CAS, R^2=0.33, p=0.006; 10 weeks: HFD-WPI vs HFD-CAS, R^2=0.32, p=0.006; PERMANOVA pairwise comparisons).



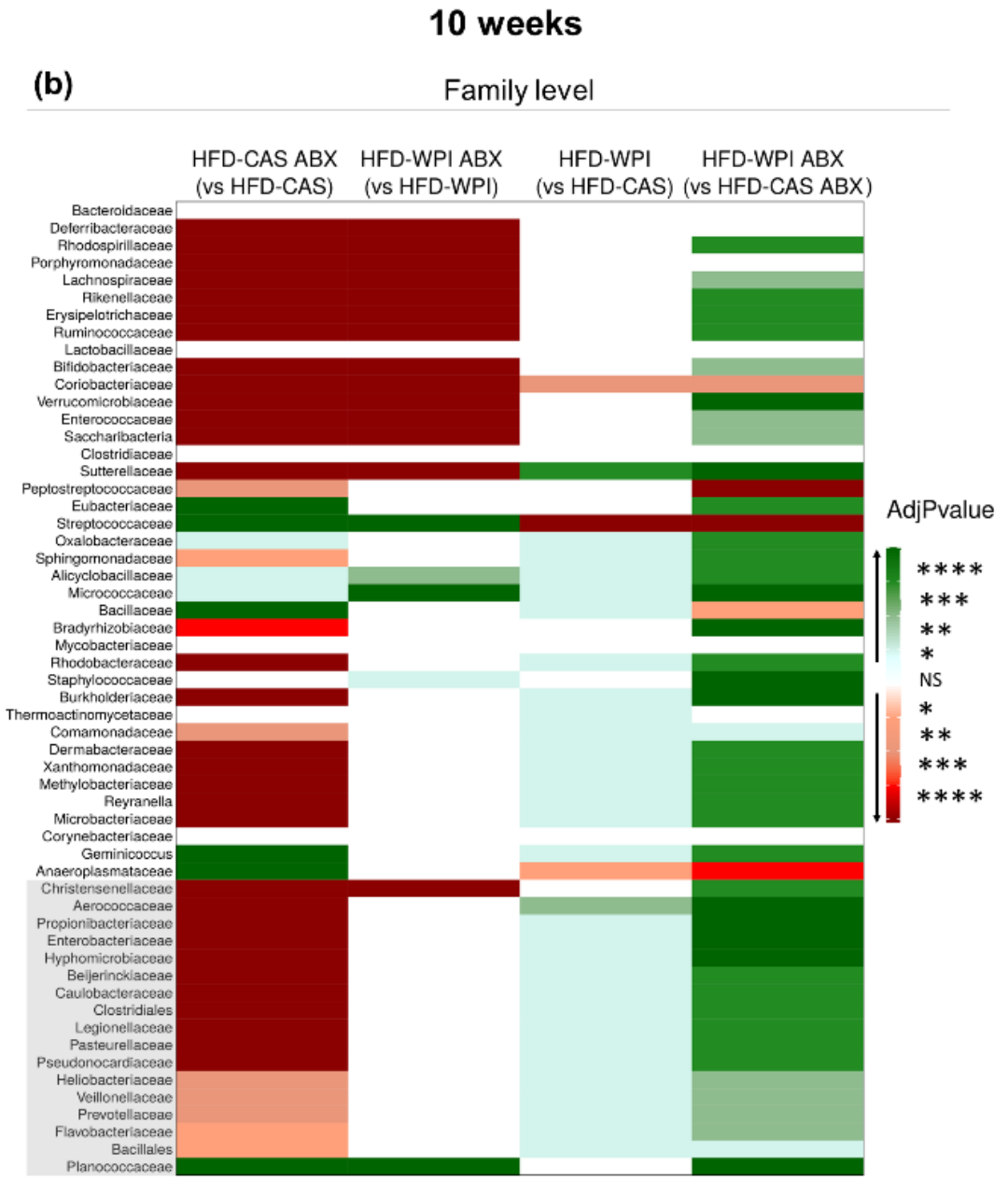
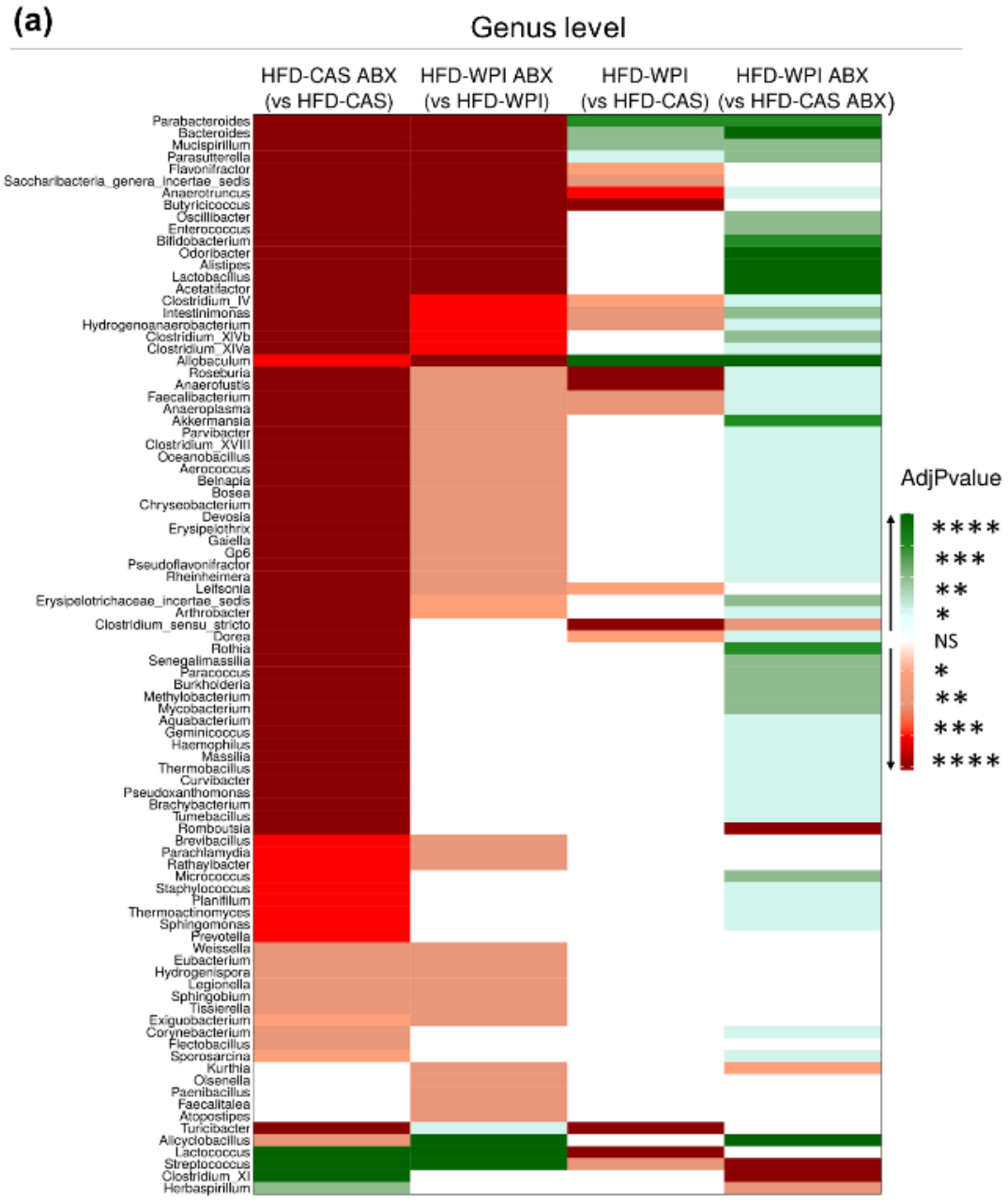


Figure 4.7: Gut microbiota analysis: taxonomic differences at family level abundance. Heatmaps representing taxonomic pairwise differences in abundance at family level across the groups, using Kruskal Wallis method **(a)** at 5 and **(b)** 10 weeks timepoint. Notably, green and red colours represent an increase and a decrease, respectively, in relative abundance of a specific group (not in brackets) compared to another group (in brackets). White colour indicates no significant differences (NS) between the two groups. The shades of each colour correspond to different p values thresholds. In (b), the families that did not show differences at 5 weeks timepoint but that showed differences at 10 weeks timepoint are indicated with a grey background. All the data were measured in mice fed with HFD-CAS and HFD-WPI, both controls and ABX-treated. Groups showing * are significant (*P<0.05 or **P<0.01 or ***P<0.001 or ****P<0.0001). A complete statistical description is detailed in Methods and Materials.

5 weeks


(a)
Genus level
HFD-CAS ABX (vs HFD-CAS)
HFD-WPI ABX (vs HFD-WPI)
HFD-WPI (vs HFD-CAS)
HFD-WPI ABX (vs HFD-CAS ABX)
Parabacteroides
Bacteroides
Mucispirillum
Parasutterella
Flavonifractor
Saccharibacteria_genera_incertae_sedis
Anaerotruncus
Butyricicoccus
Oscillibacter
Enterococcus
Bilidobacterium
Odoribacter
Alistipes
Lactobacillus
Acetatifactor
Clostridium_IV
Intestinimonas
Hydrogenoanaerobacterium
Clostridium_XlVb
Clostridium_XlVa
Allobaculum
Roseburia
Anaerofustis
Faecalibacterium
Anaeroplasma
Akkermansia
Parvibacter
Clostridium_XVIII
Oceanobacillus
Aerococcus
Belnapia
Bosea
Chryseobacterium
Devosia
Erysipelothrix
Gaiella
Gp6
Pseudoflavonifractor
Rheinheimera
Leifsonia
Erysipelotrichaceae_incertae_sedis
Arthrobacter
Clostridium_sensu_stricto
Dorea
Rothia
Senegalimassilia
Paracoccus
Burkholderia
Methylobacterium
Mycobacterium
Aquabacterium
Geminicoccus
Haemophilus
Massilia
Thermobacillus
Curvibacter
Pseudoxanthomonas
Brachybacterium
Turnebacillus
Romboutsia
Brevibacillus
Parachlamydia
Rathayibacter
Micrococcus
Staphylococcus
Planifilum
Thermoactinomyces
Sphingomonas
Prevotella
Weissella
Eubacterium
Hydrogenispora
Legionella
Sphingobium
Tissierella
Exiguobacterium
Corynebacterium
Flectobacillus
Sporosarcina
Kurthia
Osenella
Paenibacillus
Faecalitalea
Atopostipes
Turicibacter
Alicyclobacillus
Lactococcus
Streptococcus
Clostridium_XI
Herbaspirillum
AdjPvalue
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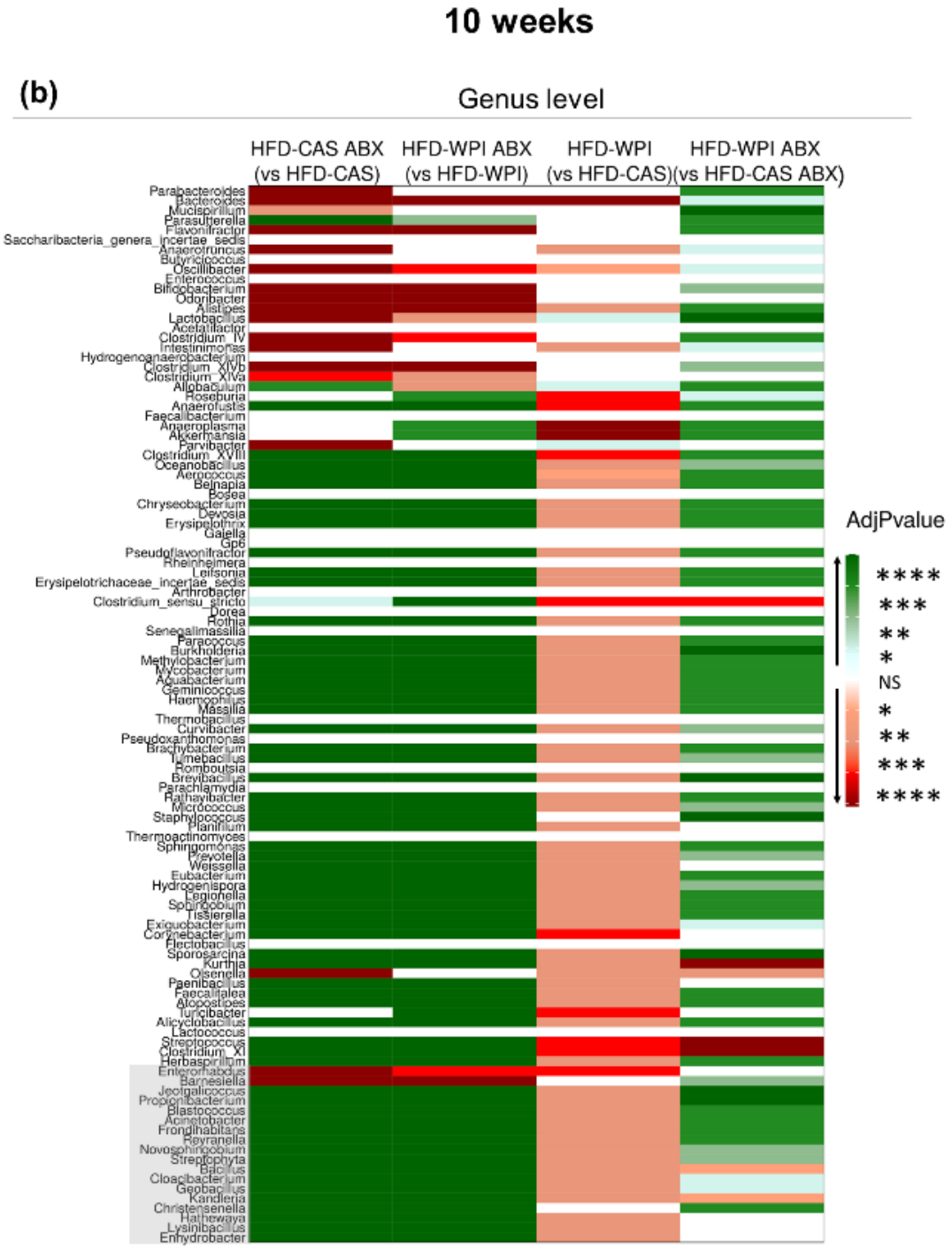


Figure 4.8: Gut microbiota analysis: taxonomic differences in genera abundance. Data show the heatmaps representing taxonomic pairwise differences in abundance at genus level across the groups, using Kruskal Wallis method **(a)** at 5 and **(b)** 10 weeks timepoint. Notably, green and red colours represent an increase and a decrease, respectively, in relative abundance of a specific group (not in brackets) compared to another group (in brackets). White colour indicates no differences between the two groups. The shades of each colour correspond to different p values thresholds. In (b), the genera that did not show differences at 5 weeks timepoint but that showed differences at 10 weeks timepoint are indicated with a grey

background. All the data were measured in mice fed with HFD-CAS and HFD-WPI, both controls and ABX-treated. Groups showing * are significant (*P<0.05 or **P<0.01 or ***P<0.001 or ****P<0.0001). A complete statistical description is detailed in Methods and Materials.

Both short- and long-term administration of ABX clearly caused a marked disruption of the gut microbiota at all the taxonomic levels (i.e., phylum, family and genus) (**Fig. 4.S3a-f**, **4.7a** and **4.7b**, and **4.8a** and **4.8b**). This was not surprising because the ABX cocktail used in this study was reported to effectively deplete gut microbiota in other studies (Cani et al 2008, Rabot et al 2016, Thackray et al 2018). Intriguingly, here we observed that taxonomic alterations due to ABX treatment differed depending on the protein quality of the diet (i.e., CAS or WPI) (**Fig. 4.S3e** and **4.S3f**, **4.7a** and **4.7b**, and **4.8a** and **4.8b**).

WPI supplementation also had a significant impact on the relative abundance of bacterial taxa at different levels.

At family level, HFD-WPI gut microbiome was characterized by an increase in 4 taxa (*Bacteroidaceae*, *Deferribacteraceae*, *Rhodospirillaceae* and *Sutterellaceae*), and a decrease in 7 taxa (among these we found *Saccharibacteria*, *Clostridiaceae*, *Eubacteriaceae*, *Streptococcaceae* and *Staphylococcaceae*) at 5 weeks timepoint (**Fig. 4.7a** and **4.S3a**). However, after 10 weeks of intervention, 31 families were more abundant (among these *Sutterellaceae* and *Aerococcaceae*) and 3 families were less abundant (*Coriobacteriaceae*, *Streptococcaceae* and *Anaeroplasmataceae*) in HFD-WPI mice compared to HFD-CAS mice (**Fig. 4.7b** and **4.S3b**).

At genus level, 4 (*Parabacteroides*, *Bacteroides*, *Mucispirillum* and *Allobaculum*) and 17 (among these *Anaerotruncus*, *Butirycoccus*, *Roseburia*, *Anaerofustis*, *Clostridium_sensu_stricto*, *Turicibacter* and *Streptococcus*) genera were increased and decreased, respectively, in HFD-WPI mice compared to HFD-CAS mice at 5 weeks timepoint (**Fig. 4.8a** and **4.S3c**). Instead, the abundance of 3 (*Lactobacillus*, *Allobaculum* and *Parvibacter*) and 74 (among these *Bacteroides*, *Roseburia*, *Anaerofustis*, *Anaeroplasma*, *Akkermasia*, *Clostridium_XVIII*, *Clostridium_sensu_stricto* and *Streptococcus*) genera were higher and lower,

respectively, in HFD-WPI group relative to HFD-CAS group at 10 weeks timepoint (**Fig. 4.8b** and **4.S3d**).
Notably, at every level, a greater number of taxa were changed in abundance between the groups after 10 weeks of intervention with respect to 5 weeks.

In conclusion, ABX treatment caused a dramatic decrease in alpha-diversity and profoundly affected beta-diversity together with a significant change in the taxonomical composition of the gut microbiota after 5 and 10 weeks of intervention.
In non-treated groups, supplementation of WPI in HFD significantly affected alpha- and beta-diversity as well as taxonomical structure of the intestinal microbiota.

