

METABONOMIC INVESTIGATIONS OF THE MAMMALIAN HOST-GUT MICROBIOME INTERACTIONS IN OBESITY AND METABOLIC DISEASES

Thesis submitted by

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ABSTRACT

Obesity is known to contribute to the risk of developing type 2 diabetes mellitus, non-alcoholic fatty liver diseases, cancer, osteoarthritis and cardiovascular diseases. Obesity results from an imbalance between energy intake and energy expenditure but its aetiology is complex and not completely understood. Genetic and environmental factors, (high calorie diet, lack of physical activity) strongly influence the onset of obesity and metabolic diseases. Modifications of gut microbial–host metabolic interactions by dietary interventions also contribute to obesity and are associated with metabolic disturbances. Noticeably, some obese individuals seem protected from the development of metabolic diseases which highlight individual predispositions to obesity associated metabolic disturbances. It is therefore important to underpin the molecular mechanisms linked to obesity and the role of gut microbiota in the degradation of a healthy phenotype in an appropriate biological model.

¹H nuclear magnetic spectroscopy and mass spectrometry analysis were employed to measure the abundant metabolites in C57BL/6 mouse urine, feces, and plasma. Metabolic profiles of mice subjected to dietary interventions were compared using multivariate statistics. Alterations of gut microbial ecosystem by dietary interventions were also assessed by 16s rRNA pyrosequencing of mouse fecal microbiota.

When given a high fat diet, the obesity prone and obesity resistant mice showed differences in leucine catabolism and tricarboxylic cycle. This may indicate specific regulations of mitochondrial oxidative pathways during diet induced obesity (DIO), influencing obesity development and metabolic disturbances. Possible roles of gut microbiota in the regulation of mitochondrial oxidative pathways were also envisaged. Gut microbial remodelling by the supplementation of different prebiotic blends (galactosyl-oligosaccharides, fructosyl-oligosaccharides and inulin or galactosyl-oligosaccharides and cow milk oligosaccharides) during a high fat challenge also decreased obesity and low grade inflammation and affect mouse metabolism. Decreased gut bacterial amino acids fermentation by prebiotic supplementation may contribute to the improvement of mouse physiology during DIO.

STATEMENT OF ORIGINALITY

I certify that this thesis, and the research to which it refers, are the product of my own work, and that any ideas or quotations from the work of other people, published or otherwise, are fully acknowledged in accordance with the standard referencing practices of the discipline.

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To my family

To the memory of my grand father

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TABLE OF CONTENTS

CHAPTER I: INTRODUCTION	33
1. Obesity	33
1.1. Definition of obesity and metabolic syndrome	33
1.2. Aetiology of obesity and MetS.....	36
1.3. Obesity and Energy homeostasis.....	38
1.3.1. Fatty acid metabolism.....	40
1.3.2. Carbohydrate digestion and glucose metabolism	42
1.3.3. Amino acid metabolism	44
1.3.4 Obesity and insulin resistance: the underpinning mechanisms of obesity development.....	47
1.4. Obesity and low grade inflammation.....	50
1.4.1. Basic mechanisms of innate immunity	50
1.4.2. Basic mechanisms of adaptive immunity.....	53
1.4.3. Obesity associated low grade inflammation.....	55
1.4.4. Links between low grade inflammation and impaired insulin signalling.....	56
1.5. Obesity, MetS and metabolic diseases	58
2. Role of the gut microbiota in the development of obesity and the MetS.....	59
2.1. The biological role of symbiotic bacteria.....	59
2.2. Gut microbiota modulation and disease development.....	61
2.3. Gut microbial remodelling by nutritional interventions.....	62
3. A metabonomic strategy to understand the interactions between diet and microbiota in obesity and MetS development	65
4. Scope of the thesis.....	66

CHAPTER II: ANALYTICAL METHODS	68
1. Nuclear Magnetic resonance:	68
1.1. Fundamental principles of NMR spectroscopy	69
1.2. Chemical shifts	71
1.3. The spin-spin coupling	71
1.4. 1D NMR spectroscopy.....	72
1.5. 2D NMR spectrometry	73
2. Liquid Chromatography Mass Spectrometry	75
2.1. Principles of Liquid Chromatography.....	76
2.2. Principle of Mass spectrometry	77
2.3. Multiplex targeted MS/MS assay: the Biocrates kit	79
3. Statistical analysis	83
3.1. Unsupervised method: Principal Component Analysis (PCA).....	83
3.2. Supervised methods.	85
3.3. Biomarker identifications.....	88
4. 16S ribosomal RNA gene sequencing	89

**CHAPTER III: EARLY METABOLIC ADAPTATION IN C57BL/6 MICE RESISTANT TO HIGH FAT DIET
INDUCED WEIGHT GAIN INVOLVES AN ACTIVATION OF MITOCHONDRIAL OXIDATIVE PATHWAYS** **92**

1. Introduction	92
2. Materials and methods	93
2.1. Animal handling procedure and sample preparation	93
2.2. Measurement of body weight gain and identification of NR and SR mice	94
2.3. ¹ H NMR spectroscopy	94

2.4.	Data processing and multivariate data analysis	95
2.5.	Pathways-specific analysis using univariate statistical approach	96
3.	Results	97
3.1.	Body weight gain variability in C57BL/6 mice fed a HF diet	98
3.2.	¹ H NMR urine metabolic profiling of LF- and HF-fed mice identifies a sustained metabolic signature associated to DIO	99
3.3.	¹ H NMR urine metabolic profiling of NR and SR mice highlights a specific metabolic adaptation associated to obesity prone and obesity resistant phenotype.	102
3.4.	Univariate analysis of intermediate metabolites from candidate pathways pointed out specific deregulations of mitochondrial metabolism in HF-fed mice that may be exacerbated in SR mice.....	108
4.	Discussion.....	111
4.1.	HF diet-induced obesity is associated with a rapid up-regulation of fatty acid breakdown and BCAA catabolism	111
4.2.	SR and NR mice develop specific metabolic adaptations to HF diet based on BCAA catabolism and β -oxidation.	115
4.3.	Influence of gut microbiota on host energy metabolism	116
4.4.	Fatty acid overflow is associated with saturation of mitochondrial oxidative pathways which might be involved in obesity development.....	117
5.	Conclusions	119

CHAPTER IV: THE PREBIOTIC STUDY

<u>A TOP-DOWN METABONOMIC APPROACH TO INVESTIGATE THE LONG TERM EFFECTS OF PREBIOTICS ON C57BL/6 MOUSE PHYSIOLOGY AND METABOLISM DURING DIO</u>		120
1.	Introduction.....	120
2.	Materials and methods.....	125

2.1.	Animal handling procedure and sample preparation	125
2.2.	Measurement of body weight, body weight gain, fat mass, food intake and food efficiency.....	126
2.3.	Measurement of body composition	127
2.4.	Glucose tolerance and insulin secretion test.....	127
2.5.	Measurement of circulating cytokines with Multiplexed Elisa assay (Meso Scale platform)	128
2.6.	Sample preparation and ¹ H NMR spectroscopy	128
2.6.1.	Urine.....	129
2.6.2.	Plasma	129
2.6.3.	Feces	129
2.7.	¹ H NMR spectra pre-processing	129
2.8.	¹ H NMR Data processing	130
2.9.	MS targeted profiling using the Biocrates kit	130
2.10.	¹ H NMR metabolic data and MS targeted data analysis	131
3.	Results	131
3.1.	Physiological data:	131
3.1.1.	Body weight gain, food intake, cumulative food efficiency and fat mass	131
3.1.2.	Systemic insulin and glucose homeostasis.	135
3.1.3.	Inflammatory markers	137
3.2.	Metabonomic data.....	139
3.2.1.	Characterisation of ¹ H NMR urinary, fecal and plasma metabolic profiles of C57BL/6 mice.....	139

3.2.2.	Effect of prebiotics supplemented HF diet at D70 after the diet switch in C57BL/6 mice.....	146
3.2.3.	Global OPLS-DA models of urine and feces samples describe metabolic adaptations associated with nutritional interventions.....	147
3.2.4.	Comparison of urinary, fecal and plasma metabolic profiles of LF, HF and each HF + prebiotic groups using OPLS-DA emphasize prebiotics induced metabolic rescue.....	149
3.2.5.	¹ H NMR spectral data of urine, feces and plasma samples at D70 were compared between the diet groups using pair-wise OPLS-DA models.....	155
3.2.6.	Targeted LC/direct infusion MS analysis of plasma samples reveals strong variations in lipid and amino acid metabolism with prebiotic supplementation after 70 days of HF challenge	160
4.	Discussion.....	165
4.1.	HF ingestion induce a saturation of hepatic lipid metabolism	165
4.2.	HF challenge alter mitochondrial oxidation efficiency	167
4.3.	Prebiotic supplementation during HF diet limit dyslipidemia improves mitochondrial oxidative pathways.	168
4.4.	FOS-IN activate amino acid metabolism involved in insulin signalling and mitochondrial metabolism	169
4.5.	Prebiotics supplementation during HF challenge improves mouse health by modulating colonic bacterial fermentation.	171
4.6.	GOS supplementation alleviated inflammatory responses induced by the combination of HF and lactose mal-absorption	173
5.	Conclusion.....	175

CHAPTER V: THE PREBIOTIC STUDY

CORRELATIVE ANALYSIS OF FECAL MICROBIOTA, ¹H METABOLIC PROFILES AND CIRCULATING CYTOKINE LEVELS

177

1. Introduction	177
2. Materials and Methods.....	180
2.1. Animal handling procedure and sample preparation.....	180
2.2. Measurement of circulating cytokines with Multiplexed Elisa assay (Meso Scale platform).	180
2.3. Sample preparation and ^1H NMR spectroscopy.	180
2.4. ^1H NMR spectra pre-processing and ^1H NMR data processing.	180
2.5. ^1H NMR data binning using statistical recoupling of variables.	180
2.6. 16S rRNA gene sequencing of fecal microbiota.....	181
2.7. Multiplatform data analysis	182
3. Results.....	183
3.1. HF diet and prebiotic supplementation during HF challenge induce significant changes in the composition of the fecal microbiota	183
3.2. Integration of D70 family-level fecal microbiota, metabolomic (^1H NMR urine, plasma and feces) and plasma cytokine data.....	188
3.2.1. MFA highlights similar patterns of variation in the composition of the fecal microbiota, metabolic profiles and circulating inflammatory markers in the different diet groups.....	190
3.2.2. Correlation analyses of the family-level microbiota and metabolic (^1H NMR urine, plasma and feces) data from D70 confirm microbiome-metabolome associations.....	192
4. Discussion.....	197
4.1. HF diet induced rapid changes in the composition of the gut microbiota	198
4.2. HF-induced modulation of the gut microbiota accompanies changes in host lipid storage and metabolism	199

4.3. Prebiotic supplementation during HF diet leads to increased representation of <i>Bifidobacteriaceae</i> or <i>Lactobacillaceae</i> in the fecal microbiota.....	201
4.4. Prebiotic supplementation during HF challenge changes the proportions of families belonging to the phyla <i>Bacteroidetes</i> and <i>Firmicutes</i>	203
4.5. Correlation analysis highlights associations between <i>Lachnospiraceae</i> , <i>Bacteroidaceae</i> , <i>Porphyromonadaceae</i> , <i>Rikenellaceae</i> , <i>Coriobacteriaceae</i> , or <i>Erysipelotrichaceae</i> and host mitochondrial oxidative pathways.....	205
4.6. Correlation analysis highlighted an association between circulating inflammatory status of mice and gut microbial activity/composition	206
4.7. Increased gut bacterial proteolysis contribute to inflammation in HF-fed mice.....	208
5. Conclusion.....	210

CHAPTER VI: THE PREBIOTIC STUDY

IDENTIFICATION OF CANDIDATE MARKERS PREDICTING THE LONG-TERM EFFECT OF PREBIOTICS ON MOUSE PHYSIOLOGY DURING DIET-INDUCED OBESITY

1. Introduction	211
2. Materials and methods.....	214
2.1. Animal handling procedure and sample preparation.....	214
2.2. Measurement of body weight, body weight gain, fat mass, food intake and food efficiency	214
2.3. Measurement of organ weights	214
2.4. Measurement of body composition	215
2.5. Glucose tolerance and insulin secretion test.....	215
2.6. Measurement of circulating cytokines with Multiplexed Elisa assay (Meso Scale platform)	215
2.7. Sample preparation and ¹ H NMR spectroscopy	215

2.8.	¹ H NMR spectra pre-processing and ¹ H NMR data processing	215
2.9.	¹⁶ S rRNA gene sequencing of fecal microbiota.....	216
2.10.	Prediction of physiological outcomes of mice fed HF and HF + prebiotics by OPLS regression models.....	216
3.	Results.....	216
3.1.	Prebiotic supplementation during HF diet limits energy storage and adipose accumulation, and affects organ weights.....	216
3.2.	Multivariate prediction of the long-term (D70) prebiotic efficiency in limiting HF induced obesity and metabolic disorders.....	217
3.2.1.	¹ H NMR urinary metabolic profiles from D7 after the diet switch predict prebiotic efficiency in reducing long-term calorie storage during HF challenge	220
3.2.2.	¹ H NMR fecal metabolic profiles do not consistently predict the long-term prebiotic efficiency in reducing calorie storage during HF challenge	226
3.2.3.	Fecal microbial profiles at D7 and D70 after the diet switch predict the long-term prebiotic efficiency in reducing calorie storage during HF challenge.....	227
3.2.4.	Early, stable candidate markers commonly predict the long-term efficiency of GOS, GOS_CMO and FOS_IN in reducing calorie storage.....	231
3.2.5.	Urinary abundance of PAG, PCG, PCS and fecal <i>Streptococcaceae</i> at D7 after the diet switch predicts long-term beneficial effect of prebiotic on calorie storage, organ weights and other physiological disorders during HF challenge.....	232
4.	Discussion.....	236
4.1.	Rapid changes in colonic bacterial amino acid fermentation influence mouse physiology during DIO	237
4.2.	PCS, PCG and PAG may confer pharmacological effects on host fat storage and insulin signalling pathways.....	238
4.3.	PCS, PCG and PAG may contribute to host IL-1 β related inflammatory pathways	239

4.4. HF associated renal dysfunction may contribute to increase PCS, PCG or PAG in systemic circulation	240
4.5. Variability in fecal metabolic profiles does not allow predicting the physiological outcomes	241
4.6. The predictive models present some limitations	241
5. Conclusion.....	242
 CHAPTER VII: GENERAL DISCUSSION	 243
1. Key findings	243
2. Statements of novelty.....	244
3. Strengths and weaknesses of the experiments	246
4. Obesity development of C57BL/6 mice under nutritional interventions.	248
4.1. The role of mitochondrial oxidative pathways in obesity development	250
4.2. Influence of gut microbiota on host mitochondrial metabolism.....	251
4.3 Interactions between gut microbial tyrosine fermentation, host inflammatory pathways and host metabolism during diet induced obesity	253
5. Perspectives on human health.....	257
 REFERENCES	 260

TABLE OF FIGURES

Figure 1 Energy supplying pathways connecting glucose, fatty acids and amino acids (Ile: isoleucine, Asn: Asparagine, Asp: Aspartate, phe: Phenylalanine, Tyr: Tyrosine, Val: Valine, Leu: leucine). TGs: triglycerides, *neoglucogenesis only occurs in the liver 36

Figure 2: Summary of energy metabolic pathways during the postprandial period. A: Fatty acids metabolism, B: carbohydrate metabolism, C: Protein metabolism. 1: TG synthesis, 2: glyceroneogenesis, 3: TCA cycle, 4: Fatty acid synthesis, 5: glycolysis, 6: oxidative phosphorylation, 7: galactose conversion into glucose (Leloir pathway), 8: Fructose metabolism, 9: pentose phosphate pathways, 10: gluconeogenesis, 11: transamination, 12: urea cycle, 13: lipolysis, 14: protein synthesis, 15: TG degradation, 16: glycerolysis, 17: Cori cycle, 18: Ketogenesis, 19: proteolysis 43

Figure 3: Summary of energy metabolic pathways during a fasting period. 1: TG synthesis, 2: glyceroneogenesis, 3: TCA cycle, 4: Fatty acid synthesis, 5: glycolysis, 6: oxidative phosphorylation, 7: galactose conversion into glucose (Leloir pathway), 8: Fructose metabolism, 9: pentose phosphate pathways, 10: gluconeogenesis, 11: transamination, 12: urea cycle, 13: lipolysis, 14: protein synthesis, 15: TG degradation, 16: glycerolysis, 17: Cori cycle, 18: Ketogenesis, 19: proteolysis 44

Figure 4: Schema of insulin signalling pathways inspired by R. Saltiel *et al*⁴² 46

Figure 5: Induction of inflammatory signals in pro-inflammatory macrophages. A: Bacterial LPS activate the cytokines transcription *via* the Toll like receptor-4 (TLR-4) activation. B: Other pattern-recognition receptors involved in inflammasome pathways and leading to IL-1/IL-8 maturation 49

Figure 6: Important pro and anti inflammatory cytokines secreted by M1 or M2 activated macrophages. 52

Figure 7: Theory of NMR spectroscopy. (A) .Energy level and transition for a nucleus having a nuclear spin $I=1/2$ in a magnetic field B_0 . (B). Motion of net nucleus magnetisation which relax at the Larmor frequency ω_0 in a magnetic field B_0 . The spins resultant gives a stationary bulk magnetization vector prior to radiofrequency (RF) perturbation. (C) Effect of RF irradiation with a frequency ω_0 on bulk magnetisation vector M_0 67

Figure 8: Multiplicity and relative intensities of NMR signals from equivalent coupled nuclei with spin number $I=1/2$ (such as ^1H coupling) 69

Figure 9: Standard one pulse sequence (RD: relaxation delay pw: pulse width) 70

Figure 10: NMR pulse sequence of a standard COSY experiment (RD: relaxation delay, p1: first pulse width to reach 90° ; t1: evolution time, p2: second pulse width to reach 90° , t2: acquisition time) 71

Figure 11: Diagram of mass spectrometry. HPLC: high pressure liquid chromatography, UPLC: ultra high pressure liquid chromatography, gas chromatography, CE: capillary electrophoresis chromatography, EI/CI:

electronic or chemical ionisation, APCI: atmospheric pressure chemical ionisation, FAB: Fast atom bombardment, ESI: electrospray ionisation MALDI: matrix-assisted laser desorption ionisation, FT ICR: Fourier transform-ion cyclotron resonance..... 74

Figure 12: Example of ions fragmentation in the MS analyser 77

Figure 13: Protocol for sample preparation using the Biocrates kit..... 78

Figure 14: Construction of the first principal component (PC1) explaining the main variation within the dataset. (var: variable). The score (T) of one observation (i), its corresponding residual (E) and the loading of the variable 1 for this observation (Pvar1) is also geometrically represented. 81

Figure 15: Geometric approach of principal component analysis ¹¹⁴ : Variations within a X matrix containing 13 samples and 3 variables are approximate in a lower dimension model plane. Scores plot and loadings plot obtained from the model respectively provide information on sample classification and on variable influence in the model. 82

Figure 16: Principle of PLS model. The multidimensional X matrix (top left) represents the predictor and the multidimensional Y matrix (top right) represents the responses to predict. In each multi-variate space, the orientation of the first component must approximate as well as possible the shape of the point cloud and the scores t1 and U1 should be maximally correlated. The second component is build perpendicularly to the first component using the same principle. 83

Figure 17: Scheme of 16S rRNA gene sequence containing hypervariable regions (V) and highly conserved regions (C) adapted from Petrosino *et al* ¹³⁴. 87

Figure 18: Principles of pyrosequencing and chemical reactions involved in the determination of DNA sequences adapted from Petrosino *et al* ¹³⁴. 3 main steps are involved in one sequencing cycle and several cycles are performed to identify the nucleotide sequence 88

Figure 19: Variability of body weight gain in a population of n=56 mice fed a HF diet. (A) Experimental design. (B) Body weight distribution of mice after 7 days and 60 days of HF diet feeding. (C) Body weight and (D) body weight gain monitoring of control (LF, n=24), NR (n=10), and SR (n=14) mice before the diet (t0), after 7 days (t1) and 60 days (t2), after diet switch. (n=average ± standard error, significant differences in body weight and body weight gain between LF fed mice and NR mice or LF fed mice and SR mice were evaluated using non parametric Wilcoxon-Mann-Whitney test, *p value*: *<0.05, **<0.001, ***<0.0001.) 94

Figure 20: Correlation between mouse BWG after 1 week of HF diet and mouse BW 60 days after diet switch..... 9

Figure 21: ^1H NMR urine metabolic profile of C57BL/6 HF fed or LF fed mice 7 days and 60 days after the diet switch. (A) Mean ^1H NMR spectrum of urine from LF fed mice or (B) HF fed mice. (C) OPLS-DA score plot of urine metabolic profile of LF and HF fed mice at 7 days (D) OPLS-DA score plot of urine metabolic profile of LF and HF fed mice at 60 days. (E) Heatmap obtained from the OPLS-DA coefficient plots showed metabolites found to be significantly different in HF and LF fed mice. Correlation values of the metabolites are displayed by color code (Gradient of red color for metabolites positively correlated with HF-fed mice and gradient of blue colors for metabolites negatively correlated). 98

Figure 22: Specific metabolic signatures of NR and SR mice. (A) Mean of ^1H NMR spectra of urine from NR mice or (B) SR mice. (C) OPLS-DA score plot of urine metabolic profile of NR and SR mice at 7 days (D) and 60 days. (E) Heatmap obtained from the OPLS-DA coefficient plots showed metabolites found to be significantly different in NR and SR mice. Correlation values of the metabolites are displayed by color code. (Gradient of red color for metabolites positively correlated with SR mice and gradient of blue colors for metabolites negatively correlated). * Tentative assignment..... 100

Figure 23: Loadings plot of OPLS-DA models showing the comparison of the urine metabolic profile between HF and LF fed mice at 7 days (A), at 60 days (B) and OPLS-DA models showing comparison between urine metabolic profile of NR and SR mice at 7 days (C) and 60 days (D)..... 102

Figure 24: Mapping of urinary metabolite excretion pattern involved in BCAAs, NAD metabolism, TCA cycle, β -oxidation, ketogenesis and gluconeogenesis. The bar plots showed the mean ratio with standard error of metabolite integrals at day 7 to day 0 or day 60 to day 0. The Y axis indicates the value of the mean for LF, HF, NR and SR mice (arbitrary unit). Significant difference between mean ratios of LF and HF or NR and SR were calculated with non parametric Wilcoxon-Mann Whitney test: * <0.05, **<0.001, ***<0.0001 (values of mean, standard errors and *p* value in Appendix 1 Tables S5, and Table S6). Indirect metabolic reactions are highlighted with dash arrows. PEP: phosphoenolpyruvate 107

Figure 25: Summary of mitochondrial oxidative pathways and energy production. Glycolysis, β -oxidation, and BCAAs oxidation enable the production of reduced co-enzymes, which in turn are used to synthesize ATP. These pathways are also interconnected with TCA cycle, which contributes in energy production. Mitochondria nutrient overload leading to an up-regulation of oxidative pathway might increase the level of mitochondrial reactive oxygen species (ROS) formation, which triggers damage in mitochondrial (mt) DNA, proteins and lipids. Alternatively ROS can be detoxified *via* the glutathione pathway. This figure highlights in a simplified way, the general metabolic pathways occurring in the mitochondria and does not reflect any tissue-specific mitochondrial activity. GSSG: oxidized glutathione, GSH: reduced glutathione, LCFA: long chain fatty acid, TG; triglycerides. Cyt: cytochrome, CPT: citrate protein transporter, MPC: mitochondrial pyruvate carrier, BCAT: branched-chain aminotransferase, BCKDH: branched-chain keto acid dehydrogenase 112

Figure 26: Experimental design to investigate the effect of prebiotic supplementation on physiological and metabolic adaptation of C57BL/6 mice during HF challenge. LF (low fat), HF (high fat), HF_S (HF + glucose-

galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin) 119

Figure 27: Impact of different diet interventions on (A.) mouse weight gain, (B.) food intake and (C.) cumulative food efficiency during the overall course of the experiment. Cumulative food intake (CFE) = body weight gain (BWG) /cumulative food intake. Wilcoxon-Mann-Whitney tests are presented at the last timepoint. 1 star for p -value<0.05, 2 stars for p -value<0.01, 3 stars for p -value<0.001 (x: HF vs HF+GOS, ¥ HF vs HF +FOS_IN, °: HF_S vs HF+GOS_CMO, ‡: LF vs HF+GOS, +: LF vs HF+FOS_IN, #:LF vs HF+GOS_CMO). LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin) 130

Figure 28: Body fat composition of mice fed with different diets at 56 days (D56). Fat mass (red), lean mass (blue) and total mass (green) levels of different diet groups are shown. Values are expressed as mean $\pm$ SEM. Differences between diet groups were assessed by non parametric Wilcoxon-Mann-Whitney tests . $P<0.05^*$, $p<0.01^{**}$, $p<0.001^{***}$. LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin) 132

Figure 29: Time-dependent effect of different dietary interventions on mouse fasting glycaemia. Concentration of glucose was obtained at 7 days (D-7) before the diet switch, at 35 (D35) and at 49 (D49) days after the diet switch and the values are expressed as mean $\pm$ SEM. Differences between groups were assessed by non parametric Wilcoxon-Mann-Whitney tests , $P<0.05^*$, $p<0.01^{**}$, $p<0.001^{***}$. LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin) 134

Figure 30: Circulating inflammatory status of mice fed different diets at 70 days (D70) after diet switch. Plasma level of IFN- γ , IL-1 β , IL-6, IL-10, IL-12, KcGRO, TNF- α were measured in each dietary group at D70 after diet switch with the MesoScale Discovery platform. Results are expressed as mean concentration (pg/ml) $\pm$ SEM. For each cytokine, the differences between groups were assessed by non parametric Wilcoxon-Mann-Whitney tests, $P<0.05^*$, $p<0.01^{**}$, $p<0.001^{***}$. LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin) 136

Figure 31: Mean of ^1H NMR spectra of urine from LF, HF and HF + prebiotic fed mice from D7 to D70 The aromatic region of the spectrum (6.8-7.8ppm) has been zoomed to improve the spectral assignment. 4PY : *N*-methyl-4-pyridone-3-carboxamide, 2PY: *N*-methyl-2-pyridone-5-carboxamide*: tentative assignment. 138

Figure 32: Mean of ^1H NMR spectra of feces from LF, HF and HF + prebiotic fed mice from D7 to D70. The spectrum has been zoomed in three different regions (0-2.2, 2.3-4.5 and 4.7-8.55ppm) to identify peak multiplicity and improve the spectral assignment.*: tentative assignment.....	139
Figure 33: Mean of ^1H NMR spectra of plasma from LF, HF and HF + prebiotic fed mice from D7 to D70. (A.) ^1H NMR CPMG spectra (B.) ^1H NMR standard spectra	140
Figure 34: Global urinary (A) and fecal (B) metabolic profile of each diet group. All urine (n=826) or fecal (n=785) samples acquired from 7 days (D7) to 70 days (D70) after the diet switch were used to calculate OPLS-DA models. For each matrix, 3D score plots showed the three dimensional dispersion of the scores of each diet group across time. The 2D projections indicate the biggest variability in the dataset along the first and the second component. Both models were built with 6 predictive and 2 orthogonal components. $Q^2Y=0.46$ and $R^2Y=0.49$ for urinary metabolic profile. $Q^2Y=0.24$, $R^2Y=0.46$ for fecal metabolic profile: LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin).....	145
Figure 35: OPLS-DA score plot from global urinary (A.) and fecal (B.) metabolic profile highlighting the time-dependent trajectories: Urine (n=826) or fecal (n=785) samples acquired from 7 days (D7) to 70 days (D70) after the diet switch were used to calculate OPLS-DA models. For each matrix, 3D score plots showed the three dimensional dispersion of the scores on the 4th, 5th and 6th component which highlights the time trajectory.....	156
Figure 36: Identification of specific metabolic effects and partial normalisation of metabolic profiles by prebiotic supplementation in urine (A, B, C.), feces (D, E, F.) and plasma (G, H, I) after 70 days of HF diet. For each biological matrix, OPLS-DAs were calculated with LF, HF and HF + GOS groups (A, D, G), or LF, HF and HF + FOS_IN (B, E, H) or LF, HF_S and HF + GOS_CMO (C, F, I). Q^2Y and R^2Y for each model are given in Table 6.. LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin).....	148
Figure 37: Evaluation of the “metabolic rescue” of different prebiotics during HF challenge in urine, feces and plasma. The metabolic rescue of FOS_IN and GOS was estimated (in percentage) by predicting the score of HF + GOS and HF + FOS_IN groups onto the first component of OPLS-DA model built with urinary (A), fecal (C) or plasma (E) metabolic profile of HF and LF groups. The “metabolic rescue” of GOS_CMO was calculated by predicting HF + GOS_CMO scores onto the first predictive component of the OPLS-DA models built with urinary (B), fecal (D) or plasma (F) metabolic profile of LF and HF groups. Negative control group (LF), positive control group (HF, or HF_S) and test groups (HF + FOS_IN , HF + GOS or HF + GOS_CMO) LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin).....	151

Figure 38: Comparison of metabolic signatures associated to different diet groups. (A) Standard ^1H NMR plasma metabolic profiles (B) CPMG ^1H NMR plasma metabolic profiles (C) Standard ^1H NMR urinary metabolic profiles (D) Standard ^1H NMR fecal metabolic profiles. Correlation values (R) of the metabolites are displayed by color code. Gradient of red color are for metabolites positively correlated with HF or HF_S (in LF vs HF or LF vs HF_S models) or HF + prebiotics (in LF vs HF+prebiotics or HF vs HF + prebiotic models). The gradient of blue colors are for metabolites negatively correlated with the same diet groups LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin) (PC: predictive components, OC: orthogonal components).....156

Figure 39: Global OPLS-DA model comparing plasma lipid and amino acid variations between different diet groups measured by targeted LC/direct infusion MS. OPLS-DA score plot (A.) showed the main variations in the dataset while OPLS-DA loadings plot (B.) indicated metabolite contributions to the separation between diet groups. LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin).....159