WPI supplementation and ABX treatment affect metabolites within the caecum

Considering the importance of gut microbial-derived metabolites in regulating host metabolism to maintain good health, we carried out a broad profiling of the metabolites present in the caecum in mice that underwent 10 weeks of dietary intervention and ABX-treatment. The 10 week-treatment timepoint was selected because we observed more marked differences in body weight gain, adiposity, inflammation and gut permeability across the groups. The analysis showed a wide variety of metabolites to be affected by ABX-treatment and/or protein quality. For this reason, we grouped the data in different heatmaps that show metabolome changes caused by ABX treatment only, protein quality only and a combined effect of ABX treatment and protein quality.
The ABX treatment brought about a corresponding effect in the presence of CAS and WPI, with respect to CAS and WPI non-treated mice, in the change in the abundance of 50 metabolites belonging to different categories such as SCFAs, amino acids, sugars, amino acids metabolism components and vitamins (**Fig. 4.9a**). It was particularly notable that the concentration of four SCFAs (i.e., 2-methyl propanoic acid, acetic acid, 3-methyl-butanoic acid and butanoic acid), as well as three tryptophan metabolism-related metabolites (i.e., 3-indolepropionic acid, 3-(4-hydroxyphenyl)propionic acid and glutaric acid) were decreased during ABX treatment (**Fig. 4.9a**).
The presence of WPI within HFD, both in ABX-treated and non-treated mice, caused an increase in the abundance of five amino acids (i.e., glycine, tryptophan, serine,

proline and lysine), two modified amino acids (i.e., N6-Acetyl-L-lysine and prolyl-leucine), the tryptophan metabolism-related skatole, vitamin B1 as well as an increase in the nucleoside 7-methylguanine (**Fig. 4.9b**).

Previously, we noted that both ABX-treated groups and HFD-WPI group were characterized by lower body weight gain, adiposity and inflammation with respect to HFD-CAS group. Thus, in those groups, we searched for metabolites that were changed in a similar way. We observed that the amino acids threonine and asparagine, cis aconitic acid (from tricarboxylic acid cycle, TCA) and 3-indoleacrylic acid (from tryptophan metabolism) were more abundant in both ABX-treated groups, relative to non-treated groups, and also in HFD-WPI-fed mice (ABX treated and non-treated), relative to their CAS counterparts (**Fig. 4.S4a**). In addition, in the same groups (except in HFD-WPI+ABX compared to HFD-CAS+ABX), a decrease in 2-oxindole (from tryptophan metabolism), 2,3-pyridinecarboxylic acid and quinolinic acid (from kynurenine pathway) was detected (**Fig. 4.S4a**).

In conclusion, here we showed that the metabolic profile of caecal content can be affected by both ABX treatment and protein quality. In addition, a similar change in the abundance of seven well known metabolites (i.e., specific amino acids, TCA metabolites and tryptophan-related metabolites) were found in mice that showed lower body weight gain, adiposity and inflammation.

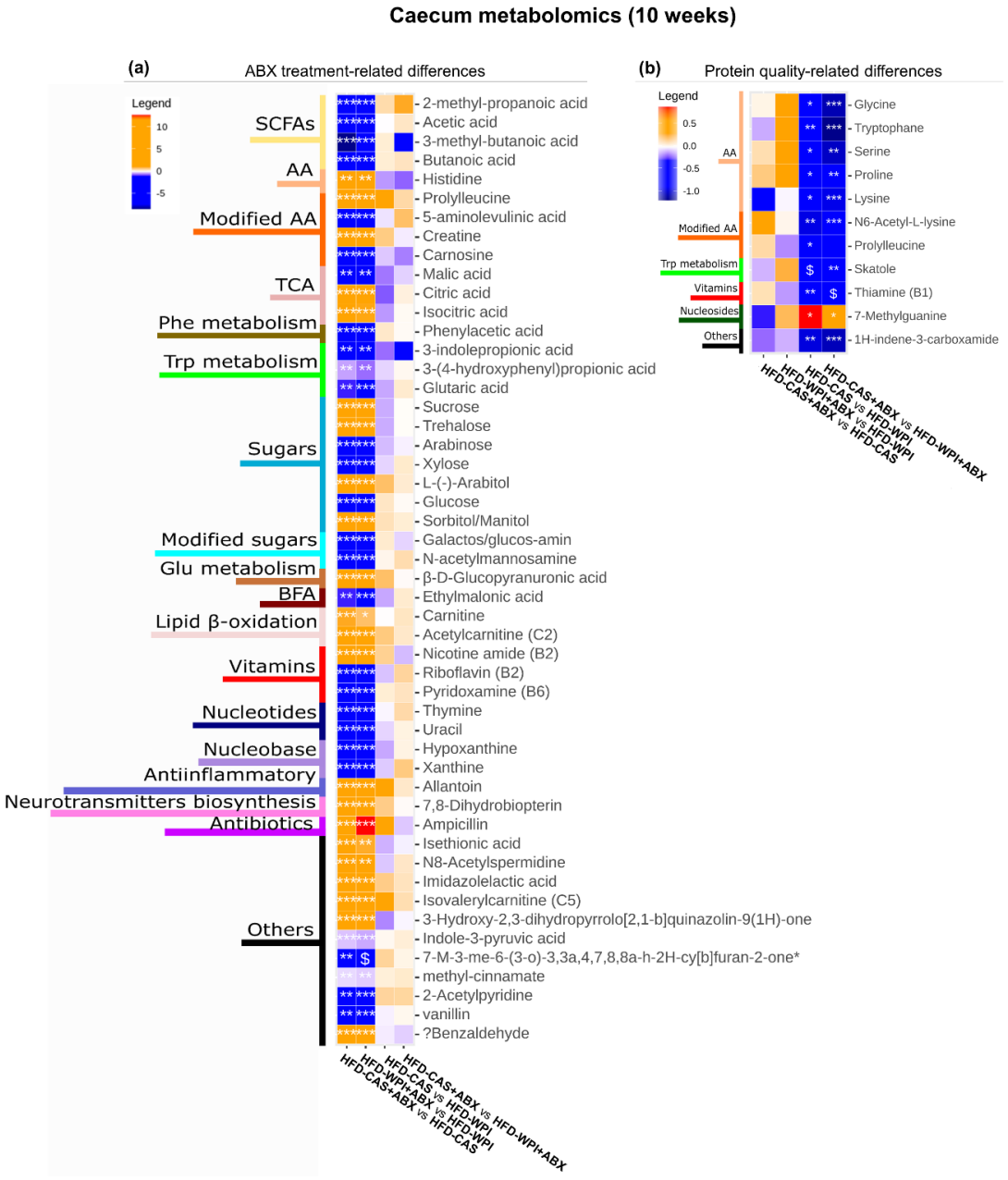


Figure 4.9: Metabolites changes in the caecum at 10 weeks timepoint. The heatmaps show metabolites measured within the caecum content, where the changes observed across the groups are related to **(a)** ABX treatment and **(b)** protein quality. Notably, orange and blue colours represent an increase and a decrease, respectively, in abundance of group 1 compared to group 2 (below in each heatmap: Group 1 vs Group 2). On the left side of the heatmaps are indicated the categories in which the metabolites belong to. *Abbreviations:* 7-M-3-me-6-(3-o)-3,3a,4,7,8,8a-h-2H-cy[b]furan-2-one; 7-Methyl-3-methylene-6-(3-oxobutyl)-3,3a,4,7,8,8a-hexahydro-2H-cyclohepta[b]furan-2-one. Groups showing * are significant (* P<0.05 or ** P<0.01 or *** P<0.001) and $ indicates a trend (0.05< P >0.07). A complete statistical description is detailed in Methods and Materials.

Discussion

Recently, there has been a growing interest in deciphering the mechanism underpinning the anti-obesity actions of WPI. We previously observed that WPI given as part of HFD caused a decrease in body weight gain and adiposity, modulating lipid metabolism-related enzyme expression, as well as gut microbiota composition and function in adolescent mice, relative to HFD-CAS mice (Boscaini et al 2020) (see Chapter 3). These observations led to the hypothesis that the anti-obesity effects of WPI could be, at least in part, mediated by the growth of beneficial bacterial taxa in the gut. Thus, we hypothesised that a gut microbiota depletion with a broad-spectrum ABX would reduce or neutralize the positive effects exerted by WPI on body weight and metabolic outcomes during HFD feeding. However, the data generated do not support this hypothesis. This is possibly due to the fact that microbiota depletion *per se* will have a robust anti-obesity effect in HFD-fed mice. Indeed, we showed that HFD-CAS+ABX administration resulted in a reduction of body weight gain, adiposity, leptin availability and inflammation, both after short- and long-term intervention (**Fig. 4.1** and **4.2**). Not surprisingly, we did not observe significant differences between HFD-WPI and HFD-WPI+ABX groups either. In light of this, it is hard to disentangle the effects of WPI with or without microbiota depletion on body weight. To this end, we investigated whether WPI utilises the gut microbiota to protect the host from HFD-induced obesity. However, we did observe an additive effect of WPI and ABX treatment towards adiposity and leptin availability following long-term intervention. These findings suggest that WPI might exploit other mechanisms, independent of gut microbiota, to protect the host from HFD-induced obesity.

Several studies have demonstrated that HFD consumption affects the composition of the gut microbiota along with an increase in metabolic syndrome-related derangements (i.e., body weight gain, dysfunction of adipose tissue, dysregulation of energy balance, leptin and insulin resistance) and low-grade inflammation (Davis 2016, Ellulu et al 2017, Uranga & Keller 2019). It was shown that microbiota depletion through ABX administration during HFD feeding reduced glucose intolerance, body weight gain, fat mass development, inflammation, and macrophage infiltration within the adipose

tissue (Cani et al 2008). Similarly, germ-free mice were resistant to HFD-induced metabolic dysfunction such as insulin resistance, inflammation, glucose tolerance and hypercholesterolemia (Rabot et al 2010). These evidences suggest the importance of the gut microbiome in the pathogenesis of obesity. Accordingly, in the present study, we observed a decrease in metabolic syndrome-related outcomes, such as body weight gain, adiposity, gene expression of inflammatory markers and leptin availability, in HFD-CAS+ABX mice relative to HFD-CAS non-treated counterparts, at both timepoints. On the contrary, no one of these differences was observed between HFD-WPI+ABX mice and HFD-WPI non-treated mice after 5 weeks of intervention. After 10 weeks of HFD-WPI feeding, ABX-treated group showed less adiposity and lower leptin level than non-treated groups, without showing differences in body weight gain and inflammation outcomes. In general, these results highlighted the independent action exerted by WPI and ABX during HFD-feeding.

Next, we reported the marked anti-obesity effects of WPI in HFD-fed mice. At both 5 and 10 weeks of dietary intervention, a standard percentage (20%) of WPI contained in 45% fat HFD caused a decrease in body weight gain, adiposity and leptin levels compared to HFD-CAS-fed mice. Similar findings were observed in a previous study conducted in our group in which mice were fed with the aforementioned diet for 5 weeks (from 5 weeks-old until 10 weeks-old), 8 weeks (from 8 weeks-old until 16 weeks-old) and for 21 weeks (from 6 weeks-old until 27 weeks-old) (Boscaini et al 2020, McAllan et al 2013, McAllan et al 2014) (see Chapter 3).

In addition, in this study, we acquired novel insights relating to the effect of WPI on inflammation and intestinal permeability. Previously, two *in vitro* studies have highlighted the anti-inflammatory and anti-oxidant features of a modified form of WPI (i.e., hydrolysate) on endothelial cells (Da Silva et al 2017, Piccolomini et al 2012). In rodents, a specific component of WPI, namely alpha-lactalbumin (LAB), exerted a positive effect on inflammation in the presence of HFD. In particular, LAB affected TNFα, IL6 and MCP-1 gene expression within the adipose tissue and colon as well as their serum levels (Gao et al 2018, Li et al 2019a). Here, we found that WPI reduced the inflammatory response associated with HFD. In particular, WPI caused a decrease

in MCP1 plasma levels (at 10 weeks timepoint only) and a decrease in adipose tissue and ileal expression of *Mcp-1* (both at 5 and 10 weeks), and adipose tissue expression of *Tnfα* and *Cd68* genes. These observations suggest the anti-inflammatory action of WPI on the intestinal and adipose tissues (both at 5 and 10 weeks). A lower expression of *Cd68* suggests that a decreased inflammation within the adipose tissue might be due to a decrease in macrophages infiltration.

With respect to intestinal permeability, we demonstrate what is to our knowledge for the first time that supplementation with WPI effectively protects the gut from the impairment of the epithelial barrier function associated with HFD-induced obesity. Indeed, HFD consumption, as well as ABX treatment, have been previously shown to compromise intestinal epithelial permeability (Lendrum et al 2016, Murakami et al 2016). In agreement with these reports, we observed an increased epithelial permeability in the small intestine (distal ileum) of ABX-treated mice. The effect was more robust following 10 week of ABX administration. In addition, we also observed an increase in ileal permeability in HFD-CAS-fed mice at the 10-week timepoint, compared to the 5-week, indicating intestinal permeability perturbation overtime. Maintenance of epithelial integrity in the gut is critically important for the health. The phenomenon of "leaky" gut has been tightly associated with gastrointestinal disorders, such as inflammatory bowel syndrome, and has also been recently extended to metabolic and psychiatric diseases (Chakaroun et al 2020, Kelly et al 2015, Michielan & D'Incà 2015). Here, we observed that the presence of WPI prevented epithelial barrier disruption and decreased the gene expression of the inflammatory marker MCP-1 in the small intestine. These data suggest that WPI might have a positive effect on intestinal health and function. For this reason, supplementation of WPI can be considered as an adjunctive dietary therapeutic approach in inflammatory bowel diseases (IBD). In support of this idea, 2-month whey protein administration improved intestinal permeability (measured as lactulose mannitol excretion ratio in urine) and morphology in Crohn's disease patients (Benjamin et al 2012).

Notably, after a long-term intervention, ABX cancelled the beneficial effect of WPI on gut permeability. This suggests that WPI protects form HFD-induced ileal permeability alteration through the gut microbiota. In accordance with this finding, in

the present study, we observed a decrease in plasma MCP-1 in the presence of WPI in HFD-fed mice and this change was abolished during gut microbiota depletion. This might suggest that also the action of WPI on systemic inflammation (and not anti-inflammatory cytokines expression at the tissue-level) is gut microbiota-mediated. In the future, a deeper analysis of systemic inflammation is required to confirm this first evidence of gut microbiota-dependent effects of WPI as part of HFD on gut permeability and systemic inflammation. Additionally, a more specific investigation focused on targeting the specific members of the gut microbiota involved in WPI action will be required.

Notably, the presence of WPI within HFD had a significant effect on the gut microbiota composition. In accordance with our previous study (Boscaini et al 2020) (see Chapter 3), HFD-WPI and HFD-CAS groups showed significant differences in beta-diversity and the relative abundance of multiple bacterial taxa at the phylum, family and genus levels after 5 weeks or 10 weeks of dietary intervention. Notably, different duration of the dietary intervention, from adolescence to adulthood, had a differential impact on the microbiota composition. Previously, it was shown that WPI caused an increase in the relative abundance of *Lactobacillus* and *Bifidobacterium* spp. and a decrease in *Clostridium* compared to HFD-CAS-fed rodents (McAllan et al 2014, Sprong et al 2010). Here, we found an increase in *Lactobacillus* only after 10 weeks of dietary intervention, without finding any differences in *Bifidobacterium* proportions.
Both after 5 and 10 weeks, in HFD-WPI groups we found an increase in Deferribacteres at phylum level, an increase in *Sutterellaceae* and a decrease in *Streptococcaceae* at family level, and an increase in *Allobaculum* and a decrease in *Anaerotruncus*, *Saccharibacteria_genera_incertae_sedis*, *Intestimonias*, *Roseburia*, *Anaerofusits*, *Anaeroplasma*, *Lefsonia*, *Clostridium_sensu_stricto*, *Turicibacter* and *Streptococcus* at genus level. This is to our knowledge the first evidence of an increase in *Allobaculum* in the presence of HFD-WPI. Notably, in figure **4.S3c** and **4.S3d** is clearly *Allobaculum* much more present in the presence of WPI compared to CAS in non-treated mice. A decrease in *Allobaculum* was found in obesity-prone mice fed with HFD and an increase of this genera has been linked with a reduction in body weight in obese mice (Huazano-García et al 2017, Qiao et al 2014, Ravussin et al

2012). Furthermore, unlike our previous results (Boscaini et al 2020) (see Chapter 3), here we observed a decrease in alpha-diversity in HFD-WPI vs HFD-CAS group. However, it is worth noting that direct comparison of data between both studies is challenging due to the different approaches to gut microbiota analysis utilised (shotgun metagenomics vs 16s rRNA-based sequencing).

Here, we also observed some differences between the ABX groups after 10 weeks intervention. For instance, HFD-WPI+ABX mice showed a lower adiposity and leptin level compared to HFD-CAS-ABX mice. This suggests an additive effect of WPI and ABX treatment on those parameters. Differences between these two groups were also found within the gut microbiota. The HFD-WPI+ABX group showed a higher alpha-diversity, differences in beta-diversity, lower faecal bacterial load and an increase in relative abundance of multiple taxa at phylum, family and genus level, compared to HFD-CAS+ABX group. These intriguing observations clearly indicate that protein quality impacts on body weight-related parameters and gut microbiota despite the very low microbial abundance caused by 10-week ABX exposure.

In the presence of WPI, both in ABX-treated and non-treated mice, several amino acids were more abundant in the caecal content, such as tryptophan, N6-acetyl-L-lysine and prolyl-leucine. The two latter ones are modified amino acids containing branched-chain amino acids (BCAAs). This is in line with the fact that whey protein is naturally enriched in essential BCAAs, which are very important for muscle repair, recovery and protein synthesis (Gorissen et al 2018). In addition, WPI has a higher proportion of tryptophan compared to other dietary proteins. Particularly, LAB contained in WPI has a very high percentage of tryptophan. This amino acid is the precursor of serotonin, a neurotransmitter that acts in the brain modulating mood, cognitive function, appetite and sleep (Layman et al 2018a). A higher percentage of vitamin B1 was also found in the caecal content of WPI-fed mice. Several *Lactobacillus spp*. are vitamin B1-producers, thus the higher relative abundance of *Lactobacillus* genus within the gut of HFD-WPI-fed mice (both ABX-treated and non-treated) can explain the vitamin B1-related data observed (Yoshii et al 2019). Vitamin B1 is an essential dietary requirement for the host due to its role in conversion of ingested food by the host into

energy as well as in proper functioning of peripheric and central nervous system (Osiezagha et al 2013).

In mice with lower body weight, adiposity and leptin levels (i.e., both ABX-treated groups and HFD-WPI non treated group), we observed a similar increase in threonine, asparagine, cis aconitic acid and 3-indoleacrylic acid, and a similar decrease in 2-oxindole and quinolinic acid. Interestingly, 3-indoleacrylic acid is a tryptophan-derivate indole which have been associated with suppression of inflammation (Wlodarska et al 2017). This suggests that the high abundance of tryptophan within WPI might contribute at least in part the anti-inflammatory action observed within the intestine.