Figure 40: Comparison of the plasma metabolic signature acquired by MS targeted profiling analysis among the different diet group at D70 after the diet switch. The correlation matrix summarises the pair-wise OPLS-DA models between the different diet groups. Each column represents an OPLS-DA model comparing the two groups indicated above each column, and illustrates the variation in circulating levels of lipids, amino acids, acylcarnitines and other metabolites. For each model, correlation coefficients were obtained from the OPLS-DA coefficient plot. Correlation values (R) of the metabolites are displayed by color code. Gradient of red colors is for metabolites positively correlated with HF or HF_S (in LF vs HF or LF vs HF_S models) or HF + prebiotics (in LF vs HF+prebiotics or HF vs HF + prebiotic models). The gradient of blue colors is for metabolites negatively correlated with the same diet groups (Cx:y means x :the number of carbons in the side chains, Y: number of insaturations). LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin). LPC (lysophosphocholine) PC/PC-O (phosphocholine) SM (sphingomyelin).....160

Figure 41: Phylum-level analysis of the fecal microbiota of the isogenic C57BL/6 mice at (A) D-7, (B) D7 and (C) D70 of the different dietary interventions. Prior to D-7, animals were fed the LF diet for 2 weeks. Diets: LF, low fat; HF, high fat; HF_S, high fat + glucose-lactose-galactose (positive control for GOS_CMO); HF + GOS, high fat + galactosyl-oligosaccharides; HF + FOS_IN, high fat + fructo-oligosaccharides-inulin; HF + GOS_CMO, high fat + galactosyl-oligosaccharides-cow milk oligosaccharides (glucose-lactose-galactose). The stacked bar graphs show the mean percentage the major phyla which contribute to the total fecal microbiota of the mice at each time point.182

Figure 42: Family-level analysis of the fecal microbiota of the isogenic C57BL/6 mice at D7 and D70 of the different dietary interventions. Diets: LF, low fat; HF, high fat; HF_S, high fat + glucose–lactose–galactose (positive control for GOS_CMO); HF + GOS, high fat + galactosyl-oligosaccharides; HF + FOS_IN, high fat + fructo-oligosaccharides–inulin; HF + GOS_CMO, high fat + galactosyl-oligosaccharides–cow milk oligosaccharides (glucose–lactose–galactose). Bar plot intensities for each family of bacteria (arbitrary unit: au) correspond to the mean percentage of the total detected bacteria at family level. Significant differences in the relative abundances of detected taxa between different diet groups were evaluated using the non-parametric Mann–Whitney tests (*p* value: *<0.05, **<0.001, ***<0.0001). #: NaN.....183

Figure 43: Individual matrix scaling in MFA, adapted from Dumas *et al.*3 (A) XNMR and Xmicrob correspond to NMR and microbiota datasets with KNMR and Kmicrob variables, respectively. (B) PCA of each dataset, which enables calculation of the largest eigenvalue (γ). (C) All datasets were individually scaled with $1/\gamma$ and merged in one block-dataset. (D) Global PCA using the new block-dataset.....185

Figure 44: PCA model of the combined data from the fecal microbiota (family level), relevant metabolites (^1H NMR urine, plasma and feces) and circulating inflammatory markers at D70. (A) The PCA scores plot shows the dispersion of scores for each diet group: 20 % of the variance in the merged matrix (*X*) is explained by the first component, while 17 % of the variance in *X* is explained by the second component. (B) The PCA loadings plot indicated the contribution of variables from the different datasets in the separation of the diet groups. For instance, HF and HF_S groups present in the dotted circle in the score plots showed an increase in several metabolites, cytokines and bacterial families highlighted by the dotted circle in the loadings plot.187

Figure 45: Heatmaps showing bidirectional clustering of Spearman correlation data for families of fecal bacteria and (A) plasma, (B) fecal and (C) urinary metabolites at D70. Heatmaps were produced using Spearman correlation values generated from family-level microbiota data and statistical recoupling of variables (SRV) clusters of ^1H NMR peak intensities²²³ (see Material and Methods). Each SRV cluster was in turn assigned as a metabolite, but several clusters remained unidentified (UA metabolites). Sign of correlation: red, positive correlations; green, negative correlations. *p* Values were determined for Spearman correlations; *p* value <0.05 was taken as representing statistically significant correlations192

Figure 46: Bipartite graph showing the correlation network for IL-1 β , metabolites (urine, plasma and feces) and members of the fecal microbiota. The correlation network was built using significant Spearman correlation results using an arbitrary cut-off of IRI >0.3 and *p* value <0.05. Each node corresponds to an entity (i.e. a metabolite, family or cytokine). The nodes colors are associated with the type of entity: brown, fecal metabolites; yellow, urinary metabolites; pink, plasma metabolites; blue, fecal bacteria at family level; white, circulating cytokine. The edge between two nodes represents a significant Spearman correlation (*R*) between the two entities the nodes represent. The colors of the edges are dependent on the sign of *R*: green, positive

correlation; red, negative correlation. The thickness of the lines indicates the absolute value of R: thinnest lines for $IRI \leq 0.49$; medium thickness lines for $0.5 \leq IRI \leq 0.599$; and thickest lines for $IRI > 0.6$ 193

Figure 47: Identification of metabolic/biological information from urine and feces samples predicting the long-term prebiotic effect on obesity development during HF challenge. BWG monitoring of negative control group (LF), positive control groups (HF and HF_S) and prebiotic test groups (HF + GOS, HF + GOS_CMO, HF + FOS_IN) indicated 2 phases in the physiological adaptation to prebiotic supplementation. Time-dependant metabolic and gut microbial adaptation to each diet intervention underlying the mouse physiological variations was also suggested. Therefore, ^1H NMR metabolic profiles for urine and feces at each time-point, and fecal microbiota profiles at D7 and D70 after the diet switch were used to describe the long-term effect of prebiotic on obesity development and metabolic disorders. LF: low fat, HF: high fat, HF_S : HF and glucose–lactose–galactose (positive control of HF + GOS_CMO group,) GOS : galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose). 208

Figure 48: Cumulative food efficiency of negative control group Wilcoxon-Mann-Whitney tests are presented at the last timepoint. 1 star for p -value<0.05, 2 stars for p -value<0.01, 3 stars for p -value<0.001 (x: HF vs HF+GOS, ¥ HF vs HF +FOS_IN, °: HF_S vs HF+GOS_CMO, ‡: LF vs HF+GOS, +: LF vs HF+FOS_IN, #:LF vs HF+GOS_CMO) (LF), positive control groups (HF, HF_S) and prebiotic test groups (HF + GOS, HF + GOS_CMO and HF + FOS_IN) overtime. LF: low fat, HF : high fat, HF_S : HF and glucose–lactose–galactose (positive control of HF + GOS_CMO group,) GOS : galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose)..... 212

Figure 49: OPLS regression models predicting mouse calorie storage at D70 after the diet switch from urinary metabolic profiles of HF and HF + GOS fed mice (.A ,B), HF_S and HF + GOS_CMO fed mice (.C, .D) or HF and HF + FOS_IN fed mice (E, F). For each model, the score plots indicate a good relationship between early metabolic profiles and CFE_D70 (.A, C, E). The OPLS-loadings plot highlights metabolites that are positively or negatively correlated with CFE_D70 (.B, .D, .F). LF: low fat, HF: high fat, HF_S : HF and glucose–lactose–galactose (positive control of HF + GOS_CMO group,) GOS: galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose) 217

Figure 50: Goodness of fit (R^2Y) and goodness of prediction (Q^2Y) of OPLS-regression models predicting CFE_D70 with urinary metabolic profiles of HF and HF + GOS groups (A.), HF_S and HF + GOS_CMO groups (B.) or HF and HF + FOS_IN groups (C.) at each time point. The models were calculated with 1 predictive component and various numbers of orthogonal components. For each model, the number of components is indicated at each time point on top of the R^2Y value. All models were validated with multiple permutation testing. (see Appendices) LF: low fat, HF : high fat, HF_S : HF and glucose–lactose–galactose (positive control

of HF + GOS_CMO group,) GOS : galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose)..... 218

Figure 51: Heatmaps highlighting discriminatory metabolites at each timepoint being positively or negatively correlated with mouse calorie storage at D70 after the diet switch. Correlation coefficients were obtained from the OPLS-DA coefficient plot of predicting models from urinary metabolic profiles of HF and HF + GOS fed mice (A), HF_S and HF +GOS_CMO fed mice (B) or HF and HF + FOS_IN fed mice (C) at each timepoint. Positive values are color-coded in red shades while negative values are color-coded in blue shades. *: tentative assignment LF: low fat, HF : high fat, HF_S : HF and glucose–lactose–galactose (positive control of HF + GOS_CMO group,) GOS : galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose).....22

1

Figure 52: Goodness of fit (R^2Y) and goodness of prediction (Q^2Y) of OPLS-regression models predicting CFE_D70 with fecal metabolic profiles of HF and HF + GOS groups (A), HF_S and HF + GOS_CMO groups (B) or HF and HF + FOS_IN groups (B) at each time point. The models were calculated with 1 predictive component and various numbers of orthogonal components. For each model, the number of components is indicated at each time point on top of the R^2Y value. *: indicates the models validated with multiple permutation testing. (see Appendix 2, Table S7). LF: low fat, HF : high fat, HF_S : HF and glucose–lactose–galactose (positive control of HF + GOS_CMO group,) GOS: galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose) 223

Figure 53: OPLS-coefficient plots indicating correlation coefficients between bacteria families at D7 and D70 and GOS (A, B), GOS_CMO (C, D) or FOS_IN (E, F) efficiency in reducing mouse calorie storage at D70 after the diet switch. OPLS-DA coefficient plots were obtained from models with HF and HF + GOS fed mice(A,B), HF_S and HF + GOS_CMO fed mice (C,D) or HF and HF + FOS_IN fed mice (E,.F) at D7 and D70 after the diet switch. Correlation values (R^2) color-coded in red shades are close to 1 while those color-coded in blue shades are close to 0..... 226

Figure 54: Venn diagram indicating the number of specific and common markers of calorie storage at D70 after the diet switch among the different prebiotics. GOS: galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose) 227

Figure 55: OPLS-regression models predicting long-term physiological outcomes based on the abundance of *Streptococcaceae* (St), *p*-cresol sulfate (PCS), *p*-cresol glucuronide (PCG) and phenylacetyl glycine (PAG) at D7

after the diet switch. For each model, the scores plots indicate a significant relationship between a specific pattern of excretion of these markers and the physiological outcomes (Figure 54.A for CFE_D70, Figure 54.D for fat mass, Figure 54.G for circulating IL-1 β , Figure 54.J for fasting insulinemia). The OPLS-loadings plot indicated the correlation coefficients between the variables and the physiological outcomes Figure 54.B for CFE_D70, Figure 54.E for fat mass, Figure 54.H for circulating IL-1 β , Figure 54.K for fasting insulinemia). All the models were validated with multiple permutations testing (*p value* <0.05) (Figure 9.C for CFE_D70, Figure 54.F for fat mass, Figure 54.I for circulating IL-1 β , Figure 54.K for fasting insulinemia) 231

Figure 56: OPLS-regression models predicting organ weight at sacrifice based on the abundance of *Streptococcaceae* (St), *p*-cresol sulfate (PCS), *p*-cresol glucuronide (PCG) and phenylacetylglycine (PAG) at D7 after the diet switch. For each model, the scores plots indicate a significant relationship between a specific pattern of excretion of these markers and organ weights (Figure 55.A for mesenteric fat, Figure 55.D for epididymal fat, 55.G for caecum weight,). The OPLS-loadings plot indicated the correlation coefficients between the variables and the physiological outcomes (Figure 55.B for mesenteric fat, Figure 55.E for epididymal fat, Figure 55.H for caecum weight). All the models were validated with multiple permutations testing (*p value* < 0.05) outcomes (Figure 55.C for mesenteric fat, Figure 55.F for epididymal fat, Figure 55.I for caecum weight) 232

Figure 57: Possible mechanisms linking gut bacterial tyrosine degradation, host inflammatory pathways and host physiological disturbances in diet induced obesity and its regulation with prebiotics..... 251

TABLE OF TABLES

Table 1: Gradient and solvents for the FIA-MS/MS analysis of the Biocrates kit 79

Table 2: Gradient and solvents the HPLC MS/MS analysis of the Biocrates kit 79

Table 3: List of metabolites assigned in the loadings plot of OPLS-DA models (shown in Figure S3). **Chemical shifts of carbons and protons were assigned only from resolved peaks present in ¹H NMR standard spectrum and ¹H-¹³C HSQC spectrum..... 103

Table 4: Treatment and sample collection during the experiment..... 120

Table 5: ¹H NMR chemical shifts from metabolites assigned in urinary, fecal or plasma metabolic profiles. (Only resonances identified in the spectra are reported and signals of unassigned metabolites are not included in the table)..... 14

Table 6: Statistics of global OPLS-DAs models and OPLS-DAs models with three groups generated with ¹ H NMR urinary, fecal or plasma metabolic signatures. (PC: predictive components, OC: orthogonal components).....	149
Table 7: Significance (<i>p values</i>) of the differences between the predicted scores (FOS_IN, GOS, or GOS_CMO) and the negative control group (LF) or the positive control group (HF or HF_S), <i>p values</i> were calculated by non parametric Wilcoxon-Mann-Whitney test.....	152
Table 8: Statistics of the OPLS-DAs models calculated with MS targeted metabolic profiles of plasma samples at D70 after the diet switch (PC: predictive components, OC: orthogonal components)	161
Table 9: Metabolites that varied significantly between feeding groups after a 70-day dietary intervention....	175
Table 10: Effect of long-term dietary interventions on physiological outcomes (<i>p values</i> calculated with non parametric Wilcoxon-Mann-Whitney tests)	214
Table 11: Summary of the main metabolic adaptations to dietary interventions in each experiment.....	245

ABBREVIATIONS

2PY	<i>N</i> -1-methyl-2-pyridone-5-carboxamide
4PY	<i>N</i> -1-methyl-4-pyridone-3-carboxamide
AMPK	Adenosine Monophosphate Activated Protein Kinase
anglp4	Angiopoetin Like Protein 4
APCI	Atmospheric Pressure Chemical Ionisation
ATP	Adenosine Tri-Phosphate
BCAA	Branched Chain Amino Acid
BCAT	Branched Chain Aminotransferase
BCKDH	Branched-Chain Keto Acid Dehydrogenase
BMI	Body Mass Index
BMRDB	Biological Magnetic Resonance Data Bank
BW	Body Weight
BWG	Body Weight Gain
CEC	Capillary Electrophoresis Chromatography
CI	Chemical Ionisation
CID	Collision Induced Dissociation
CMO	Cow Milk Oligosaccharide
CPMG	Carr-Purcell-Meilboom-Gill
CV	Cross Validation
CVD	Cardiovascular Disease
DIO	Diet Induced Obesity
DNTP	Deoxynucleotide Tri-Phosphate
EI	Electronic Ionisation
ESI	Electrospray Ionisation
ERK	Extracellular Signal Kinase
FA	Fatty Acid
FAB	Fast Atom Bombardment
FAS	Fatty Acid Synthase
FFMI	Fat Free Mass Index
FIA	Flow Injection Analysis
FID	Free Induction Decay
FMI	Fat Mass Index
FOS	Fructosyl-Oligosaccharide
FTICR	Fourrier Transform-Ion Cyclotron Resonance
GC	Gas Chromatography
GF	Germ Free
GOS	Galactosyl-Oligosaccharide
GPR	G-Coupled Receptor
HDL	High Density Lipoprotein
HF	High Fat
HILIC	Hydrophilic Interaction Liquid Chromatography
HMO	Human Milk Oligosaccharide
HPLC	High Pressure Liquid Chromatography

IDL	Intermediate Density Lipoprotein
IFN	Interferon
IGF-1	Growth Factor Receptor-1
IL	Interleukin
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
JNK	c-Jun-N Terminal Kinase
LCFA	Long Chain Fatty Acid
LF	Low Fat
LPC	Lysophosphatidylcholine
LPL	Lipoprotein Lipase
LPS	Lipopolysaccharide
MAD	Median Absolute Deviation
MALDI	Matrix-Assisted Laser Desorption Ionisation
MAP	Mitogen-activated Protein
MetS	Metabolic Syndrome
MFA	Multivariate Factor Analysis
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MTTP	Microsomal Triglyceride Transfer Protein
MyD88	Myeloid Differentiation Factor 88
NAD	Nicotinamide Adenine Nicotinamide
NMR	Nuclear Magnetic Resonance Spectroscopy
NLR	Nucleotide Oligomerisation Domain like Receptor
NR	Non-Responder
NRC	Nestlé Research Center
OGTT	Oral Glucose Tolerance Test
OSC	Orthogonal Signal Correlation
OTU	Operational Taxonomic Group
PAG	Phenylacetylglycine
PC	Principal Component
PC(Cx:y/Cx:y)	Phosphatidylcholine (x:carbon number, y:unsaturation)
PCA	Principal Component Analysis
PCG	<i>p</i> -Cresol Glucuronide
PCR	Polymerase Chain Reaction
PCS	<i>p</i> -Cresol Sulfate
PDK 1	Phosphoinositide-dependent Kinase 1
PI3-K	Phosphoinositol-3-OH-Kinase
PLS	Partial Least Squares
PPAR	Peroxisome Proliferator Activated Receptor
Q	Simple Quadrupole
REE	Resting Energy Expenditure
ROS	Reactive Oxygen Species
SCFA	Short Chain Fatty Acid

SM	Sphingomyelin
SNP	single nucleotide polymorphism
SOS	Son-of-Sevenless
SPE	Solid Phase Extraction
SR	Strong Responder
SRV	Statistical Recoupling of Variables
STOCSY	Statistical Total Correlation Spectroscopy
T2DM	Type II Diabetes Mellitus
TCA	Tricarboxylic
TG	Triglyceride
TOF	Time Of Flight
TLR	Toll Like Receptor
TMA	Trimethylamine
TMAO	Trimethylamine- <i>N</i> -Oxide
TNF- α	Tumor Necrosis Factor- α
TSP	Trimethylsilyl Propionic Acid
UA	Unassigned Metabolite
UPLC	Ultra-High Pressure Liquid Chromatography
VLDL	Very Low Density Lipoprotein

OBJECTIVES

This PhD thesis has two main objectives:

- 1) To identify specific metabolic adaptations to obesity prone and obesity resistant C57BL/6 mice to high fat diet.
- 2) To investigate the effects of gut microbial remodelling by prebiotics on C57BL/6 mouse physiology, metabolism and immune system during obesity development.

THESIS STRUCTURE



Chapter I : Introduction

- Aetiology of obesity
- Metabolic disturbances and low grade inflammation in obesity
- Role of gut microbiome on health and obesity development
- Advantages of metabolomics in studying obesity and metabolic diseases

Chapter II : Analytical methods

- ¹H NMR spectroscopy and MS targeted analysis
- Multivariate statistics
- 16S rRNA gene sequencing

EXPERIMENT 1 : the body weight variability study

Chapter III :

Early metabolic adaptation in C57BL/6 mice resistant to high fat diet induced weight gain involves an activation of mitochondrial oxidative pathways

EXPERIMENT 2: the prebiotic study

Chapter IV:

A top-down metabolomic approach to investigate the long term effects of prebiotics on C57BL/6 mouse physiology and metabolism during DIO

Chapter V:

Correlative analysis of fecal microbiota, ¹H metabolic profiles and circulating cytokine levels in mice fed HF and prebiotics

Chapter VI:

Identification of candidate markers predicting the long-term effect of prebiotics on mouse physiology during diet-induced obesity



Chapter VII : General discussion

- Key Findings and statements of novelty
- Role of gut microbiota in mitochondrial oxidative pathways
- interactions of gut microbiota, bacterial amino acid fermentation and inflammation

CHAPTER I: INTRODUCTION

1. OBESITY

Obesity is one of the most important global healthcare problems of the 21st century. Studies carried out by the World Health Organization (WHO) revealed that obesity increased over the last 20 years reaching pandemic proportions¹. The prevalence of obesity in industrialised countries continues to rise in part due to sedentary lifestyle. Interestingly, the obesity epidemic is not restricted to industrial countries. In particular, it is significantly rising in several countries of the Middle East, the Pacific islands and in Africa. Children are not excluded from this epidemic with 22 million overweight children under the age of 5 in the world¹.

The WHO estimated that around 1 billion adults are overweight and more than 115 million are heading obesity-related problems worldwide¹. Obesity occurs when there is an imbalance between energy intake and energy expenditure. The development of obesity depends on genetic and environmental factors and is associated with various metabolic disorders, impaired hormonal signals, and chronic inflammation. Obesity also increases the probability of developing cardiovascular diseases (CVDs), type 2 diabetes, hypertension, cancer, respiratory problems and osteoarthritis²⁻⁴. Infantile obesity might also increase the risk of developing obesity related diseases in the adulthood⁵. Medical services required for prevention, diagnosis and treatment of obesity account for a considerable part of the budget in the healthcare system of the western countries. As an example, obesity in the European Union costs 59 billion euros every year⁶. Hence, reducing prevalence of obesity has become economically crucial and one of the primary healthcare aims worldwide.

The scope of this thesis aims to expand scientific knowledge on the short-term and long-term physiological and metabolic effects of obesity in order to help healthcare providers in the evaluation and treatment of obesity.

1.1. Definition of obesity and metabolic syndrome

Obesity is defined by an excess of adipose tissue and is characterised in human by a body mass index (BMI) equal or superior of 30 kg/m²¹. BMI is calculated by dividing the weight (in

kilograms) of the individual by its height squared (in square meters)⁷. This weight for height assessment does not require specific/expensive tools and is the most reliable measure of adiposity which can be easily recorded in epidemiological studies and compared within and between populations.

On the other hand, defining obesity from the BMI spectrum is an arbitrary cut-off and can induce mischaracterisation. A very muscular person or a person who has lost muscle mass can be easily misclassified as overweight or underweight⁷. Hence, measures of waist to hip ratio or waist circumferences are more effective than BMI to define obesity as it assesses the proportion of abdominal fat. The proportion of lean mass and fat mass, which strongly influences metabolism and obesity-related metabolic disorders, also needs to be taken into account. For instance, in non-obese individuals, a significant decrease in fat mass has been associated with a reduction of mortality whereas a loss of lean mass have been associated with a rise in mortality⁸. Partitioning BMI into fat mass index (FMI) and fat free mass index (FFMI) turned out to be really useful to estimate the levels of obesity and leanness. These two indexes can be defined by the following equation:

$$\text{BMI (kg/m}^2\text{)} = \text{FFMI (kg/m}^2\text{)} + \text{FMI (kg/m}^2\text{)}$$

FMI is measured by skin fold or hydrodensitometry whereas FFMI is calculated by tomography or magnetic resonance imaging. However, most of these techniques are really expensive and are not necessarily available in clinics⁹. The comparison of FMI and FFMI in addition to BMI can help redefining the cut-off for obesity and avoids false diagnosis. Individuals with muscle hypertrophy, people suffering from protein energy malnutrition or sarcopenic obesity (loss of lean mass and gain of fat mass) can be distinguished with measurement of FMI and FFMI, while BMI fails to identify them. The relative proportion of fat mass and lean mass also varies with age. During the puberty, young men tend to increase lean mass whereas young women increase their proportion of fat mass. These indices enable us following a spectrum of body compositions, providing additional information about risk factors of metabolic disorders during childhood and adulthood^{8,10}.

Interestingly, lean mass are metabolically active tissues requiring significant amounts of calories even during a non-active period. This process is so called resting energy expenditure (REE) and largely contributes to the basal metabolic rate, which corresponds to the energy consumed daily⁸. However, these tissues contain metabolically heterogeneous compartments. Skeletal muscles, corresponding to 40-50 % of the lean mass, only account for 20 to 30 % of REE. By contrast, the liver, heart and kidney, being less than 6 % of lean mass, account for 60 to 70 % of the REE. Hence, subtle changes in organs mass/activity may lead to significant variation in energy expenditure⁸. Inter-

individual activity in REE may induce various proportions of leanness and fatness which may predispose the development of obesity-related metabolic diseases^{8,10}. The fat mass is also compartmentalized into upper body fat and lower body fat. The upper body fat, comprising visceral adipose tissues and subcutaneous fat, is known to have deleterious effects and have been associated with elevated risks for type 2 diabetes and CVDs⁸. In particular dense subcutaneous fat seems to be associated with insulin resistance, whereas superficial subcutaneous fat, which is more present in women, has been thought to have protective effects by lowering fatty acid storage^{11,12}. Other ectopic fat accumulation around tissues and organs can induce lipotoxicity and organ malfunctions¹³. In contrast, lower body fat, comprising leg adipose tissues and gluteofemoral fat, is viewed as a metabolic buffer enabling to maintain a good balance between energy storage and energy intake and protect against ectopic fat accumulation⁸. Higher proportions of lower body fat compared to upper body fat have been linked with reduced cardiovascular and metabolic risks¹². Hence, fat distribution is an essential criterion to consider an individual's risks of developing metabolic diseases.

Obesity is often associated with the development of several chronic complications such as (i) high fasting glucose (hyperglycemia), (ii) hypertriglyceridemia, (iii) low high density lipoprotein (dyslipidemia) and (iv) high blood pressure (hypertension)¹⁴. Individuals having at least 3 of these criteria are diagnosed with the metabolic syndrome (MetS)¹⁴. MetS was initially described by Reaven in 1988 under the name of "syndrome X" and was shown to increase the risks of type 2 diabetes¹⁵. Lately, MetS has been thought to be a good predictor of CVDs, which caused it to be renamed as cardio-metabolic syndrome^{14,16}. The underlying causes of MetS are unclear. Most of the individuals with MetS have abnormal obesity suggesting that excess of adipose tissue plays a causative role. However, this hypothesis has been contested particularly because several epidemiological studies identified people having normal BMI but presenting a cluster of metabolic disorders such as high triglycerides and high fatty liver^{14,17}. In this case, body fat distribution seemed to be a better indicator of metabolic disorders than the actual obesity threshold given by the BMI, as these non-obese but unhealthy individuals had higher level of visceral fat than healthy individuals. Some investigators including Reaven proposed that insulin resistance is the central biological mechanism driving MetS, but this remains uncertain¹⁶.

1.2. Aetiology of obesity and MetS

As previously mentioned, obesity occurs when there is an imbalance between energy intake and energy expenditure and it is closely linked with the occurrence of MetS¹⁶. However, the aetiology of obesity as well as MetS remains unclear as it results from a complex interaction between genetic and environmental factors¹⁸.

Several studies focusing on family associations pointed out the influence of the genetic background in the development of obesity. In particular, several investigators observed that individuals' risks of developing obesity rise when people have relatives who are obese¹⁹. Genes influencing BMI, sum of skin fold thickness and fat mass being involved in obesity-related phenotype have also been proved to be heritable up to 70%¹⁹. Genetic linkages for MetS have been evidenced as well. An epidemiological study on Mexican-Americans showed heritability ranged from 45% to 57% for the factors associated with levels of insulin, glucose, lipids and blood pressure. A study on monozygotic and dizygotic twins also highlighted the genetic influence on glucose intolerance, overall obesity and low high density lipoproteins cholesterol (HDL-C)²⁰. Several investigations have been performed to characterise causative genes for obesity and MetS. Zhang *et al.* identified the *ob* gene in the C57BL/6 mouse model producing the satiety hormone, the leptin which regulates food intake and energy expenditure. These findings raised the hypothesis that leptin could be key in body weight and adiposity regulation²¹. However, additional studies on humans revealed that the impact of leptin on obesity remains unclear²². Other candidate genes involved in metabolic abnormalities have been characterised from rodents and human studies such as genes coding for tumor necrosis factor α (TNF- α), peroxisome proliferator activated receptor (PPAR), angiotensin-converting enzyme and mitochondrial uncoupling proteins¹⁹. More recently, quantitative trait locus mapping analyses have revealed that polymorphism in several genes involved in neuronal processes, inflammatory pathways, TCA cycle and glycolysis also affect fat and glucose metabolism and contribute to abdominal obesity²³.

Nevertheless, as genetic background within populations/countries did not radically change over the last three decades, the rapid expansion of obesity and MetS can only be explained by environmental changes that induce weight gain and metabolic deregulations. Indeed, western societies have marked preferences for a high carbohydrate and high fat diet and a sedentary lifestyle (motorised transport, lack of physical activity) which frequently leads to an imbalance between energy intake and energy expenditure¹. This environment promoting obesity is qualified as "obesogenic" and raises the probability of developing MetS. To limit the obesity epidemic, medical

services recommend the selection of low-energy food (wholegrain, cereals, and fruits) over high-energy food (animal fat, sugary drinks, alcoholic drinks) and encourage physical activity⁶. In addition, the choice of specific diets rich in unsaturated fatty acids, vitamins and fibres are thought to have beneficial effects on energy balance and obesity²⁴.

As detailed previously, the aetiology of obesity is multi-factorial and the risk factors for weight gain vary from individual to individual leading to an individual's specific predisposition. In addition, MetS is defined as a constellation of symptoms involving a variety of risk factors across different demographic populations¹⁴. In particular age, gender, ethnicity and socio-economic status need to be taken into account to understand the onset of metabolic abnormalities. Studies on sexual-dimorphic physiology tend to indicate that for a same BMI, women have more fat mass and less lean mass compared to men and are prone to excess weight²⁵. Ageing is also positively correlated with the prevalence of obesity. Interestingly, aging and particularly menopause in women have been thought to modify fat distribution toward central adiposity promoting MetS²⁶.

With the help of new technologies, other aetiologies of obesity underlying the interactions between genetic and environment have recently emerged. In particular, epigenetic regulations provide important explanations enabling us to better understand the phenotypic differences between individuals²⁷. Epigenetic mechanisms triggering chromatin modifications such as histone acetylation, or DNA methylation regulate the expression level of several genes involved in cell differentiation and energy homeostasis^{28,29}. Epigenetic regulations already occur during foetal and neonatal life depending on maternal habits and influence the long-term expression of genes²⁷. However, environmental stimuli such as dietary changes or stress can also trigger epigenetic mechanisms during the childhood and adulthood²⁹. For instance, an animal study showed that high fat (HF) diet decreases gene expression in specific brain areas by increasing DNA methylation of gene promoters within the reward-related brain regions²⁹. The epigenome is also transmittable from one generation to the next. Hence, in the context of "obesogenic" environment, genetic background along with epigenetic regulations can predispose or protect individuals of developing obesity³⁰.