Conversely, the kynurenine pathway-derivate quinolinic acid has been shown to have neurotoxic effect and its brain levels are high in patients with depression (Bansal et al 2019, Gheorghe et al 2019, Myint et al 2012). Altogether, the caecum metabolomics readouts suggest an influence of WPI presence within the diet on amino acid content, especially on tryptophan and some tryptophan metabolites derivates produced by the gut bacteria which are involved in inflammation and brain health.

In conclusion, we have further delved into the molecular mechanism of WPI anti-obesity effects. We have shown that the protective effects of WPI dietary supplementation on body weight, adiposity and leptin levels in HFD-fed mice persist in the conditions of gut microbiota depletion with ABX cocktail. However, ABX administration *per se* had a strong anti-obesity effect with similar effect size. Therefore, in the current experimental design, it is impossible to conclude whether WPI exploits microbiota to protect the host from HFD-induced obesity. However, the additive effect of WPI and ABX on adiposity and leptin production suggest that WPI has a gut microbiota-independent protective effect against HFD-induced obesity.

In the future, it would be worth to repeat the same study but in the presence of normal chow or low-fat diet to have a complete picture on the relation between WPI effects and microbiota. Alternatively, other gut microbiota-related strategies (such as a direct intervention on *Lactobacillus* genus or faecal microbiota transplantation) can be applied to avoid the strong effect of ABX.

Moreover, we have shown that supplementation of WPI reduced HFD-induced pro-inflammatory cytokines plasma level and gene expression in the ileum and adipose tissue as well as ileal barrier disruption. In particular, the effects observed for ileal permeability and plasma level of MCP-1 seem to be mediated by the gut microbiota. These data together further support the potential of WPI as an anti-obesity agent. However, the exact mechanisms underpinning this are multi-factorial and require further investigation.

Supplemental Material

Supplemental Tables

Table 4.TS1 Sequences of qPCR primers.

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin (*ActB*)	5'-gtggtacgaccagaggcatac-3'	5'-aaggccaaccgtgaaaagat-3'
Interleukin 1 β (*Il1β*)	5'-attgttgaacatcgccactg-3'	5'-aaatgagccccagtagcactt-3'
Monocyte chemoattractant protein 1 (*Mcp1* or *Ccl2*)	5'-agccaactctcactgaagcc-3'	5'-atcctcttgtagctctccagcc-3'
Adiponectin (*Adipoq*)	5'-ggagagaaaggagatgcaggt-3'	5'-ctttcctgccaggggttc-3'
Leptin (*Ob*)	5'-ccccgcaccgctggaagtac-3'	5'-atgtgccctgaaatgcggtatg-3'
Cluster of differentiation 68 (*CD68*)	5'-cacttcgggccatgtttctcttg-3'	5'-aggggctggtaggttgattgtcgtc-3'
Tumor necrosis factor alpha (*Tnf* α)	5'-tcttctcattcctgcttgtgg-3'	5'-ggtctgggccatagaactga-3'
Proopiomelanocortin (*Pomc*)	5'-agtgccaggacctcacca-3'	5'-cagcgagaggtcgagtttg-3'
Neuropeptide Y (*Npy*)	5'-ccgctctgcgacactacat-3'	5'-tgtctcagggctggatctct-3'
Glucocorticoid receptor (*Gr*)	5'-caaagattgcaggtatcctatgaa-3'	5'-tggctcttcagaccttctt-3'
Cocaine- and amphetamine-regulated transcript protein (*Cartpt*)	5'-cgagaagaagtacggccagg-3'	5'-ctggccccttttcctcact-3'
Suppressor of cytokine signalling 3 (*Socs3*)	5'-aataccttttgacaagcggac-3'	5'-ccttcacacacccttttctc-3'
Cannabinoid receptor type 1 (*Cb1*)	5'-gggcaaatttccttgtagca-3'	5'-ggctcaacgtgactgagaaa-3'
Corticotropin-releasing hormone (*Crh*)	5'-caaatgctgcgtgctttc-3'	5'-ttgccttttcccttttctcttc-3'
Tight junction protein (*Tjp1*)	5'-gttttgagagcaagccttctg-3'	5'-cagcatcagtttcgggtttt-3'
Junctional adhesion molecule 1 (*F11* or *Jam1*)	5'-actccttgctgaatctgaacagt-3'	5'-ggacacaaagattgcgatcag-3'
Claudin 1 (*Cldn1*)	5'-agaacaaagaaagggactgcac-3'	5'-accaggaacgacgaggtct-3'
Occludin (*Ocln*)	5'-gtccgtgaggccttttga-3'	5'-ggtgcataatgattgggtttg-3'

Table 4.TS2 Hypothalamic gene expression across all experimental groups, after short- (5 weeks) or long-term (10 weeks) intervention. Relative gene expression and standard error of the mean (SEM) are indicated. Abbreviation; Pomc; proopiomelanocortin, Cartpt; Cocaine- and amphetamine- regulated transcript, Npy; neuropeptide Y, Cb1; cannabinoid receptor 1, Crh; corticotropin-releasing hormone, Gr; glucocorticoid receptor, Socs3; suppressor of cytokine signalling 3. **a**: significant differences between HFD-CAS vs HFD-CAS+ABX; **b**: significant differences between HFD-WPI vs HFD-WPI+ABX; **c**: significant differences between HFD-CAS vs HFD-WPI; **d**: significant differences between HFD-CAS+ABX vs HFD-WPI+ABX. A complete statistical description is detailed in Methods and Materials and **figures 4.S6 and 4.S7**.

	5 weeks							
	HFD-CAS		HFD-CAS+ABX		HFD-WPI		HFD-WPI+ABX	
Hypothalamic gene	Relative expression	SEM	Relative expression	SEM	Relative expression	SEM	Relative expression	SEM
Pomc	0,943	0,103	0,706	0,070	0,926 **c**	0,132	1,105	0,140
Cartpt	1,174	0,103	1,812 **a**	0,187	1,104	0,100	1,021 **d**	0,088
Npy	0,835	0,087	1,227 **a**	0,111	1,003	0,140	1,326 **b**	0,116
Cb1	1,202	0,043	1,447	0,153	1,081	0,096	1,055 **d**	0,111
Crh	0,914	0,085	1,056	0,126	1,333	0,224	1,529	0,230
Gr	0,926	0,049	0,777	0,056	1,033	0,063	0,958 **d**	0,057
Socs3	0,846	0,058	0,554 **a**	0,061	0,788	0,095	0,722	0,072
	10 weeks							
Pomc	0,878	0,077	1,304	0,144	1,043 **c**	0,111	1,082	0,114
Cartpt	0,612	0,034	0,790 **a**	0,052	0,642	0,078	0,962 **b d**	0,058
Npy	0,728	0,107	0,810	0,052	0,821	0,079	1,153 **b d**	0,189
Cb1	0,675	0,013	0,944 **a**	0,085	0,635	0,090	0,984 **b**	0,112
Crh	0,621	0,053	0,867	0,108	0,647	0,146	1,044 **b**	0,113
Gr	1,189	0,045	1,072	0,046	0,919 **c**	0,044	1,087	0,152
Socs3	1,446	0,113	1,082 **a**	0,064	1,632	0,238	0,911 **b**	0,054

Table 4.ST3: Tight junctions gene expression across all experimental groups, after short- (5 weeks) or long-term (10 weeks) intervention. Relative gene expression and standard error of the mean (SEM) are indicated. Abbreviation; Tjp1; tight junction protein 1, F11; Junctional adhesion molecule A, Cldn1; claudin 1, Ocln; occludin. **a**: significant differences between HFD-CAS vs HFD-CAS+ABX; **b**: significant differences between HFD-WPI vs HFD-WPI+ABX; **c**: significant differences between HFD-CAS vs HFD-WPI; **d**: significant differences between HFD-CAS+ABX vs HFD-WPI+ABX. A complete statistical description is detailed in Methods and Materials and **figure 4.S6**.

	5 weeks							
	HFD-CAS		HFD-CAS+ABX		HFD-WPI		HFD-WPI+ABX	
Tight junction gene	Relative expression	SEM	Relative expression	SEM	Relative expression	SEM	Relative expression	SEM
Tjp1	1,147	0,070	1,118	0,043	0,874 **c**	0,072	0,714 **d**	0,069
F11	0,875	0,074	0,749	0,041	1,027	0,083	1,010 **d**	0,057
Cldn1	0,856	0,106	0,633	0,083	1,152	0,174	0,706 **b**	0,124
Ocln	0,771	0,092	0,843	0,060	0,877	0,107	1,134 **b,d**	0,070
	10 weeks							
Tjp1	0,890	0,072	1,178	0,098	0,996 **c**	0,083	1,103	0,067
F11	1,236	0,093	1,052	0,069	1,104	0,081	0,940	0,082
Cldn1	1,757	0,188	0,871 **a**	0,129	1,282 **c**	0,197	0,888	0,178
Ocln	1,335	0,130	1,103 **a**	0,095	1,009 **c**	0,126	0,931	0,073

Supplemental Figures

Figure 4.S1 Absolute organ/tissue weights and intestinal length. Data show tissue and organ absolute weights **(a)** at 5 and **(b)** 10 weeks timepoints. Data also show small intestinal and colonic length **(c)** at 5 and **(d)** 10 weeks timepoint. All the data were measured in mice fed with HFD-CAS and HFD-WPI, both controls and ABX-treated. Groups showing * (for HFD-CAS vs HFD-CAS+ABX and HFD-WPI vs HFD-WPI+ABX) and # (for HFD-CAS vs HFD-WPI and HFD-CAS+ABX vs HFD-WPI+ABX) are significant (*/# P<0.05 or **/## P<0.01 or ***/### P<0.001). A complete statistical description is detailed in Methods and Materials and **figures 4.S5 and 4.S7**.

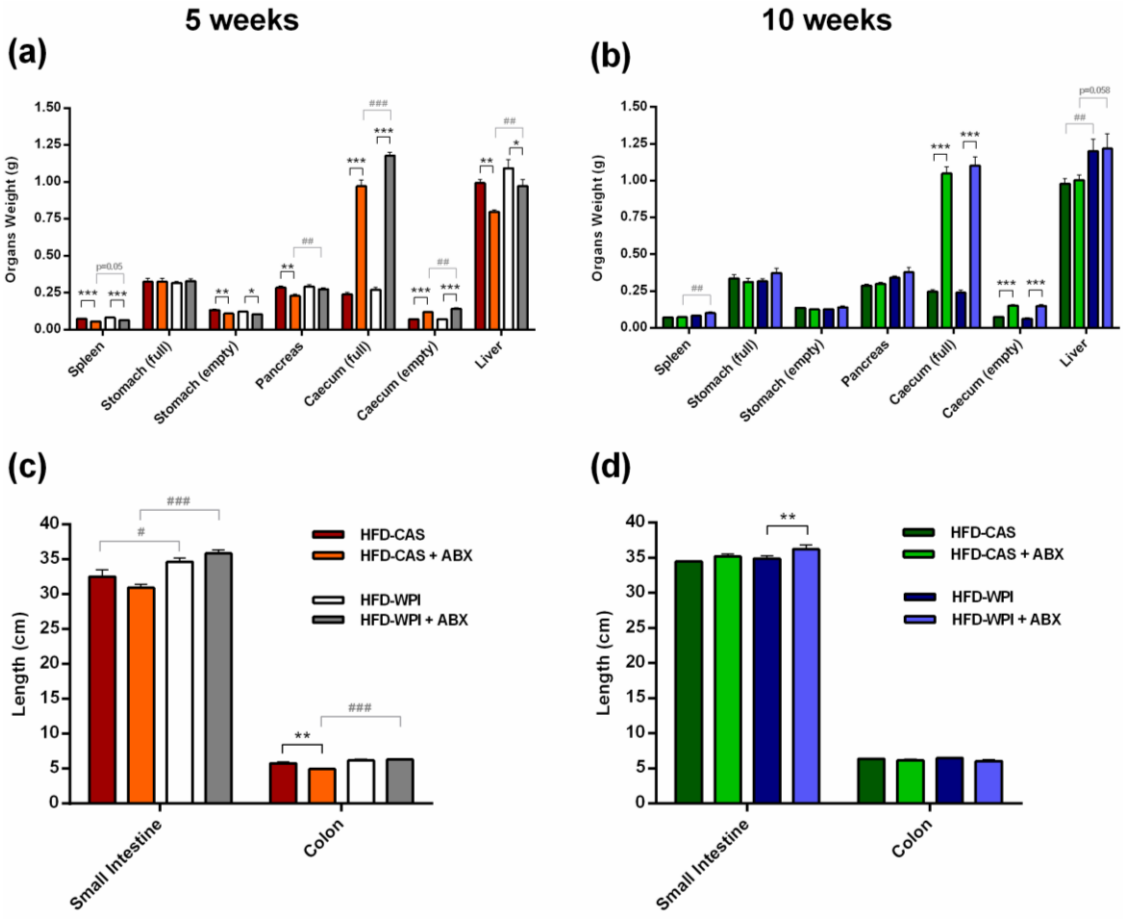
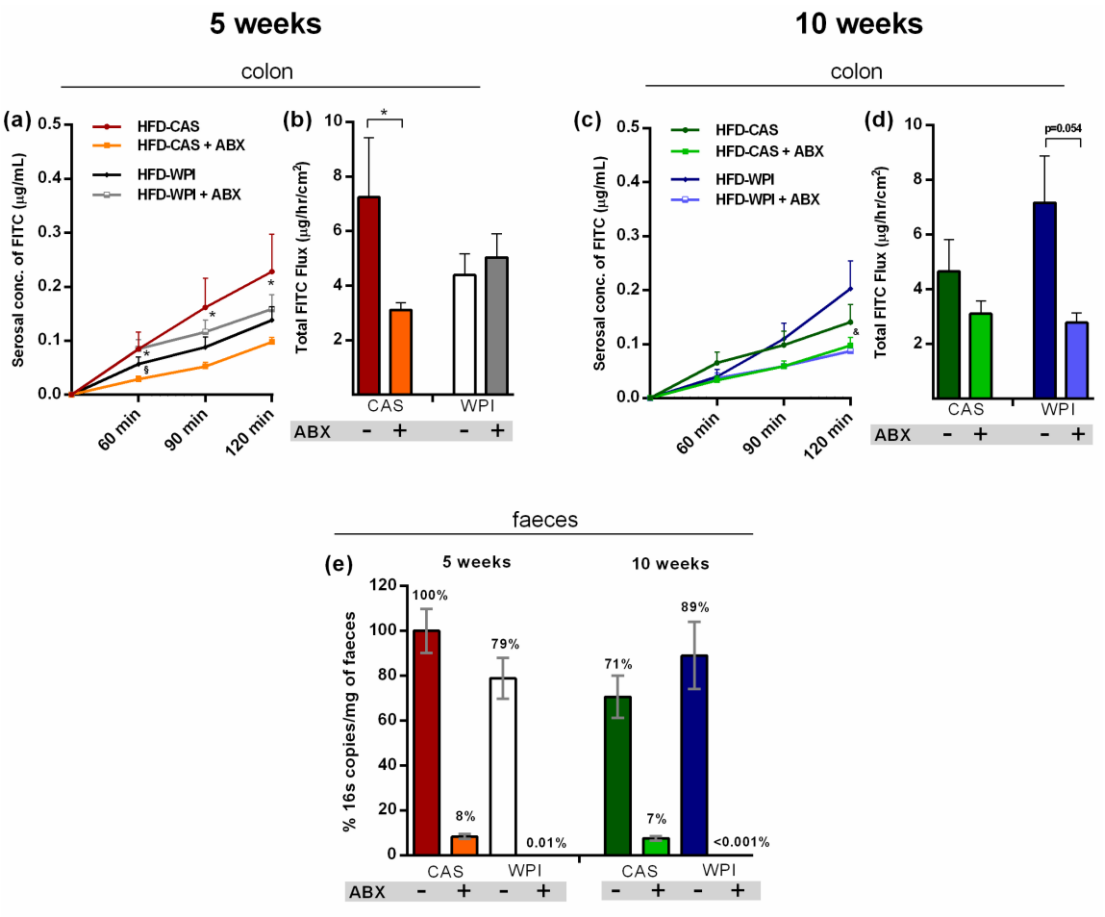


Figure 4.S2 Colonic permeability and faecal bacterial quantification. Data show **(a)** colonic FITC paracellular permeability at 60, 90 and 120 min and **(b)** the total FITC flux that passed through the colonic epithelium over 2 hours, at 5 weeks timepoint. Data also show **(c)** colonic FITC paracellular permeability at 60, 90 and 120 min and **(d)** the total FITC flux that passed through the colonic epithelium over 2 hours, at 10 weeks timepoint. **(e)** % of bacterial yield calculated at % of 16S copies present in 1 mg of faeces at 5 and 10 weeks timepoints (bacterial yield average of HFD-CAS at 5 weeks was considered as 100%; bacterial yield of all the groups was normalized by bacterial yield average of HFD-CAS at 5 weeks). Statistical analysis: **(a, c)** groups showing * (for HFD-CAS vs HFD-CAS+ABX), § (for HFD-CAS+ABX vs HFD-WPI+ABX) and & (for HFD-WPI vs HFD-WPI+ABX) are significant (*/§/& P<0.05 or **/§§/&& P<0.01 or ***/§§§/&&& P<0.001). A complete statistical description is detailed in Methods and Materials, "Supplementary Statistics" and **figures 4.S6** and **4.S7**.



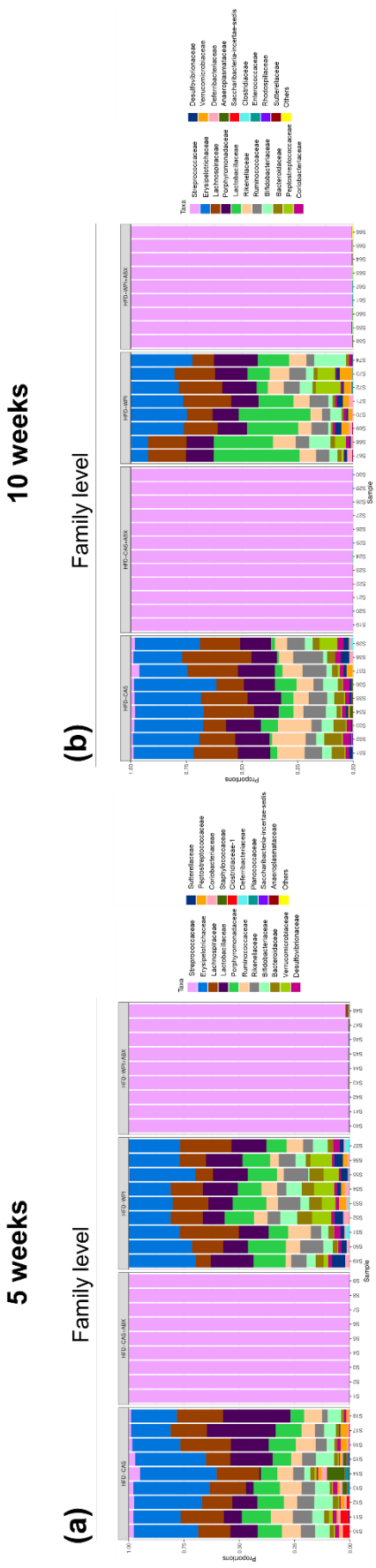


Figure 4.S3 Gut microbiota analysis: further taxonomic differences across the groups. Taxaplot showing families **(a)** at 5 and **(b)** 10 weeks timepoints and genera at **(c)** 5 and **(d)** 10 weeks timepoints within the faecal samples. Data also show the heatmaps representing taxonomic pairwise differences in abundance at phylum level across the groups, using Kruskal Wallis method **(e)** at 5 and **(f)** 10 weeks timepoint. Notably, green and red colours represent an increase and a decrease, respectively, in relative abundance of a specific group (not in brackets) compared to another group (in brackets). White colour indicates no differences between the two groups. The shades of each colour correspond to different p values thresholds. In (b), the phyla that did not show differences at 5 weeks timepoint but that showed differences at 10 weeks timepoint are indicated with a grey background. All the data were measured in mice fed with HFD-CAS and HFD-WPI, both controls and ABX-treated. Groups showing * are significant (*P<0.05 or **P<0.01 or ***P<0.001 or ****P<0.0001). A complete statistical description is detailed in Methods and Materials.

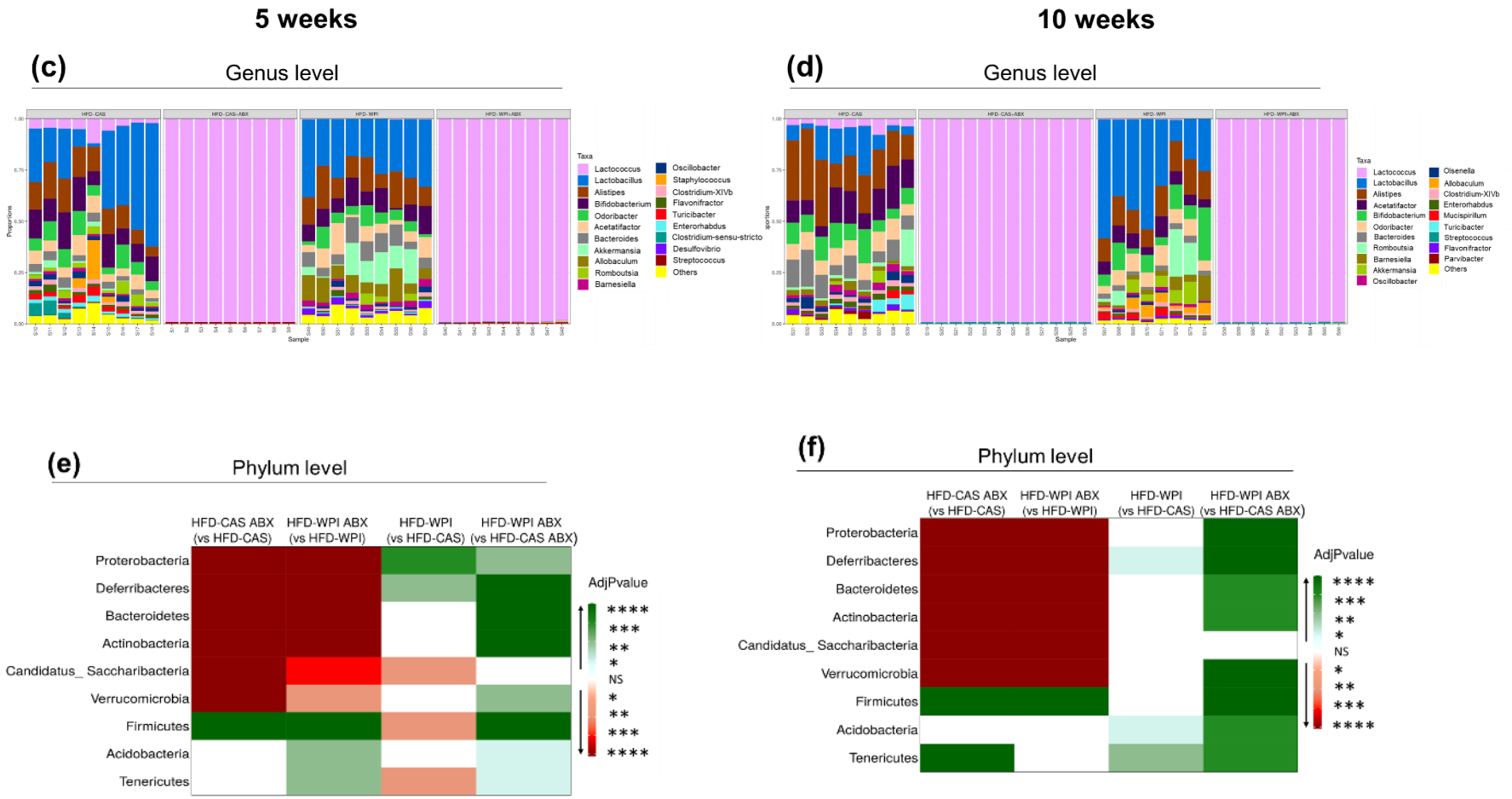

5 weeks
10 weeks
(c)
Genus level
(d)
Genus level
HFD-CAS
HFD-CAS+ABX
HFD-WPI
HFD-WPI+ABX
Proportions
Sample
Taxa
Lactococcus
Lactobacillus
Alistipes
Bifidobacterium
Odoribacter
Acetatifactor
Bacteroides
Akkermansia
Allobaculum
Romboutsia
Barnesiella
Oscillobacter
Staphylococcus
Clostridium-XlVb
Flavonifractor
Turicibacter
Enterorhabdus
Clostridium-sensu-stricto
Desulfovibrio
Streptococcus
Others
Olsenella
Mucispirillum
Parvibacter
(e)
Phylum level
(f)
Phylum level
HFD-CAS ABX (vs HFD-CAS)
HFD-WPI ABX (vs HFD-WPI)
HFD-WPI (vs HFD-CAS)
HFD-WPI ABX (vs HFD-CAS ABX)
Proterobacteria
Deferribacteres
Bacteroidetes
Actinobacteria
Candidatus_ Saccharibacteria
Verrucomicrobia
Firmicutes
Acidobacteria
Tenericutes
AdjPvalue
****
***
**
*
NS
*
**
***
****

Figure 4.S4 Further metabolites changes in the caecum at 10 weeks timepoint. The heatmaps show metabolites measured within the caecum content, where the changes observed across the groups are related to **(a)** both ABX treatment and protein quality. Also reported **(b)** an heatmaps with changes in one pairwise comparison. Notably, orange and blue colours represent an increase and a decrease, respectively, in abundance of group 1 compared to group 2 (below in each heatmap: Group1 vs Group 2). On the left side of the heatmaps are indicated the categories in which the metabolites belong to. *Abbreviations:* (8aR,12S,12aR)-12-H-4-m-4,5,6,7,8,8a,12,12a-o-2H-3-b-2,9(1H)-d; (8aR,12S,12aR)-12-Hydroxy-4-methyl-4,5,6,7,8,8a,12,12a-octahydro-2H-3-benzoxecine-2,9(1H)-dione. Groups showing * are significant (* $P<0.05$ or ** $P<0.01$ or *** $P<0.001$) and $ indicates a trend ($0.05< P >0.07$). A complete statistical description is detailed in Methods and Materials.

Caecum metabolomics (10 weeks)

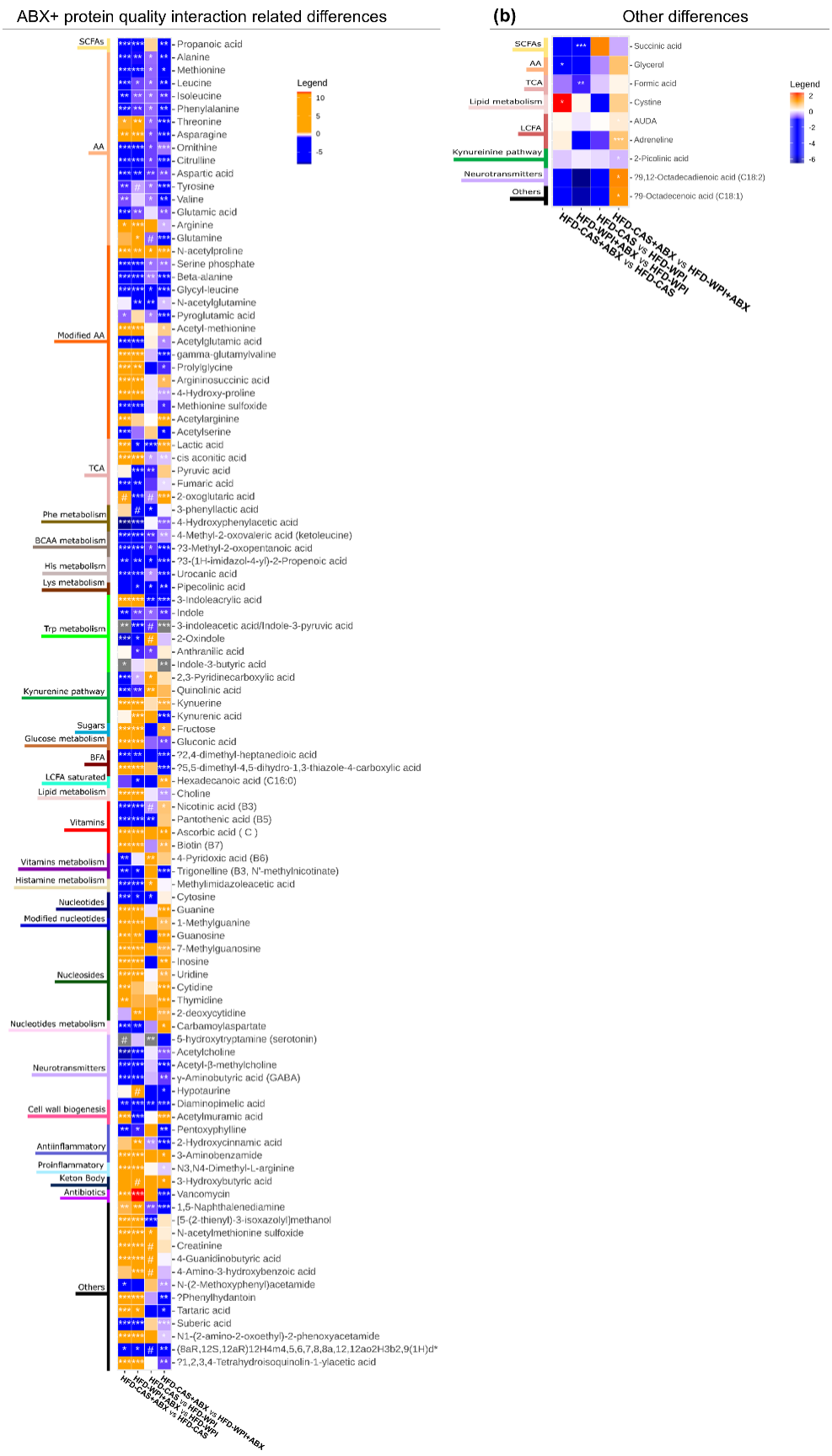


Figure 4.S5 Two-way ANOVA - Organs and tissues weight statistics. Complete statistical description of organs and tissues absolute weight data **(a)** after 5 weeks and **(b)** 10 weeks of intervention.

(a)

	ANOVA			
	Organ/Tissue (5 weeks)	**Treatment (ABX vs Ctrl)**	**Protein Type (CAS vs WPI)**	**Interaction**
Absolute	**eWAT**	$F_{(1;31)} = 30.09$, $p < 0.001$	$F_{(1;31)} = 23.08$, $p < 0.001$	$F_{(1;31)} = 16.03$, $p < 0.001$
	sWAT	$F_{(1;31)} = 11.07$, $p = 0.002$	$F_{(1;31)} = 29.90$, $p < 0.001$	$F_{(1;31)} = 14.16$, $p = 0.001$
	BAT	$F_{(1;32)} = 9.70$, $p=0.004$	$F_{(1;32)} = 0.09$, $p=0.77$	$F_{(1;32)} = 4.49$, $p=0.042$
	rAT	$F_{(1;32)} = 20.99$, $p < 0.001$	$F_{(1;32)} = 33.61$, $p < 0.001$	$F_{(1;32)} = 13.99$, $p = 0.001$
	mAT	$F_{(1;32)} = 37.05$, $p < 0.001$	$F_{(1;32)} = 13.06$, $p = 0.001$	$F_{(1;32)} = 20.56$, $p < 0.001$
	Spleen	$F_{(1;32)} = 21.14$, $p < 0.001$	$F_{(1;32)} = 6.73$, $p = 0.014$	$F_{(1;32)} = 0.05$, $p=0.82$
	Stomach (full)	$F_{(1;31)} = 0.08$, $p=0.77$	$F_{(1;31)} = 0.015$, $p=0.90$	$F_{(1;31)} = 0.08$, $p=0.77$
	Stomach (empty)	$F_{(1;31)} = 15.62$, $p < 0.001$	$F_{(1;31)} = 1.87$, $p=0.18$	$F_{(1;31)} = 0.43$, $p=0.51$
	Pancreas	$F_{(1;31)} = 10.03$, $p = 0.003$	$F_{(1;31)} = 5.37$, $p=0.025$	$F_{(1;31)} = 2.94$, $p=0.10$
	Caecum (empty)	$F_{(1;32)} = 158.36$, $p < 0.001$	$F_{(1;32)} = 5.63$, $p=0.024$	$F_{(1;32)} = 5.52$, $p=0.025$
	Liver	$F_{(1;32)} = 16.37$, $p < 0.001$	$F_{(1;32)} = 13.06$, $p = 0.001$	$F_{(1;32)} = 0.93$, $p=0.34$
	Colon Length	$F_{(1;30)} = 5.03$, $p=0.032$	$F_{(1;30)} = 29.62$, $p < 0.001$	$F_{(1;30)} = 9.05$, $p=0.005$
	SI Length	$F_{(1;32)} = 0.06$, $p=0.8$	$F_{(1;32)} = 27.87$, $p < 0.001$	$F_{(1;32)} = 4.33$, $p=0.045$

(b)

	ANOVA			
	Organ/Tissue (10 weeks)	**Treatment (ABX vs Ctrl)**	**Protein Type (CAS vs WPI)**	**Interaction**
Absolute	**eWAT**	$F_{(1;33)} = 26.80$, $p < 0.001$	$F_{(1;33)} = 24.70$, $p < 0.001$	$F_{(1;33)} = 0.09$, $p=0.76$
	sWAT	$F_{(1;33)} = 28.53$, $p < 0.001$	$F_{(1;33)} = 31.58$, $p < 0.001$	$F_{(1;33)} = 0.34$, $p=0.56$
	Stomach Full	$F_{(1;34)} = 0.35$, $p=0.56$	$F_{(1;34)} = 0.66$, $p=0.42$	$F_{(1;34)} = 2.46$, $p=0.13$
	Caecum Full	$F_{(1;34)} = 408.06$, $p < 0.001$	$F_{(1;34)} = 0.33$, $p=0.57$	$F_{(1;34)} = 0.58$, $p=0.45$
	Caecum Empty	$F_{(1;34)} = 119.56$, $p < 0.001$	$F_{(1;34)} = 0.84$, $p=0.36$	$F_{(1;34)} = 0.47$, $p=0.49$
	Colon Length	$F_{(1;34)} = 4.21$, $p < 0.05$	$F_{(1;34)} = 0.004$, $p=0.95$	$F_{(1;34)} = 0.74$, $p=0.39$
	SI Length	$F_{(1;33)} = 6.06$, $p < 0.05$	$F_{(1;33)} = 3.13$, $p=0.09$	$F_{(1;33)} = 0.54$, $p=0.47$

Figure 4.S6 Two-way ANOVA – Plasma analysis, gene expression (ileum, eWAT, Hypothalamus) and FITC flux statistics. Complete statistical description of plasma analysis, gene expression and FITC flux data **(a)** after 5 weeks and **(b)** 10 weeks of intervention.