Finally, the mammalian host-gut microbiota metabolic axis represents an important external factor involved in several host physiological processes, such as the digestion and the absorption of nutrients, and can contribute in the development of metabolic diseases^{31,32}. It has been established that specific gut microbiota composition/activity in obese individuals has better ability to extract calories from the diet which may encourage obesity development³³. These findings highlight that

specific gut microbial activity plays a significant role in the onset of obesity and may contribute to an individual's predisposition. This aspect will be further discussed in this chapter (section 2).

1.3. Obesity and Energy homeostasis

Obesity is characterised by increased triglyceride (TG) storage in adipose tissues and peripheral tissues. This phenomenon results from an imbalance between energy intake and energy expenditure. Indeed, energy required to perform vital functions can be supplied by dietary constituents (fat, carbohydrates, and proteins) or released from source stored within the cells (TGs). Glucose is the primary energy substrate as it is capable of supplying energy to all the cells. However, other alternative energy substrates, fatty acids (FAs) and amino acids are also required to carry out physiological processes including cell signalling, molecular transports, and energy metabolism. Energy supplying pathways involving glucose, fatty acids and amino acids are strongly connected as they generate common intermediate metabolites which can feed the tricarboxylic (TCA) pathways to produce ATP (Figure 1).

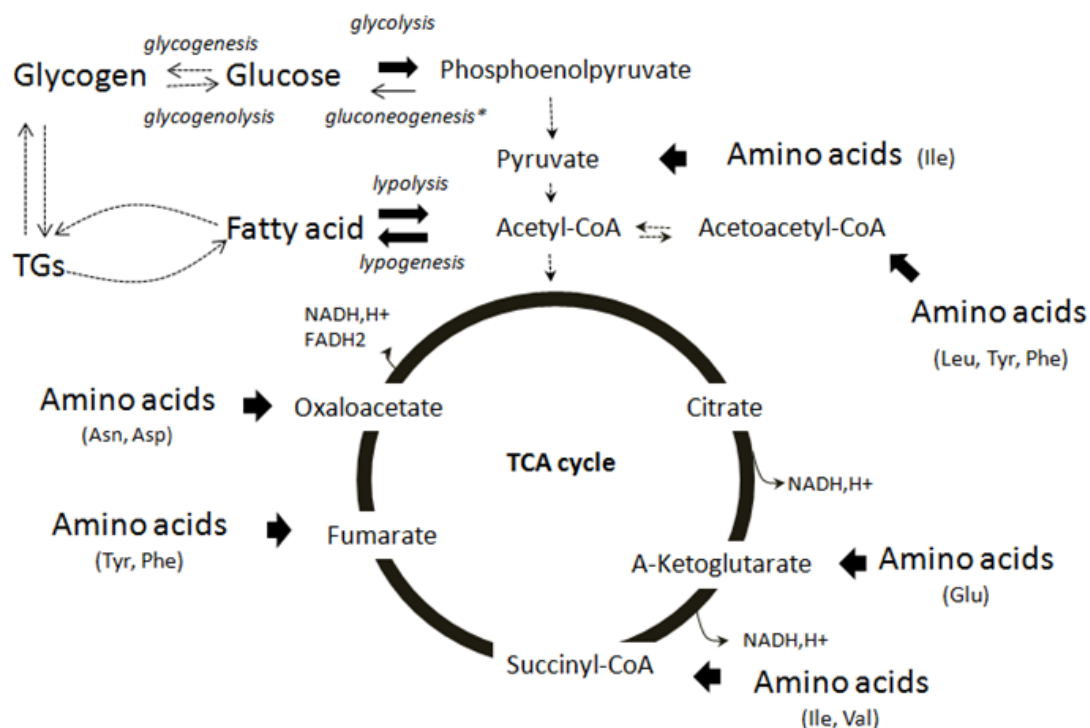


Figure 1 Energy supplying pathways connecting glucose, fatty acids and amino acids (Ile: isoleucine, Asn: Asparagine, Asp: Aspartate, phe: Phenylalanine, Tyr: Tyrosine, Val: Valine, Leu: leucine). TGs: triglycerides, *gluconeogenesis only occurs in the liver, kidney and intestine.

In normal physiological conditions, appropriate energy substrate concentration is maintained in the blood in order to supply enough energy to all cells in the body. In particular blood glucose concentration must be kept around 1g/L during fasting³⁴. A low glucose concentration can induce brain damage while a high glucose concentration is known to trigger multiple organ damages³⁵. Therefore, specific physiological mechanisms enabling the maintenance of calorie supply are required to survive starvation and over-feeding periods. In case of nutrient abundance, calories are stored in adipose tissues, liver and peripheral tissues under TG form (Figure 1). In particular:

- Glucose is converted into glycogen by glycogenesis.
- Acetyl-CoA production from glucose degradation is used for FA synthesis *via* lipogenesis.
- TGs are produced by esterification of glycerol and FAs.

When calories are needed, glycogen and TGs are degraded into glucose and free FAs respectively which provides energy across organs and other tissues (Figure 1). In particular:

- Glucose is degraded by glycolysis to provide pyruvate
- FAs are oxidised through β -oxidation to form acetyl-CoA.
- Protein breakdown is activated to form amino acids which provide intermediate metabolites of the TCA cycle.

The production of pyruvate, acetyl-CoA and other intermediate metabolites activates the TCA cycle which generate reduced coenzymes (NADH, FADH). These coenzymes are finally re-oxidised (NAD^+ , FAD^+) by oxidative phosphorylation to form adenosine tri-phosphate (ATP) which is used for intracellular energy. Energy storage and utilization are largely controlled by hormonal excretions and insulin in particular. During the post absorptive period, insulin secretion promote glucose intake from blood circulation, the activation of glycogenesis and FAs synthesis, and inhibit protein breakdown. During fasting, insulin secretion is reduced and an antagonist hormone glucagon is released. This promotes glycogen degradation into glucose *via* glycogenolysis, the up-regulation of lipolysis and down-regulation of lipogenesis. In addition, the adipocytes control lipid partitioning and energy intake by excreting hormones that regulate satiety *via* hypothalamic centres and energy metabolism³⁶.

In a context of chronic consumption of high energy food, cells are subjected to a saturation of stored energy source and aberrant blood concentration of energy substrates. This triggers a

disruption of energy homeostasis, and hormonal secretion which promote obesity development and other physiological disturbances. To better understand the onset of obesity and its associated disorders, it is important to establish the mechanisms involved in absorption, circulation and utilisation of lipids, carbohydrates and proteins.

1.3.1. Fatty acid metabolism

Several categories of lipids including TGs, sterols, phospholipids, sphingolipids, eicosanoids, can be found in the body and confer various physiological properties:

- TGs are esters of glycerol and FAs. They constitute the major dietary lipids and provide the energy source for the body.
- Sterols are essential structures of the cells membranes and are involved in the biosynthesis of steroids hormones.
- Phospholipids and sphingolipids are also components of cell membrane and lipoprotein interfaces^{34,36}.
- Eicosanoids are involved in several cell signalling processes including inflammation^{36,37}.

Most of the lipids can be synthesised endogenously or brought by dietary products. After a meal, lipids are absorbed and digested in the intestinal lumen (Figure 2.A). In particular, TGs are transformed into free FAs and 2-monoacylglycerol by lipases, which only act at the lipid-water interfaces³⁶. As lipids are relatively insoluble in water, the lipid digestion requires the presence of bile acids which allow lipid emulsification. The digested products enter intestinal epithelial cells where they are reconverted into TGs and packed in lipoproteins called chylomicrons. Chylomicrons are in turn secreted in the lymph and then in the blood circulation (Figure 2.A). When there is a surplus of energy substrates in the liver, hepatic glucose is converted into FAs which are used to synthesise TGs *via* insulin stimulated lipogenesis. TGs are ultimately stored in hepatic cells or exported from the liver to the blood circulation in very low density lipoproteins (VLDL).

TGs in chylomicrons and VLDL are finally degraded into FAs by lipoprotein lipase (LPL), an enzyme present at the border of capillary endothelial cells. FAs are taken up by the muscle and other tissues and then oxidised *via* β -oxidation to produce energy (Figure 2.A). Surplus of circulating FAs are converted in TGs and stored in the adipose tissues or liver³⁴. TGs degradation by LPL also induces the transformation of chylomicrons and VLDL into chylomicron remnants and intermediate density lipoproteins (IDLs) containing relatively low TG levels^{34,36}. These products return to the liver by

endocytosis and are degraded by lysosomal actions. A part of IDLs and other lipoproteins (low density lipoproteins (LDL) and high density lipoproteins (HDL)) are also used for cholesterol transport between peripheral tissues, adipose tissues and liver (Figure 2.A).

During fasting, TGs in adipocytes and hepatocytes are broken down into glycerol and FAs *via* lipolysis, which are released in blood circulation. The FAs bind with albumin and enter peripheral tissues (muscle, kidney) to produce energy. During prolonged fasting, ketogenesis can be activated. This mechanism converts FAs breakdown end-products into ketone bodies in the liver which are in turn used by muscle, kidney and brain to produce energy³⁴. Glycerol-3-phosphate is usually synthesised from glucose by glycolysis. However, when the organism is deficient in carbohydrates, glycerol-3-phosphate can be generated from non-carbohydrate sources such as alanine, glutamine or pyruvate *via* glyceroneogenesis³⁴. This pathway mainly occurs in liver and adipose tissues and is regulated by phosphoenol-pyruvate carboxylase (PEPCK). Its activation is particularly important in the regulation of circulating FA concentrations during fasting or starving periods³⁴. These metabolic pathways are summarised in Figure 2.

Thus during the feeding process, TG synthesis is favoured in adipocytes and liver. In addition leptin is secreted by adipocytes to reduce caloric intake, stimulate oxidation of surplus FAs *via* β -oxidation in non adipose tissues and limit lipogenesis. This process of caloric homeostasis is useful because it protects non adipose tissues (such as muscles, heart and blood vessels) against ectopic fat accumulation and FAs overload^{13,34}. For instance, individuals with adipose tissues dystrophy (lipodystrophy) suffer from an increased fat accumulation in non adipose tissues and have higher prevalence for insulin resistance (IR), type 2 diabetes mellitus (T2DM) and other symptoms of MetS^{13,38}. In case of chronic overfeeding, the surplus of energy intake provokes an expansion of the fat cell number and an increase of the fat cell size. This adipose tissue overgrowth underlying obesity often leads to dysfunction of endothelium as well as the release of adiponectin³⁸. Non adipose tissues are also constituted with a small intracellular reserve of lipid which is used to maintain essential functions such as membrane structures and intracellular signalling³⁴. In the case of obesity development, increased deposition of lipids in organs such as liver, skeletal muscle, kidney and heart occurs, which impairs organ and tissues functions in different ways. First, increased fat size around the organ can change its structure and function by applying physical compression¹³. Second, prolonged overfeeding is frequently associated with leptin resistance leading to the failure of this leptin-induced protective mechanism. Then, TGs level in non adipose tissues rises, which is associated with an excess of intracellular FAs and an activation of harmful non-oxidative metabolic pathways producing ceramides among other toxic metabolites¹³. In addition, a variety of lipid-

derived toxic metabolites from incomplete FA oxidation, such as long chain fatty acyl-CoA, were found to be elevated³⁹. This harmful phenomenon is referred as lipotoxicity and is thought to cause insulin resistance and to impair cardiac functions^{13,39}.

1.3.2. Carbohydrate digestion and glucose metabolism

Carbohydrates are other important nutrients providing energy source for the cells and other molecules necessary for biosynthesis such as reduced coenzymes (NADH, NADPH) and precursors³⁴. Carbohydrates can be constituted of monosaccharide (1 saccharide unit), disaccharides (2 saccharide units), oligosaccharides (from 3 to 10 saccharide units) and polysaccharides (more than 10 saccharide units)³⁴. Glucose is the most important monosaccharide as it is degraded by glycolysis which allows the production of energy in all the cells. Therefore, dietary carbohydrates are ultimately converted into glucose to provide energy, excepted for fructose which can feed directly the glycolysis³⁴. Starch, which is the most common dietary carbohydrate, is easily broken down by several amylases present in saliva and pancreatic secretion into oligosaccharides, such as maltose or isomaltose (Figure 2.B). Oligosaccharides and disaccharides are further degraded by several enzymes present in the brush border of intestinal lumen, forming glucose, galactose and fructose³⁴. The most common dietary disaccharides are sucrose and lactose. Sucrose can be degraded into fructose and glucose by sucrase activity, while lactose is broken down into glucose and galactose by lactase⁴⁰. Monosaccharides are able to cross the intestinal membrane by binding membrane transporters. In particular, glucose and galactose enter the enterocytes *via* sodium-glucose transport proteins (SGLT), while fructose is taken up by the GLUT 5 transporter. They are all transported out of the enterocytes by the GLUT 2 transporter and passively enter the capillary blood to reach tissues and organs (Figure 2.B). In the intracellular compartment, galactose is rapidly converted into UDP-glucose by a succession of enzymatic reactions involving galactokinase among others. Fructose, on the other hand, is degraded into glyceraldehyde-3-phosphate, which in turn may feed the gluconeogenic pathway, forming glycerol or glycolytic pathways and generating pyruvate. The polysaccharides and fibres not digested by gastrointestinal enzymes transit to the large intestine and colon where they can be fermented by the gut microbiota³⁴ (see Chapter I, section 2).

As previously mentioned, glucose storage and utilisation are largely controlled by insulin and glucagon hormones to maintain a normal glycemia^{34,41}. When the glucose availability exceeds the amount required for maintaining a normal blood glucose concentration (during the postprandial period for exemple), glucose is transformed in the liver and adipose tissues into glycogen³⁴. The

surplus of acetyl-CoA from glucose degradation can also promote FA synthesis for energy storage or for maintaining other physiological processes (cell membrane, or cell signalling) (Figure 2.B) ⁴¹. During fasting, glucose may be in short supply. In this context, glycogen is degraded into glucose *via* glycogenolysis and FAs synthesis from glucose is down regulated (Figure 3). Glucose can also be synthesized by gluconeogenesis using lactate in the liver, kidney and intestine. Research in the past 5 years has revealed the importance of intestinal gluconeogenesis in the control of glucose and energy homeostasis. The activation of intestinal gluconeogenesis induces glucose delivery in the portal vein and initiates a nervous signal *via* portal sensors, which is further transmitted to the nucleus of the solitary tract and/or the hypothalamus by autonomous afferents. The hepatportal sensing triggers a signal to the brain promoting a satiety effect, and regulates glucose homeostasis ⁴². Interestingly, high protein diet was associated with the increased expression of 3 key enzymes (glutaminase, phosphoenolpyruvate carboxykinase-cytosolic form and glucose-6-phosphatase) involved in intestinal gluconeogenesis. A recent experiment showed that 15 to 20% of the total glucose observed in protein-fed rats during a postabsorptive period was brought by intestinal gluconeogenesis. When the same proportion of glucose was infused in the portal vein, a significant drop of food intake was noticed without degrading endogenous glucose homeostasis. In addition, a better redistribution of glucose in the kidney and intestine as well as a enhanced suppression of endogenous glucose production by insulin was observed, notably due to more efficient glycogen storage in the liver⁴³. Therefore the intestinal gluconeogenesis has been considered as a key factor in the central improvement of insulin sensitivity and participates in satiety regulation in protein feeding.

Finally glucose may be transformed into other monosaccharide such as ribose-6-phosphate *via* the pentose phosphate pathway which produce NADPH and feed the glycolysis³⁴. Thus, depending on the cell requirement, glucose may be transformed into FAs, coenzymes, other monosaccharides as well as amino acids (Figure 3). However, in the context of an excess of glucose intake from the diet, insulin action is deregulated. In particular, circulating glucose is not efficiently transported into the cells and intracellular glucose is not sufficiently stored into glycogen. Therefore, blood glucose is not maintained within the physiological range which contributes to obesity development and promotes physiological disturbances. Impaired insulin action and obesity development are further discussed in the section 1.3.4 of this introduction.

1.3.3. Amino acid metabolism

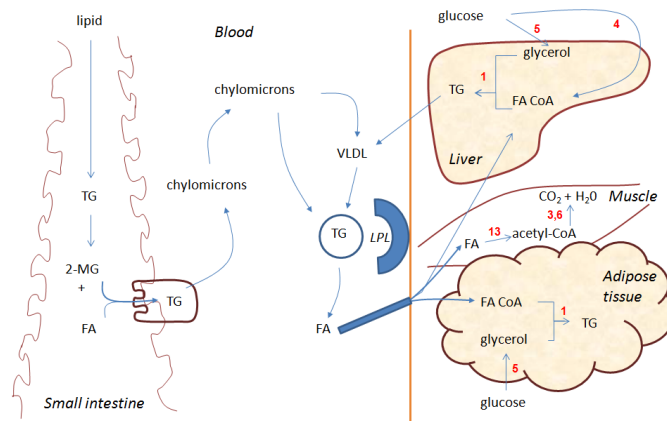
Dietary proteins and amino acids are essential nutrients as they provide the nitrogen source for the synthesis of nitrogen containing molecules (nucleic acids, proteins, amino acids) for the cells^{34,44}. Protein and amino acid degradations also produce energy for the organisms. Diets provide 20 different amino acids. Among them, eight are essential because they cannot be endogenously synthesized (leucine, isoleucine, valine, lysine, methionine, threonine, tryptophan, phenylalanine). After protein digestion by proteases in the small intestine the resulting peptides and amino acids enter the enterocytes *via* intestinal peptide transporters (PepT1) (Figure 2.C). In the intracellular compartment, peptides are further hydrolysed into amino acids, which in turn travel across the intestinal mucosa by basolateral amino acid transporters and enter the blood circulation³⁴. Amino acids are distributed among all tissues where they can be transformed into other amino acids, used for protein synthesis, or be degraded to provide nitrogen (Figure 2.C). The balance between nitrogen intake and excretion is strictly controlled to keep a constant nitrogen level in the organism. The nitrogen waste is processed by the urea cycle which finally produces urea. The final step of urea cycle only occurs in the liver, urea is then rapidly excreted in urine (Figure 2.C). Amino acid breakdown is therefore strongly regulated to maintain this homeostasis^{34,44}. Protein and amino acid metabolism are also regulated by insulin secretion which increases amino acid uptake in liver, muscle and adipose tissues and promote protein synthesis in these tissues⁴⁴. On the other hand, the drop in insulin secretion favours amino acid breakdown.

For many amino acids (leucine, isoleucine, valine, phenylalanine), the first catabolic step is a transamination, in which transaminase enzymes transfer the amino group to ketoacetate or 2-ketoglutarate forming a keto-acid³⁴. This process also produces aspartate or glutamate, respectively. Aspartate directly participates in urea synthesis. On the other hand, glutamate is deaminated to provide ammonia, which is used to produce another urea cycle intermediate, citrulline. For several amino acids (such as arginine, lysine, proline) catabolism occurs by other processes than transamination including conversion into different amino acids. Following nitrogen removal, most of the keto acids are degraded, ultimately generating TCA cycle intermediates pyruvate and acetyl-CoA^{34,44} (Figure 2C) which are used to produce ATP and CO₂. In addition, amino acids can generate glucose *via* pyruvate in all the tissues capable of gluconeogenesis and can be converted into FAs or ketone bodies *via* acetyl-CoA production. Interestingly, leucine is defined as strictly ketogenic because it does not form pyruvate and as a result cannot be converted into glucose^{34,44}. Therefore it is important to consider the dietary significance of proteins in the energy supply. Because nitrogen balance is strictly controlled *via* this protein turnover and the production of urea, a high protein diet

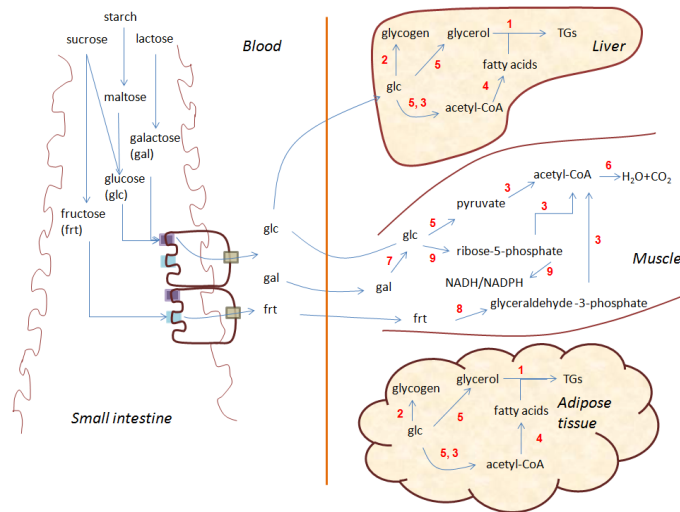
promotes amino acid degradation, which in turn may trigger uncontrolled FAs production, TGs storage and obesity³⁴.

To conclude, energy supplying pathways are strongly interconnected by common precursors which serve as “biological hubs” for various cell functions. Therefore they must be seen as an integrated set of metabolic processes which are strongly regulated by hormonal actions. In particular, insulin secretion induces a significant and global effect on energy metabolism.

A.



B.



C.

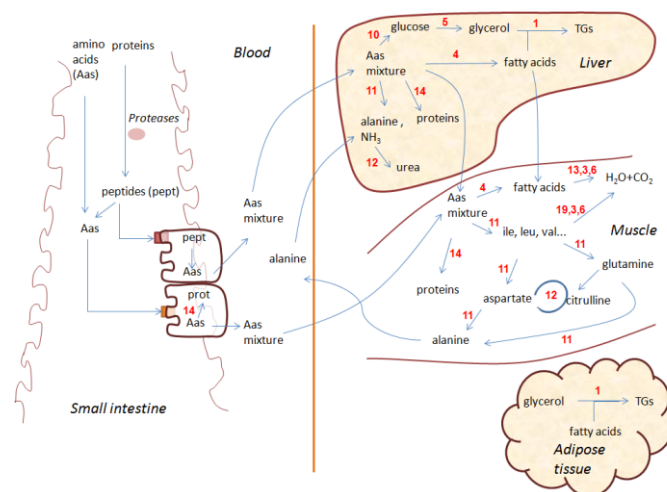


Figure 2: Summary of energy metabolic pathways during the postprandial period. A: Fatty acids metabolism, B: carbohydrate metabolism, C: Protein metabolism. 1: TG synthesis, 2: glycogenesis, 3: TCA cycle, 4: fatty acid synthesis, 5: glycolysis, 6: oxidative phosphorylation, 7: galactose conversion into glucose (Leloir pathway), 8: fructose metabolism, 9: pentose phosphate pathways, 10: gluconeogenesis, 11: transamination, 12: urea cycle, 13: lipolysis, 14: protein synthesis, 15: TG degradation, 16: glyceroneogenesis, 17: Cori cycle, 18: ketogenesis, 19: proteolysis, 20: glycogenolysis

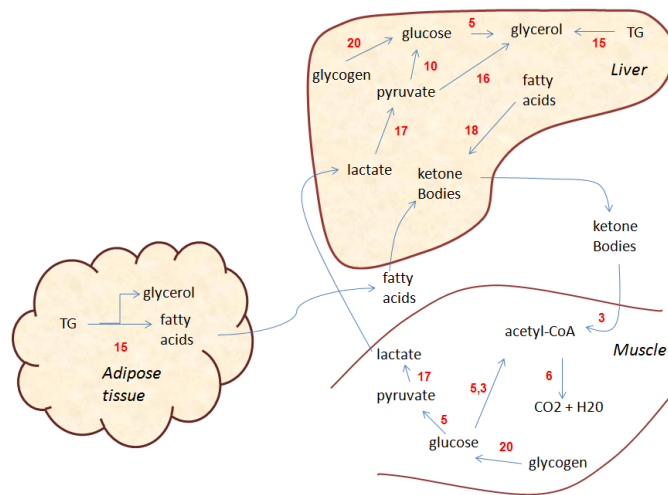


Figure 3: Summary of energy metabolic pathways during a fasting period. 1: TG synthesis, 2: glycogenesis, 3: TCA cycle, 4: fatty acid synthesis, 5: glycolysis, 6: oxidative phosphorylation, 7: galactose conversion into glucose (Leloir pathway), 8: fructose metabolism, 9: pentose phosphate pathways, 10: gluconeogenesis, 11: transamination, 12: urea cycle, 13: lipolysis, 14: protein synthesis, 15: TG degradation, 16: glyceroneogenesis, 17: Cori cycle, 18: ketogenesis, 19: proteolysis, 20: glycogenolysis

1.3.4. Obesity and insulin resistance: the underpinning mechanisms of obesity development.

Insulin is a hormone secreted by the pancreas in the β cells of the islets of Langerhans which regulate glycemia⁴⁵. Its primary role is to facilitate the uptake of glucose into cells and its secretion is driven by the rise in postprandial plasma glucose concentration. The coordinate role of insulin is to allow the storage of carbohydrates, lipids and proteins in the cells by stimulation of glycogenesis, lipogenesis, and protein synthesis. It also blocks the degradation and the recirculation of carbohydrates and lipids by inhibiting glycogenolysis and lipolysis⁴⁵. By these mechanisms, not only does insulin reduce glycemia, but also induces the accumulation of glycogen and fat in cells. The effect of insulin is mediated by a tyrosine phosphorylation cascade. Insulin binds to the extracellular α subunit domain of the insulin receptor causing the phosphorylation of the tyrosine chain of the receptor's β subunit in the plasma membrane (Figure 4). The phosphorylated receptor then binds and phosphorylates a number of intracellular proteins, namely insulin receptor substrates (IRS), which induce several additional protein kinases signalling systems. In particular, IRS-1 and IRS-2 interact with the phosphoinositol-3-OH-kinase (PI3-K) *via* their tyrosine-phosphorylated motif. PI-3K then converts the phosphatidylinositol 3,4 diphosphate into phosphatidylinositol 3,4,5 triphosphate, generating multiple signals^{34,45}. This signalling cascade involves the activation of phosphoinositide-

dependent kinase 1 (PDK1) that stimulates the coupling of the serine and threonine kinases Akt/PKB. These proteins then initiate many of the actions of insulin, such as the increase of glucose transport. They allow vesicles containing the glucose transporters (GLUT 4 and GLUT2) to fuse with the plasma membrane and enable glucose uptake^{34,45}.

Another pathway is required for insulin-stimulated glucose uptake, and involves the tyrosine phosphorylation of the Cbl proto-oncogene (Figure 4). Phosphorylated Cbl acts with the adapter protein CAP and the formed complex is translocated into the lipid raft domain of the plasma membrane. An adapter protein CrkII is then engaged in the lipid raft domain and creates complexes with the guanyl nucleotide-exchange protein C3G. This enables C3G to be translocated and to interact with the G protein T10, leading to its stimulation. Finally, activated T10 has an impact on the stabilization of cortical actin which might play an important role in GLUT 4 vesicle translocation⁴⁵.

The pathway involving the mitogen-activated protein (MAP) kinase is also activated by insulin. In this case, the tyrosine phosphorylated IRS and Shc proteins interact with different compounds such as the Son-of-sevenless (SOS) exchange proteins and the SHP2 protein inducing the activation of RAS protein. RAS then initiates the serine cascade through the activation of MEK and ERK, which results in the stimulation of a transcription factor, implicated in cell proliferation or differentiation processes. However, this latter pathway seems to have no obvious role in the metabolic actions of insulin⁴⁵.

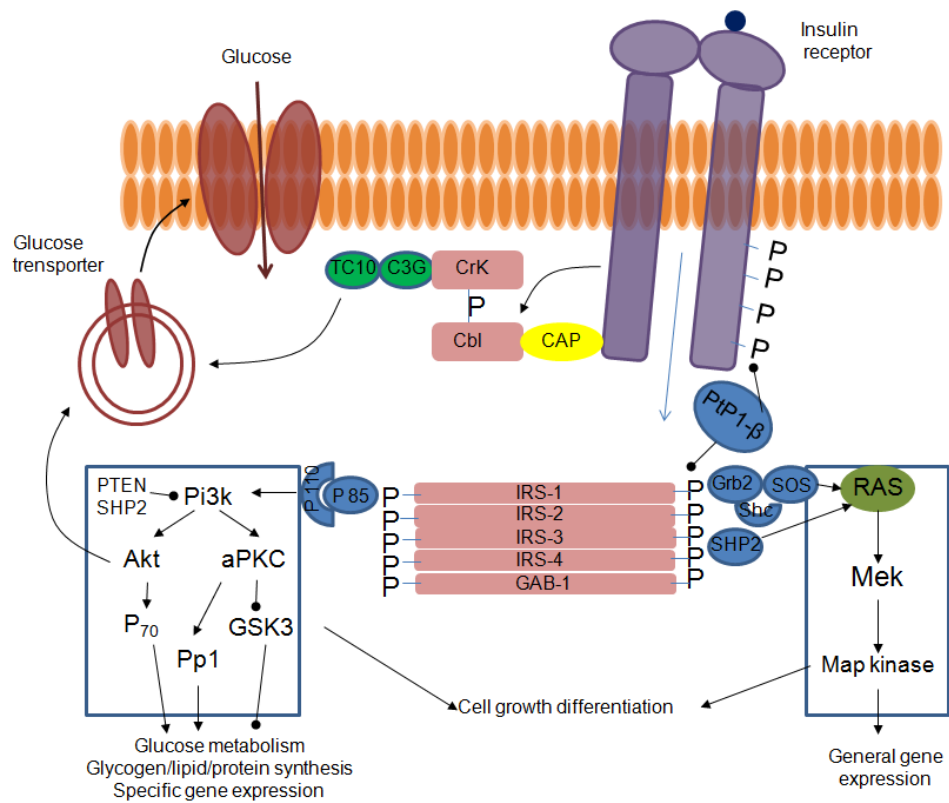


Figure 4: Schema of insulin signalling pathways inspired by R. Saltiel *et al.*⁴⁵

The loss of sensitivity to insulin leading to insulin resistance (IR) is a complex mechanism which firstly targets adipose tissues, liver and muscle. Excess insulin in blood and sustained hyperglycemia seems to be one of the contributors to IR development. One theory proposes that IR arises from an adverse effect inherent in the insulin signalling pathways. The decrease of glucose transporter levels and insulin receptor substrate levels (IRS-1, IRS-2) in the cell, or the activation of the tyrosine kinase, which in turn phosphorylate other key enzymes present in the signalling pathways, inducing a negative feedback loop mechanism reducing the glucose uptake^{34,46,47}.