(a)

		ANOVA		
	5 weeks	**Treatment (ABX vs Ctrl)**	**Protein Type (CAS vs WPI)**	**Interaction**
Plasma	**MCP-1**	$F_{(1;32)}$ = 0.012, p=0.91	$F_{(1;32)}$ = 6.40, p=0.02	$F_{(1;32)}$ = 0.68, p=0.41
	TNF-α	$F_{(1;31)}$ = 10.19, p=0.003	$F_{(1;31)}$ = 2.60, p=0.12	$F_{(1;31)}$ = 1.43, p=0.24
	Leptin	$F_{(1;29)}$ = 11.18, p=0.002	$F_{(1;29)}$ = 36.92, p < 0.001	$F_{(1;29)}$ = 10.74, p=0.003
	Insulin	$F_{(1;29)}$ = 2.03, p=0.16	$F_{(1;29)}$ = 0.07, p=0.79	$F_{(1;29)}$ = 2.61, p=0.12
	LBP	$F_{(1;31)}$ = 0.03, p=0.96	$F_{(1;31)}$ = 0.13, p=0.72	$F_{(1;31)}$ = 0.13, p=0.08
Ileum	***Il1β***	$F_{(1;35)}$ = 3.55, p=0.07	$F_{(1;35)}$ = 27.73, p < 0.001	$F_{(1;35)}$ = 1.59, p=0.22
	Tjp1	$F_{(1;35)}$ = 1.63, p=0.21	$F_{(1;35)}$ = 20.91, p < 0.001	$F_{(1;35)}$ = 0.78, p=0.38
	F11	$F_{(1;35)}$ = 1.55, p=0.22	$F_{(1;35)}$ = 12.99, p=0.001	$F_{(1;35)}$ = 0.92, p=0.34
	Cldn1	$F_{(1;35)}$ = 9.57, p=0.004	$F_{(1;35)}$ = 2.91, p=0.09	$F_{(1;35)}$ = 1.05, p=0.31
	Ocln	$F_{(1;35)}$ = 6.45, p=0.016	$F_{(1;35)}$ = 9.36, p=0.004	$F_{(1;35)}$ = 2.06, p=0.16
eWAT	***TNF-alpha***	$F_{(1;34)}$ = 9.34, p=0.005	$F_{(1;34)}$ = 0.82, p=0.37	$F_{(1;34)}$ = 24.37, p < 0.001
	Adipoq	$F_{(1;35)}$ = 0.33, p=0.57	$F_{(1;35)}$ = 4.82, p=0.036	$F_{(1;35)}$ = 1.84, p=0.18
	Leptin	$F_{(1;34)}$ = 26.05, p < 0.001	$F_{(1;34)}$ = 21.19, p < 0.001	$F_{(1;34)}$ = 23.93, p < 0.001
	CD68	$F_{(1;35)}$ = 2.29, p=0.14	$F_{(1;35)}$ = 0.40, p=0.53	$F_{(1;35)}$ = 10.76, p=0.003
Hypothalamus	***Npy***	$F_{(1;35)}$ = 9.65, p=0.004	$F_{(1;35)}$ = 1.34, p=0.25	$F_{(1;35)}$ = 0.09, p=0.77
	Pomc	$F_{(1;33)}$ = 0.06, p=0.8	$F_{(1;33)}$ = 2.63, p=0.11	$F_{(1;33)}$ = 3.11, p=0.088
	Gr	$F_{(1;34)}$ = 3.96, p=0.056	$F_{(1;34)}$ = 6.55, p=0.016	$F_{(1;34)}$ = 0.44, p=0.51
	Socs3	$F_{(1;34)}$ = 5.86, p=0.022	$F_{(1;34)}$ = 0.55, p=0.46	$F_{(1;34)}$ = 2.36, p=0.13
	Cb1	$F_{(1;34)}$ = 1.08, p=0.31	$F_{(1;34)}$ = 5.93, p=0.021	$F_{(1;34)}$ = 1.65, p=0.21
	Cartpt	$F_{(1;34)}$ = 5.22, p=0.029	$F_{(1;34)}$ = 12.52, p=0.001	$F_{(1;34)}$ = 8.80, p=0.006
	Crh	$F_{(1;34)}$ = 0.88, p=0.36	$F_{(1;34)}$ = 6.09, p=0.019	$F_{(1;34)}$ = 0.02, p=0.88
FITC Flux	**Colon**	$F_{(1;30)}$ = 2.18, p=0.15	$F_{(1;30)}$ = 0.15, p=0.7	$F_{(1;30)}$ = 4.09, p=0.052

(b)

		ANOVA		
	10 weeks	**Treatment (ABX vs Ctrl)**	**Protein Type (CAS vs WPI)**	**Interaction**
Plasma	**TNF-α**	$F_{(1;33)}$ = 1.42, p=0.24	$F_{(1;33)}$ = 0.27, p=0.61	$F_{(1;33)}$ = 1.47, p=0.23
	Insulin	$F_{(1;31)}$ = 5.95, p=0.02	$F_{(1;31)}$ = 0.007, p=0.94	$F_{(1;31)}$ = 2.35, p=0.13
	LPB	$F_{(1;33)}$ = 5.11, p=0.03	$F_{(1;33)}$ = 7.55, p=0.01	$F_{(1;33)}$ = 0.001, p=0.97
Ileum	***Il1β***	$F_{(1;35)}$ = 0.18, p=0.67	$F_{(1;35)}$ = 3.05, p=0.09	$F_{(1;35)}$ = 1.35, p=0.25
	Tjp1	$F_{(1;37)}$ = 0.03, p=0.87	$F_{(1;37)}$ = 4.36, p=0.044	$F_{(1;37)}$ = 0.92, p=0.34
	F11	$F_{(1;37)}$ = 5.94, p=0.02	$F_{(1;37)}$ = 2.92, p=0.09	$F_{(1;37)}$ = 0.02, p=0.89
	Cldn1	$F_{(1;35)}$ = 19.25, p < 0.001	$F_{(1;35)}$ = 2.46, p=0.13	$F_{(1;35)}$ = 2.83, p=0.10
	Ocln	$F_{(1;36)}$ = 3.54, p=0.07	$F_{(1;36)}$ = 9.07, p=0.005	$F_{(1;36)}$ = 0.86, p=0.36
eWAT	***MCP1***	$F_{(1;35)}$ = 12.77, p=0.001	$F_{(1;35)}$ = 42.41, p < 0.001	$F_{(1;35)}$ = 4.71, p=0.038
	Adipoq	$F_{(1;37)}$ = 1.56, p=0.22	$F_{(1;37)}$ = 1.32, p=0.26	$F_{(1;37)}$ = 0.06, p=0.81
	Leptin	$F_{(1;35)}$ = 22.29, p < 0.001	$F_{(1;35)}$ = 19,73, p < 0.001	$F_{(1;35)}$ = 1.02, p=0.32
	CD68	$F_{(1;36)}$ = 28.22, p < 0.001	$F_{(1;36)}$ = 40.52, p < 0.001	$F_{(1;36)}$ = 11.65, p=0.002
Hypothalamus	***Pomc***	$F_{(1;36)}$ = 0.06, p=0.81	$F_{(1;36)}$ = 4.07, p=0.052	$F_{(1;36)}$ = 2.83, p=0.10
	Cartpt	$F_{(1;37)}$ = 19.09, p < 0.001	$F_{(1;37)}$ = 3.13, p=0.086	$F_{(1;37)}$ = 1.56, p=0.22
	Crh	$F_{(1;37)}$ = 8.48, p=0.006	$F_{(1;37)}$ = 0.851, p=0.36	$F_{(1;37)}$ = 0.46, p=0.50
FITC Flux	**Ileum**	$F_{(1;24)}$ = 3.37, p = 0.079	$F_{(1;24)}$ = 1.44, p = 0.24	$F_{(1;24)}$ = 3.74, p=0.065
	Colon	$F_{(1;30)}$ = 10.61, p=0.003	$F_{(1;30)}$ = 1.42, p=0.24	$F_{(1;30)}$ = 2.39, p=0.13

Figure 4.S7 Non-parametric test Kruskal Wallis & Mann Whitney – Organs and tissues, plasma analysis, gene expression (ileum, eWAT, Hypothalamus) and FITC flux statistics. Complete statistical description **(a)** of organs and tissues absolute weight data and **(b)** plasma analysis, gene expression and FITC flux, after 5 weeks and 10 weeks of intervention. 1 vs 2 for HFD-CAS+ABX vs HFD-WPI+ABX, 3 vs 4 for HFD-CAS vs HFD-CAS+ABX, 1 vs 3 for HFD-CAS vs HFD-WPI and 2 vs 4 for HFD-WPI vs HFD-WPI+ ABX.

(a)

		Non parametric test (Kruskal Wallis & Mann Withney)					
	Organ/Tissue	for the effect of the ABX treatment	for the effect of the protein type	1 VS 2	3 VS 4	1 VS 3	2 VS 4
Absolute	Caecum (full) (5 weeks)	$\chi^2_{(1)} = 24.69$, p<0.001	$\chi^2_{(1)} = 1.81$, p=0.18	$U_{(16)} = 1.00$, p<0.001	$U_{(18)} = 0.00$, p<0.001	NS	$U_{(22)} = 13.5$, p=0.004
	BAT (10 weeks)	$\chi^2_{(1)} = 3.75$, p=0.053	$\chi^2_{(1)} = 4.91$, p<0.05	$U_{(21)} = 21.50$, p=0.018	NS	NS	NS
	rAT (10 weeks)	$\chi^2_{(1)} = 9.85$, p<0.01	$\chi^2_{(1)} = 9.85$, p<0.001	$U_{(20)} = 0.00$, p<0.001	$U_{(21)} = 13.00$, p=0.002	$U_{(17)} = 8.00$, p=0.006	$U_{(16)} = 1.00$, p<0.001
	mAT (10 weeks)	$\chi^2_{(1)} = 9.78$, p<0.01	$\chi^2_{(1)} = 12.73$, p<0.001	$U_{(21)} = 2.00$, p<0.001	$U_{(21)} = 17.00$, p=0.007	$U_{(17)} = 10.00$, p=0.011	$U_{(17)} = 4.00$, p=0.001
	Spleen (10 weeks)	$\chi^2_{(1)} = 0.52$, p=0.47	$\chi^2_{(1)} = 9.59$, p<0.01	$U_{(20)} = 14.00$, p=0.006	NS	NS	NS
	Stomach Empty (10 weeks)	$\chi^2_{(1)} = 0.39$, p=0.53	$\chi^2_{(1)} = 0.23$, p=0.63	NS	NS	NS	NS
	Pancreas (5 weeks)	$\chi^2_{(1)} = 0.25$, p=0.62	$\chi^2_{(1)} = 12.41$, p<0.001	$U_{(21)} = 21.50$, p=0.008	NS	$U_{(17)} = 8.00$, p=0.006	NS
	Liver (10 weeks)	$\chi^2_{(1)} = 0.17$, p=0.67	$\chi^2_{(1)} = 8.21$, p<0.01	$U_{(21)} = 27.50$, p=0.058	NS	$U_{(17)} = 12.00$, p=0.038	NS

(b)

		Non parametric test (Kruskal Wallis & Mann Withney)					
	Organ/Tissue	for the effect of the ABX treatment	for the effect of the protein type	1 VS 2	3 VS 4	1 VS 3	2 VS 4
Plasma	MCP-1 (10 weeks)	$\chi^2_{(1)} = 2.44$, p=0.12	$\chi^2_{(1)} = 11.98$, p<0.05	NS	NS	$U_{(16)} = 0.00$, p<0.001	$U_{(16)} = 9.00$, p=0.016
	Leptin (10 weeks)	$\chi^2_{(1)} = 5.05$, p<0.01	$\chi^2_{(1)} = 15.82$, p<0.001	$U_{(21)} = 6.00$, p<0.001	$U_{(18)} = 12.00$, p=0.015	$U_{(13)} = 0.00$, p=0.001	$U_{(15)} = 10.00$, p=0.05
Ileum	*Mcp1* (5 weeks)	$\chi^2_{(1)} = 13.46$, p<0.001	$\chi^2_{(1)} = 2.36$, p=0.12	NS	$U_{(18)} = 0.00$, p<0.001	$U_{(17)} = 11.50$, p=0.015	NS
	Mcp1 (10 weeks)	$\chi^2_{(1)} = 3.20$, p=0.07	$\chi^2_{(1)} = 4.55$, p<0.05	NS	$U_{(20)} = 8.00$, p=0.001	$U_{(17)} = 4.00$, p=0.001	NS
eWAT	*Mcp1* (5 weeks)	$\chi^2_{(1)} = 3.42$, p=0.065	$\chi^2_{(1)} = 8.25$, p=0.004	NS	$U_{(17)} = 7.00$, p=0.01	$U_{(18)} = 5.00$, p=0.01	NS
	TNF-alpha (10 weeks)	$\chi^2_{(1)} = 5.29$, p<0.05	$\chi^2_{(1)} = 5.29$, p<0.01	NS	$U_{(21)} = 7.50$, p<0.001	$U_{(17)} = 2.00$, p<0.001	NS
Hypothalamus	*Npy* (10 weeks)	$\chi^2_{(1)} = 3.93$, p<0.05	$\chi^2_{(1)} = 4.53$, p<0.05	$U_{(21)} = 21.00$, p=0.018	NS	NS	$U_{(17)} = 15.00$, p=0.046
	Gr (10 weeks)	$\chi^2_{(1)} = 0.06$, p=0.81	$\chi^2_{(1)} = 5.57$, p<0.05	NS	NS	$U_{(16)} = 3.00$, p=0.001	NS
	Socs3 (10 weeks)	$\chi^2_{(1)} = 13.60$, p<0.001	$\chi^2_{(1)} = 1.20$, p=0.27	NS	$U_{(20)} = 14.00$, p=0.006	NS	$U_{(17)} = 11.00$, p=0.015
	Cbl (10 weeks)	$\chi^2_{(1)} = 9.59$, p<0.01	$\chi^2_{(1)} = 0.37$, p=0.54	NS	$U_{(20)} = 19.00$, p=0.025	NS	$U_{(17)} = 14.00$, p=0.036
FITC Flux	Ileum (5 weeks)	$\chi^2_{(1)} = 3.88$, p<0.05	$\chi^2_{(1)} = 1.40$, p=0.24	$U_{(21)} = 11.00$, p=0.054	NS	NS	NS

Supplemental Statistics

Repeated Measurement

Delta body weight 5 weeks: $F_{(5;160)} = 610.12$, $p < 0.001$ for the effect of the time, $F_{(1;32)} = 11.44$, $p < 0.01$ for the effect of the treatment, $F_{(1;32)} = 5.25$, $p < 0.05$ for the effect of the protein type, $F_{(1;32)} = 3.93$, $p = 0.056$ for the effect of the treatment x protein type interaction, $F_{(5;160)} = 12.15$, $p < 0.001$ for the effect of the time x treatment interaction, $F_{(5;160)} = 6.00$, $p < 0.01$ for the effect of the time x protein type interaction, $F_{(5;160)} = 4.48$, $p < 0.05$ for the effect of the time x treatment x protein type interaction.

Delta body weight 10 weeks: $F_{(10;340)} = 571.68$, $p < 0.001$ for the effect of the time, $F_{(1;34)} = 4.54$, $p < 0.05$ for the effect of the treatment, $F_{(1;34)} = 6.44$, $p < 0.05$ for the effect of the protein type, $F_{(1;34)} = 0.99$, $p = 0.33$ for the effect of the treatment x protein type interaction, $F_{(10;340)} = 6.96$, $p < 0.01$ for the effect of the time x treatment interaction, $F_{(10;340)} = 14.85$, $p < 0.001$ for the effect of the time x protein type interaction, $F_{(10;340)} = 1.42$, $p = 0.25$ for the effect of the time x treatment x protein type interaction.

Serosal FITC concentration ileum 5 weeks: $F_{(3;78)} = 36.49$, $p < 0.001$ for the effect of the minute passed, $F_{(1;26)} = 5.92$, $p < 0.05$ for the effect of the treatment, $F_{(1;26)} = 0.30$, $p = 0.59$ for the effect of the protein type, $F_{(1;26)} = 0.57$, $p = 0.46$ for the effect of the treatment x protein type interaction, $F_{(3;78)} = 7.75$, $p < 0.01$ for the effect of the minutes passed x treatment interaction, $F_{(3;78)} = 0.25$, $p = 0.63$ for the effect of the minutes passed x protein type interaction, $F_{(3;78)} = 0.61$, $p = 0.45$ for the effect of the minutes passed x treatment x protein type interaction.

Serosal FITC concentration ileum 10 weeks: $F_{(3;75)} = 39.27$, $p < 0.001$ for the effect of the minute passed, $F_{(1;25)} = 1.21$, $p = 0.28$ for the effect of the treatment, $F_{(1;25)} = 1.43$, $p = 0.24$ for the effect of the protein type, $F_{(1;25)} = 3.61$, $p = 0.07$ for the effect of the treatment x protein type interaction, $F_{(3;75)} = 1.01$, $p = 0.35$ for the effect of the minutes passed x treatment interaction, $F_{(3;75)} = 2.99$, $p = 0.084$ for the effect of the minutes passed x protein type interaction, $F_{(3;75)} = 2.28$, $p = 0.13$ for the effect of the minutes passed x treatment x protein type interaction.

Serosal FITC concentration colon 5 weeks: $F_{(3;90)} = 62.16$, $p < 0.001$ for the effect of the minute passed, $F_{(1;30)} = 1.71$, $p = 0.20$ for the effect of the treatment, $F_{(1;30)} = 0.003$, $p = 0.95$ for the

effect of the protein type, $F_{(1;30)} = 4.94$, $p < 0.05$ for the effect of the treatment x protein type interaction, $F_{(3;90)} = 2.24$, $p = 0.14$ for the effect of the minutes passed x treatment interaction, $F_{(3;90)} = 0.51$, $p = 0.50$ for the effect of the minutes passed x protein type interaction, $F_{(3;90)} = 4.21$, $p < 0.05$ for the effect of the minutes passed x treatment x protein type interaction.

Serosal FITC concentration colon 10 weeks: $F_{(3;93)} = 73.25$, $p < 0.001$ for the effect of the minute passed, $F_{(1;31)} = 6.18$, $p < 0.05$ for the effect of the treatment, $F_{(1;31)} = 0.059$, $p = 0.81$ for the effect of the protein type, $F_{(1;31)} = 0.15$, $p = 0.7$ for the effect of the treatment x protein type interaction, $F_{(3;93)} = 7.38$, $p < 0.01$ for the effect of the minutes passed x treatment interaction, $F_{(3;93)} = 1.14$, $p = 0.31$ for the effect of the minutes passed x protein type interaction, $F_{(3;93)} = 2.23$, $p = 0.14$ for the effect of the minutes passed x treatment x protein type interaction.

Chapter 5

General Discussion

5.1 Overview and Summary

In this thesis, I explored the multiple effects exerted by bovine WP (as a whole and specific WP, i.e., LAB) in the context of HFD-induced obesity in male mice (C57BL/6J) between 5-weeks and 15-weeks old. Additionally, I investigated the possibility of a relationship amongst various WP-related anti-obesity parameters, with a focus on those involved in controlling weight gain.

Our data showed that HFD containing the specific WP, LAB, as the protein source altered energy balance and gut-related functions, such as intestinal nutrient absorption and gut microbiota composition (*Chapter 2*). Specifically, mice fed with HFD-LAB for 10 weeks had a similar body weight and energy expenditure compared to controls, HFD-CAS-fed mice, but showed an increase in cumulative energy intake. This energy imbalance suggested a loss of energy in the presence of LAB within the diet. In the small intestine, we observed a decrease in *Glut2* and *Cd36* (i.e., glucose and fatty acid transporters, respectively) expression within the ileum and a decrease and a trend towards an increase, respectively, in *Cd36* and *Lat4* (an essential amino acids transporter) expression within the jejunum of mice fed with HFD-LAB. These results suggested that, following LAB consumption, changes in gene expression of important intestinal nutrient transporters within the small intestine could be one of the causes of the energy mismatch observed in HFD-fed mice. The presence of LAB within HFD significantly affected the gut microbiota configuration. Beta-diversity of HFD-LAB-fed mice showed a significant separation from HFD-CAS-fed mice and, at genus level, the relative abundances of *Parabacteroides*, *Bifidobacterium*, *Parvibacter* and *Lactobacillus* were significantly higher in the HFD-LAB group. Together these data highlight the specificity with which WP components act to alter different aspects of energy balance.

In *Chapter 3*, we examined WPI as a whole and we focused on the effect of WPI given with HFD on energy balance, lipid metabolism and gut-related functions at two different stages of life (i.e., adolescence and early adulthood). A 5-week HFD-WPI dietary intervention carried out in younger mice (from 5 to 10-weeks old) caused a

decrease in body weight gain and adiposity. In addition, differences in enzymes involved in lipid catabolism were found. In particular, *Cpt1a* and *Ucp2* expression increased and *Hsl* and *Lpl* expression decreased within the eWAT of adolescent HFD-WPI-fed mice. These data suggest that the presence of WPI decreases adiposity, possibly through a modulation of lipid metabolism within the adipose tissue. In addition, WPI caused a reduction in plasma level of leptin and *Ob* expression (i.e., gene that codes for leptin) within eWAT in younger mice fed with WPI, which is a sign of attenuation of the metabolic syndrome caused by HFD. A decrease in *Cd68* expression within the eWAT in younger HFD-WPI-fed mice also suggests a lower macrophage recruitment in the adipose tissue. Through gut microbiota analysis, we observed a significant separation in beta-diversity at species level between HFD-WPI and HFD-CAS younger mice. Moreover, *Lactobacillus murinus* relative abundance and 49 *L. murinus*-related functions were more abundant in the HFD-WPI group.

All these changes, observed after dietary intervention in younger mice, were not observed in mice that started the 5-week HFD-WPI dietary intervention at an older age (from 10- to 15-weeks old).

These results suggest that WPI, when given as part of HFD, impacts differently on body weight, adipose tissue and gut microbiota, depending on the stage of life during which the diet is provided.

Following on from the results found in *Chapter 3*, we next examined (*Chapter 4*) if WPI exerts a beneficial effect on HFD-induced metabolic and physiological dysregulations through the modulation of the gut microbiota. HFD-WPI or HFD-CAS-fed mice were treated with ABX to deplete the gut microbiota for a short-(5 weeks) or long-term (10 weeks) intervention. HFD-WPI-fed mice, both ABX-treated and non-treated, showed a reduction in body weight gain, adiposity, leptin availability and gene expression of selected pro-inflammatory markers (MCP-1, TNFα and CD68) within the eWAT and ileum, compared to non-treated HFD-CAS-fed mice. In particular, WPI and ABX had an additive effect in lowering adiposity and leptin production in HFD-WPI+ABX mice that underwent long-term intervention. These data indicated that WPI acts on body weight and adipose-related functions in a gut microbiota-independent manner in HFD-fed mice. Conversely, WPI supplementation protected from HFD-

induced ileal permeability disruption and high MCP-1 level in the plasma and these effects were abolished by ABX administration. These results suggest that WPI action on gut permeability and systemic inflammation required an intact gut microbiota. Gut microbiota composition and caecum metabolomic analysis showed differences between HFD-WPI and HFD-CAS-fed mice, both non-treated and, surprisingly, ABX-treated. In this regard, WPI modulated the gut microbiota differently compared to CAS, even in a microbiota depleted condition.

Altogether, these results demonstrate that WPI beneficially modulates HFD-induced obesity dysfunctions in different ways, which can be gut microbiota-dependent or independent.

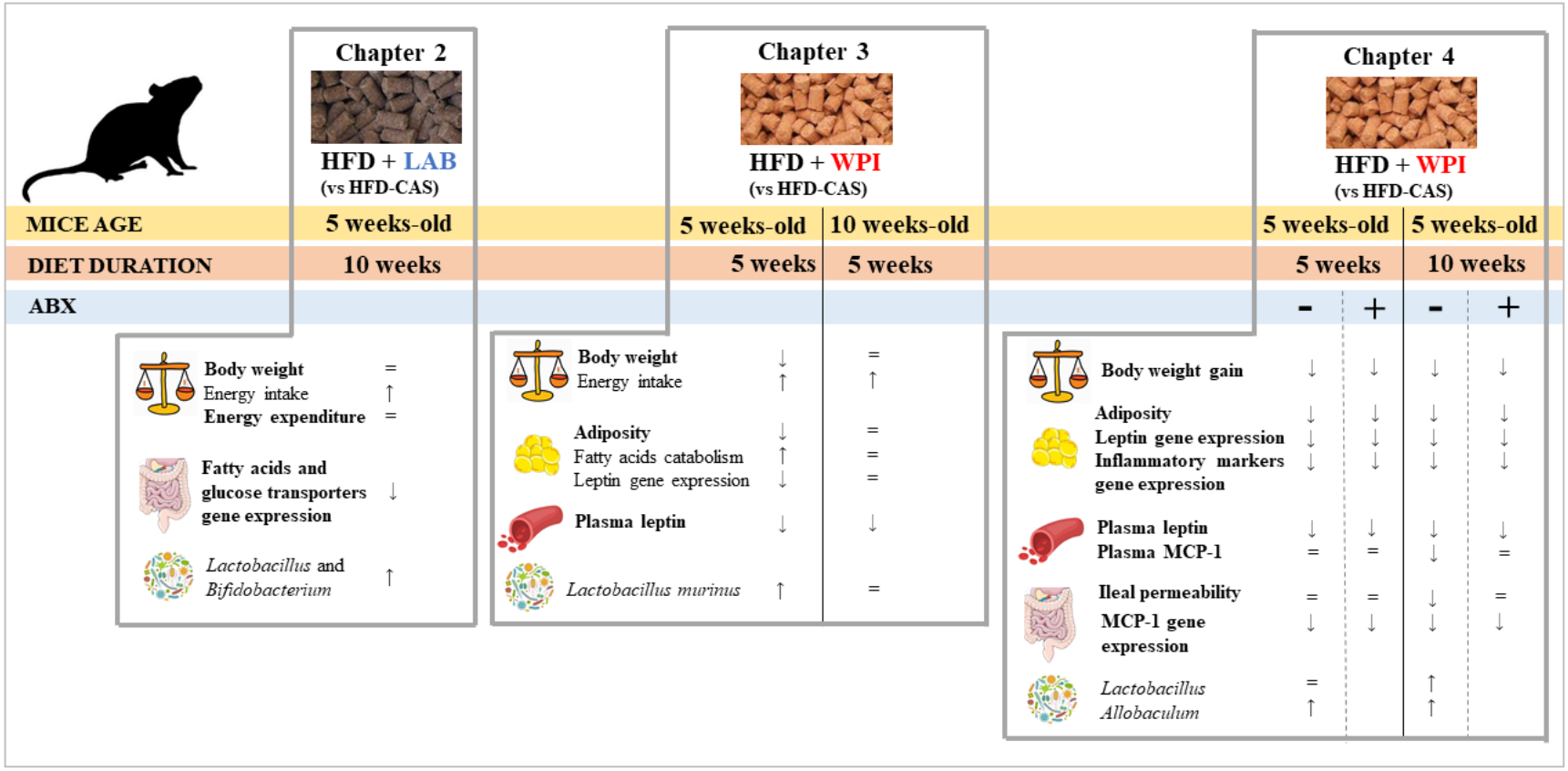


Figure 5.1 Summary of the findings of this thesis. *Abbreviations: HFD, high-fat diet; LAB, alpha-lactalbumin; WPI, whey protein isolate; CAS, casein; ABX, antibiotic cocktail; MCP-1, monocyte chemoattractant protein 1.*

5.2 Whey protein: a dietary strategy to fight obesity

Obesity has become a global healthcare challenge of the 21st century, with worldwide obesity levels increasing 4-fold in the past 20 years. Data from Irish Health Service Executive (HSE) show that only 37% of Irish population have a normal weight, whereas 37% are overweight and 23% obese (www.hse.ie). Other countries, such as USA, UK and Australia, show a similar situation, as reported by the World Health Organization (WHO) (www.who.int). The fact that more than half of the population in these countries is overweight or obese means that this condition represents the "new normal". This phenomenon has a huge personal and financial cost because of the wide list of health complications that come together with obesity, which reduces the life expectancy, and, consequently, increases treatment and hospitalization costs. This highlights why we must be concerned and drive the scientific effort to overcome the problem.

The most common approach adopted to lose body weight is through dietary strategies, which include a wide range of diet programs that allow the consumption of fewer calories compared to the calories expended. However, these approaches have yielded limited results because of the adaptions of energy balance-related mechanisms to counteract energy deficits, such as changes in hypothalamic neuropeptides that seek to drive food searching behavior and over-eating when food becomes available. In recent decades, studies have focused on the importance of macronutrients in the diet and changing their proportions to manipulate energy balance in a way that could overcome the calorie deficit. It has emerged that, in a situation of equal calorie intake, diets with a high content of protein are more efficient in decreasing body fat and increasing lean mass. However, this effect can be different depending on the protein source. In this regard, WP has been emerged as a good candidate because it is a rich source of essential amino acids, including BCAA, which are responsible for rapid digestibility and positive effects on metabolism and adiposity (Siddik & Shin 2019).
This thesis focuses on the multi-organ effects of WP supplementation (present in a standard amount of 20% of energy) in mice under HFD-induced obesity condition. The implications of our findings will be discussed in this section.

5.2.1 Whey protein consumption impacts on energy balance, gut physiology and the gut microbiota during obesity

It has been shown many times that WP exerts a potent satiety effect accompanied by an increase in plasmatic anorexigenic hormones and a decrease in plasmatic orexigenic hormones produced along the GIT, both in lean humans and rodents (Hall et al 2003, McAllan et al 2015, Pal & Ellis 2010a, Veldhorst et al 2009a). However, this is not the case for obese individuals or HFD-induced obese mice, despite WP action lowering body weight and adiposity. The studies in obese individuals have contrasting end points. In some clinical trials in obese individuals, WP supplementation shows a positive effect on satiety, whereas in others, WP either has no effect on satiety or has a short-term effect that then disappears after a long-term intervention (Bowen et al 2006b, Mollahosseini et al 2017, Pal et al 2014, Wiessing et al 2015). In HFD-fed mice, WPI does not show a satiety effect but, as we observed in *Chapter 3*, WPI-fed mice show a higher energy intake compared to CAS counterparts (McAllan et al 2014, Tranberg et al 2013). We observed the same scenario in *Chapter 2*, where mice fed with HFD containing LAB (i.e., a whey protein component) showed a higher energy intake compared to HFD-CAS-fed mice, without showing any differences in body weight. This clearly suggest an energy loss in the presence of WP within HFD. In this thesis, we explored the energy loss by looking at the gene expression of different nutrient transporters within the small intestine. In *Chapter 2*, the presence of LAB within HFD showed a decrease in the gene expression in FAs and glucose transporters (CD36 and GLUT2) within the ileum and jejunum. This would explain, in part, the energy balance mismatch in these mice. However, in *Chapter 3*, in HFD-WPI-fed mice, we did not find relevant changes in the gene expression of ileal nutrient transporters or through the investigation of TAGs content in the caecum. Thus, the reason for the discrepancy between energy intake and body weight in HFD-fed mice supplemented with WPI or LAB remains unsolved. One possibility is that this relates to AA compositional differences between WPI and LAB, and how they are digested by the host and microorganisms and made available to the host. In the future, more investigations on caloric content within the faeces or a detailed analysis on energy expenditure such as body temperature, BAT functionality and energy dissipation are

required. It would also be useful to assess how an imposed change in the AA composition of the protein source affects the host.

Furthermore, in *Chapter 4*, we showed that WPI can also protect from HFD-induced intestinal barrier disruption. Other studies reported the beneficial effect of WPI on intestinal repair, integrity and maturation in rodents and pigs (Boza et al 1996, Kotler et al 2013, Xiao et al 2016). However, these studies were not performed during HFD consumption. Thus, we showed for the first time WPI action on gut permeability that protects from HFD-induced derangements. Moreover, we observed that this effect is mediated by the gut microbiota, since the depletion of the gut microbiota though ABX treatment abolished the protective effect on ileal permeability during HFD consumption. However, more studies are required to confirm this evidence. Currently, the effect of WPI on the gut health in obese individuals remains unexplored.

WP presence in the diet influences the gut microbiota composition. It was previously shown that WP feeding in rodents increases the abundance of *Lactobacillus* and *Bifidobacterium* genera (McAllan et al 2014, Sprong et al 2010). Accordingly, in *Chapter 3*, we observed an increase in abundance of *Lactobacillus murinus* in the gut of younger mice fed with HFD-WPI. In *Chapter 4*, we observed a significant increase in the relative abundance of *Lactobacillus* following 10-week HFD-WPI intervention in mice. Accordingly, HFD-WPI-fed mice showed a higher caecal concentration of vitamin-B1, which is produced by several *Lactobacillus spp*. (Yoshii et al 2019). However, both in *Chapter 3* and *4*, we did not observe significant differences in *Bifidobacterium*. In addition, in *Chapter 4*, we found that both 5-week and 10-week intervention with HFD-WPI provoked an increase in *Allobaculum* and a decrease in *Anaerotruncus*, *Saccharibacteria_genera_incertae_sedis*, *Intestimonias*, *Roseburia*, *Anaerofusits*, *Anaeroplasma*, *Lefsonia*, *Clostridium_sensu_stricto*, *Turicibacter* and *Streptococcus* at genus level. This is the first evidence of an increase in the relative abundance of *Allobaculum* in the presence of HFD-WPI.

Moreover, in mice fed with HFD, some individual WP components were able to modulate the gut microbiota. In particular, Lf supplementation led to an increase in

the proportion of *Bifidobacterium spp*., and LAB supplementation caused an increase in relative abundance of *Lachnospiraceae* and *Blautia* (Li et al 2019a, Sun et al 2016). In *Chapter 2*, we observed that LAB caused an increase in *Parabacteroides*, *Bifidobacterium*, *Parvibacter* and *Lactobacillus* within the gut of HFD-fed mice.
Together, these results show clearly that WP or single WP components can modulate the composition of the gut microbiota, promoting the growth of selected taxa. Since certain strains of *Lactobacillus* and *Bifidobacterium* are among the most important probiotics, WP consumption can be beneficial for HFD-induced gut microbiota alteration. Accordingly, a decrease in *Allobaculum* was found in obesity-prone mice fed with HFD and an increase of this genera has been linked with a reduction in body weight in obese mice (Huazano-García et al 2017, Qiao et al 2014, Ravussin et al 2012). Variation between different studies might be due to animal strain, diet kind and duration, age and, very importantly, the gut microbiota analysis approach adopted.
While there are few studies looking at the gut microbiota following WP intake related to exercise in humans (Cronin et al 2018, Moreno-Pérez et al 2018), studies that explore the impact of WP supplementation on the gut microbiota of obese/overweight people are not currently available. To better explore the relationship between WP intake, obesity and gut microbiota, evidence in humans are necessary.

In light of the multiple effects on host physiology observed during WP administration, we sought to find a link between gut microbiota modulation and physiological outcomes during HFD-WPI administration. In *Chapter 4*, through a microbiota depletion with ABX cocktail, we observed that WPI action in lowering body weight, adiposity and leptin level seems to be independent from the modulation of the gut microbiota. Thus, we hypothesized that adipose tissue lipid metabolism, which might be the cause of lower adiposity (see paragraph 5.2.2), would not be affected by the microbiota manipulation. However, in the future, different strategies to better understand if the gut microbiota mediates WPI effects, such as experiments in GF mice or specific bacterial taxa manipulation (for example, for studies that involve WPI, it would interesting to target specifically *Lactobacillus spp.*) or FMT studies are required. Also, to have a complete picture of the effect of WPI during microbiota depletion, it would be worth carrying out a microbiota depletion experiment using the

same ABX cocktail in normal diet/LFD-fed mice supplemented with WPI. In this way, the direct effect of WPI on the gut microbiota, independently of HFD, can be investigated.

5.2.2 Whey protein ameliorates HFD-induced adiposity *via* modulations of lipid metabolism

From this thesis, and from previous studies carried out in rodents, the ability of WPI to decrease adiposity and body weight gain in a context of HFD feeding has been revealed (Abbas et al 1989, McAllan et al 2013, McAllan et al 2014, Pezeshki et al 2015, Pilvi et al 2007, Pilvi et al 2008). In some of the cited studies, the decrease in fat mass was accompanied by an increase in the lean mass (enhancement of muscle protein synthesis). Notably, considerations of lean body mass were omitted from this thesis. Clinical studies that involve WP supplementation in overweight and obese humans on either a normal diet or an energy-restricted diet have been carried out (Baer et al 2011, Frestedt et al 2008a, McGregor & Poppitt 2013, Mojtahedi et al 2011). From those studies, it emerged that WP (both under WPI and WPC form) had a positive effect on reducing body weight and adiposity when supplemented at approximately 50g/day for at least six months. In particular, WP supplementation in obese humans had a strong impact on body fat loss, which is not always associated with body weight loss. Together, these findings in HFD-fed rodents and obese humans highlight the impact of WP on lipid accumulation and the way of action was demonstrated to be through a regulation of lipid metabolism. It was shown that an upregulation of *Adrb3* expression, which codes for β-3 adrenergic receptor, occurs during WP supplementation in the eWAT of HFD-fed mice (Pilvi et al 2008). Catecholamines (adrenaline and noradrenaline) bind to this receptor and trigger the hydrolyzation of TAG stored within the adipocytes so that TAG can be mobilized as fuel for other organs. This suggests that WP might promote fat catabolism. In support of this evidence, in this thesis we have shown that WPI as part of a HFD given in younger mice decreased the gene expression of HSL, an enzyme involved in lipolysis, which can be regulated by the stimulation of the β-3 adrenergic receptor (Collins

2012). In parallel, we observed an increase in the gene expression of two genes involved in fatty acids β-oxidation (*Cpt1a* and *Ucp2*) in HFD-WPI-fed mice. These results indicated that different pathways (i.e., lipolysis and β-oxidation) involved in fat catabolism are affected by WP consumption. Moreover, a lower expression of *Lpl* was observed in the eWAT of HFD-WPI-fed mice. Given the importance of LPL for FAs uptake into adipocytes, it seems that WP suppresses fat gain by acting on adipose tissue lipid metabolism, both preventing the entrance of FAs into the adipocytes and enhancing fat storage mobilization. Moreover, it was shown that specific WP components could alone impact on fat metabolism. Unlike LAB, which had no impact on either eWAT or sWAT (*Chapter 2*), the WP component BSA was shown to reduce sWAT, where expression of genes involved in fat catabolism (i.e., *Pparα*, *Cpt1b* and *Ucp3*) were increased (McManus et al 2015b).

Future work should focus on investigating the impact of lipid metabolism in other fat depots, such as mAT and rAT. In this regard, future studies are also required to investigate the impact of WPI on the browning of the WAT by looking at "browning markers" such as UCP1 and PGC-1α.

In previous studies, it was shown that WP also has an impact on genes involved in lipid metabolism in other organs, such as muscle and the liver (Shi et al 2011, Tauriainen et al 2011). In the liver, WPI supplementation was associated with a suppression of hepatic accumulation of lipids in rodents and an improvement of hepatic steatosis in obese women (Bortolotti et al 2011, Hamad et al 2011, Shi et al 2011). In this thesis, we did not focus on the impact of WPI on the liver, however, in *Chapter 3*, we observed a lower hepatic TAG concentration. Additionally, in both *Chapter 3* and *Chapter 4*, we observed a significant increase in liver weight after WPI supplementation. Since WPI protects from lipid accumulation in the liver, the reason for our observation is still unknown. This unanswered question can be addressed in future studies through histological approaches and, perhaps, through metabolomics or transcriptomics analysis to better elucidate lipid metabolism-related pathways and pathways of another nature.