Another aspect has emerged, that the accumulation of fat in tissues induces local inflammatory responses and the release of cytokines in the circulation, which then contribute to desensitizing the insulin signalling pathways in the peripheral tissues⁴⁸. The impact of obesity-associated low-grade inflammation on IR will be further discussed in the introduction (section 1.4.4).

The impairment of insulin action is characterised by hyperlipidemia and fasting hyperglycemia and is often associated with other disorders such as dyslipidemia, hypertension and fat accumulation in adipose tissues which are characteristic of MetS^{45,49}. Therefore, the triggering event of IR is a complex mechanism and its understanding requires further investigation. However, it can be assumed that IR is one of the underpinning factors linking obesity and MetS.

The gastrointestinal tract also releases a number of hormones, including ghrelin, leptin, glucagon and pancreatic polypeptides, which signal the peripheral and central nervous system and in turn regulate food intake, energy expenditure, and body composition⁵⁰. Using gut hormones as therapeutic treatment may represent a promising new area of research to struggle against obesity and obesity associated diseases. However, the real efficacy of hormones in the regulation of metabolic diseases has not been proven yet.

1.4. Obesity and low grade inflammation.

Strong evidence demonstrated that obesity is associated with an aberrant activation of the immune system leading to the development of low grade inflammation. The emergence of chronic inflammation in obese individuals has been suggested to promote the clinical progression of obesity-related pathologies such as T2DM and hepatic steatosis. The inflammatory disorders are characterised by the overproduction of inflammatory effectors such as cytokines and by immune cell infiltration in adipose and peripheral tissues^{51 52}. These effector molecules and cells are primary components of the innate immune system but can be involved in the set up and the recruitment of the long lasting adaptive immune system.

1.4.1. Basic mechanisms of innate immunity

The innate immune system provides the front line of host defense against pathogenic microorganisms⁵³. Several immune cells immediately recognize chemical structures present on the surface of pathogens and activate a non-specific immune signal. This innate response requires the presence of mononuclear phagocytes that are derived from common myeloid progenitor cells present in the bone marrow. These cells mature in the blood, giving granulocytes (monocytes) and neutrophils⁵³. Monocytes reach the tissues to mature into mast cell, dendritic cells and macrophages. The monocyte-macrophage cells are highly heterogeneous so as to adapt the immune response according to the exposure of the micro-environment^{52,53}. These immune cells recognize the pathogens *via* receptors also known as pattern recognition molecules, which mediate the innate activation signals. Several classes of receptors located in the cell surface can identify and bind a specific pattern present in the bacterial cell wall, such as a specific repetition of carbohydrates or lipid moieties. For instance, the mannan-binding lectins are well defined receptors that recognize mannose and fructose residuals on the bacterial surface^{52,53}. The downstream signal directly initiates the phagocytosis of the external body leading to its ingestion and destruction by the macrophages. Pathogen recognition and phagocytosis sometimes require the activation of intermediate pathways

involving the protein complements. The protein complements system induces a cascade of reactions around the pathogen surfaces producing active components that allow pathogens to be identified and phagocytosed by the immune system⁵³. A second class of phagocytic cell receptors does not bind a specific pathogen pattern but is involved in the recognition of microbial components and triggers the appropriate antifungal and antibacterial responses. The most studied family of transmembrane receptors in mammals is named Toll like receptors (TLRs)^{52,53}. Their activation mediates a large range of inflammatory signals including the myeloid differentiation dependant pathways (MyD88). The downstream responses trigger the activation of mitogen activated protein kinases pathways including extracellular signal kinases (ERK1/2), c-Jun-N terminal kinase (JNK), p38 which are also involved in insulin signalling and the inhibitor of K kinase (IKK β)^{51,52}. Finally, these pathways participate in the activation of transcription factors NF- κ B and AP-1 and cytokine production (Figure 5)^{52,53}. For instance lipopolysaccharide (LPS) is a cell wall component of gram negative bacteria and can stimulate an inflammatory response *via* TLRs activation. LPS binds the plasma LPS-binding protein which in turn enables the fixation of the receptor protein CD14 located in the plasma or on the cell surface of the macrophage. The generated complex then binds the TLR4, which trigger the transduction signals in the nucleus activating NF κ B and AP1 and the gene expression of several inflammatory effectors (chemokines and cytokines) (Figure 5A)^{53,54}.

Other intracellular receptors are present in macrophages and dendritic cells, such as nucleotide oligomerisation domain like (NOD-like) receptors, to cooperate with TLRs in the activation of inflammatory signals. The NOD-like receptors recognize peptidoglycans from gram – and gram + bacteria and have been found to be up-regulated in contact with LPS and TNF- α . NOD-like receptor pathway also induces NF- κ B and is involved in the recruitment of other effector molecules such as those in inflammasome pathways⁴⁸. Inflammasomes are multi protein complexes composed of three proteins: nucleotide binding domain leucine rich protein (NLR), adaptor protein (ACS) and caspase-1. Their activation participates in the maturation of the cytokines IL-1 β and IL-8(Figure 5.B)⁵⁵.

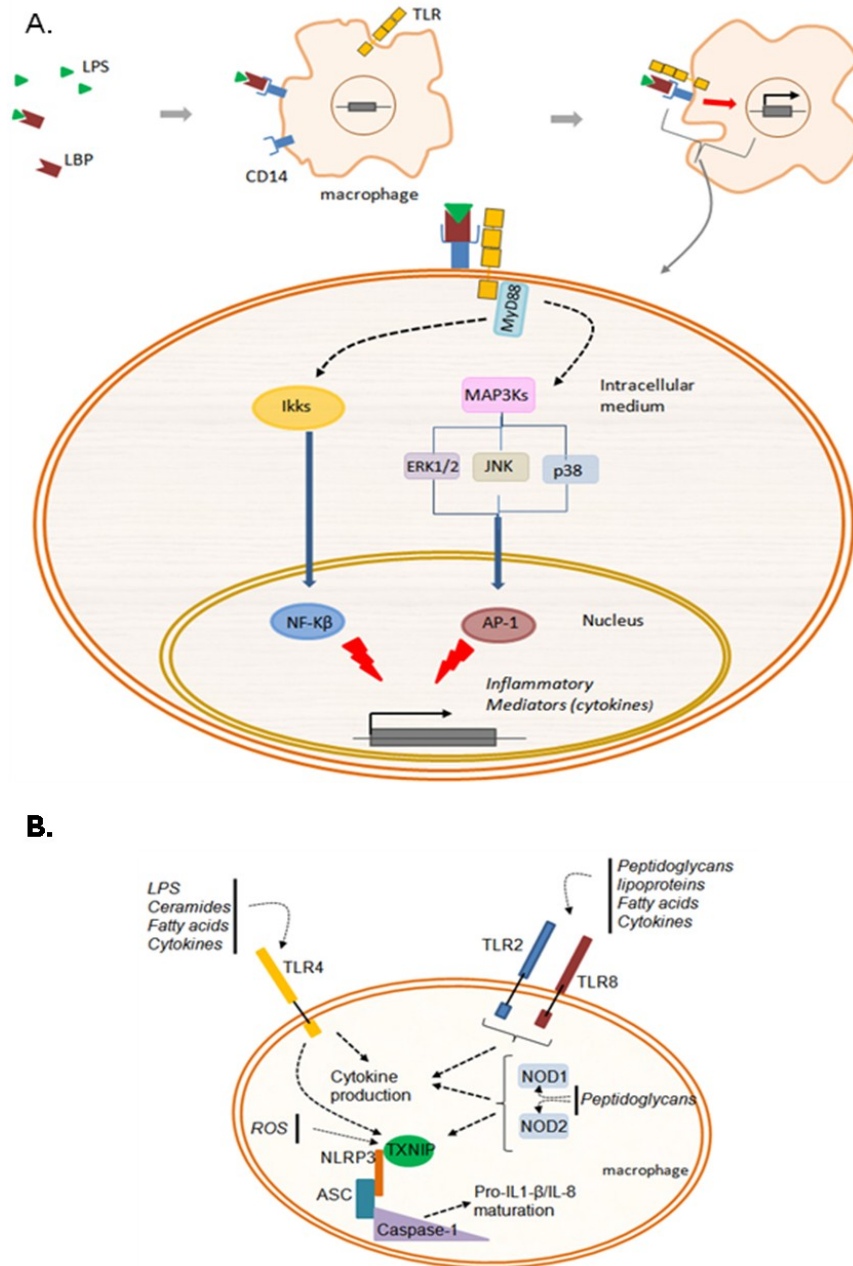


Figure 5: Induction of inflammatory signals in pro-inflammatory macrophages. A: Bacterial LPS activate the cytokines transcription *via* the Toll like receptor-4(TLR-4) activation. B: Other pattern-recognition receptors involved in inflammasome pathways and leading to IL-1 β and IL-8 maturation.

The early innate responses to infection initiate the production and the recruitment of further effector molecules and cells to reinforce the line of defence. The release of pro-inflammatory cytokines in turn modifies the cell behavior in a paracrine and autocrine manner and amplifies the

inflammatory reactions⁵². Cytokines are classified in various structural families: the hematopoietin family including growth hormones and many interleukins such as interleukin 6 (IL-6), the TNF family including TNF- α , and the chemokine family (interleukin 8 (IL-8)). In addition, interleukin 1 (IL-1) and interleukin 12 (IL-12) are grouped in a structurally distinct family^{53,56}. The recognition of different pathogens may result in the production of different cytokines and chemokines. They all have important local and systemic effect and both contribute to the activation of the innate and adaptive immune systems. In particular, chemokines attract cells containing chemokine receptors such as neutrophils from the bloodstream into the site of infection^{53,56}. The cytokines also induce fever, the production of acute phase response proteins and the mobilization of antigen-presenting cells for adaptive immune responses. Other co-stimulatory molecules, such as interferon induced by viral infection, trigger the activation of natural killer (NK) cells which exclusively target infected cells and induce program cell death⁵³. Therefore, NK cells play an important role in the inhibition of viral replication. Interferon γ is not exclusively produced in the context of viral infection and has an impact on the stimulated responses to intracellular pathogens, in particular by polarizing macrophage into pro-inflammatory M1 type macrophage^{53,57}. The major classes of inflammatory cytokines are summarised in Figure 6.

1.4.2. Basic mechanisms of adaptive immunity

Innate immunity is an efficient system for preventing the establishment of pathogens. However, if it fails to remove the infection, cytokines, chemokines, NK cells, macrophages and neutrophils are employed to initiate the development of the adaptive immune system leading to a more specific and long lasting immune response to pathogens⁵³. In particular, lymphocyte progenitors are requisitioned and give rise to T cells and B cells that recognize a great variability of antigens from bacteria and virus. The antigen-recognition molecules of B cells are immunoglobulins (Ig) which directly bind specific antigens. On the other hand, antigen recognition receptors of T cells are membrane-bound proteins that exclusively recognize short peptides fragments of pathogen proteins⁵³. Fully differentiated B cells are able to release their Ig into the circulation as antibodies, which mediate the main effectors of adaptive immune system. In particular, this allows the clonal expansion and production of highly specific antibodies⁵³. These highly diversified receptors can be generated *via* small mutations into genes that encode for a large proportion of receptors. The most important strength of the adaptive immune responses is the ability to produce memory T and B cells which will be highly efficient in removing invading agents encountered previously⁵³. Activated T cells require the production of cytokine molecules, and particularly the T cell growth factor interleukin-2 (IL-2) to proliferate and differentiate into armed T cells.

Alternatively, the monocyte-macrophages can be matured into type II activated macrophages (M2) to promote anti-inflammatory responses⁵³. These macrophages are generally characterised by low production of pro-inflammatory cytokines (IL-1, TNF, IL-6) and high production of anti inflammatory cytokines, such as interleukin-10 (IL-10) and glucocorticoid hormones, which induce various transcriptional programs. For instance, IL-10 secretion deactivates inflammation by lowering cytokine production and activating leukocyte molecule signalling involved in adaptive immunity⁵⁷. It also inhibits the expression of TLR4 in human cells, downregulates NFkB transcription and alleviates the production of inflammatory chemokines involved in the pro-inflammatory immune system. On the other hand, chemokines induced by M2 macrophages improve the recruitment of leukocytes present in tissue repair, as well as remodelling mechanisms and tumor progression. In fact, several sub-populations of activated M2 macrophages exist, inducing a wide spectrum of cytokine secretion and leading to various effects such as killing and encapsulating parasites, reducing allergy responses, immuno-regulation and tissue remodelling⁵⁷. Therefore, generation of M2 macrophages are essential to tune the inflammatory responses triggered by the innate and adaptive pro-inflammatory responses. When the pro-inflammatory signal overcomes the systemic circulation, the type II alternative system generates negative signals which inhibit the recruitment of mononuclear phagocytes at the site of inflammation and help tissue repair^{53,57}.

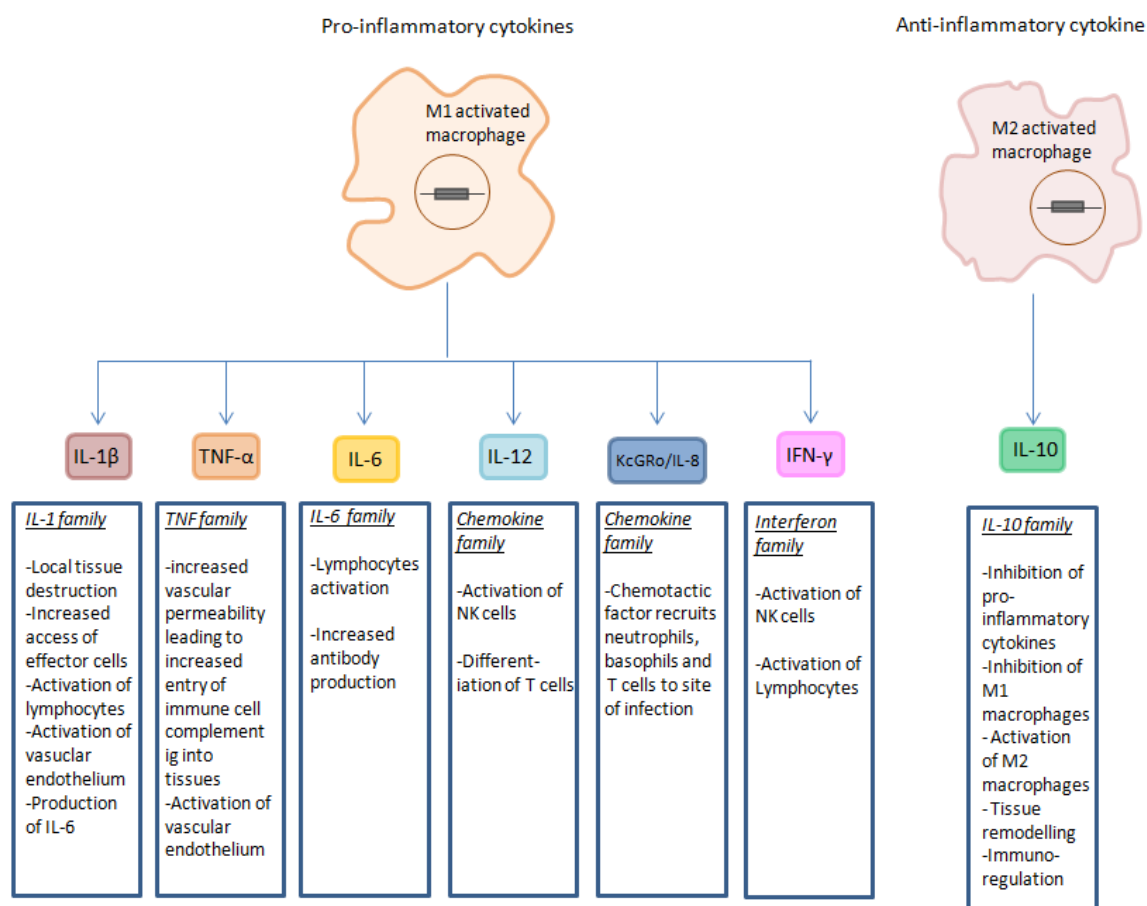


Figure 6: Important pro and anti inflammatory cytokines secreted by M1 or M2 activated macrophages.

1.4.3. Obesity associated low grade inflammation

Unlike the classical immune response induced by pathogens, obesity associated inflammation is triggered by the excess components of nutrients and/or by a specific type of nutrient^{48,51}. As previously mentioned, the hallmark of obesity induced inflammation is characterised by an overproduction of effector molecules and cells primarily involved in innate immunity⁵¹. Recently, several evidences have also shown increased adipose tissue T cell populations in obesity highlighting an environment conducive to adaptive immune activation^{51,58}. In addition, unlike the pathogen-induced immune signals, metabolic overload triggers a low grade inflammatory response which is maintained as a chronic level and lowers the basal metabolic rate⁵¹.

The starting point of the inflammatory signals is still unclear but nutrient overload has been found to stimulate pathogen sensors in metabolic cells such adipocytes, hepatocytes and myocytes

by multiple signalling pathways^{48,54}. In addition, in the overfeeding context, several nutrients can be identified as biologically harmful molecules which activate inflammatory signals^{51,54}. For instance, ectopic fat accumulation and lipotoxicity have been associated with a rise in inflammatory effectors. Saturated FAs have been found in particular to activate TLR4 in adipocytes, hepatocytes and skeletal muscle, resulting in the activation of intracellular pro-inflammatory pathways (including the MyD88/PI3k/Akt pathways) and macrophage stimulation. The direct binding of saturated FA onto TLR4 required the presence of the liver-derived circulating glycoprotein, Fetuin-A (Fet-A) which serves as an adaptor protein linking FAs and TLR4^{48,54}. Interestingly, wild type mice infused with saturated FAs exhibit IR while Fet-A knockdown mice are protected from these symptoms. Therefore Fet-A may confer therapeutic potential in improving glucose homeostasis and insulin sensitivity in obese and T2DM patients. Saturated FAs have also been proven to directly stimulate TLR4 and TLR2 in β -cells leading to the accumulation of monocyte and macrophage in the islet and β cell dysfunction. In addition, several *in vitro* studies on monocytes have also reported an increase in IL-6 and TNF- α production, an increase in TLR4 and TLR2 expression, as well as an activation of the NF κ B pathway under high glucose conditions (Figure 5.A)^{54,59}.

Nucleotide oligomerisation domain (NOD) 1 and 2 are also sensors found to be activated in obese hepatocytes and adipocytes⁵³. In particular, NLRp3 and caspase-1 have been found to increase in overweight people and obese mice^{48,55}. The triggering factors of NOD and inflammasome pathways in the context of obesity remain to be defined. Adipose tissue expansion due to high energy storage can modify cell vascularisation, promote endoplasmic reticulum stress, hypoxia and cell damage, which in turn may contribute to further inflammation responses^{48,51}. Several groups have reported that FAs, ceramides, ATP, glucose, oxidized LDL, uric acid, cholesterol as well as an increase in ROS could activate the NLRp3 inflammasome⁵⁵. Because all these factors are elevated in obesity, it can be suggested that inflammasome signalling pathways are an important link between metabolic disruption and obesity induced inflammation. Finally, increased intestinal permeability and alteration of gut microbiota activity in obesity promote the entry of LPS into blood circulation. This phenomenon is defined as metabolic endotoxemia and has been found to trigger TLR related pro-inflammatory responses *via* the above mentioned mechanism (Section 1.4.1, Figure 5)^{51,54}.

1.4.4. Links between low grade inflammation and impaired insulin signalling.

Several pieces of evidence have shown that activation of effector molecules of inflammation contribute to desensitizing the insulin signalling pathways⁶⁰. At the molecular level, several

mechanisms come into play: IK β and JNK activation in inflammatory tissues of obese individuals have been found to induce uncontrolled phosphorylation of the inhibitory serine site of IRS, triggering a decrease of IRS tyrosine phosphorylation and insulin signal attenuation⁴⁸. However, the consequence of JNK and IK β activation on the obesity and insulin resistance varies depending on the tissue site^{48,54}. In particular, tissue specific JNK -/- mice displayed a conditional phenotype. JNK1 invalidation in myeloid cells decreases obesity-induced inflammation and may promote insulin signalling. On the other hand, JNK invalidation in hepatocytes disturbs liver vital function⁴⁸. Other inflammatory kinases involved in various metabolic regulations have been thought to target the insulin receptor signalling cascade. Extracellular signal-regulated kinases (ERK) 1 and 2, which belong to the MAP kinase pathways, are activated by NOD immune sensors as well as inflammatory cytokines such as IL-1 β . Besides, ERK1 and ERK2 actions have been involved in a negative feedback mechanism altering the interaction between IRS-1 and PI3k as well as IRS-1 and insulin receptors, resulting in a disturbed insulin signalling and glucose transport⁴⁸. Therefore, ERK1 and ERK2 might be a contributing factor of insulin resistance in the context of obesity induced inflammation. In addition, the production of cytokines such as TNF- α , or IL-1 β in visceral adipose tissues within rodents and human affect insulin sensitivity by altering the expression of genes for insulin receptor IRS-1, GLUT4, and PPAR- α ^{61,62}.

The emergence of obesity-induced inflammatory response leading to the loss of insulin sensitivity may occur in adipose tissues as it is a secretory organ producing many inflammatory proteins⁵⁴. Mediators of inflammation are then released to the portal vein and can reach other tissues including liver, pancreas, brain or muscle which contribute to maintain inflammatory signals and develop systemic insulin resistance. However, Cai *et al.* have demonstrated that a local inflammation in the liver associated with high levels of stored lipids can be a primary cause of insulin resistance⁶³. Therefore, the causal relationships between inflammation and IR during obesity require further investigation.

To conclude, the metabolic cells trigger the inflammatory signalling pathways by several mechanisms activating the downstream inflammatory responses, which in turn alter metabolic homeostasis and insulin signalling. Over time, metabolic overload triggers a low-grade inflammatory response which is maintained at a chronic level. This induces the recruitment and activation of many mature immune cells in metabolic tissues such as adipocytes (mast cells, macrophages, dendritic cells) that modify tissue milieu and reinforce inflammation⁶⁴.

1.5. Obesity, MetS and metabolic diseases

As previously detailed, obesity is a chronic disorder characterised by a disruption of glucose mediated insulin action and increased circulating lipoproteins and FAs which all together increase morbidity and mortality^{13,65}. In particular, these metabolic abnormalities have been found to raise the probability of developing metabolic diseases including T2DM, CVDs, NAFLD, cancer and polycystic ovarian syndrome^{1,16,65}. Low grade inflammation and impaired insulin action, which both have a central role in the lipid, glucose and protein metabolism dysregulation, strongly influence the development of these metabolic diseases^{2,48}. In the context of IR, an increased insulin production by the β cells is needed to meet the increased insulin demand. This results in higher systemic insulin levels. However, in the context of prolonged IR, the β cells may not be able to meet the insulin demand, leading subsequently to higher circulating glucose levels and lower peripheral glucose levels indicative of T2DM.

Increased fat accumulation leading to low grade inflammatory responses also affects β cells functions and may thus contribute to the development of T2DM². Visceral fat and ectopic fat accumulation have been correlated with central IR, T2DM and the development of CVDs. Fat accumulation and lipotoxicity in myocytes have been found to promote myocyte degeneration, myocyte apoptosis and cardiac dysfunction⁶⁶. In addition, increased adipose tissue deposits around vasculature, releasing local mediators of inflammation, was associated with endothelial lesions and vascular dysfunction, promoting CVDs such as coronary artery diseases⁶⁶. Finally, lymphatic overload is known to increase intravascular volume, and peripheral vascular resistance due to an exacerbated amount of insulin, cytokines, fat and other nutrients in circulation, which promote hypertension^{66,67}.

In addition, hepatic fat accumulation leading to NAFLD is considered as an important risk for CVDs and has been associated with T2DM^{3,13}. Hepatic TGs accumulation under steatosis is the first step in the full spectrum of NAFLD. NAFLD may subsequently progress from steatosis on to hepatitis, fibrosis and finally hepatic cirrhosis³. NAFLD alone can be a major cause of mortality and can exacerbate the inflammatory status of obese individuals and IR, which contribute to the occurrence of T2DM and CVD^{2,66}.

Yet, although obesity is significantly associated with severe pathologies, many obese individuals do not exhibit metabolic dysfunction and therefore seem protected from the development of T2DM, NAFLD or CVDs risk. This category of individuals is the subject of intensive research to underpin the molecular mechanisms linking obesity and the degradation of the healthy

phenotypes. For example, Wildman *et al.* noted that ethnic differences in healthy middle-age women are associated with differential metabolic activity of visceral and subcutaneous adipose tissues, which may influence the predisposition of certain ethnicities to develop obesity, CVDs and T2DM⁴. Given the multiplicity of factors that determine the likelihood of developing severe pathologies in obese individuals, there is a crucial need to identify early metabolic markers of susceptibility to obesity-related metabolic disorders, to prevent the development of metabolic diseases. Thus, by measuring prognostic biomarkers or early metabolic signature to stimuli, drug efficiency and potential adverse effects could be estimated in a stratified population. This opens the field of personalised healthcare, for which clinicians may be able to tailor therapy for a selected group of people based on patient evaluation before intervention⁶⁸. The concept of personalised healthcare goes beyond therapeutic treatment and could be used to anticipate the most efficient cancer treatment, nutritional intervention or training set for individuals or group of individuals^{68,69}.

2. ROLE OF THE GUT MICROBIOTA IN THE DEVELOPMENT OF OBESITY AND THE METS

The mammalian host-gut microbial metabolic axis represents an important factor involved in several host physiological processes and can contribute, in some cases, to the development of metabolic diseases. A deeper understanding of the mechanisms underlying this crosstalk could prove to be important in the treatment of obesity and obesity associated diseases³¹.

2.1. The biological role of symbiotic bacteria

The term “gut microbiota” refers to the trillions of bacteria living in the intestine. Owing to their symbiotic interaction with the host, they play an essential role in the maintenance of human physiology. Indigenous or commensal bacteria are involved in the development of the digestive tract, can increase the number and maturation of crypts and can also promote the education of the immune system throughout the lifetime of the host^{70,71,72}. For instance, *B. thetaiotaomicron*, one of the best characterised members of the normal human distal small intestine and colonic microbiota, promotes intestinal angiogenesis and stimulates the development of paneth cells. In particular, *B. thetaiotaomicron* regulates the production of epithelial glycans necessary for the lineage of

intestinal cells and the development of mucosal barrier which in turn promotes the gut's innate immune system^{71,73}. Studies on Germ Free (GF) animals also highlight the role of the gut microbiota on the physiological, biochemical and immunological development of the host. Rodents without gastro-intestinal (GI) microbiota have a larger caecum, a reduction in size of many organs including the liver, heart and adrenal glands, and the complexity of their intestinal capillary network is reduced and much more primitive than conventional mice. The efficacy of the immune system as well as the inflammatory responses are also reduced in the GF animal⁷⁴. Therefore, the presence and activity of commensal bacteria is thought to be essential in maintaining the physiology of the digestive tract and in preventing the colonization of pathogenic microorganisms⁷⁵.

Intestinal bacteria operate as a metabolic “organ”, actively taking part in digestion and degradation of nutrients otherwise undigestible⁷⁶. Indeed, polysaccharides are not digested in the proximal human intestine, but instead, they are transformed into digestible compounds such as sugars or short chain fatty acids (SCFAs) by gut bacteria in the distal intestine, providing energy substrates usable by colonocytes in particular, and the host in general. In addition, microbiota actively regulates fat storage by promoting lipid absorption through the chemical modulation of bile acids. The enhanced absorption of monosaccharides also contributes to lipogenesis, leading to an increase of hepatic triglyceride production. Backhed *et al* demonstrated that GF mice re-conventionalized with bacteria of mouse cecal origin undergo an increase of 60% of the total body fat content⁷⁷. Other metabolic processes undertaken by the gut microbiota involve dietary oxalate degradation and vitamin synthesis⁷³. Therefore, the gut microbiota help to enhance extraction of calories from the diet and are involved in the control of energy and lipid metabolism of the host⁷⁸.

The diversity and functions of the gut microbiome was also explored by analyzing the composition of the gut microbial community of healthy human populations^{79,80}. The sequencing of bacterial 16s ribosomal RNA genes from gut biopsies and feces showed that human gut microbiome contain members of seven divisions of bacteria (*Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Cyanobacteria*) 2 bacterial divisions (*Firmicutes* and *Bacteroidetes*) mainly occupy distal gastro-intestinal tract and influence host phenotype³³. Several studies have also demonstrated significant differences in gut microbial composition between healthy individuals^{79,81}.

Recently the full sequencing of the gut metagenome has brought new insights in the role of the gut microbiome on human health⁸². In particular; Qin *et al* established a gut microbial gene catalogue by encoding the total DNA of fecal samples from 124 adults. They detected more than

1000 bacterial species encoding for 3364 non redundant genes. Interestingly, 57 species were found in common in more than 90% of the individuals⁸². Although they found a significant variability of bacterial species among individuals, bacterial gene clustering allowed the identification of a core function of the metagenome. Gut microbiome is involved in essential functions of gut microbiome-host interactions, including synthesis of SCFAs, amino acids, vitamins and the degradation of polysaccharides and other undefined functions which may have an important role in gut ecosystem and physiology^{80,82}.

2.2. Gut microbiota modulation and disease development

Microbiota colonize mammalian gut after birth and over the first years of life and the acquisition of adult-type microbiota is the consequence of several host and external factors^{72,73}. Transient changes in the intestinal ecosystem occur throughout life through the influence of dietary and environmental factors, leading in some cases to the alteration of bacterial-host symbiosis⁸³. Gut bacteria also influence the development of various diseases such as Crohn's disease, irritable bowel syndrome, food allergy, and CVDs among others^{77,84,85}. In particular, stress is correlated with a higher bacterial translocation and increase of gut permeability. This may trigger inflammatory episodes and contribute to enteric inflammatory disorders⁸³. In addition, the release of adrenaline and noradrenalin has been shown to encourage the growth of putative pathogenic bacteria such as *Helicobacter pylori* or *Clostridium difficile*⁸⁶. In some cases, introduction of drugs, particularly antibiotics, also leads to ecosystem alterations and increase the number of pathogenic microorganisms in the intestinal lumen. Pathogen overgrowth is often correlated with more putrefaction-associated metabolites such as ammonia, amines and indoles and increased bacterial translocation, which can be harmful for the host organism. The return to a symbiotic ecosystem may be achieved by selective growth and activation of commensal bacteria which can potentially be promoted by prebiotic or probiotic supplementation. This aspect will be further discussed in the section 2.3 of this introduction.