5.2.3 Whey protein: a possible solution for leptin resistance?

Historically, leptin is the hormone considered to be the master regulator of satiety, aside from its role in regulating energy expenditure, metabolism and immune function (Park & Ahima 2015). Indeed, mice and humans carrying a mutation in either *Ob* gene (leptin production from the adipose tissue) or *Db* gene (leptin receptor within the brain) develop severe obesity. While for obese people with *Db* mutate there is no cure, leptin replacement therapy is very efficient for obese *Ob* deficient people. Unfortunately, the same therapy cannot be applied in common obesity (which covers the majority of cases) because the over production of leptin, which overstimulates leptin receptors, causes a desensitization (leptin resistance). For these reasons, the scientific community is looking for therapies that can increase leptin sensitivity or reduce leptin resistance. This last strategy would be ideal since people affected by non-genetic obesity already have elevated leptin levels that can act on more sensitized receptors. In this context, results that emerged from this thesis are promising.

In *Chapter 3* and *Chapter 4*, WPI supplementation clearly showed a drastic neutralization of HFD-induced leptin production (both at gene expression level within the eWAT and at concentration level in the plasma). This observation is in agreement with other studies in rodents (McAllan et al 2013, McAllan et al 2014, Zapata et al 2017b). Moreover, McAllan *et al* showed a decrease in the expression of the gene that codes for hypothalamic leptin receptor LepRb in HFD-WPI-fed mice, compared to HFD-CAS-fed mice. This suggests that WPI influences adipose leptin production, as well as the leptin receptor within the hypothalamus. Unlike LAB (*Chapter 2*), a beneficial effect on leptin production was also observed in HFD-induced obese mice supplemented with the WP components Lf and BSA (McManus et al 2015a, McManus et al 2015b). Notably, while WPI and BSA caused a decrease in adiposity together with leptin production, Lf acted on leptin levels without affecting adiposity. This suggests that changes in leptin production upon consumption of WP components are not always related to changes in adiposity.

In *Chapter 4*, we further investigated the hypothalamic leptin receptor signaling pathway by measuring the gene expression of Socs3, which is involved in feedback inhibition on the leptin receptor and is a potential mediator of physiologic leptin resistance (Bjorbak et al 2000). However, no differences were found between HFD-

WPI and HFD-CAS-fed mice, and thus a deeper investigation on the impact of WPI on leptin receptor functionality is necessary. This should include all the components of the LepRb cascade signaling, since they are all important regulators of LepRb functionality. Since these components are constantly activated and inactivated by post-translational modifications, a proteomics analysis, rather than a gene expression analysis, would be ideal. Also, further investigations on the impact of WPI on leptin production and leptin resistance in humans are required.

5.2.4 Whey protein and its anti-inflammatory effect

Obesity is associated with chronic low-grade inflammation. This condition, over a longer term, triggers the development of obesity-related comorbidities, such as adipose tissue dysregulation, cardiovascular diseases and insulin resistance (Ellulu et al 2017, Lee et al 2013). Thus, finding dietary strategies that could ameliorate this inflammatory status would be useful to prevent the development of obesity-related conditions. Although several studies on obese individuals showed no differences in the circulatory inflammatory cytokines after WP administration, other studies showed a beneficial effect of WP components, such as Lf and immunoglobulins, during obesity (Brimelow et al 2017, Pal & Ellis 2010b, Pal & Ellis 2011). In HFD-induced obesity in rodents, WP supplementation showed a decrease in the adipose tissue inflammation but this intervention was coupled with aerobic exercise (Ahmadi-Kani Golzar et al 2018). Among WP components, LAB has been shown to decrease HFD-induced inflammatory markers, such as TNFα, MCP-1 and IL6 in the adipose tissue, colon and in the serum, together with a decrease of LPS in the serum (Gao et al 2018, Li et al 2019a). In *Chapter 2*, we did not carry out a detailed analysis of the anti-inflammatory properties of LAB contained in HFD. Our only measurement of relevance related to the level of gene expression of CD68, which is a marker of macrophage infiltration within the adipose tissue, without observing any differences compared to the HFD-CAS control group. On the contrary, in *Chapter 3* and *4*, we observed a decrease in *Cd68* gene expression in HFD-WPI-fed mice. In addition, in *Chapter 4*, we found a decrease in the gene expression of MCP-1 and TNFα in the adipose tissue. These

results suggest that WPI decreases the expression of pro-inflammatory cytokines in the adipose tissue, in parallel with a decrease in macrophage recruitment and infiltration in HFD-induced obese mice. In the future, histological analyses of the adipose tissues will be required to further confirm this finding.

In *Chapter 4*, HFD-WPI-fed mice showed a decrease in gene expression and concentration of MCP-1 in the ileum and in the plasma, respectively. Notably, according to the positive effect of WPI on ileal permeability, WPI caused a decrease of MCP-1 concentration in the presence of HFD, but this effect is abolished by gut microbiota depletion.

These findings might suggest that WPI decreases HFD-induced systemic inflammation through a protective effect on gut permeability and, potentially by gut microbiota modulation. Interestingly, our previously described data suggests that this mechanism of action of WPI is independent from the WPI effect on adiposity, fat metabolism control, and local tissue inflammation.

Notably, in *Chapter 4*, we observed a higher caecal content of tryptophan and 3-indoleacrylic acid, which is a tryptophan-derived indole associated with suppression of inflammation, in HFD-WPI-fed mice (Wlodarska et al 2017). Thus, the high tryptophan content in WP might contribute to ameliorating the inflammation caused by HFD.

In the future, further studies to investigate the beneficial effects of WP on inflammation in obese individuals are required. Since the human studies mentioned in this section lasted for 12 weeks, it is necessary to carry out a WPI supplementation for longer periods. According to studies showing WPI efficacy in lowering adiposity, discussed in paragraph 5.2.2, WP supplementation should last at least 6 months. Ultimately, analyses on the inflammatory status should be coupled with analyses of intestinal permeability.

5.2.5 Anti-obesity effects of whey protein: when and for how long?

Previous studies in our lab focused on WPI administration in HFD and LFD, taking into account the duration of the dietary intervention. Both short-term (7-8 weeks) and long-term (15-21 weeks) dietary interventions have been carried out and, based on the diet's macronutrient composition, WPI action can be different. In the presence of HFD, for example, WPI causes an improvement in insulin sensitivity only after a short-term dietary intervention and causes an alteration of the gut microbiota only after a long-term intervention. However, WPI given with HFD affects body weight gain, liver TAG and lean mass both after short- and long-term interventions (McAllan et al 2013, McAllan et al 2014, McAllan et al 2015). In this thesis, we also consider the duration of WPI intervention, both in *Chapter 3* and *Chapter 4*, keeping in both studies a WPI administration that lasted 5 and/or 10 weeks. In *Chapter 4*, we have seen that WPI caused lower body weight gain, adiposity, leptin availability and inflammation markers expression after both 5 and 10 weeks of administration. However, the efficacy of WPI in ameliorating systemic inflammation and gut permeability was significant only after 10 weeks of WPI administration. These results suggest that different durations of WPI intervention lead to different effects.

Another factor that is worth considering is the age, or the stage of life, of the individual. In *Chapter 3*, we showed that 5-week HFD-WPI intervention brought about beneficial effects in adolescent mice but not in mice during early adulthood. However, this study is limited by the fact that early adult mice, before the intervention with HFD-WPI, were fed with LFD-WPI that had a different % of sucrose compared to HFD. Despite this, the study highlights when in life WP supplementation can be more effective. Future studies that will focus on the effect of HFD-WPI on adult rodents are planned in our lab.

Thus, both duration and stage of life are two important parameters to consider during WP administration, to maximize the desired effect. Further work is needed to better explore the present topic.

5.3 Beyond obesity: other applications of whey protein

In this thesis, we focused on the beneficial effects of WP during obesity. However, it has been shown that WP supplementation could help to ameliorate other pathological conditions, such as type 2 diabetes, cardiovascular diseases and inflammatory bowel diseases.

Type 2 diabetes is a metabolic condition characterized by chronic hyperglycaemia. This is due to impaired insulin secretion and/or insulin resistance in peripheral organs (Olokoba et al 2012). Although, in this thesis, we did not find important differences in plasma insulin and glucose concentrations in the fasting condition between mice fed with HFD-WPI and HFD-CAS, several studies support a positive effect of WPI during diabetes. In rodents, it was shown that WP consumption causes a decrease of postprandial plasma glucose and fasting plasma insulin, which indicates an improvement in insulin sensitivity (Belobrajdic et al 2004, Kavadi et al 2017). In support of this evidence, in *Chapter 3*, WPI as part of a LFD (10% fat diet with 70% carbohydrates) was shown to reduce glucose levels in mice, and this could be related to changes in GLUT2 within the ileum (Nilaweera et al 2017). Similarly, in both healthy and diabetic humans, daily supplementation with WP had comparable effects to drugs used to manage hyperglycaemia in type 2 diabetes. Indeed, WP consumption lowered fasting insulin level. In postprandial analysis, humans consuming WP had a lower plasma glucose concentration and higher insulin, confirming an increase in insulin responsiveness (Frid et al 2005, McGregor & Poppitt 2013, Pal & Ellis 2010a, Pal et al 2010). It seems that this insulinotropic effect exerted by WP is due to its high BCAA and tryptophan content (Nilsson et al 2004, von Post-Skagegård et al 2006). These essential amino acids promote the release of satiety hormones within the gut, such as GLP-1, which is known to be an incretin hormone (Mignone et al 2015).

Cardiovascular diseases include numerous pathological conditions of the cardiovascular system. They can be grouped into coronary-artery diseases, cerebrovascular diseases, peripheral artery diseases and aortic atherosclerosis (Benjamin et al 2018). Although, in this thesis, we did not investigate any cardiovascular-related parameters, WP has been demonstrated to have a beneficial

effect against cardiovascular-related conditions. WP consumption in hypertensive and/or overweight humans resulted in a postprandial decrease of both systolic and diastolic blood pressure (Pal & Ellis 2010a, Pal & Ellis 2010b). This anti-hypertensive effect exerted by WP seems to be mediated by the inhibition of angiotensin-converting enzyme (ACE), which catalyses the conversion of angiotensin 1 to angiotensin 2 and controls arterial vasoconstriction (FitzGerald & Meisel 1999). Moreover, long-term consumption of WP results in a significant decrease in the augmentation index (AI), which is an indicator of arterial stiffness (Pal & Ellis 2010b). Very often, AI is high in people with hypercholesterolemia (Wilkinson et al 2002). This effect might be linked with role of WP in lowering blood pressure (Blacher & Safar 2005).

In this thesis, we provided further insight into the anti-inflammatory properties of WPI together with gut permeability protection. Thus, WP could be a beneficial dietary approach also for individuals affected by inflammatory bowel disease (IBD), which is a multifactorial immune disorder characterised by chronic relapsing inflammation of the intestine. IBD primarily consists of two different disorders: Crohn's disease (CD) and ulcerative colitis (UC). Disrupted intestinal permeability and gut microbiota alteration are two common conditions found in IBD patients (Khan et al 2019, Michielan & D'Incà 2015). The causes of IBD are still not very well defined. However, it is believed that an impairment of the immune system, together with an imbalanced interaction between gut microbiota and intestinal epithelium, leads to the development of chronic intestinal inflammation when genetically susceptible hosts come across certain environmental factors (Kim & Cheon 2017, Podolsky 1991). Consumption of WP caused an increased secretion of faecal mucin and the abundances of *Lactobacillus* and *Bifidobacterium* in rats administered with the inflammatory colitis inductor dextran sulfate sodium (DSS). This was accompanied by a decrease in the gene expression of IL1β, calprotectin, and inducible nitric oxide synthase. In addition, WP diminished diarrhea symptoms and faecal blood loss (Sprong et al 2010). Similarly, low-temperature processed WPC supplementation increased mucin production, decreased intestinal lymphocyte infiltration and epithelial morphology distribution in DSS-induced colitis in BALB/c mice (Jayatilake et al 2014). These evidences suggest a protective and anti-inflammatory role exerted by WP within the intestine. Accordingly, a study carried out in humans with Crohn's disease showed that WP

supplementation significantly decreased intestinal permeability and ameliorated intestinal morphology parameters, such as the villi ratio (Benjamin et al 2012).

In conclusion, a dietary supplementation with WP can be beneficial for multiple diseases, thanks to their wide variety of multiorgan effects. The composition of WP, which is rich in essential AA, is one of the main driving forces of the multiple actions of WP.

5.4 Whey protein and obesity: Future Directions

Different experimental strategies can be employed to further investigate the beneficial effects of WP in obese individuals. More effort is required to seek the mechanism of action of WP, to identify one or more possible therapeutic targets. Moreover, the relationship between gut microbiota and WP consumption in the context of obesity needs to be better elucidated. This section describes different experimental approach that could help to identify new aspects of the role of WP consumption on counteracting the development of obesity.

5.4.1 Dietary interventions

Macronutrient quality within precast diet

It is well known that the precast diet purchased to study obesity in rodents has limitations due to the very high fat contents (usually 40-45% and 60%), which do not always match with the fat content in human HFD. In addition, the percentage of sucrose of HFD compared to LFD/normal diet is different. This can lead to a misinterpretation of the effect of fat contained in HFD. Another, limitation of the precast diet is the quality of the macronutrients (i.e., fats, proteins and carbohydrates). To make precast diet more similar to a human diet, it is now possible to change the quality of the FA composition (i.e., saturated/mono-unsaturated/polyunsaturated FAs)

within the diet, and to vary the percentage of sucrose and protein (Hu et al 2018, Speakman 2019).

To this end, the use of precast diets with different macronutrient compositions that better mimic human diets can be employed to confirm and better understand the effect of WPI in HFD-induced obese mice. In addition, experiments with a fixed percentage of sugar in both HFD and LFD/normal diet containing WPI are required.

Use of isotope of carbon to measure energy expenditure

The most common way to measure energy balance, i.e., the combined impact of energy intake and energy expenditure, in rodents is using metabolic cages. Some important metabolic parameters detected by these cages are: oxygen consumption (VO_2) carbon dioxide production (VCO_2), respiratory exchange ratio (RER, VO_2/ VCO_2) and locomotor activity (Speakman 2013). However, the energy expenditure measurement is general and it is not possible to precisely quantify the utilization (oxidation) of a specific macronutrient (MacAvoy et al 2006).

A more detailed measurement of energy expenditure can be done by using macronutrients (fat or sugars) labeled with an isotope of carbon, ^{13}C. Practically, mice are provided with a diet (LFD or HFD) supplemented with WPI and then a macronutrient that contains the carbon isotope can be administered to the mice. Through this method, it is possible to measure the oxidation efficiency of the specific macronutrient through the quantity of ^{13}C in breath (contained in CO_2) (Welch Jr. et al 2016). This would provide a better understanding of how WPI impacts on energy demand and metabolic rates.

Whey protein-derived bioactive peptides and obesity

Once in the stomach, dietary proteins are exposed to an acidic environment, which promote their denaturation. In this status, the proteins are more accessible to proteolytic enzymes. The high concentration of HCl in the stomach allows the autocatalytic activation of pepsinogen in the aspartic protease pepsin, which is active in a pH range of 1-5 (Lin et al 1992). Pepsin can hydrolyse the bonds between the aromatic amino acids phenylalanine, tryptophan and tyrosine, breaking down the proteins into peptides (four to nine amino acids) (Antonov et al 1974). The next step of the protein degradation process occurs in the first tract of the small intestine, the

duodenum, in which are released other proteolytic enzymes, together with bicarbonate, by the pancreas. The presence of bicarbonate allows an increase of the pH in the duodenum, creating a favorable environment for the action of pancreatic proteases. Trypsin, chymotrypsin, elastase and carboxypeptidases are initially secreted by the pancreas as inactive zymogens that, within the duodenum, are cleaved in their active form by enterokinases (Layer & Keller 1999). Trypsin and chymotrypsin cleave peptide bonds adjacent to lysine or arginine and hydrophobic amino acids, respectively (Ma et al 2005). Instead, elastase cleaves peptide bonds adjacent to alanine, glycine and serine and carboxypeptidases cleave one amino acid at a time from the carboxyl ends of peptide chains (Feher 2017). At the end of the protein digestion, the final products are compose of 30% free amino acids and 70% by oligopeptides (Matthews 1971).

Most of the peptides that come from bovine milk intestinal hydrolysis are considered "bioactive" because of their capability to exert a range of biological activities such as antihypertensive, antioxidative, antithrombotic, hypocholesterolemia, satiety and antimicrobial actions (Park & Nam 2015). In addition, whole WP and each WP component are the source of different kinds and quantities of bioactive peptides (Madureira et al 2010). Through a deep investigation of WP-derived bioactive peptides, it might be possible to better understand the anti-obesity effect attributable to WP.

WP-derived bioactive peptides can be obtained by performing *in vitro* digestion and selected peptides can be directly injected in the small intestine (to avoid further digestion) of obese mice. In this way, the anti-obesity potential of each WP-derived bioactive peptide (or a combination of more bioactive peptides) can be assessed. This approach would prevent the interference of WP-derived bioactive peptide activity with food formulation. For instance, micronutrients, macronutrients, and other ingredients present in the food that contains WP could interfere with the digestion, absorption and activity of the bioactive peptides.

It is worth noting that the gut microbiota can ferment proteins and peptides that escape from digestion (Diether & Willing 2019). Thus, the effect of specific WP-derived bioactive peptides on the gut microbiota can be studied and can be linked with the WP biopeptide-related biological function.

5.4.2 Microbiota-related experimental approaches

Probiotics, prebiotics and synbiotics combined with WPI supplementation

Probiotics are living bacteria that confer beneficial health effects on the host, when ingested in adequate amounts (Butel 2014). Several studies have demonstrated the wide range of effects exerted by different strains of probiotics in improving host immunity, metabolism and endocrine function (El Aidy et al 2015, Patterson et al 2016). Several *Lactobacillus* and *Bifidobacterium* spp. are among the most common probiotics. As we showed in this thesis, these correspond to the genera that mainly increase in abundance within the gut in the presence of WP. To this end, administration of WPI or specific WP components can potentially amplify the beneficial effect exerted by specific probiotic strains on host metabolism and health.