It has also been established that ingestion of a diet rich in fat and carbohydrates modulates the gut microbial composition and encourages obesity development. Turnbaugh *et al* have observed differences in the distal gut microbiota of obese *versus* lean humans and the relative abundance of *Bacteroidetes* increased as individuals lost weight on either a fat- or carbohydrate-restricted low calorie diet³³. The lower *Bacteroidetes/Firmicutes* ratio characterised in obese people is thought to be more efficient at hydrolysing non-digestible polysaccharides in the intestinal lumen and may be able to extract more calories and fat compared to lean individuals⁷⁷.

As previously mentioned, increase in body weight is frequently accompanied by IR and MetS. Substantial evidence suggests that gut microbiota also play an active role in IR and obesity associated diseases, especially through immune mediated mechanisms. Indeed, the proliferation of gram-negative bacteria associated with HF feeding in mice generates hepatotoxic substances such as endotoxins and increases production of cytokines which favour liver injury⁸⁷. It has been shown that individuals with relatively increased gram-negative bacteria counts also have elevated levels of circulating LPS – being key components of bacterial cell walls. Higher circulating levels of these molecules has been correlated with an increase in fasting glucose and insulin, weight gain and steatosis in mice^{73,84,87}. Therefore physiological processes associated with imbalanced gut microbiota work as a vicious cycle, triggering and promoting the initiation and the progression of obesity^{72,74,78,88}.

Dumas *et al* established a link between reduced choline bioavailability for the host associated to a up-regulation of microbial choline pathways in mice fed with a HF diet and the development of IR and NAFLD⁸⁹. These results confirm that microbial metabolic activity is significantly involved in metabolism of endogenous and exogenous compounds, and is associated with host welfare. It is therefore essential to understand the role of the gut microbiota-host interactions in obesity development, and metabolic diseases (NAFLD, T2DM, CVDs).

2.3. Gut microbial remodelling by nutritional interventions

Therapeutic impacts of gut microbiome remodelling have been explored using several strategies. Antibiotic therapy confers the abilities to remove pathogenic bacteria involved in the production of endotoxins and infections⁹⁰. As an example, supplementation of the antibiotic rifaximin has been found to successfully reduce hepatic encephalopathy which is partly caused by gut derived products (neurotoxins, ammonia, indoles) in sub-clinical trials⁹¹. However, antibiotic utilization may compromise gut microbial ecosystem and promote the growth of new resistant strains. Several studies have reported an increase in *Clostridium difficile* infections causing diarrhea after antibiotic treatments⁹². Fecal transplantation is another strategy to shape the gut microbiota. It turns to be an efficient way of restoring gut microbial ecosystem after antibiotic treatment. In particular, mouth fecal transplantation was found to reduce symptoms associated with *Clostridium difficile*⁹³.

The selection and enhancement of specific gut microbiota may also represent a new approach to control energy intake and reduce the prevalence of obesity. The main strategies to improve gut microbial-host metabolic interactions in obesity, and metabolic diseases (T2DM, NAFLD) are based on the use of probiotics and prebiotics⁹⁴. Probiotics or microbial food supplements beneficially affect the host by improving intestinal microbial balance and changing the composition of the colonic microbiota⁹⁵. Originally, probiotics were added in yoghurt to improve lactose digestion and eliminate symptoms of intolerance for people who were unable to efficiently digest lactose. Nowadays, they are used to reduce other digestive symptoms such as diarrhea or bloating and help re-establishing a symbiotic ecosystem after drug therapy such as antibiotic treatment⁷⁰. By contrast, prebiotics are food ingredients that beneficially affect the host by selectively stimulating growth and/or activity of one or a restricted number of bacteria present in the colon⁷⁰. Prebiotics are composed of oligosaccharides or short chain polysaccharides. They are easily found in common dietary products such as, vegetables and whole grain cereals and can be added in yoghurt⁹⁵.

Recent studies have explored the potential therapeutic use of probiotics in the treatment of metabolic diseases. Specific bacterial efficacy has been demonstrated to enhance host energy metabolism, for example *Bifidobacterium spp* have been shown to improve glucose homeostasis, reduce weight gain, fat mass and restore glucose mediated insulin secretion in mice fed with HF diet⁸⁸. Martin *et al* also illustrated the contribution of the probiotic *Lactobacillus rhamnosus* and *Lactobacillus paracasei* in lipid and amino acid homeostasis through the decreased lipoprotein levels in plasma, the increased deconjugation of bile acids, and the up-regulation of branched chain amino acids (BCAAs) catabolism in mice fed with chow diet³¹. It has been established, the reduction of fat storage and the increased production of SCFAs due to probiotic supplementation enhance anti-inflammatory responses, and reduce hepatic injury⁸⁷.

The most characterised prebiotics are fructosyl-oligosaccharides (FOS), inulin (long chain fructosyl-oligosaccharide), galactosyl-oligosaccharides (GOS) and other oligosaccharides from milk (MO), which are transformed by the gut microbiota in SCFAs and simultaneously promote proliferation of selected commensal bacteria in the colon⁹⁶⁻⁹⁹. For instance, inulin, has been found to stimulate *Bifidobacteria* and may reduce calorie intake and fat mass in animals⁹⁷. It has been recently confirmed that prebiotic stimulation of *Bifidobacteria* is correlated with increased glucose tolerance, improved glucose-induced insulin secretion, and normalisation of inflammation⁸⁸. GOS also modify the monosaccharide uptake from the gut by changing the activity of host monosaccharide transporters which in turn results in activation of glycolytic pathways⁹⁸. Prebiotic consumption has been associated with a reduction of hepatic, kidney and plasma lipids in

rodents^{100,101}. In particular, GOS supplementation in healthy mice decreased hepatic TGs by lowering lipogenic enzyme activity, fatty acid synthase (FAS), and the microsomal triglyceride transfer proteins (MTTP) which is involved in VLDL synthesis^{97,100}. Furthermore, higher carnitine, and acyl-carnitine levels were observed in the urine of prebiotic fed mice, which suggested an increased FA oxidation¹⁰². Therefore, prebiotic ingestion may promote a shift toward less lipogenic activity and more lipolytic activity.

Human Milk oligosaccharides (HMO) also present various biological activities and provide a range of beneficial effect beyond nutritional needs. They are mainly composed of glucose and galactose but they also contained fucose, *N*-acetylglucosamine, *N* acetylneuramic acids (sialic acids). Fucosyl- and silyl-saccharides cannot be digested by intestinal enzymes. Thus HMO can reach the large intestine and colon, and may confer prebiotic effects¹⁰³. For instance, HMO may selectively promote the growth of certain *Bifidobacteria* strains only present in breast fed infant. However, given their structural complexity, the large scale industrial production of HMO is unfeasible. Alternatively, cow milk oligosaccharides (CMO) may be used as another oligosaccharides source for infant formula. Additionally commercial GOS products are not naturally found in human and bovine milk. Therefore new mixtures combining CMO and GOS are under development to propose nutritional supplements with fermentable properties closer to human breast milk¹⁰³. Although lactose present in milk is the main source of carbohydrates for new borns and infants, 75% of the world adult population are unable to digest lactose¹⁰⁴. Symptoms of lactose intolerance are bloating, cramp, diarrhea and inflammation, and its ingestion may promote inflammatory bowel diseases¹⁰⁴. Several studies have demonstrated that probiotic supplementation in dairy products (animal milk, yogurt, cheese) improves lactose digestion in grownups. Probiotic and prebiotic supplementation may also reduce symptom of lactose intolerance¹⁰⁵.

Finally, the concept of a 'synbiotic', based on the synergetic combination of prebiotic and probiotic is another approach which provides alternative solutions to control gut microbial composition and to reduce the risk of developing metabolic diseases such as obesity and type II diabetes⁹⁶. Therefore, by promoting beneficial commensal bacteria, probiotic, prebiotic, and synbiotic products may confer nutritional benefits and protect organisms against colonic and systemic disorders. In this regard, they are defined as health-enhancing food ingredients and have potential pharmaceutical-like applications.

3. A METABONOMIC STRATEGY TO UNDERSTAND THE INTERACTIONS BETWEEN DIET AND MICROBIOTA IN OBESITY AND METS

DEVELOPMENT

Given the complex crosstalk between carbohydrate, protein and amino acid metabolism in the context of over-nutrition, and the multitude of genetic and environmental factors affecting these metabolic pathways, it is necessary to investigate the onset of obesity and the MetS from a holistic point of view. A large number of techniques including genomics, transcriptomics or proteomics provide a “top-down” integrated view of the molecular basis of health disorders^{106,107}. However, genomic and proteomic studies usually offer limited information about metabolic modulations because several biochemical mechanisms cannot be explained by differences in concentration of proteins or gene expression levels alone. By contrast, one definition of metabonomics is “the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification”. This systemic approach allow the characterisation of the metabolic signature associated with a genetic modification as well as toxicological interventions or dietary influences¹⁰⁸. Because metabolic concentrations are dependent on gene expression, protein activity, cell kinetics and metabolic flux, metabonomics provide a “global picture” of the physiological regulations considering all the influencing factors. By using a combination of molecular analytical tools (mass spectrometry (MS) and nuclear magnetic resonance (NMR)) and multivariate data analysis, it is possible to capture a ‘snapshot’ of an individual metabolic profile from biological matrices and tissues, this profile being defined as a “metabotype”¹⁰⁹.

Nutritional metabonomics investigating the metabolic responses to various diet interventions has been defined as nutrimetabonomics and turn to be an efficient approach to investigate the role of nutrients on obesity and metabolic diseases, whilst also considering genetic and environmental factors⁶⁹. For instance, ¹H NMR spectroscopy based metabonomic study has characterised a range of metabolic variations to HF diet across five mouse strains susceptible or resistant to HF induced IR. The hierarchical metabonomic clustering were found to closely match the single nucleotide polymorphisms (SNPs) based phylogenic tree of the 5 inbreed strains which supports the concept that individual metabotypes mirror the genetic susceptibility of adaptation to HF diet¹¹⁰. Metabolic phenotyping of obese and lean subjects in mouse and human studies, have contributed to our overall understanding of the onset of T2DM and IR by highlighting the

contribution of metabolites involved in energy metabolism such as TCA cycle, lipid and amino acid processing, as well as inflammatory markers to these pathological processes¹¹⁰⁻¹¹².

Metabonomics is also suitable for investigating the continuum of mammals-microbiome interactions as metabolic profiles capture endogenous metabolites as well as “mammalian-microbial co-metabolites”³². These exogenous compounds which originate from diet and/or gut microbiota, can enter the host’s system where they can be further processed in the liver and other tissues before being excreted in the urine. Thus, urine is an ideal matrix for studying in a non-invasive manner, the impact of dietary intervention and gut microbiota activity on host physiology^{69,113}. Several metabonomic studies have emphasised the importance of the human symbiotic relationship with the gut microbiota and its role in the development of metabolic disorders. As previously mentioned, Dumas *et al* identified in a metabonomic study, the link between mitochondrial choline metabolism and the development of NAFLD and IR in the host⁸⁹. A recent human study combining ¹H NMR metabolic profiles and 16S rRNA sequencing of fecal microbiota showed that gut microbiota remodelling by inulin-type fructans tend to decrease circulating phosphatidylcholines levels and fat mass in obese women¹¹⁴. Hence, metabonomic studies supported by other “omics” technologies are useful to investigate the effect of dietary interventions on the gut microbiota-host metabolic axis.

A recent metabonomic approach named ‘pharmacometabonomics’ illustrated the concept of predicting final physiological outcome of a pharmaceutical intervention from predose metabolic profiles⁶⁸. This novel idea could be also transposed to nutritional studies to anticipate the metabolic alterations associated to specific diet and set up personalised healthcare solutions¹¹¹. Therefore, given that gut microbiota activity and host immune system have been found to influence metabolic phenotypes, metabonomics is a promising strategy to investigate the link between overfeeding, gut microbiota activity, and host inflammatory responses, in the onset of obesity and its metabolic diseases.

4. SCOPE OF THE THESIS

The complex aetiology of obesity and MetS, combining genetic and environmental factors has been largely documented in the literature. This explains the large variability in host responsiveness to rich (high fat and/or carbohydrate) diets and the presence of a large spectrum of metabolic deregulations and diseases associated with obesity. Substantial evidence has also

demonstrated the role of gut microbiota into the host's metabolic regulation and its potential roles in metabolic disorders. Thus, two hypotheses can be formulated:

- Individual responsiveness to HF diet may be predicted from early or prior metabolic profiles
- Modulation of gut microbiota activity by prebiotics during HF diet-induced obesity development may improve host physiology.

The scope of this thesis intends to investigate these questions using a metabonomic approach. In a first experiment, we aimed to identify the difference in metabolic profiles of obesity prone and obesity resistant C57BL/6 mice at 7 days and 60 days after HF feeding. In a second experiment, we evaluated the impact of different prebiotics in the modulation of the interactions between host's metabolism, host's immune system and the gut microbiota in C57BL/6 mice during HF-induced obesity development.

CHAPTER II: ANALYTICAL METHODS

Metabolic profiling techniques are applied to a wide range of complex samples such as biofluids and tissues. Profiles derived from urine yield insight to specific metabolic changes and provide indirect information about gut microbial activity as urine contains specific metabolites produced by host-microbiotal interactions³². Urine is also particularly useful to investigate gradual metabolic changes as its collection is minimally invasive to the subject. Metabolic profiles of collected specimens are obtained using nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS)¹¹⁵, which are particularly useful tools for elucidating biomarkers without prior knowledge of the sample composition¹¹⁶. Summarising various multivariate metabolic profiles by mathematical models improves the understanding of host metabolic modulation influenced by genetic and external factors¹¹⁷. Targeted approaches using MS are also used to identify and quantify known metabolites of interest, often present in lower concentrations. Integrating information generated by metabolic profiling obtained from different biofluids such as plasma and urine can provide a more systemic view of mechanisms underlying the microbiota-host metabolism interaction under different diet intervention^{32,118}. Metabonomic data can be also integrated with other omic data to provide a better “picture” of the interactions between genetic, metabolic and physiological changes in a perturbed system. In this thesis, 16S rRNA gene sequencing data has been correlated with metabonomic data to identify the link between gut microbiota composition and host metabolic adaptation during dietary interventions¹¹⁵.

1. NUCLEAR MAGNETIC RESONANCE:

High resolution NMR spectroscopy is a quantitative, non sample destructive, and reproducible analytical method used to simultaneously detect a wide range of low molecular weight compounds (<1000 Daltons) in a complex sample such as tissue extracts or biofluids. NMR-based metabolic profiling is a suitable tool for metabonomic applications as it provides a metabolic “snapshot” representative of the sample at the collection time and requires few sample preparation steps¹⁰⁸. The current generation of commercially available high-field spectrometers enables easy detection of metabolites in the micromolar range within a short experiment time¹¹³.

1.1. Fundamental principles of NMR spectroscopy

The principle of NMR is based on the fundamental properties of the atomic nucleus which is composed of protons and neutrons. These particles, also called nucleons, are strongly linked together and confer spin properties. The atomic nucleus can also have a nuclear spin which is defined as the particular vectorial sum of nucleons spins. The nuclear spin value (I) partly depends on the number of nucleons (atomic mass) and the number of protons (atomic number)¹¹⁹.

All nuclei with a non-zero nuclear spin are observable by NMR. However the most important nuclear isotopes used in biological approaches are those with a spin $\neq 1/2$ i.e. ^1H , ^{13}C , ^{15}N , ^{31}P . In particular, we focused on ^1H NMR spectroscopy as the hydrogen nucleus is the most abundant element in biological molecules and, apart from ^3H , is the best detected by NMR¹¹⁹.

When a magnetic field (B_0) is applied, the nuclear moments of spins orient themselves and the number of possible orientations is given by the equation: $2I+1$. For instance if $I = 1/2$, there are two different orientations of the nuclear spins in the external field. The spin corresponding to $1/2$ is the lower energy, is more stable, and is oriented in the same direction as B_0 (position α). On the other hand, the spin equal to $-1/2$ is the higher energy with an opposite direction to B_0 (position β). For both orientations, the spins move around the magnetic field with a frequency ν (Hz) and an angular speed ω_0 (Rad.s^{-1}), this motion is defined as the Larmor precession¹²⁰ (Figure 7).

The population of 2 energy levels are given by the Boltzmann distribution:

$$N_{\text{upper}}/N_{\text{lower}} = e^{-\Delta E/KT}$$

N_{upper} and N_{lower} represent the number of nuclei in the upper or the lower energy state. K is the Boltzmann constant and T is the absolute temperature in Kelvin (K). ΔE corresponds to the energy difference between the two orientations and the energy required to flip nuclear spins. It can be calculated by the following equation:

$$\Delta E = \gamma \hbar B_0 / 2\pi$$

Where $\hbar$ is the Planck's constant, γ is the gyromagnetic ratio (nuclear magnetic moment) and B_0 the NMR magnetic field. The slight excess of spins in α position compared to spins in β position entails a net absorption of energy which in turn gives NMR signal. As the coefficient of absorption is a constant for any nucleus, the intensity of the signal is proportional to the total number of nuclei in the sample^{119,120}.

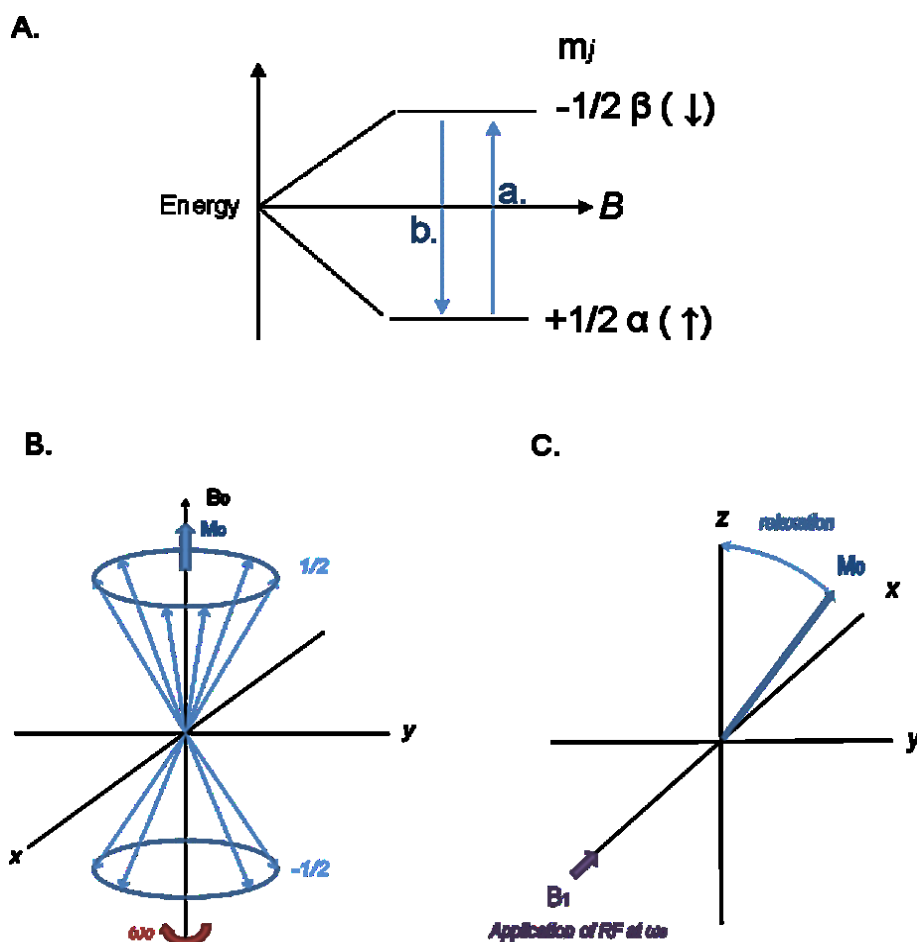


Figure 7: Theory of NMR spectroscopy. (A) .Energy level and transition for a nucleus having a nuclear spin $I=1/2$ in a magnetic field B_0 . (B). Motion of net nucleus magnetisation which relax at the Larmor frequency ω_0 in a magnetic field B_0 . The spin resultant gives a stationary bulk magnetization vector prior to radiofrequency (RF) perturbation. (C) Effect of RF irradiation with a frequency ω_0 on bulk magnetisation vector M_0 .

The difference of population between these two energy levels gives rise to a resultant magnetisation vector M_0 following the same direction as B_0 (on the Z axis). When a specific pulse of radiofrequency radiation B_1 is applied at the resonant frequency ω , some nuclear spins in the lower energy are forced to adopt higher energy levels leading to a new orientation of M_0 . When the pulse stops, the perturbed spins relax back to recover their equilibrium condition. During the precession movement, the component of magnetisation releases an oscillating voltage which gives the NMR signal, also called free induction decay (FID). If B_1 power is applied such that M_0 is reoriented by 90 degrees, this results in recording the maximum voltage. The relaxation of the nuclear spin magnetisation is described by 2 processes: The longitudinal (spin-lattice) relaxation time T_1 is the time constant for the return of M_0 to its equilibrium position (on the z axis). The transverse (spin-

spin) relaxation time T2 is the the time constant for the nuclear spins to lose phase in the X-Y plane. A Fourier Transform is then applied to convert the time domain signal of FID in the frequency domain and obtain an NMR spectrum^{119,120}.

1.2. Chemical shifts

NMR spectroscopy provides extensive information on molecular structure and conformation because each nucleus is directly influenced by its chemical environment. Indeed, electric current around the nuclei (dependent on electronegativity, local chemical environment, electron density, and circulating electron ring currents) generates a local magnetic field in the opposite direction to B_0 and shields the nuclei from the external applied field. Therefore, the nuclear shielding corresponds to the difference between the applied field and the field at the nucleus. The chemical shift is then defined as the nuclear shielding divided by the applied magnetic field. In the case of an experiment in which the exact field is applied and a reference nucleus is used, the chemical shift can be characterised by the equation:

$$\delta \text{ (ppm)} = [(\nu - \nu_{\text{ref}}) / \text{oscillator frequency (Hz)}] * 10^6$$

Where ν is the frequency of the nucleus, and ν_{ref} is the resonance frequency of a chosen nucleus in a standard molecule^{119,120}. The new chemical shift scale is independent from the nominal field B_0 .

1.3. The spin-spin coupling

Inter- and intra-molecular interactions among individual groups of nuclei in proximity may occur, leading to magnetisation transfer. In a liquid solution, molecules are constantly tumbling because of thermal motion so that the intermolecular magnetic interactions are averaged to zero. Within the molecule, magnetic nuclei can also interact with each other through space or *via* spin-spin coupling involving electrons polarisation exchange between atomic bonds. Again, the spatial interactions within the molecules are averaged due to the fact that molecules in solution are highly subjected to Brownian motion. However, spin-spin couplings from neighbouring groups in the same molecule results in a splitting of NMR signals into two or more components that are slightly shifted from the original frequency/ chemical shift (in ppm) measured in the absence of coupling. Therefore, 2 spin $\frac{1}{2}$ magnetic nuclei that mutually interact have their resonance signals split into 2 peaks with

equal intensity and separated by a scalar coupling constant J (in Hz). In the case where more than 1 nucleus is involved in the coupling, further splitting of the resonance signals have to be considered leading to doublet, triplet or multiplet with various intensities^{119,120}. In a simple system, the peak pattern is given by Pascal's triangle. The spin-spin coupling is usually transmittable up to 3 bonds, but the presence of unsaturated bonds may extend the electrons exchange further¹²¹. The J coupling is independent of the external magnetic field and gives indications on the molecular structures as it depends on the chemical bonds, the the coupled nuclei, the length of interacting bonds and the dihedral angles between the bonds¹²⁰.

Multiplicity of observed resonance		Relative intensities of components of multiplets	
Number of adjacent nuclei			
0	singlet	1	
1	doublet	1 1	
2	triplet	1 2 1	
3	quartet	1 3 3 1	
4	quintet	1 4 6 4 1	
5	sextet	1 5 10 10 5 1	
6	septet	1 6 15 20 15 6 1	

Figure 8: Multiplicity and relative intensities of NMR signals from equivalent coupled nuclei with spin number $I=1/2$ (such as ^1H coupling).

1.4. 1D NMR spectroscopy

A single pulse sequence used for 1D NMR experiment contains at least 3 parts: the preparation time, also called relaxation delay (in seconds), the pulse width (in microseconds) and the acquisition time (in seconds). The preparation time is an important period where the spins reach their equilibrium state. It should last about three to five times T_1 relaxation time for a complete spin relaxation. The pulse width corresponds to the length of time to apply a radiofrequency power pulse to perturb the spin system. After that, the excited spins relax back to their original state generating the FID which is acquired during the acquisition time. Because NMR is a relatively insensitive method compared to other spectroscopic tools, it is often required to repeat the pulse sequence several times so as to sum up the FID signals. In fact FID is constituted of coherent resonance signals from spin

relaxation and incoherent noise signals. Therefore, signal/noise ratio increases by the square root of the number of scans^{119,120}.

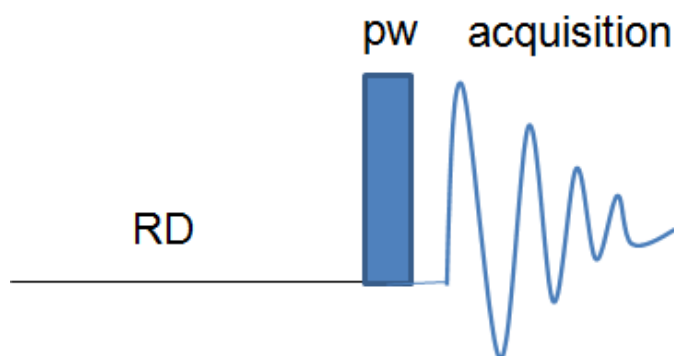


Figure 9: Standard one pulse sequence (RD: relaxation delay ,pw: pulse width)

In metabonomic studies, three 1D NMR experiments using more complex single pulse sequences are frequently employed depending on the biofluids or tissues we want to investigate¹²².

- A standard 1-D experiment allows to acquire the resonance of all proton containing molecules in the samples including sharp peaks from small molecules (amino acids, simple carbohydrates, organic acids, nucleic acids) and broad signals from the macromolecules (proteins, long chain fatty acids). This experiment is widely used for acquiring urinary metabolic profiles which do not contain large amounts of macromolecules.
- The Carr-Purcell-Meiboom-Gill (CPMG) experiment attenuates the signals of macromolecules and allows a better definition of small molecules signals. This sequence is of particular interest for tissues extract and plasma metabolic profiles which contain a large amount of proteins and/or lipids.
- The diffusion edited experiment selectively detects the signals of macromolecules.

1.5. 2D NMR spectrometry

Due to a large number of overlapped peaks in 1 D NMR metabolic profiles, it is important to perform 2D NMR experiments which provide more information about NMR peak connectivity and thus help structural elucidation of unknown metabolites in complex biological matrices. Unlike 1D

NMR experiment, the signals from 2D NMR experiments are dependent on two time variables t_1 and t_2 and the following FT (which is applied twice) give a spectrum with two frequency variables F_1 and F_2 . The scheme of a 2D NMR sequence (standard COSY) is presented below¹²⁰.

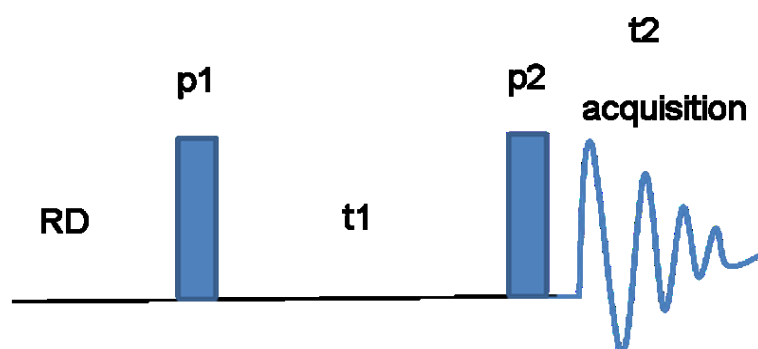


Figure 10: NMR pulse sequence of a standard COSY experiment (RD: relaxation delay, p1: first pulse width to reach 90° ; t1: evolution time, p2: second pulse width to reach 90° , t2: acquisition time)

After the relaxation delay, several pulses are applied to induce spin excitation. In turn, the spin relaxation evolves during the time t_1 . A second set of pulses are applied during the mixing time. The resulting signal is then recorded during the second time variable t_2 . The two dimensional signal is actually recorded by repeating the pulse sequence several time with increasing t_1 value. Usually the whole process requires 50 to 500 increments of t_1 . For each pulse sequence FID is recorded during the time t_2 only. The nature and the time of pulses during the relaxation delay and mixing time determine the information present in the spectrum. A generic 2D NMR spectrum can be interpreted as following^{119,120}:

- A cross-peak is observed with a frequency coordinate of 20 Hz on F_1 and 80Hz on F_2 which means that a resonance signal evolve at 20 Hz during t_1 and during the mixing time, the same signal transfers its magnetisation to another signals which evolves at 80Hz on t_2 .
- If a cross peak has a frequency coordinate of 20 Hz on F_1 and F_2 , it means that the resonance signal is not affected by the mixing time and it evolves at 20 Hz during t_1 and 20Hz during t_2 .

Therefore, pulses during the mixing time are crucial in creating a 2D NMR signal. In the case of no J coupling or inefficient mixing time, a diagonal of cross peaks with equal frequency values on F_1 and F_2 can be observed¹²⁰. Several 2D NMR experiments are frequently employed as aid for assignment in metabonomic studies¹²²:

- J-Resolved spectroscopy allows the separation of ^1H chemical shift and J coupling into two dimensions. The first dimension (F2) is quite similar to a 1D NMR spectrum although each proton appears as single peak. On the second dimension (F1), proton signals are separated by an offset according to the J coupling value. This method helps the interpretation of peak multiplicity in complex metabolic profiles¹²³.
- Correlation spectroscopy (COSY) is used to identify correlation between two ^1H which are J-coupled with each other. The cross peaks present on a COSY spectrum provide connectivity information of coupled protons separated by two or three bonds in general¹²⁴.
- Heteronuclear single quantum coherence (HSQC) experiments consist in transferring the magnetisation by one bond coupling between ^1H and ^{13}C . The signal of ^1H which is affected by the frequency of the singled bonded carbon is directly recoded on the first dimension (F2) while the ^{13}C signal is indirectly detected *via* ^1H signal. The generated spectrum provides cross peaks with co-ordinates corresponding to chemical shift of J coupled ^1H (on F2 dimension) and chemical shift of ^{13}C (on F1 dimension). This experiment is particularly useful for structural elucidation as it gives additional information on the atomic connectivity and allows a better dispersion of NMR peaks along the ^{13}C dimension¹²⁵.

2. LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

Mass spectrometry (MS) is an analytical technique in which molecules in ionised gaseous form are separated in space and time according to their mass to charge ratio prior to detection. Detected ions from one or more distinct molecular species are recorded as a mass spectrum which can provide information related to both the chemical formula and structure. The analysis of biological mixtures such as urine, serum or tissue extracts produces complex spectra with a wide range of molecular masses. Therefore, a separation step on a time dimension using gas chromatography, high performance liquid chromatography (HPLC) or recently ultra-high performance liquid chromatography (UPLC) is frequently employed prior MS to reduce sample complexity and improve the ionisation of molecules^{121,126}.

2.1. Principles of Liquid Chromatography

In liquid chromatography, molecules in solution enter a column containing a microparticle stationary phase and are eluted out with solvents (also called mobile phase) at different migration rates. The relative elution order depends on the chemical structure of the molecules and their relative affinity for the stationary phase and the mobile phase. The mobile phase usually consists of two miscible solvents that are exchanged to first bind the molecular content of a sample to the stationary phase, and then elute it cleanly with sufficient resolution between species of interest. The stationary phase is generally composed of particles of silica or cross-linked co-polymers of styrene or benzene. While these materials may be used by themselves, they often act as a support structure for additional chemically bonded molecules which greatly influence the selectivity of the chromatographic separation. The size of stationary phase particles is related to the efficiency of the achievable separation, and therefore the use of small particles is particularly important in high performance applications such as those required for biofluid profiling. Ultra high performance liquid chromatography (UPLC) is achieved with particle sizes $<2\mu\text{m}$, allowing for rapid high resolution separations^{121,126}. Such system has been engineered to withstand the high backpressure (up to 15,000 psi) that results from the use of these small particles.

In metabonomic studies, the most common UPLC-MS analyses employ a reversed phase separation system and gradient elution which allows the separation of medium to low polarity solutes. This chromatographic method most commonly uses a non-polar stationary phase of octadecyl groups (C_{18}) bound to silica or polymeric particles. Solute elution is performed by applying a gradient of solvents, starting at aqueous conditions for solute binding, and gradually introducing an organic solvent such as acetonitrile or methanol to facilitate solute elution. The solvent gradient allows changing the polarity of the mobile phase over time which improves the resolution of complex mixtures over a wide range of polarities. However, reversed phase chromatography is not particularly adapted for the analysis of high polarity solutes because these molecules are not well retained on the stationary phase and tend to be eluted with the void volume of the column¹²¹. Where polar analytes are the analytical focus, alternative chromatographic strategies must be sought. Hydrophilic interaction liquid chromatography (HILIC) columns have been developed to preferably retain small polar molecules such as amino acids and organic acids. HILIC methods have recently been developed which allow for high quality separations over short analysis times with excellent detection sensitivity when mated to MS detection¹²⁷.

2.2. Principle of Mass spectrometry

A basic mass spectrometer is capable of accepting molecular content from an inlet system, converting that material to gas phase ions, performing mass analysis of the molecular species present, and detecting the signals produced by each. Such a mass spectrometer is thus composed of an ion source, a mass analyser, and ion detector¹²¹.

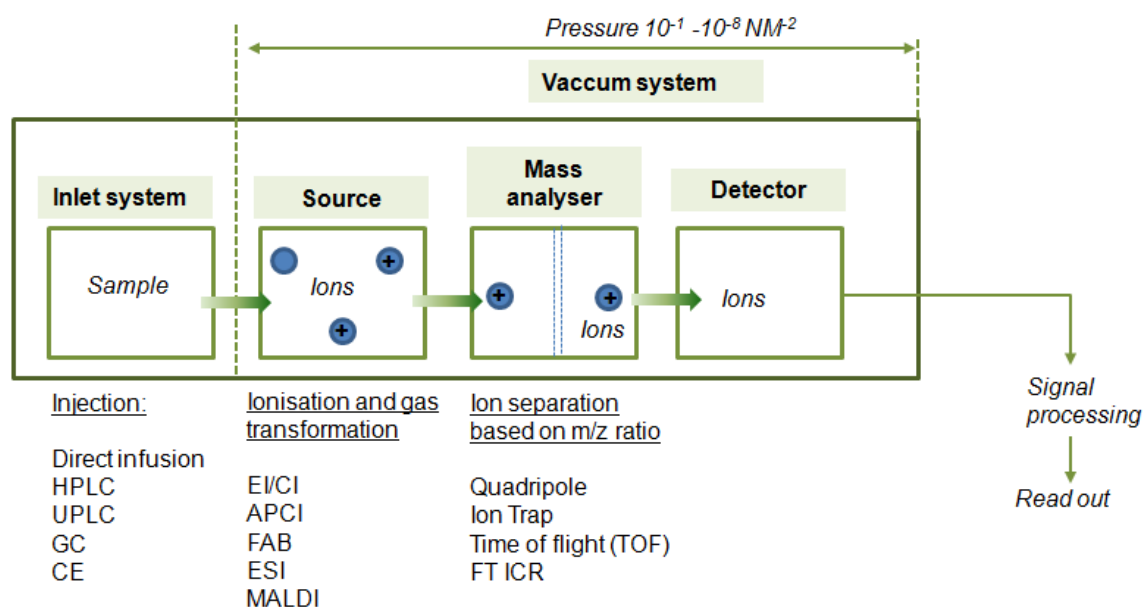


Figure 11: Diagram of mass spectrometry. HPLC: high performance liquid chromatography, UPLC: ultra high pressure liquid chromatography, gas chromatography, CE: capillary electrophoresis chromatography, EI/CI: electronic or chemical ionisation, APCI: atmospheric pressure chemical ionisation, FAB: Fast atom bombardment, ESI: electrospray ionisation MALDI: matrix-assisted laser desorption ionisation, FT ICR: Fourier transform-ion cyclotron resonance.

The sample inlet allows a controlled introduction of gaseous or vaporised liquid samples into the ion source which is subjected to high vacuum. This injection system can be interfaced with gas chromatography (GC), capillary electrophoresis chromatography (CEC), HPLC, UPLC or performed by direct infusion. These techniques are chosen according to the physiochemistry of metabolites which need to be studied. For instance, GC is preferred for the analysis of small volatile metabolites while HPLC and UPLC are adapted for liquid samples¹²¹.

The ion source which operates under high voltage induces ionisation of the molecular content. From this module, the ions are subjected to high vacuum (10^{-4} to 10^{-7} Nm⁻²), except for atmospheric pressure ionisation techniques. Several ways of ionizing molecules exist depending on the nature of the matrix and the analytical requirement. Electronic ionisation (EI), chemical ionisation (CI) and atmospheric pressure chemical ionisation (APCI) are preferably used for the analysis of small volatile and thermo-resistant molecules. On the other hand, fast atom bombardment (FAB) is more suited for the analysis of small molecules (lower than 6000 Da). In biological applications, soft ionisation methods such as electrospray ionisation (ESI) and matrix-assisted laser desorption ionisations (MALDI) inducing little fragmentation are also frequently employed as they conserve molecule integrity and non-covalent interactions.

The most common method used in LC-MS is ESI because it provides high sensitivity and it directly generates ions from liquid phase to gas phase. During ESI ionisation, the analytes in solution travel in a capillary tube subjected to a high electric field. This high potential triggers the accumulation of charges at the surface of the capillary tip, which are in turn drawn out to form droplets. Nitrogen gas and heat are introduced in the source to promote solvent evaporation and the formation of smaller highly charged droplets. Finally, when the charge density at the droplet surface is high enough, ion desorption occurs leading to the formation of gas phase ions. This process usually yields singly charged ions from small molecules (<1000 Da) but it may result in the production of multi-charged ions from bigger molecules. ESI can produce both positive and negatively charged ions which are typically measured in separate analyses, as each requires the mass analyser and related ion optics to be in one or the other polarity. By doing so, the coverage of detected molecules may be dramatically improved as many molecules are only visible in a single polarity¹²¹. ESI is a competitive process, however, whereby multiple ions may challenge each other for available charge. The resulting suppression phenomenon represents a significant drawback in ESI LC-MS of complex biological matrices¹²¹.

The mass analyser enables the separation of ions according to their mass to charge ratio. The ions formed in the source are accelerated towards the mass analyser by passing through series of ion optics with applied voltages. The mass analyser can be a simple quadrupole (Q), time of flight (TOF), ion trap or Fourier transform-ion cyclotron resonance (FT-ICR) system. Several analysers can also be combined to perform multi-stage separations. The most common hybrid analyser configurations are the Q-TOF, the triple quadrupole (QQQ), and the Qtrap¹²¹.

Once the ion species have been separated, they are passed to a detector which records the current produced when the ions hit the detector's surface or pass close to it. The abundance of each ion species is detected and the resulting signal of all the ions from a single measurement (known as a "scan") gives a mass spectrum displaying the ion abundance (on the Y axis) and the m/z ratio (on x axis). The most intense peak is defined as the base peak. The mass spectrum encodes information reflecting the molecular formula and distribution of heavy isotopes in the molecular species. Where molecular fragmentation is used (typically in hybrid mass spectrometers such as the Q-ToF or QQQ), the mass spectrum can also encode structural information in the form of a characteristic fragmentation pattern.

2.3. Multiplex targeted MS/MS assay: the Biocrates kit

While LC-MS is often employed for metabolic profiling whereby all observable ions are detected, it can be also used for targeted analysis when molecular species of interest are known in advance of the analysis. In this case, the system is configured for the selective detection of only the target analytes, with known masses, fragments, and retention times. The use of standard reference curves generated from the purified materials of interest allows for the translation of signal intensity to absolute concentration. Use of stable isotope-labelled standards in the sample corrects for any ionisation suppression effects that may confound absolute quantification. In this thesis, the Biocrates kit containing numerous isotope labelled standards has been used for targeted LC-MS analysis using a Qtrap mass spectrometer operating in ESI+ and ESI-.

The Biocrates "AbsoluteIDQTM p 180 kit allows the identification and quantification of 186 metabolites from a small volume of sample (10 μ l). Several acylcarnitines, amino acids, biogenic amines, hexose and phospho- and sphingo- lipids can be analysed quantitatively with the addition of stable isotope labelled or chemically homologous internal standards. Multiple reaction monitoring (MRM) is preceded by a combination of flow injection analysis (FIA) and liquid chromatography (HPLC) and provides high sensitivity and high selectivity analysis¹²⁸. The MRM analysis consists in selecting a target molecular species in the first MS analyser (for instance the Q1 in QQQ or Qtrap) and excluding all others based on nominal m/z value. That species is passed on to a collision cell, where fragmentation occurs by collision induced dissociation (CID). A diagnostic fragment ion species is selected in the second MS analyser (for instance the Q3 in the QQQ or the trap in the Qtrap) and passed on to the detector which measures the amount of incoming material and

translates that into signal intensity. The selection of a molecular species (parent ion) and its diagnostic fragment (fragment ion) in the first and the second MS analyser respectively is called an MRM transition. An example of ion fragmentation is given in Figure 12.

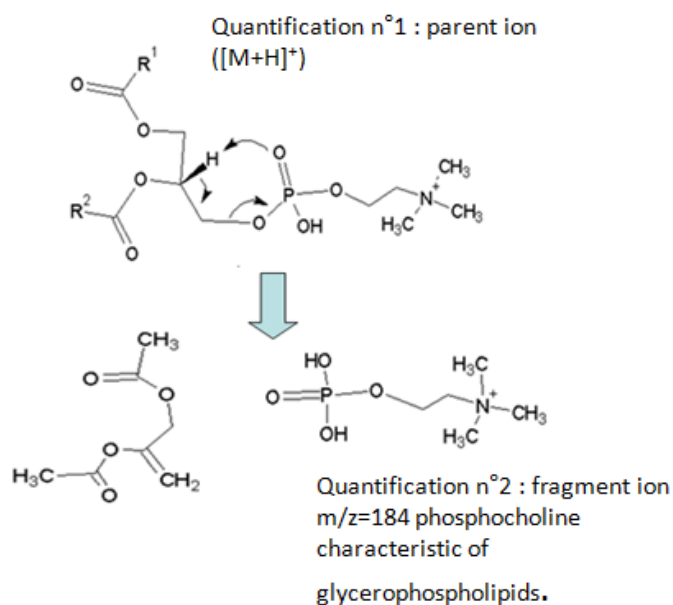


Figure 12: Example of ions fragmentation in the MS analyser.

The kit is particularly designed for MS/MS analysis on triple quadrupole or hybrid quadrupole ion trap platforms which provide a wide linear dynamic range. Several operating procedures to assess the analysis quality are also carried out during the experiment. The assay work flow is separated in 7 modules¹²⁸.

1. Sample lists are imported and registered in the Biocrates analyst software using a 96 well plate format template.
2. The assay is prepared using a specific 96 well plate design as follows:

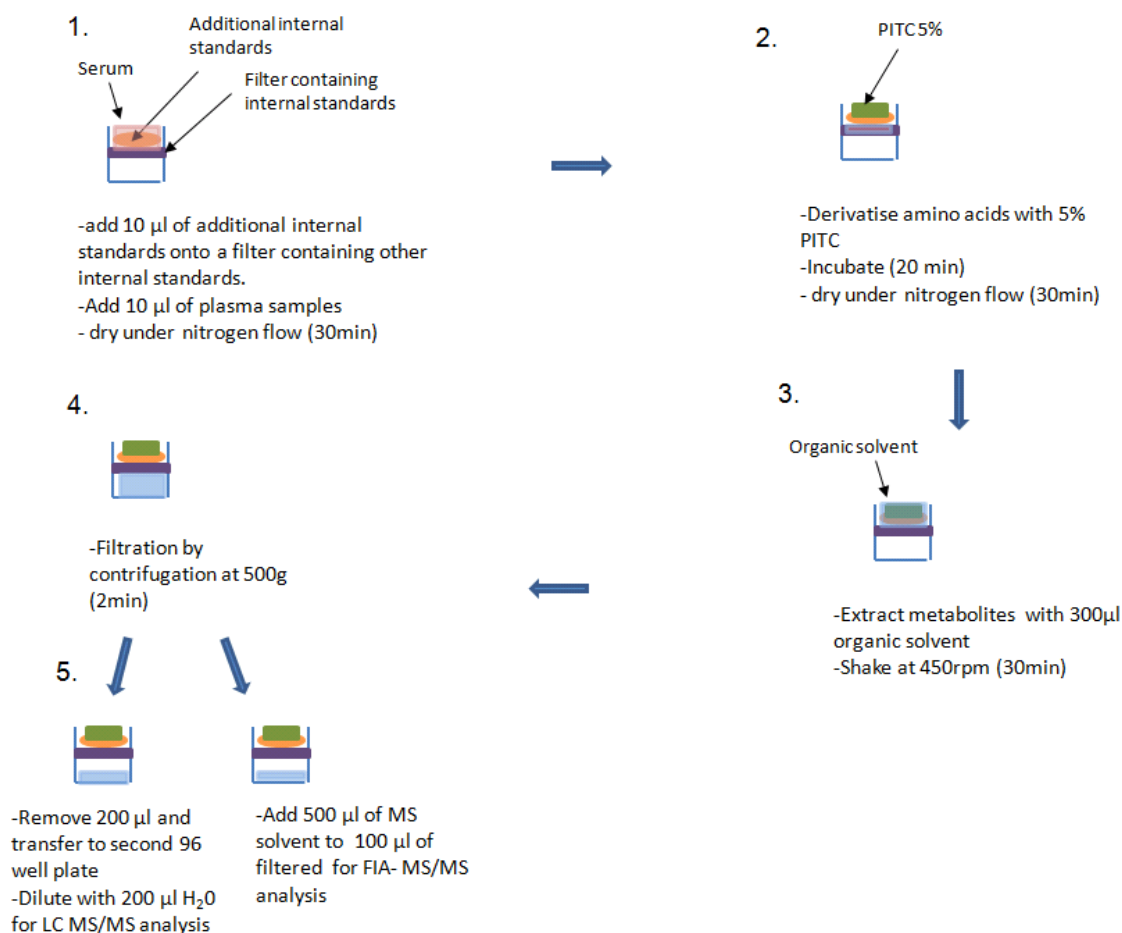


Figure 13: Protocol for sample preparation using the Biocrates kit

3. The assay is run with a standardised MS method:

Acylcarnitines, hexoses, phospho- and sphingo-lipids are analysed by FIA-MS/MS analysis. Analysis time is 3 minutes using either positive or negative ion mode electrospray and detection. One hundred and fifty-eight MRM transitions are measured in the positive ionisation mode while 2 are measured in the negative mode. Therefore, two injections of 20 μ l are necessary to perform the complete analysis. The flow rate and the gradient of the solvents are detailed in Table 1. below:

Table 1: Gradient and solvents for the FIA-MS/MS analysis of the Biocrates kit

Step	Time (minutes)	Flow (µl/min)	Biocrates solvent + Methanol %
0	0	30	100
1	1.6	30	100
2	2.4	200	100
3	2.8	200	100
4	3	30	100

Amino acids and biogenic amines are analysed using a HPLC-MS/MS method. Each sample is separated by reversed phase chromatography utilising a 9.5min separation and analysed using positive mode electrospray ionisation, monitoring 65 MRM transitions. A 10 µl injection is necessary for the analysis and the flow and the gradient of the solvents are detailed in Table 2 below :

Table 2: Gradient and solvents the HPLC MS/MS analysis of the Biocrates kit

step	Time (minutes)	flow (µl/min)	Water + 0.2 % formic acid A%	Acetonitrile + 0.1% formic acid B%
0	0	500	100	0
1	0.5	500	100	0
2	5.5	500	5	95
3	6.5	500	5	95
4	7	500	100	0
5	9.5	500	100	0

- LC-MS based quantification is performed on the Biocrates Analyst Software. After manual review of peak integration, the metabolites are automatically quantified using the provided quantification methods and known calibrator concentrations.
- The data generated from FIA MS/MS analysis are imported and automatically converted into concentrations in the Analyst software. The total ion current (TIC) trace is used to help identify the time range for integration of MRM results.
- Each plate analysis is validated in the Analyst Software by assessing the signal reproducibility and accuracy using quality controls (QCs) and blanks. The Biocrates QCs correspond to human plasma samples with analytes of known concentrations. Several QCs with different concentrations of analytes are used.

7. Metabolite concentration from samples and QCs are evaluated and each metabolite under a set limit of quantification is discarded. The final results can be exported into open source file formats for further analysis.

This multiplex targeted MS/MS analysis allows the rapid identification of polar (amino acids, biogenic acids) and unpolar metabolites (acyl-carnitines, lipids and hexoses) but presents some drawbacks. In particular, the analysis gives the degree of unsaturation in the detected lipids but does not provide the exact positions of the unsaturations. In addition, the biocrates kit detects the overall hexose contents present in the plasma samples but cannot specifically quantify the different types of hexoses such as galactose or glucose.

3. STATISTICAL ANALYSIS

NMR and MS analyses produce thousands of signals from metabolic profiles, so multivariate analyses methods are useful approach to analyse and interpret such data: The data analytical objectives are summarizing and visualizing the main sources of variation as well as performing multivariate classification and discriminant analysis. Therefore, unsupervised and supervised pattern recognition methods are routinely used for modelling two (or multiple) classes of data to drive class separation, simplify interpretation and find potential biomarkers^{129,130}.

3.1. Unsupervised method: Principal Component Analysis (PCA).

PCA is an unsupervised method because no hypothesis on the data is required for the construction of the model. This projection method is based on the representation of a multivariate data containing n rows (observation) and p columns (e.g. variables of NMR spectrum) in a lower dimension space. By calculating principal components (PC) in the p -dimensions, the model defines axes or directions describing the dominant pattern in the data matrix. The first PC is built from linear least square analysis to maximise variance harvesting in the data and the second PC orthogonal to the first one is added to improve the modelling of the data variance. This overview of the data uncovers all the information in the data matrix but may reveal groups of observation, trends and outliers^{117,129,130}.

From a geometric point of view, K-dimensions space is constructed from the K principal components and the n observations are projected onto this space according to the value of its respective variables. The scores (T) represent the co-ordinate values of the observations in the K-dimensions space and the loadings (P) represent the directions of the PC in the initial p -dimension space, they corresponds to the weight of the variable in the model. (Figure 14) The part of the matrix that is not explained by the PCA also called residuals (E) is characterised by the distance between each observation and its projection on the PC space. In summary, the matrix X containing the data is modelled by the following equation:

$$X=TP^T+E$$

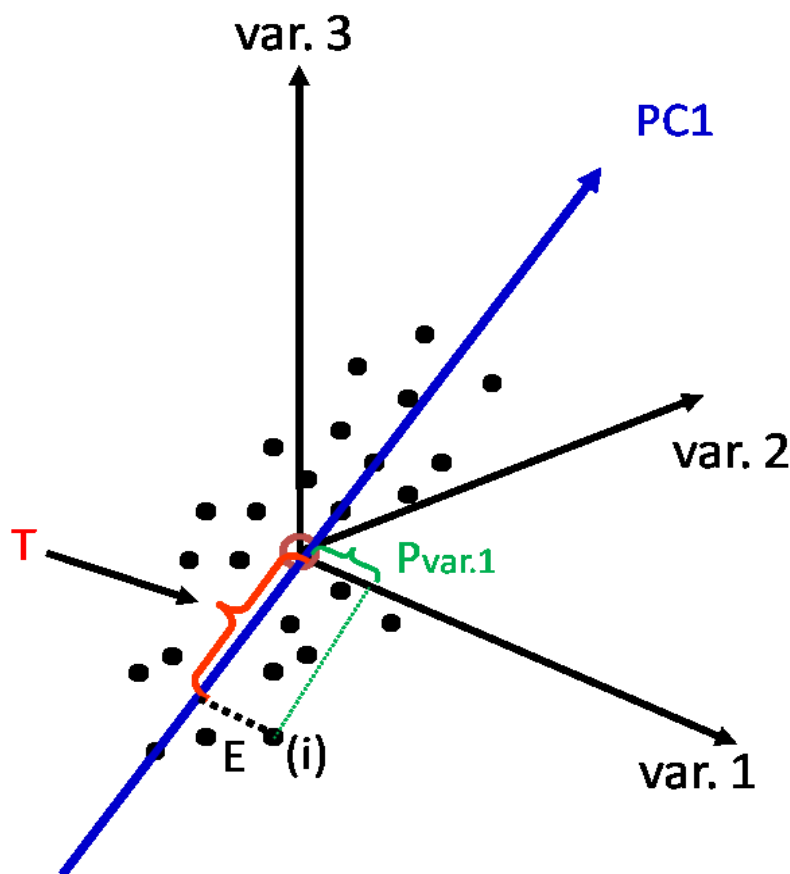


Figure 14: Construction of the first principal component (PC1) explaining the main variation within the dataset. (var: variable). The score (T) of one observation (i), its corresponding residual (E) and the loading of the variable 1 for this observation (Pvar1) is also geometrically represented.

Information contained in the PCA can be graphically interpreted by the scores plot and the loadings plot (Figure 15). On the one hand, the scores plot highlights the relation among the observations in the new co-ordinate system built by the PCs and points out patterns in the data. On the other hand, the loadings plot provides the degree of influence of the variables in the model and permits to identify which variables are responsible for this specific pattern^{117,130}.

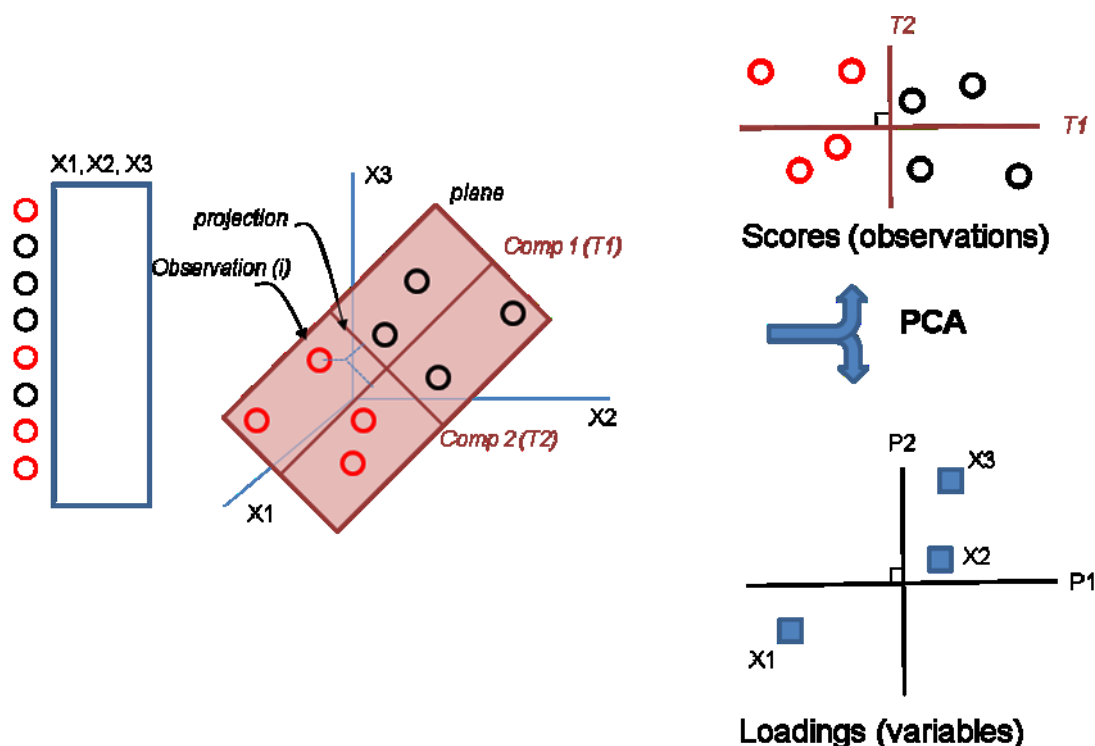


Figure 15: Geometric approach of principal component analysis¹¹⁷: Variations within a X matrix containing 8 samples and 3 variables are approximate in a lower dimension model plane. Scores plot and loadings plot obtained from the model respectively provide information on sample classification and on variable influence in the model.

3.2. Supervised methods.

Most projects in metabonomics are focused on group classification such as treated and control groups, or stratification of pathological status therefore it is common to use supervised methods where *a priori* information about class of samples is employed to perform the analysis¹³⁰.

Partial least squares (PLS) regression is a method that relates two matrices (X and Y) to each other by linear regression. Indeed, PLS components are built in the X space in order to explain as much as possible the multidimensional covariance between X and Y . Matrix X generally represents spectral or chromatographic data whereas matrix Y contains quantitative values such as time or concentration of endogenous metabolites. PLS model can be also used in a discriminant analysis (PLS-DA) in which Y matrix comprises dummy variables defining classes or gender. The Y dimension can also be a 1D vector. This method is particularly suitable in metabonomics because it provides a good predictability even when the number of variables exceeds the number of observations^{117,130}.

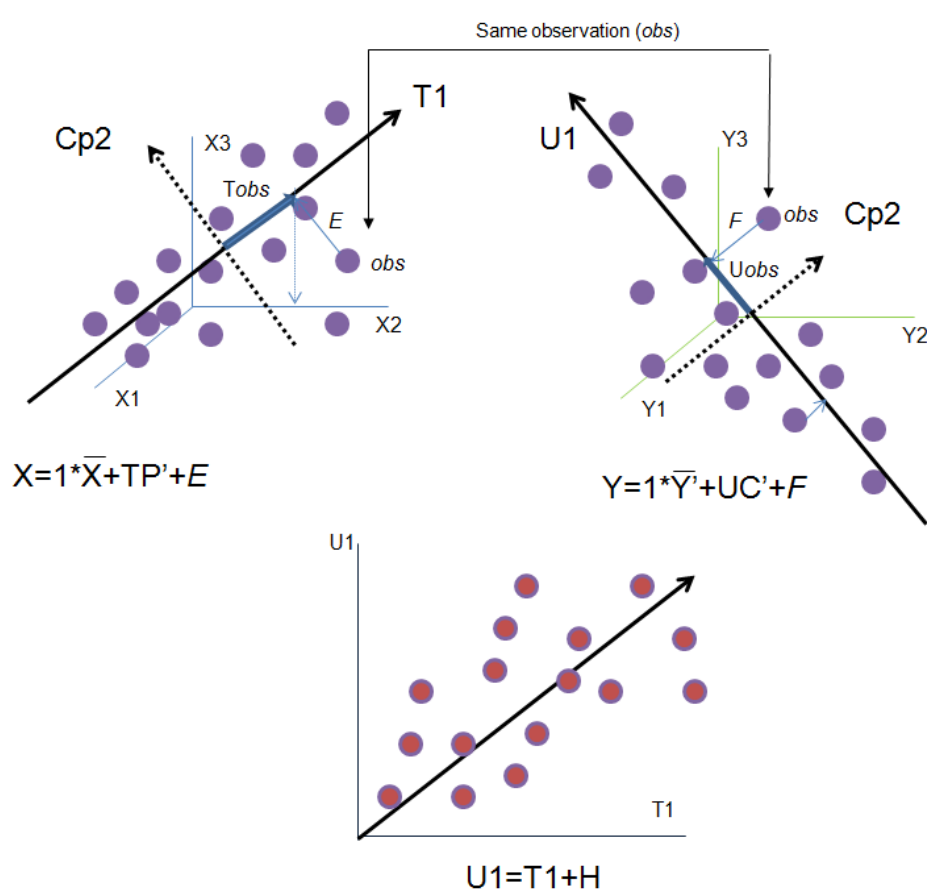


Figure 16: Principle of PLS regression. The multidimensional X matrix (top left) represents the predictor and the multidimensional Y matrix (top right) represents the responses to predict. In each multi-variate space, the orientation of the first component must approximate as well as possible the shape of the point cloud and the scores $T1$ and $U1$ should be maximally correlated. The second component is build perpendicularly to the first component using the same principle.