The same concept is valid for prebiotics, which are substrates that are selectively utilized by the host's microorganisms and which confer a health benefit (Gibson et al 2017). Dietary fibers represent the main class of prebiotics, which include inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS) and resistant starch. The consumption of prebiotics, which helps the growth of beneficial gut bacteria, has been associated with the improvement of obesity and associated co-morbidities, thanks to their influence on lipid metabolism, glycemic control and regulation of the secretion of satiety hormones (Parnell & Reimer 2012, Xavier-Santos et al 2020). As with probiotics, prebiotics, together with WP supplementation, could enhance or participate in the shaping of the gut microbiota. The co-administration of WP and prebiotics could be a strategy to improve WP's efficacy in promoting health benefits.

In general, the administration of probiotics, prebiotics or a combination of both (i.e., synbiotics) can be an adjunctive microbiota modulation-therapy to complement the WP effects on host metabolism, adiposity, immunity and intestinal health. These investigations should ideally be carried out both in preclinical and clinical studies.

Faecal microbiota transplantation and WP supplementation

Another way to investigate the relationship between microbiota, metabolic syndrome and WP supplementation could be through FMT. FMT consists of the transfer of

intestinal microbiota from one individual to another via oral administration of faecal material. This technique results in a donor-like microbiome in the GIT of the recipient, opening new strategies for the understanding of the host-microbiome relationship (Aron-Wisnewsky et al 2019, Lai et al 2018). Previously, it was demonstrated that GF mice colonized with an obesity-associated microbiota increased body fat and energy harvest compared to mice colonized with lean donor microbiota (Ley et al 2005b, Turnbaugh et al 2006a). Thus, we could potentially test the effects of WP supplementation in mice that develop metabolic syndrome not induced by HFD but through FMT of an obesity-associated microbiota. This can potentially be one strategy to study the effect of WPI in metabolic syndrome without using precast HFD.

In addition, it is possible to further investigate the microbiota's role in various clinical conditions via "humanization" of the rodent microbiota (FMT from human to rodents) (Staley et al 2017, Wrzosek et al 2018). This would provide mechanistic insights into the relationship between human microbiome and host. In our case, it would be very interesting to assess the effect of WP supplementation in rodents with metabolic syndrome caused by FMT from obese humans. However, whether FMT from obese human donors could cause the development of obesity in a lean rodent recipient remains to be elucidated. A study examined FMT from twin pairs of adult females, discordant for obesity, into GF mice. GF mice receiving FMT from the obese individuals caused a more evident increase in body weight and obesity-associated metabolic phenotypes. Co-housing mice with an "obese" microbiota with mice containing the lean co-twin's microbiota prevented the development of increased body mass and obesity-associated metabolic phenotypes (Ridaura et al 2013). In a recent study, it was shown that FMT from human obese donors did not cause the development of obesity in the lean rodent recipient (Rodriguez et al 2019). Thus, it seems that FMT from obese subjects performed into mice reproduces the metabolic syndrome defects only in a GF condition. More studies are needed to elucidate this aspect.

Germ-free models

In this thesis, we studied the relationship between WPI supplementation and gut microbiota by depleting the gut microbiota using an ABX cocktail. This method is rapid, inexpensive, and rapidly accessible; however, the degree of depletion depends

on which antibiotic is employed. Thus, only specific taxa will be eliminated by specific antibiotics. Even treatments with ABX cocktails do not completely clear the entire gut microbiota. In addition, the systemic side effects of a chronic antibiotic treatment must be taken into account (Kennedy et al 2018).

As an alternative, GF rodents can be used to study the host-microbiota relationship (Williams 2014). These rodents are generated via aseptic caesarean section (C-section) and are not exposed to microorganisms from birth and throughout the lifespan. GF mice differ with respect to development and physiology, relative to animals with commensal bacteria; for example, they are characterized by a lower body weight and have impaired intestinal function and immune system (Belkaid & Hand 2014, Jeppsson et al 1983, Savage et al 1981). Moreover, their metabolism is altered as well as their hormone signaling regulation (Kawase et al 2017, Weger et al 2019)

GF mice can be a useful tool to confirm/further investigate the host-microbiota crosstalk upon WP supplementation. A limitation of GF mice is that they do not develop obesity upon HFD consumption.

Additionally, gnotobiotic mice can be useful in the study of WP influence on specific gut microbiota taxa (Gordon & Pesti 1971). These mice are initially GF and are subsequently colonized with a few specific strains of bacteria of interest. For example, in our case, we could select specific strains belonging to *Lactobacillus*, *Bifidobacterium* or *Allobaculum*, since they were the genera that increased relative abundance following consumption of WP or WP components. In this way, it would be possible to investigate the communication between specific members of the gut microbiota and the host physiology, linked with nutrition.

5.4.3 Other preclinical interventions

Preclinical genetic models

In this thesis, we have shown that WP possibly exerts its effect on body weight through the regulation of adipose tissue metabolism, in the presence of HFD. However, the precise molecular mechanism and all the specific enzymes involved remain to be

elucidated. As described in paragraph 5.2.1, WPI can influence the expression of several genes coding for enzymes or transporters involved in different pathways that characterize adipose tissue metabolism: lipolysis (such as *Adrb3* and *Hsl*), fatty acids β-oxidation (such as *Cpt1a* and *Ucp2*) and lipid biogenesis (such as *Lpl*).

First, the analysis of the protein production profile of these components is necessary to verify if WPI acts on the gene expression level and on protein production, or both. This can be done by performing Western blots or, to have a wider picture, through a proteomic analysis (Gulcicek et al 2005, Mahmood & Yang 2012).

Second, to get further insight on the specific mechanism of action of WPI on adipose tissue metabolism, conditional mouse models can be employed. In conditional mice it is possible to perform a knockout or an inactivation of a specific gene of interest in a specific tissue. The knockout or the inactivation of a gene can be performed anytime, based on the study design and adopting different techniques (Bockamp et al 2008). In general, a transgenic DNA construct, equipped with all the elements necessary for gene expression, is inserted into a pronucleus or embryonic stem cells (which are subsequently injected into the blastocysts). Then, the inserted construct (present in every cell of the organism) will be activated in a selected way and during a selected time (Haruyama et al 2009). Recently, another technique has been developed, called "clustered regularly interspaced short palindromic repeats-Cas9" (CRISPR-Cas9), which has several advantages, such as reduced mutational experimentation, reduced cost and the ability to generate a homozygous mutant in the founder generation. The sensitivity of this system also allows researchers to investigate subtle genetic factors such as point mutations, and gene clusters, as well as allowing the specific targeting of the genomes rather than just total gene deletion or up-regulation (Hsu et al 2014, Young et al 2015).

Conventional gene targeting strategies have been successfully applied to animal models for the study of lipid metabolism, lipid disorders and adipose tissue biology. For example, mouse models of conditional CD36, TNFα, UCP1, PPARγ, PGC-1β, APOE, SCAP (i.e., escort protein of SREBP) and many others, have been developed to study important pathways linked to atherosclerosis, lipid transport and neurodegenerative diseases (Blüher 2005, Bock et al 2007).

Generating and using conditional transgenic mice for genes that might be important during WPI consumption could be a good strategy to precisely define mechanism of action of WPI within the host. Thus, the effect of WPI on inflammation and on intestinal physiology can be studied through this approach.

Sex differences

In healthy mice (of different strains) there are sex-dependent physiological differences. Indeed, it was shown that males and females have different regulation of energy metabolism and physiological parameters, such as body weight and leptin production, from weaning (Wang et al 2011). These sex differences are also accompanied by strain- and sex-specific differences in the gut microbiota and in the immune system (Baars et al 2018, Elderman et al 2018). In this thesis, we carried out all studies in male mice, and thus it would be necessary to validate/compare our findings in females.

5.4.4 Human-targeted interventions

Whey protein and anti-obesity drugs, drugs that induce weight gain and bariatric surgery

Different drugs to control satiety-related hormones are available as a help for obese individuals. These drugs act on food intake and energy expenditure regulation. A few examples include:

- Liraglutide: GLP-1 analogue that acts as GLP-1 receptor agonist (Mehta et al 2017).
- Natrexon/Bupropion: a combination between an opioid antagonist (Natrexon), that acts in the hypothalamic melanocortin, and an inhibitor of catecholamines uptake, that reduces dopaminergic tone and stimulates POMC neurons within the hypothalamus (Billes et al 2014).
- Orlistat: inhibitor of pancreatic lipases (Drew et al 2007).

- Pioglitazone: activator of PPARs. It improves glycemic control and adiponectin production. In addition, it causes redistribution of lipid storage and a decrease in inflammation of the adipose tissue (Smith 2001).

However, none of these drugs alone can cure obesity. Moreover, they are accompanied by collateral effects such as GIT disturbances and chronic usage of some of these drugs can lead to a "drug-resistance", which can lead to weight re-gain.
In light of WP potentialities described in this thesis, WP could be administered as a coadjutant of anti-obesity drugs. This may cause an additive effect of drug and WP that could potentially increase the beneficial effects on the individuals, especially if coupled with exercise and calorie restriction.

Similarly, WP administration can be beneficial for patients that undergo bariatric surgery. Currently, bariatric surgery is the only long-term solution for obesity because of its action on body fat as well as metabolic changes. However, bariatric surgery can lead to malabsorption and/or gastric acid reduction that can cause nutrient deficiency. This can lead to further complications in the long-term (Cornejo-Pareja et al 2019). In addition, bariatric surgery can cause severe muscle loss (Davidson et al 2018). Thus, WP consumption can be tested as an approach to maintain lower body weight and further improve metabolic-related conditions, as well as to help prevent muscle loss in patients that underwent bariatric surgery. In support of this hypothesis, WP supplementation caused increased body weight loss in women with long-term weight regain following bariatric surgery (Lopes Gomes et al 2017).

Moreover, different drugs may induce weight gain as a collateral effect. A few examples of these drugs are (Verhaegen & Van Gaal 2000, Wharton et al 2018):

- Drugs for diabetes (i.e., thiazolidinediones and sulfonylureas);
- Antipsychotic drugs (i.e., haloperidol, clozapine, risperidone, olanzapine, and lithium);
- Antidepressant drugs (i.e., amitriptyline, imipramine, paroxetine, and sertraline);
- Epilepsy drugs (i.e., valproate, carbamazepine, and gabapentin);
- Corticosteroids drugs (i.e., prednisone);

- Antihypertensive drugs (i.e., metoprolol, propranolol).

WP, consumed before or during administration of these drugs, might have a positive effect in preventing or ameliorating weight gain and, thus, reducing the side effects of the drugs.

Whey protein, exercise and obesity

As previously mentioned, the anti-obesity effects of WP are variable in different human studies (see paragraph 5.2). A possible way to improve the WP effect in obese individuals would be to couple WP consumption with exercise. It has been shown that physical activity exerts a large list of beneficial effects on health, such as decreased adiposity, improved cardiorespiratory function, improved metabolism and improved mental health (Vina et al 2012). In addition, both exercise and WP consumption promote muscle health and exercise performance, and decreased inflammation (Davies et al 2018, Ertek & Cicero 2012, Warburton et al 2006). Thus, the combined action of WP consumption and exercise might offer an efficient long-term solution for obesity.

Whey protein and the microbiota-gut-brain axis

WP consumption has been shown to have a beneficial effect on mental health, particularly on anxiety, stress, cognition and sleep (see paragraph 1.6.5). However, whether the WP effect on mood and behavior is linked with changes within the gut microbiome is still to be elucidated. Thus, clinical studies looking at the effect of WP on mental health and microbiota are now warranted. These examinations could be carried out in healthy people as well as in obese people (for whom mental health is very often affected (Djalalinia et al 2015)) and people that suffer from psychiatric disorders.

5.5 Overall conclusions

This thesis presents data showing that bovine whey protein (WPI) and its components modulate energy balance, adiposity, metabolism, intestinal physiology and the compositon of the gut microbiota. Therefore, we have demonstrated that this dietary milk protein source can reduce weight gain and adiposity, which have implications for ameliorating several disturbances linked with HFD-induced obesity in preclinical models.

Initally, we have shown that the WP component LAB modulates energy balance partially through regulation of the gene expression of some important nutrient tranporters within the small intestine. Moreover, LAB presence within HFD promotes the growth of specific members of the gut microbial population, such as *Lactobacillus* and *Bifidobacterium*.

Subsequently, we focused on the effect of whole WP (WPI) as part of HFD given at different ages and with different durations of the dietary intervention. We showed that WPI exerts several beneficial effects on HFD-induced body weight gain, leptin availability, adiposity and adipose tissue metabolism, gut health and inflammation. WPI impacts on gut microbiota diversity and composition, (in particular on *Lactobacillus* and *Allobaculum* taxa). Importantly, we demonstrated that WPI's positive effects on gut permeability and systemic inflammation seems to be mediated by the gut microbiota. However, more investigation on the relationship between obesity, WP and gut microbiota are needed to reveal the precise mechanisms that underlie the WP anti-obesity effects.

This thesis represents an appropriate starting point for a deeper investigation of the beneficial effects of consumption of specific macronutrients in individuals affected by metabolic syndrome and more research on the implications for clinical practice is now warranted. Importantly, WP is an attractive addition to obesity treatment as it is a natural dietary supplement, which would facilitate compliance with its administration. Overall, the work presented here may help to focus future studies exploring the weight management effects of WP and its components.

Appendix

Diets information sheet from Research Diets

D12450B, D12451 and D13081701

Formulated by Research Diets, Inc.

Product #	D12450B		D12451		D13081701	
%	gm	*kcal*	gm	*kcal*	gm	*kcal*
Protein	19.2	*20*	23.7	*20*	23.7	*20*
Carbohydrate	67.3	*70*	41.4	*35*	41.4	*35*
Fat	4.3	*10*	23.6	*45*	23.6	*45*
Total		*100*		*100*		*100*
kcal/gm	3.8		4.7		4.7	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	200	*800*	0	*0*
Lactalbumin-α	0	*0*	0	*0*	200	*800*
L-Cystine	3	*12*	3	*12*	3	*12*
Corn Starch	315	*1260*	72.8	*291*	72.8	*291*
Maltodextrin 10	35	*140*	100	*400*	100	*400*
Sucrose	350	*1400*	172.8	*691*	172.8	*691*
Cellulose, BW200	50	*0*	50	*0*	50	*0*
Soybean Oil	25	*225*	25	*225*	25	*225*
Lard	20	*180*	177.5	*1598*	177.5	*1598*
Mineral Mix S10026	10	*0*	10	*0*	10	*0*
DiCalcium Phosphate	13	*0*	13	*0*	13	*0*
Calcium Carbonate	5.5	*0*	5.5	*0*	5.5	*0*
Potassium Citrate, 1 H2O	16.5	*0*	16.5	*0*	16.5	*0*
Vitamin Mix V10001	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*	2	*0*
FD&C Yellow Dye #5	0.05	*0*	0	*0*	0.05	*0*
FD&C Red Dye #40	0	*0*	0.05	*0*	0	*0*
FD&C Blue Dye #1	0	0	0	0	0	0
Total	**1055.05**	***4057***	**858.15**	***4057***	**858.15**	***4057***

Research Diets, Inc.
20 Jules Lane
New Brunswick, NJ 08901 USA
info@researchdiets.com

D13081701.for

D12450B, [redacted] D12081601, [redacted]

Rodent Diets With 10 kcal% Fat
and 20 kcal% Protein from Casein or Whey Protein

Product #	D12450B		[redacted]	D12081601		[redacted]
%	gm	*kcal*		gm	*kcal*	
Protein	19.2	*20*		19.2	*20*	
Carbohydrate	67.3	*70*		67.3	*70*	
Fat	4.3	*10*		4.3	*10*	
Total		*100*			*100*	
kcal/gm	3.8			3.8		
Ingredient	**gm**	***kcal***		**gm**	***kcal***	
Casein	200	*800*		0	*0*	
Whey Protein	0	*0*		200	*800*	
L-Cystine	3	*12*		3	*12*	
Corn Starch	315	*1260*		315	*1260*	
Maltodextrin 10	35	*140*		35	*140*	
Sucrose	350	*1400*		350	*1400*	
Cellulose, BW200	50	*0*		50	*0*	
Soybean Oil	25	*225*		25	*225*	
Lard	20	*180*		20	*180*	
Mineral Mix S10026	10	*0*		10	*0*	
DiCalcium Phosphate	13	*0*		13	*0*	
Calcium Carbonate	5.5	*0*		5.5	*0*	
Potassium Citrate, 1 H2O	16.5	*0*		16.5	*0*	
Vitamin Mix V10001	10	*40*		10	*40*	
Choline Bitartrate	2	*0*		2	*0*	
FD&C Yellow Dye #5	0.05	*0*		0	*0*	
FD&C Red Dye #40	0	*0*		0	*0*	
FD&C Blue Dye #1	0	0		0.05	0	
Total	**1055.05**	***4057***		**1055.05**	***4057***	

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NilaweeraK12.for

January, 2015

D12450B, D12451 and D11040501

Formulated by
Research Diets, Inc.
June 2018

Product #	D12450B		D11040501		D12451	
%	gm	*kcal*	gm	*kcal*	gm	*kcal*
Protein	19.2	*20*	23.7	*20*	23.7	*20*
Carbohydrate	67.3	*70*	41.4	*35*	41.4	*35*
Fat	4.3	*10*	23.6	*45*	23.6	*45*
Total		*100*		*100*		*100*
kcal/gm	3.8		4.7		4.7	
Ingredient	**gm**	***kcal***	**gm**	***kcal***	**gm**	***kcal***
Casein	200	*800*	0	*0*	200	*800*
Whey Protein	0	*0*	200	*800*	0	*0*
L-Cystine	3	*12*	3	*12*	3	*12*
Corn Starch	315	*1260*	72.8	*291*	72.8	*291*
Maltodextrin 10	35	*140*	100	*400*	100	*400*
Sucrose	350	*1400*	172.8	*691*	172.8	*691*
Cellulose, BW200	50	*0*	50	*0*	50	*0*
Soybean Oil	25	*225*	25	*225*	25	*225*
Lard	20	*180*	177.5	*1598*	177.5	*1598*
Mineral Mix S10026	10	*0*	10	*0*	10	*0*
DiCalcium Phosphate	13	*0*	13	*0*	13	*0*
Calcium Carbonate	5.5	*0*	5.5	*0*	5.5	*0*
Potassium Citrate, 1 H2O	16.5	*0*	16.5	*0*	16.5	*0*
Vitamin Mix V10001	10	*40*	10	*40*	10	*40*
Choline Bitartrate	2	*0*	2	*0*	2	*0*
FD&C Yellow Dye #5	0.05	*0*	0.025	*0*	0	*0*
FD&C Red Dye #40	0	*0*	0.025	*0*	0.05	*0*
FD&C Blue Dye #1	0	0	0	0	0	0
Total	**1055.05**	***4057***	**858.15**	***4057***	**858.15**	***4057***

Research Diets, Inc.
20 Jules Lane
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NilaweeraK03.for

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