However, X matrices such as NMR data or MS data usually contain a certain level of variation uncorrelated to the Y matrix which come from other biological variation source or analytical noise and can decrease the interpretability of PLS model. Hence, orthogonal partial least square discriminant analysis (OPLS-DA) is a recent extension of PLS-DA in which an integrated Orthogonal Signal Correlation (OSC) filter is added in the construction of the model¹³⁰⁻¹³². The filter leads to partitioning the X variance into a predictive and orthogonal component to the Y matrix. This enhances the variation of interest while it minimizes other variation uncorrelated to Y. Hence, X data splitting facilitates model interpretation and the analysis of scores plot and loadings plot of the orthogonal component can bring about additional information on subgroup structure within the data^{117,131}.

In OPLS regression and OPLS-DA models, R^2_X and R^2_Y indicate the proportion of explained variance from the X matrix and Y matrix and need to be taken into account to estimate the robustness of the model. However, in any multivariate modeling, it is easy to obtain an apparently good model fitting the training dataset very well but which has not the ability to predict external data. This phenomenon is called overfitting. To overcome this, a cross validation (CV) step is systematically implemented into the data modelling to assess the real predictability of the model. CV is generally performed by keeping out the seventh of the observation and predicts them back into the model to test the performance of the model. This step is repeated until every data has been once kept out and it results in the determination of the cross validation parameter (Q^2_Y) indicating the proportion (in percentage) of the predicted variance from the Y vector in the model. Generally when $Q^2 > 0.5$, the model is considered as strong enough to be used for data interpretation, however this subjective criteria is limited and is often re-evaluated as it depends on the condition of the experiment (number of samples, size of dataset)^{129,130}. A permutation test which estimates the significance of the estimated predicted power (Q^2_Y) can be also used for the model validation. The method consists in generating a number of parallel models based on the fit of randomly re-ordered Y data. (at least 1000 iterations) Then the real Q^2_Y is considered as significant if its value is out of the distribution of Q^2_Y values generated with random models^{129,130}.

As described by Cloarec *et al*, the OPLS regression coefficient can be displayed using a back-scaling method and is really useful to interpret NMR data^{131,133}. The coefficients from a model fitted with unit-variance scaling are multiplied back by the standard deviation of the corresponding variable in order to display them in their initial and more natural scaling. For NMR it produces a pseudo spectrum which relates to the covariance between X and Y. The squared regression coefficients (R^2), proportional to the correlation coefficients between X and Y for a model fitted with

unit-variance scaling are then mapped onto the pseudo spectrum by using a colour code starting from red to blue¹³¹. The hot colours are allocated to high correlated variables whereas the cold colours represent variables with non significant correlation. Hence the pseudo spectrum helps the interpretation of the loadings by facilitating the determination of important group of variables which represent peaks and individuals variables corresponding to the noise in the spectrum.

Loadings plot interpretation from MS data is based on the same principle as for NMR data but the visualisation tool used for MS analysis is different due to the 2 dimensional structures of the data (one feature is defined by its m/z value and its retention time). In this context, the S plot is preferably employed to identify significant features in the OPLS models. This scatter plot is based on the same principle as for OPLS regression coefficient plot as it consists in modelling modelled correlation (p corr) of each feature versus the modelled covariance between the same feature and the predictive score tp. The features that combine high correlation and high covariance are considered of potential interest¹³⁰.

3.3. Biomarker identifications:

NMR and MS techniques associated with multivariate statistics are applied in metabolomics to extract from complex biofluids containing thousands of metabolites, including specific biomarkers responsible for group discrimination. These biomarkers can provide indications on time dependant biological processes underlying physiological perturbations such as disease development, drugs effects or dietary intervention outcome.

Molecule identification from NMR data is commonly achieved by determining the peak shape, coupling values (J) from 1D NMR spectra and cross correlation peaks from 2D NMR spectra. The attribution is performed by comparing this information with available literature and existing databases such as the Human Metabolome Data Base (HMDB): <http://hmdb.ca/>, or the Biological Magnetic Resonance Data Bank (BMRB): <http://www.bmrwisc.edu>. However, for several regions, the 1D NMR peaks overlap and are not sufficiently distinct to permit a clear assignment. STOCSY (Statistical Total Correlation Spectroscopy) is recent technique proposed by Cloarec *et al*, which exploits the correlation between data point amplitudes in the NMR spectra. By calculating the correlation matrix of a data point corresponding to the maximum value in a peak, it is often possible to extract other data points in the spectrum highly correlated to this selected variable. Therefore STOCSY is able to reveal highly correlated variables corresponding to the whole NMR peaks of a

single metabolite, but it also highlights in a lesser extent, correlated and anti-correlated variables from different molecules involved in the same pathways¹³³.

Alternative approaches based on biofluid separation methods using solid phase extraction (SPE) chromatography or high pressure liquid chromatography (HPLC) has been found useful to improve the identification of complex biological sample composition. For instance, a biological matrix in solution can be loaded in C₁₈ SPE cartridge and diluted with a methanol/water gradient and/or pH gradient. This method allows a reduction of sample complexity by separating the molecules according to their hydrophobicity. The first aliquots contain highly hydrophilic molecules while the last aliquots contain less hydrophilic molecules. Each aliquot can be in turn analysed in 1D and 2D NMR to perform metabolite identification¹³⁴.

4. 16S RIBOSOMAL RNA GENE SEQUENCING

Microbiome-host interaction has been found to play a crucial role in mammalian health and disease development^{32,88}. Therefore studying the microbial communities present in various mucosal surfaces and mammalian skin has contributed to increase knowledge about the development of infectious and immune mediated diseases and diseases susceptibilities^{135,136}. Metagenomics, which is defined as the culture-independent study of a set of genome from microbial population or the exploration of the entire microbial genome, has largely expanded due to the growing interest on the role of the microbiome on human biology¹³⁷. Improved DNA-sequencing technologies have also enabled enhanced capabilities for sequencing large scale data sets. However the complexity of microbial populations makes metagenomic studies challenging. The genetic content of the metagenome can be up to approximately 100 times greater than the human genome and a large part of bacterial taxa are still unknown. In addition, around 80 % of the human gut bacteria sequenced by metagenomic studies are not cultivable *in vitro*¹³⁷.

In this thesis, fecal microbiota from C57BL/6 mice fed with different diets have been investigated using pyrosequencing of bacterial 16S ribosomal RNA gene (16S rRNA). This new deep sequencing method has been proved to be a convenient platform for analysing the human microbial composition by carefully targeting short length of DNA. Pyrosequencing employs the polymerisation chain reaction technology (PCR) which consists in the amplification of a piece of DNA sequence in several orders of magnitudes. In a PCR experiment several thermal cycles occur where a short DNA fragment or primer containing the complementary sequenced to the targeted DNA piece is fixed to

the genetic material and DNA polymerase and dNTP are used to replicate the targeted sequence. In particular, 16S rRNA gene contains highly conservative regions (C1-C9) interspaced by highly informative hypervariable regions (V1-V9) which allow PCR primer to be designed in such way that they are complementary to universally conserved regions flanking the variable region. Then the amplification, sequencing and comparison with database allow the identification of bacterial lineages and their proportion in community. It is important to note that sequencing different hypervariables regions introduces various degrees of variability and efficacies in identification of bacterial species. For instance V2 and V3 regions have been the most effective for bacterial identification at genus level. In addition, the identification of bacterial classes, families or genus may differ according to the taxonomic classifications employed¹³⁷.



Figure 17: Scheme of 16S rRNA gene sequence containing hypervariable regions (V) and highly conserved regions (C) adapted from Petrosino *et al*¹³⁷.

The pyrosequencing procedure is detailed as follows: A specific primer is hybridised to a DNA template which is immobilized into streptavidin coated beads and incubated with DNA polymerase, deoxynucleotide triphosphates (dNTPs), ATP sulfurylase, adenosine-5-phosphosulfate (APS), luciferase and apyrase, and luciferin. The sequencing procedure is performed by a stepwise elongation of primer strand upon the sequential incorporation of the different dNTPs. The PPi released due to the nucleotide incorporation is converted into ATP by the presence of ATP sulfurylase and APS. ATP is then consumed by luciferin to produce oxyluciferin. This process catalysed by the luciferase generates visible light which is measured by a camera and processed by a specific program^{137,138}. The luminescence is directly proportional to the number of bases that have been incorporated. After the addition of each base, a washing step is carried out in which incorporated nucleotides and ATP are eliminated by the apyrase. These steps are repeated in cycles and the DNA sequence is deduced based on the light sequence that has been detected¹³⁷.

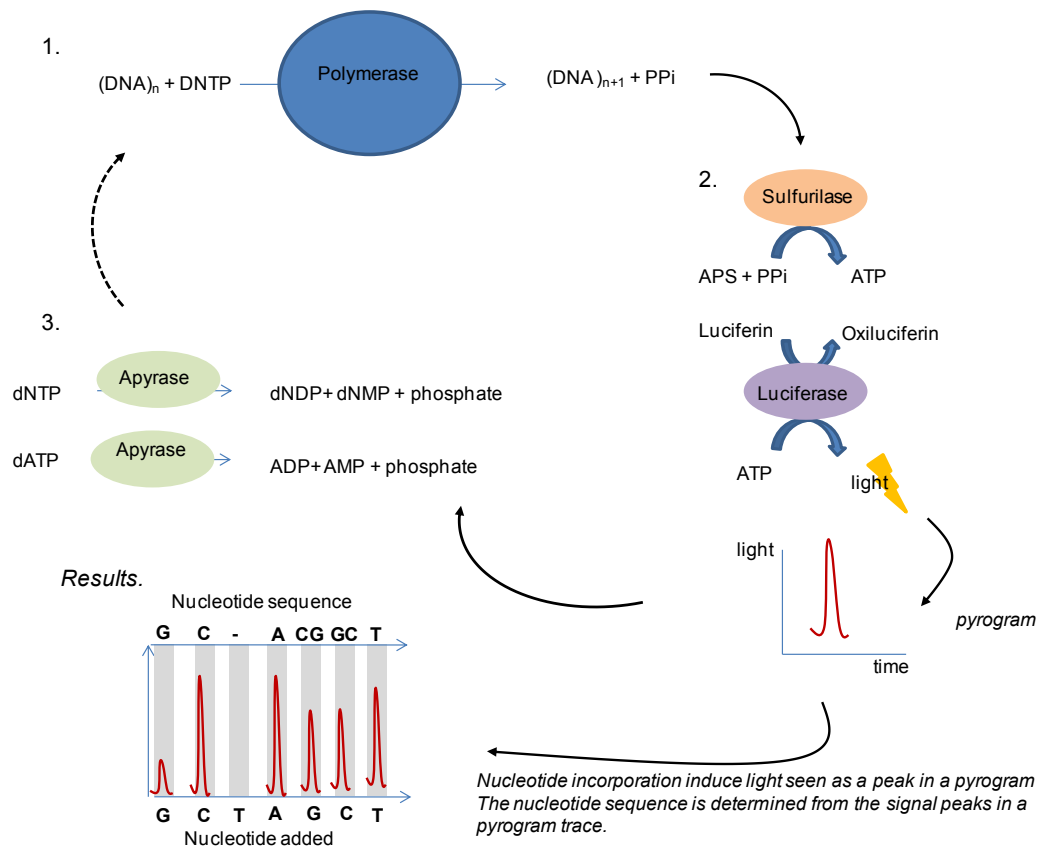


Figure 18: Principles of pyrosequencing and chemical reactions involved in the determination of DNA sequences adapted from Petrosino *et al.*¹³⁷. 3 main steps are involved in one sequencing cycle and several cycles are performed to identify the nucleotide sequence.

CHAPTER III: EARLY METABOLIC ADAPTATION IN C57BL/6 MICE RESISTANT TO HIGH FAT DIET INDUCED WEIGHT GAIN INVOLVES AN ACTIVATION OF MITOCHONDRIAL OXIDATIVE PATHWAYS.

1. INTRODUCTION

Obesity results from a complex interaction between genetic and environmental factors, such as a high calorie diet, and lack of physical activity¹⁸ and recent research has also suggested that the gut microbiota may play a role in the development of obesity^{94,139,140}. Ectopic fat deposition, particularly in a central distribution, is thought to contribute to a range of metabolic disorders such as hypertriglyceridaemia, hypertension, high fasting glucose and insulin resistance (IR)^{3,17,20}. Yet, although obesity is significantly associated with severe pathologies, many obese individuals do not exhibit metabolic dysfunction and therefore seem protected from the development of type 2 diabetes (T2DM), non-alcoholic fatty liver diseases (NAFLD) or cardiovascular diseases (CVDs) risk. This category of individuals defined as “clinically healthy obese” are the subject of intensive research to underpin the molecular mechanisms linking obesity and the degradation of the healthy phenotypes.

Given the multiplicity of factors that determine the likelihood of developing severe pathologies in obese individuals, there is a crucial need to identify early metabolic markers of susceptibility to obesity related metabolic disorders to prevent the development of metabolic diseases. McKay *et al.* used genetically identical strains of mice as they are useful models to investigate the impact of environmental and epigenetic factors on metabolic disease development regardless of genetic background¹⁴¹. Burcelin *et al* demonstrated that isogenic C57BL/6 mice can be partitioned in 3 sub-phenotypes based on body weight and blood glucose distributions after being fed a high fat diet for 2 months: obese diabetic, lean diabetic and lean non-diabetic¹⁴². Transcriptomic analysis of each sub-phenotype indicated that specific activation of lipogenic genes contributed to the obese phenotype¹⁴³. These transcriptional differences associated with weight gain variability in a genetically identical mouse population suggest the existence of an altered metabolic phenotype. However, the metabolic heterogeneity of HF-fed mice in these sub-populations of C57BL/6 mice has not been determined.

The C57BL/6 mouse is particularly predisposed to diet induced obesity due to its genetic background¹⁴⁴. Indeed C57BL/6 mice fed with a HF diet develop obesity, mild to moderate glycemia and hyper-insulinemia¹⁴⁵. In that regard, this strain of mice is a suitable model to investigate obesity development during HF challenge.

In this study, the longitudinal metabolic response to high-fat (HF) diet-induced obesity (DIO) in the C57BL/6 mouse strain, which is predisposed to IR and obesity has been investigated. The short-term (7 day) and long-term (60 day) metabolic response of mice fed a low fat (LF) diet (n=24) or high fat (HF) diet (n=56) was recorded using ¹H NMR spectroscopy of urine samples collected at these time points. Based on the observation of a significant correlation between body weight after one week and final body weight, the early metabolic signatures associated with the high phenotypic variability in the response to high fat diet was explored. Using the variability in weight gain of mice at 7 days and 60 days post HF diet intervention, strong responders (SR) (n=14) and non-responders (NR) (n=10) to diet induced obesity (DIO) were selected and the specific metabolic differences underlying these sub-phenotypes were interrogated.

2. MATERIALS AND METHODS

2.1. Animal handling procedure and sample preparation

The experiment was carried out under appropriate national guidelines at the Nestlé Research Center (NRC, Switzerland). Animals were permanently maintained in individual cages under 12h-12h of light-dark regime and fed ad libitum during the overall experiment. A total of 80 C57BL/6 female mice aged of 6 weeks, firstly received a standard chow diet (Baseline 3437, see Appendix 1 for diet composition Table S1) for 7 days and during this period, baseline samples of urine were collected (d0). Mice were then split in 2 groups: 24 mice were fed a LF diet (Low Fat 12450B, Research Diet, New Jersey USA, see Appendix 1 Table S1 for detailed composition) in which the rate of proteins, vitamins, minerals and carbohydrates differed from the acclimatization standard diet. 56 other mice were fed with HF diet (High Fat 12492, Research Diet, New Jersey USA, see Appendix 1, Table S1 for detailed composition) in which the dietary composition apart from the level of carbohydrate and fat, were comparable to LF diet. Urine samples were then collected 7 days (d7) and 60 days (d60) after the diet switch. All the samples were snap-frozen at -80 °C until analysis.

Urine collection was performed in the morning by gently pressing the abdomen¹⁴⁶. All mice were also weighted at d0, d7 and d60 in order to monitor the weight gain in both HF- and LF-fed mice. Differences in body weight and body weight gain between HF and LF mice were assessed by non-parametric Wilcoxon-Mann-Whitney tests. Individual food intake (FI) and cumulative energy intake of each mouse was also recorded at d7 and d60. There was a significant decrease of FI in HF-fed mice compared to LF-fed mice overtime (see Appendix 1 Table S2 and Table S3 for detailed FI values). The statistical difference of FI between groups was calculated by Wilcoxon-Mann-Whitney tests (see Appendix 1 Table S2 and Table S3 for *p values*).

2.2. Measurement of body weight gain and identification of NR and SR mice

Body weight gain (BWG) for each mouse was calculated by subtracting mouse weight at a given timepoint (7 days or 60 days) from mouse weight before diet intervention (d0). Identification of NR and SR mice was based on BWG distribution of HF-fed mice at 7 days and 60 days. Mice consistently found within the first tercile of the BWG distribution at both 7 days and 60 days after diet switch were designated as NR mice (n=10). Mice consistently in the third tercile of the BWG distribution were designated as SR mice with the exception of 4 mice being in the third tercile of BWG distribution at d7 and in the second half of the BWG distribution at d60 (n=14). These mice were added to increase sample size in multivariate models both at 7 days and at 60 days. This point was crucial to follow the time dependant metabolic changes of the same animals. Significant difference in body weight and BWG was noticed (using Wilcoxon-Mann-Whitney test) between NR and SR mice at d7 and d60 (Figure 19). SR mice also had higher FI and cumulative energy intake than NR mice at both time points (see Appendix 1 Tables S2 and S3 for detailed FI values).

2.3. ¹H NMR spectroscopy

A volume of 40 µL of urine was diluted in 20 µL of phosphate buffer solution (NaHPO₄, 0.6M pH=7.0, 100% D₂O) containing sodium azide (3 mM) and trimethylsilyl propionic acid (TSP) (0.5 mM). After centrifugation, samples were transferred into 1.7 mm diameter NMR tubes by using a syringe. ¹H NMR spectra were then recorded on a 600MHz Avance III Bruker NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 600.13 MHz, by performing 64 scans of a sequence (noesypr1d) with a mixing time of 100 ms and 98K data-points. The temperature of NMR experiment

was maintained at 300 K. Processing of urine spectra was carried out by using the software TOPSPIN 2.0 (Bruker Biospin, Rheinstetten, Germany). For each spectrum, the free induction decays were multiplied by an exponential function corresponding to a line broadening of 1 Hz, prior to undergo Fourier transformation. Spectral phase and baseline were manually corrected and the chemical shifts were calibrated on TSP signal at $\delta 0.0$. Metabolite assignments were achieved by matching the recorded signals with NMR spectral databases, published assignments and also using the technique of Statistical TObal Correlation SpectroscopY (STOCSY¹³³) to facilitate the extraction of putatively coupled signals from the complex spectra of urine samples^{102,113,122}. In addition 2D NMR spectroscopy was performed on selected samples using 600.13 MHz DRX Bruker NMR spectrometer using homonuclear (^1H - ^1H COSY¹⁴⁷ and ^1H - ^1H TOCSY¹⁴⁸) and heteronuclear (^1H - ^{13}C HSQC) coupling experiments for identification.

2.4. Data processing and multivariate data analysis

The spectral data (from 0.2 to 9.5 ppm) were finally imported into Matlab software (2011 version, the Mathworks Inc, Natwick MA) and were digitalized onto 22K data-points. Water peak resonance (4.7-5.05 ppm) was removed from each spectrum in order to eliminate the variability linked to the water resonance presaturation. ^1H NMR spectra were first normalized on total area to account for global dilution effects and scaled to unite variance to emphasize the contribution of minority compounds. Pre-processed spectra were analyzed with multivariate data analysis methodologies such as Principal Component Analysis (PCA), Orthogonal Projection on Latent Structures (OPLS) and Discriminant Analysis based on Orthogonal Projection on Latent Structures (OPLS-DA). To identify relevant features in the metabolic profiles, OPLS regression coefficients were back-scaled using a back-scaling method which allows the estimation of the proportion of variance for each NMR variable. Correlation heatmaps were generated by compiling the Pearson correlation coefficient values for discriminating metabolites between HF and LF-fed mice or SR and NR mice at each timepoint. To facilitate their reading, there was set arbitrarily a cut off at 0.3 to keep the most relevant features.

2.5. Pathways-specific analysis using univariate statistical approach

Intermediates metabolites from β -oxidation, BCAA catabolism, TCA cycle and NAD pathways assigned in urine ^1H NMR spectra were relatively quantified (see Appendix 1 Table S5 for chemical shifts and mean of integral values) in order to assess the urinary excretion of these metabolites 7 days and 60 days after diet switch in LF and HF-fed mice or NR and SR mice. For each metabolite, the integral at 7 days and 60 days was divided by the integral at t0 (during the pre-intervention period) in order to normalize the urinary excretion of these metabolites to the baseline. The ratio obtained for each metabolite was compared between LF, HF, NR and SR groups at each time point using Wilcoxon-Mann-Whitney tests (see Appendix 1 Table S6 for detailed *p values*).

3. RESULTS

3.1. Body weight gain variability in C57BL/6 mice fed a HF diet

In order to study the contribution of diets to the development of obesity, 80 C57BL/6 mice were fed with a chow diet during a pre-intervention period of 1 week, followed by a diet switch where mice were fed with a LF diet (n=24) or a HF diet (n=56) for 60 days. Body weights were measured during the pre-intervention period and 7 and 60 days after the diet switch (Figure 19.A). Body weight monitoring showed a significant increase of weight in HF-fed mice compared to LF-fed mice through the course of the study. In particular, the average weight of HF-fed mice was 1.5 g higher ($p = 3.9 \times 10^{-7}$) at 7 days and 4.5 g higher ($p = 2.36 \times 10^{-8}$) at 60 days compared to control mice. The body weight distribution also revealed a strong heterogeneity within the HF group at 7 days (coefficient of variation CV = 0.05), which was even more noticeable at 60 days (CV = 0.120) (Figure 19.B). In addition, a significant correlation between mouse body weight gain (BWG) at 7 days and mouse body weight at 60 days (Figure 20) was observed. This result highlights the existence of a strong and sustainable phenotypic variability within the HF group. It also suggests the existence of early and specific metabolic signatures associated to these obese-sub phenotypes.

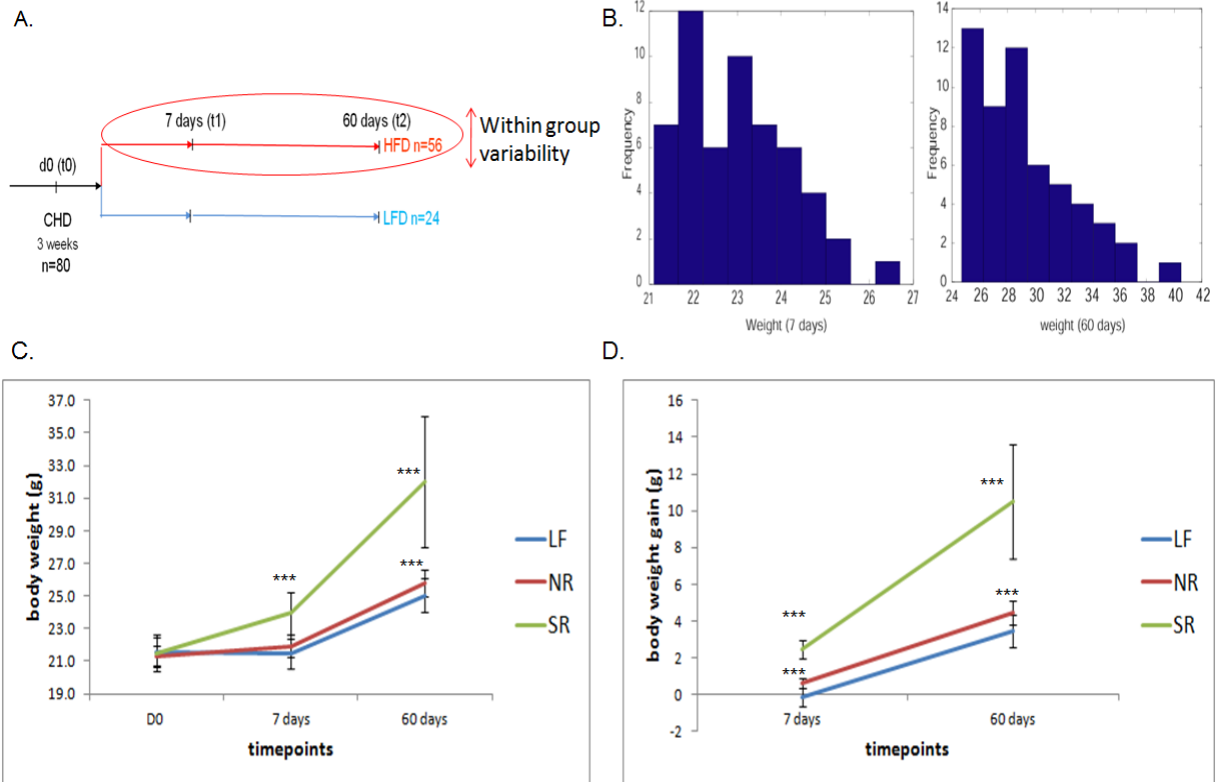


Figure 19: Variability of body weight gain in a population of $n=56$ mice fed a HF diet. (A) Experimental design. (B) Body weight distribution of mice after 7 days and 60 days of HF diet feeding. (C) Body weight and (D) body weight gain monitoring of control (LF, $n=24$), NR ($n=10$), and SR ($n=14$) mice before the diet (t_0), after 7 days (t_1) and 60 days (t_2), after diet switch. (n =average $\pm$ standard error, significant differences in body weight and body weight gain between LF-fed mice and NR mice or LF-fed mice and SR mice were evaluated using non parametric Wilcoxon-Mann-Whitney test, p value: * <0.05 , ** <0.001 , *** <0.0001 .)

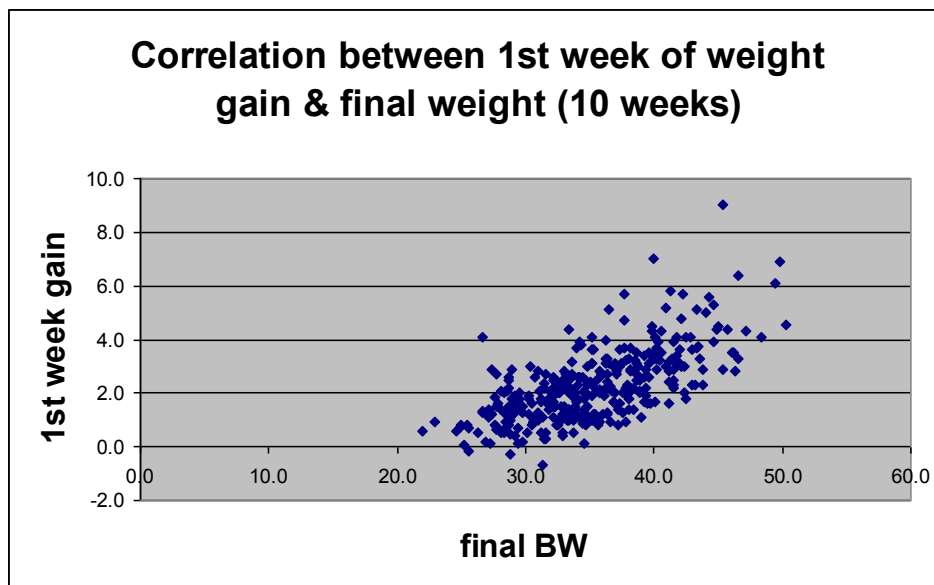


Figure 20: Correlation between mouse BWG after 1 week of HF diet and mouse BW 60 days after diet switch

In order to characterise the “Strong-Responders” (SR) and “Non-Responders” (NR) to HF feeding, the mouse population was stratified according to BWG after 7 days and 60 days of HF feeding. Mice being consistently found within the first and the third terciles of the BWG distribution were designated as NR and SR mice respectively. In addition, 4 mice being in the third tercile of BWG distribution at 7 days and at the second half of BWG distribution at 60 days were included in SR mice group. These thresholds were selected in order to obtain enough samples in each group (NR mice $n=10$ SR mice=14) and to perform powerful statistical tests. The average body weight trajectory overtime for NR, SR and LF-fed mice (Figure 19) revealed that SR mice gained significantly more weight than NR mice and LF-fed mice during the experiment. Interestingly, there was no significant difference in body weight between the NR group and the LF diet group at 7 days ($p = 0.10$), but significant difference in body weight was identified at 60 days ($p = 7.67 \times 10^{-5}$). In addition, the NR mice body weight gain trajectory (Figure 19.D) (β -coefficient of the slope=3.85) was similar to LF-fed mice (β -coefficient of the slope=3.57) whereas SR mice tended to gain weight faster (β -coefficient of the slope =8.05). This early and sustained inflexion of body weight gain trajectory, defining strong responder and non-responder subgroups suggests the existence of a differential predisposition to DIO in C57BL/6 mice. Hence, the ability to discriminate NR and SR mice based on early (7 days) and late (60 days) metabolic profiles was tested.

3.2. ¹H NMR urine metabolic profiling of LF- and HF-fed mice identifies a sustained metabolic signature associated to DIO

To investigate the specific metabolic signature associated with diet-induced obesity development, urinary metabolic profiles were acquired at 1 week before, at 7 days and at 60 days after the dietary intervention using ¹H NMR spectroscopy (Figure 21.A, Figure 21.B). Urine metabolic profiles from LF and HF-fed mice were then compared at each time point by using OPLS-DA models. Each model was calculated by using one predictive component and one or several orthogonal components. The optimal number of orthogonal components was determined by R²Y (goodness of fit) and Q²Y (goodness-of-prediction) statistics (see Appendix 1, Table S4). The OPLS-DA score plots for models at 7 days (Figure 21.C) and 60 days (Figure 21.D) showed strong metabolic variations associated with HF feeding highlighted along the X axis (predictive component, T_{pred}). At the same time, the Y axis, (first orthogonal component, T_{orth}) reflected within-group variations related to diet-independent effects.

For pair-wise OPLS-DA models, the metabolites with the highest correlation coefficients between LF and HF-fed mice were identified and summarised in a heatmap (Figure 21.E). The OPLS-DAs loadings plots of each model are also presented in the Figure 23.A and Figure 23.B, and the metabolite assignments are summarised in Table 3. Specifically, urinary excretion of carnitine, hexanoylglycine, and intermediates of branched chain amino acid (BCAA) catabolism (isovalerylglycine, α-keto-β-methylvalerate and α-ketoisovalerate) were significantly increased in the HF group at 7 days and 60 days. Conversely, the levels of trimethylamine (TMA) and trimethylamine-*N*-oxide (TMAO) respectively produced from microbial bioconversion of choline and mammalian phase I detoxification reaction of the former, as well as phenylacetylglycine which is a phase II detoxification conjugate of gut bacterial phenylalanine degradation end-products were decreased in the HF group during the whole experiment. In particular, the degree of variation in the urinary TMAO level between 7 days and 60 days suggests a time-dependent shift in the conversion of TMA to TMAO under HF feeding. Hence, HF diet may involve significant changes in gut microbial activity. Time-dependent metabolic adaptation to HF feeding was also characterised by a significant reduction of indoxylsulfate in urine of HF-fed mice for 7 days. *N*-1-methyl-2-pyridone-5-carboxamide (2PY) and *N*-1-methyl-4-pyridone-3-carboxamide (4PY), which are end products of nicotinamide adenine dinucleotide (NAD) pathways, were also positively correlated with mice fed a HF diet for 60 days. Isovalerylglycine, α-keto-β-methylvalerate and α-ketoisovalerate excretion was significantly

and consistently increased in the HF-fed group compared to the LF-fed group overtime, so they constitute qualitative and stable candidate biomarkers of DIO.

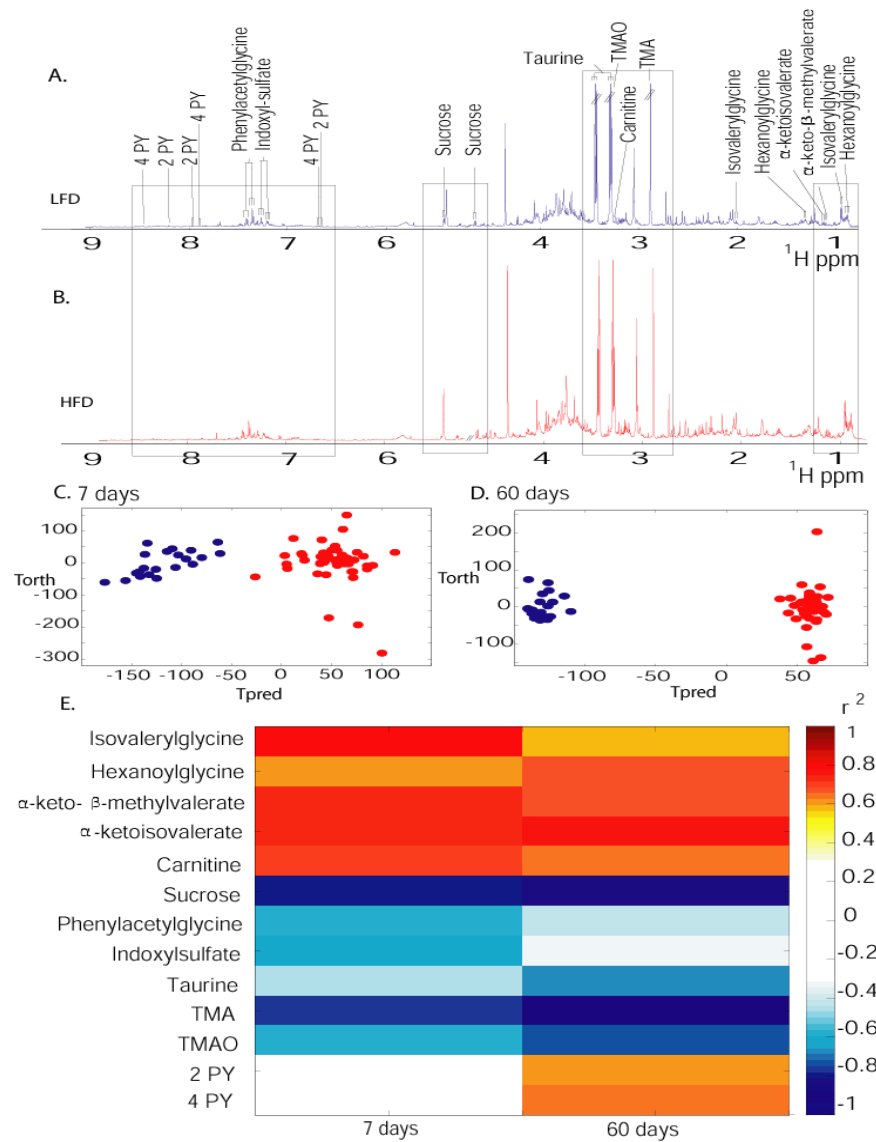


Figure 21: ^1H NMR urine metabolic profiles of C57BL/6 HF-fed or LF-fed mice 7 days and 60 days after the diet switch. (A) Mean ^1H NMR spectrum of urine from LF-fed mice or (B) HF-fed mice. (C) OPLS-DA score plot of urine metabolic profiles of LF and HF-fed mice at 7 days (D) OPLS-DA score plot of urine metabolic profile of LF and HF-fed mice at 60 days. (E) Heatmap obtained from the OPLS-DA coefficient plots showed metabolites found to be significantly different in HF and LF-fed mice. Correlation values of the metabolites are displayed by color code (Gradient of red color for metabolites positively correlated with HF-fed mice and gradient of blue colors for metabolites negatively correlated).

3.3. ^1H NMR urine metabolic profiling of NR and SR mice highlights a specific metabolic adaptation associated to obesity prone and obesity resistant phenotype.

The acquisition of distinct metabolic signatures between SR and NR mice enabled to identify metabolites associated with the highest divergence in BWG. Comparisons of ^1H NMR spectral data between NR and SR were performed using pair-wise OPLS-DA models at 7 days and 60 days (Figure 22.A, Figure 22.B). Results addressing the suitability of both models are displayed in supplementary information (See Appendix 1, Table S4). OPLS-DA score plots at 7 days (Figure 22.C), and 60 days (Figure 22.D) displayed a good discrimination (as confirmed by Q^2Y and R^2Y values in see Appendices Table S4) between NR and SR mice along the predictive component (T_{pred}). The Y axis illustrates orthogonal variation to strong obesity-associated-response. Interestingly, no differences in urinary metabolic profiles of NR and SR mice were identified before the diet switch, which highlights that C57BL/6 mice all have similar phenotypes and metabotypes when they were fed a chow diet.

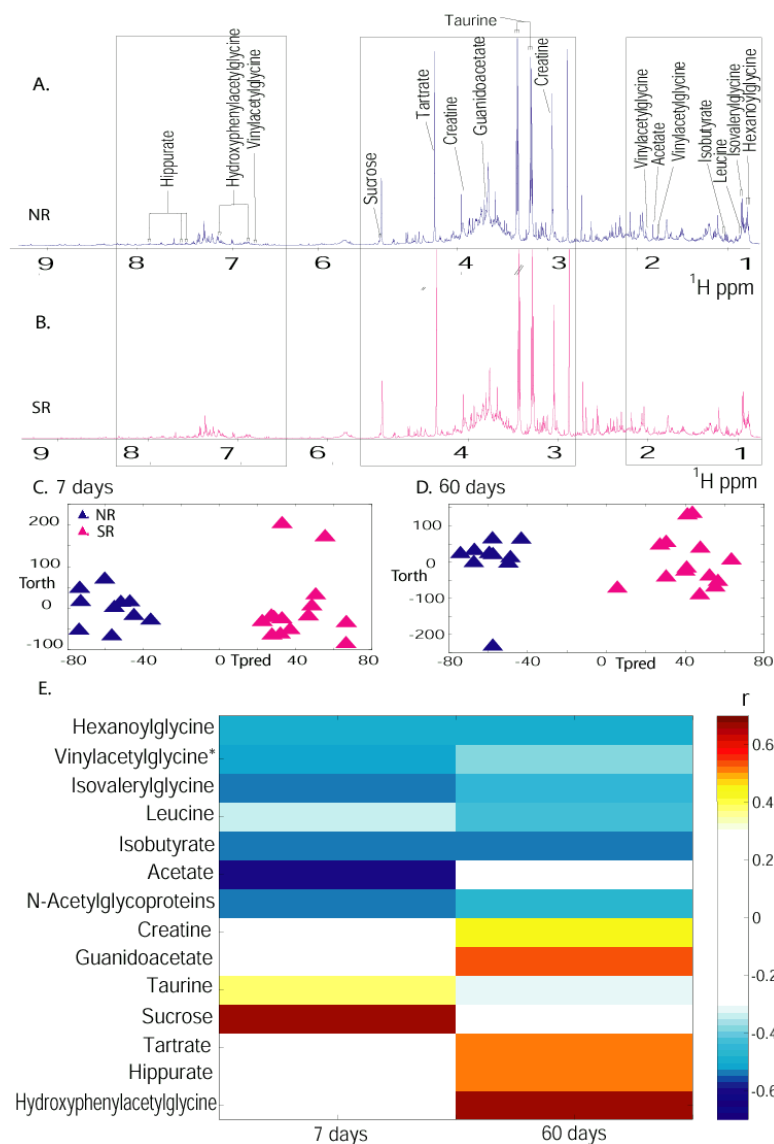


Figure 22: Specific metabolic signature of NR and SR mice. (A) Mean of ^1H NMR spectra of urine from NR mice or (B) SR mice. (C) OPLS-DA scores plot of urine metabolic profile of NR and SR mice at 7 days (D) and 60 days. (E) Heatmap obtained from the OPLS-DA coefficient plots showed metabolites found to be significantly different in NR and SR mice. Correlation values of the metabolites are displayed by color code. (Gradient of red color for metabolites positively correlated with SR mice and gradient of blue colors for metabolites negatively correlated). * Tentative assignment

A heatmap (Figure 22.E) summarised discriminant metabolites between NR and SR mice during short-term (7 days) and long-term (60 days) HF feeding. The OPLS-DA loadings plots of each model are shown in Figure 23C and Figure 23.D and metabolite assignments are summarised in Table 3. In particular, a specific metabolic signature involving leucine catabolism, β -oxidation and production of short chain fatty acids, was associated with BWG variability. Indeed, hexanoyl-glycine,

isovalerylglycine, leucine, acetate and isobutyrate were negatively correlated with SR mice during the overall experiment. As these metabolites are consistently down-regulated in SR mice, they constitute stable candidate markers of obesity-resistant phenotype. An unknown metabolite tentatively assigned as vinylacetyl glycine (see Appendix 1, Figure S4 for structural assignment) also increased in NR mice compared to SR mice. Vinylacetyl glycine is likely to be the glycine conjugate of vinylacetyl-CoA, however existence of vinylacetyl glycine and vinylacetyl-CoA in rodents and human have been poorly reviewed in the last decades and their origin in urine remains uncertain^{149,150}. Metabolic signatures between SR and NR mice at 7 days and 60 days also showed a time dependant metabolic signature associated with BWG variability. A lower urinary excretion of acetate was observed in SR mice after 7 days of HF diet. By contrast, a higher urinary excretion of sucrose was noticed in SR mice at the same period. Surprisingly, taurine was positively correlated with SR mice 7 days after of HF feeding and negatively correlated with SR mice after 60 days. The urine metabolic profile of SR mice after 60 days of HF diet was also marked by an increase in creatine, guanidinoacetate, tartrate, hippurate, and hydroxyphenylacetyl glycine. Interestingly, hexanoylglycine and isovaleryl glycine, which were initially characterised as qualitative candidate markers positively associated with DIO in this model, were also identified as stable candidate markers of obesity-resistant phenotype. These results point out that leucine catabolism and β -oxidation taking place in the mitochondria are key metabolic pathways in onset of DIO and their specific regulation might contribute in obese phenotype variability.

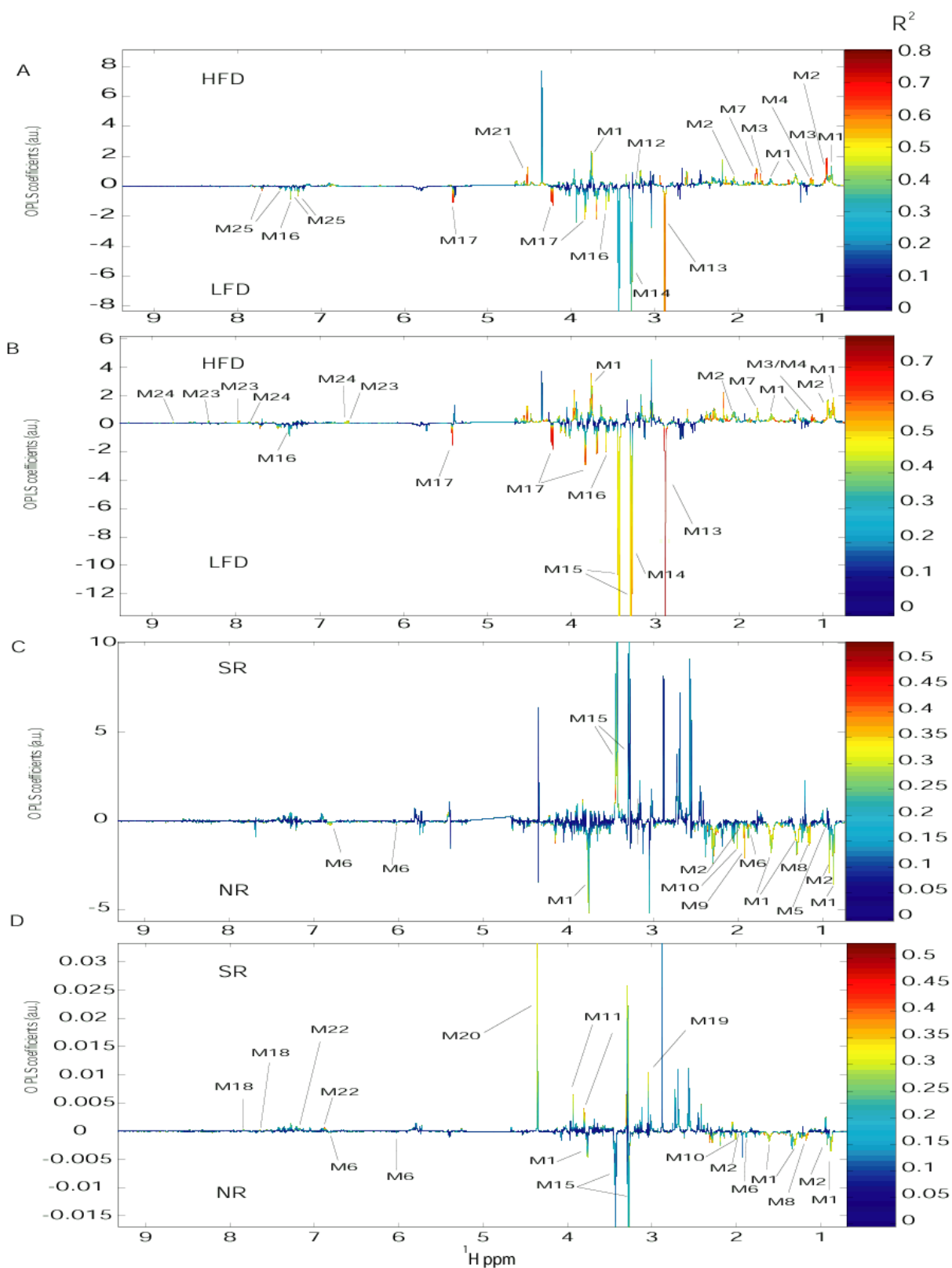


Figure 23: Loadings plot of OPLS-DA models showing the comparison of the urine metabolic profile between HF and LF-fed mice 7 days (A), at 60 days (B) or OPLS-DA models showing comparison between urine metabolic profile of NR and SR mice at 7 days (C) and 60 days (D).

R^2 : square of Pearson correlation coefficient

Table 3: List of metabolites assigned in the loadings plot of OPLS-DA models (shown in Figure S3). **Chemical shifts of carbons and protons were assigned only from resolved peaks present in ^1H NMR standard spectrum and ^1H - ^{13}C HSQC spectrum

metabolite key	metabolites	**Chemical shift (^1H ppm)	**Chemical shift (^{13}C ppm)
M1	Hexanoylglycine	(CH ₃) 0.88 (t), (CH ₂) 1.32 (m), (CH ₂) 1.66(m), (CH ₂) 3.76 (d)	(CH ₃) 13.8, -, (CH ₂) 22.2, (CH ₂) 46.5
M2	Isovalerylglycine	(CH ₃) 0.92 (d), (CH ₂) 2.04 (m), (CH ₂) 2.17 (d),	(2CH ₃) 24.5, (CH ₂) 27.2, (CH ₂) 46.32
M3	α -Keto- β -methylvalerate	(CH ₃) 1.10 (d)	(CH ₃) 16.60
M4	α -Ketoisovalerate	(2CH ₃) 1.11 (d)	(2CH ₃) 19.20
M5	Leucine	(2CH ₃) 0.96 (t)	(2CH ₃) 25.06
M6	Vinylacetylglycine	(CH ₂) 1.88 (d), (CH ₂) 6.05 (d), (CH) 6.82 (m)	(CH ₂) 24.1,-,-,-
M7	Putrescine (tentative assignment)	(2CH ₂) 1.75 (m)	(2CH ₂) 27.74
M8	Isobutyrate	(2CH ₃) 1.157 (d)	(2CH ₃) 19.1
M9	Acetate	(CH ₃)1.92 (s)	(CH ₃) 30.4
M10	<i>N</i> -acetylglycoproteins	(CH ₃) 2.07	-
M11	Creatine	(CH ₃) 3.03 (s), (CH ₂) 3.93 (s)	(CH ₃) 39.80, (CH ₂) 56.68 (3CH ₃) 47.5

M12	Carnitine	(CH ₃) 3.21 (s)	(CH ₃) 56.83
M13	Trimethylamine	(3CH ₃) 2.88 (s)	(3CH ₃) 47.5
M14	Trimethylamine- <i>N</i> -oxide	(3CH ₃) 3.27 (s)	(3CH ₃) 62.3
M15	Taurine	(CH ₂) 3.27 (t), (CH ₂) 3.44 (t)	(CH ₃) 50.2, (CH ₂) 38.40
M16	Phenylacetyl glycine	(CH) 3.66 (s), (CH) 7.35 (m), (CH) 7.36 (m), (CH) 7.40 (m)	(CH) 45.1, (CH) 132.15, (CH) 130.36, (CH) 132.15
M17	Sucrose	(CH) 3.83 (m), (CH) 4.23 (d), (CH) 5.42 (d)	(CH) 65.57, (CH) 79.4, (CH) 95.2
M18	Hippurate	(CH ₂) 3.96 (d), (2CH) 7.54 (m), (2CH) 7.81 (m)	(CH ₂) 46.7, -, (2CH) 130.0
M19	Guanidoacetic acid	(CH ₂) 3.80 (s)	(CH ₂) 46.26 (s)
M20	Tartrate	(2CH) 4.34 (s)	(2CH) 76.81
M21	Unassigned metabolite	4.53 (m)	-
M22	Hydroxyphenylacetyl glycine	(2CH) 6.88 (d), (2CH) 7.17 (d)	(2CH) 118.54, (2CH) 133.52
M23	<i>N</i> -methyl-2-pyridone-5-carboxamide: 2 PY	(CH) 6.66 (d), (CH) 7.83 (dd), (CH) 8.32 (d), (CH ₃) 3.64 (d)	(CH) 120.7, (CH) 130.0, (CH) 144.52, (CH ₃) 45.08
M24	<i>N</i> -methyl-4-pyridone-3-carboxamide : 4 PY	(CH) 6.69 (d), (CH) 7.97 (dd), (CH) 8.54 (d), (CH ₃) 3.90 (s)	(CH) 123.0, (CH) 142.74, (CH) 149.29, (CH ₃) 56.59
M25	Indoxylsulfate	(CH) 7.27 (m), (CH) 7.36 (s), (CH) 7.50 (d), (CH) 7.70 (d)	(CH) 124.5, -, (CH) 115.8, (CH) 120.43

3.4. Univariate analysis of intermediate metabolites from candidate pathways pointed out specific deregulations of mitochondrial metabolism in HF-fed mice that may be exacerbated in SR mice

The variation in mitochondrial metabolism in HF-fed mice was further investigated by a complementary univariate data analysis using Wilcoxon-Mann-Whitney tests (Figure 24). Urinary excretion of β -oxidation intermediates: hexanoylglycine, carnitine and acylcarnitine was consistently increased in urine of HF-fed mice compared to LF-fed mice which suggests an increase in fatty acid overflow in the mitochondria and an activation of β -oxidation¹⁵¹. The end product of NAD pathways (2PY, 4PY) also constantly increased in mouse urine after HF feeding which indicates an increase in NAD/NADH recycling. This red-ox complex enabling electron transport is required in TCA cycle and oxidative phosphorylation in the mitochondria to produce energy¹⁵². The relative quantifications confirmed that leucine, valine, isoleucine as well as intermediates of BCAA catabolism (isovalerylglycine, α -keto- β -methylvalerate and α -ketoisovalerate) were significantly and consistently increased in HF-fed mice (see Appendix 1 Table S6 for detailed *p values*) supporting the fact that HF diet induced an up-regulation of BCAA catabolism (Figure 24). TCA cycle was also partly regulated in HF-fed mice as noted with a short-term urinary increase of succinate in mouse 7 days after HF diet compared to LF-fed mice¹⁵². These results support the hypothesis that valine and isoleucine catabolism is up-regulated inducing the formation of succinyl-CoA and the production of the following TCA cycle intermediates^{153,154}. Interestingly the other TCA cycle intermediates (citrate, cis-aconitase, α -ketoglutarate) were not significantly different (*p value* > 0.05, see Appendix 1 Table S6 for detailed *p values*) between LF and HF-fed mice suggesting a disconnection between BCAA catabolism and β -oxidation producing acetyl-CoA, and TCA cycle. A part of TCA cycle flux could be diverted toward other metabolic pathways (Figure 25). In particular oxaloacetate may be redirected toward phosphoenolpyruvate (PEP) to produce glucose *via* gluconeogenesis¹⁵². Hence, in case a concomitant activation of BCAA catabolism and gluconeogenesis occurs, TCA cycle may be partly up-regulated between succinyl-CoA and oxaloacetate¹⁵². These results confirm that HF diet induce an up-regulation of mitochondrial oxidative pathways which might lead to partial saturation of TCA cycle.

Univariate data analysis using Wilcoxon-Mann-Whitney tests also enabled us to better understand the link between β -oxidation, BCAA catabolism and TCA cycle in the context of BWG

variability. Integration of BCAA catabolism intermediates showed that only isovalerylglycine was significantly higher (*p value*=0.030 at 7 days, *p value*=0.018 at 60 days) in urine of NR mice compared to SR mice or LF-fed mice indicating that obesity-resistant mice were associated with up-regulation of leucine catabolism exclusively. Leucine catabolism mainly produces acetoacetate which in turn induce the formation of ketone bodies (acetone and 3-D-hydroxybutyrate)¹⁵². Leucine degradation also forms a small amount of acetyl-CoA which may enter TCA cycle, but this pathway cannot produce substrate for gluconeogenesis¹⁵². Hence NR mice may preferably activate ketogenic pathways. Hexanoylglycine was also significantly higher (*p value* at 7 days: 0.023, *p value* at 60 days: 0.001) in urine of NR mice compared to SR mice during the overall experiment whereas the urinary excretion of carnitine and acylcarnitine stayed unchanged. Hence, even though the carnitine shuttle system which is essential for mitochondrial oxidation of fatty acids seemed to be unaffected, β -oxidation might be disturbed in NR mice compared to SR mice¹⁵². Interestingly, no difference in TCA cycle activity between NR and SR mice was observed after 7 days of HF diet. At 60 days, succinate was significantly higher in urine of SR mice suggesting an up-regulation of TCA cycle. In addition, the level of 2PY, 4PY and nicotinurate tend to increase in SR mice compared to NR mice after 60 days of HF diet, which support the hypothesis of an exacerbated utilization of mitochondrial oxidative pathways in obesity-prone mice. However, no significant difference in the variation of these metabolites was observed between NR and SR mice (See Appendix 1 Table S6 for detailed *p values*), which may be due to the small number of samples and the significant within group variability (Figure 24). Our results indicate that after a long-term period of HF diet, obesity prone mice are associated with an impairment of energy metabolism^{152,155}.

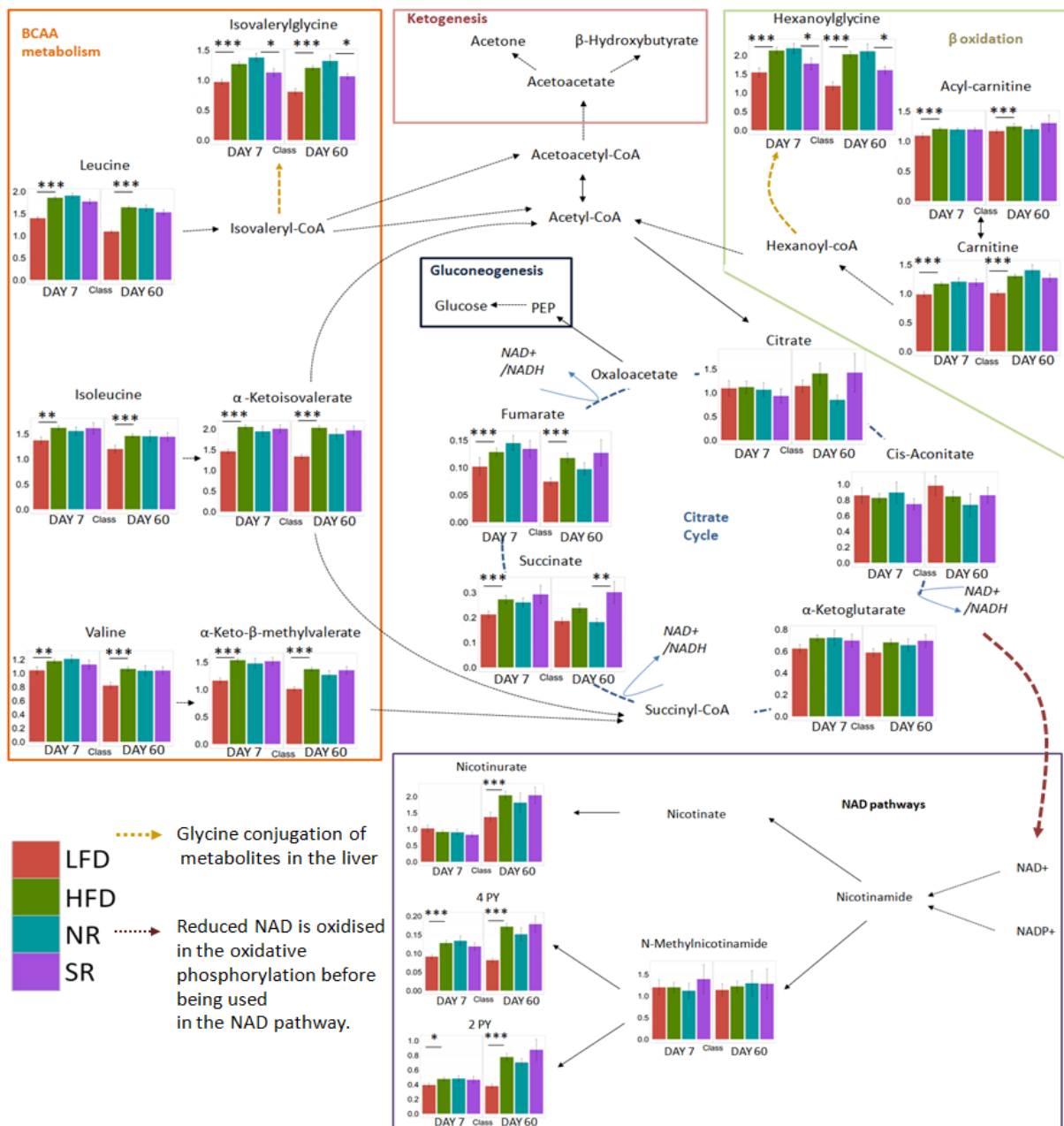


Figure 24: Mapping of urinary metabolite excretion pattern involved in BCAAs, NAD metabolism, TCA cycle, β-oxidation, ketogenesis and gluconeogenesis. The bar plots showed the mean ratio with standard error of metabolite integrals at day 7 to day 0 or day 60 to day 0. The Y axis indicates the value of the mean for LF, HF, NR and SR mice (arbitrary unit). Significant difference between mean ratios of LF and HF or NR and SR were calculated with non parametric Wilcoxon-Mann Whitney test: * <0.05, ** <0.001, *** <0.0001 (values of mean, standard errors and *p* values in Appendix 1 Tables S5, and Table S6). Indirect metabolic reactions are highlighted with dash arrows. PEP: phosphoenolpyruvate.

4. DISCUSSION

This study, showed that HF feeding led to a rapid development of obesity in C57BL/6 mice, but isogenic mice responded differently to HF diet, generating within-group variability of the obesity phenotype.¹⁴² Burcelin *et al.* already reported that dietary or/and unidentified environmental factors may be involved in the differential regulation of glucose metabolism and may contribute to induce variable obesity outcomes in C57BL/6 mice. Our metabonomic results also demonstrated that specific activation of mitochondrial metabolic pathways including β -oxidation, and BCAA metabolism under HF diet has a determinant role in weight gain variability.

4.1. HF diet-induced obesity is associated with a rapid up-regulation of fatty acid breakdown and BCAA catabolism

The abnormal fatty acid influx in the organism generated by HF diet induces obesity development and a strong change in energy metabolism through the modulation of fatty acid and BCAA metabolism^{156,157}. In this experiment, mice fed a HF diet displayed a urinary increase in hexanoylglycine, carnitine and acylcarnitine over time. Hexanoylglycine is respectively obtained from the conjugation of hexanoyl-CoA with glycine in the liver and are then excreted in urine¹⁵². Hexanoyl-CoA is an intermediate metabolite of the β -oxidation¹⁵⁸ and carnitine is responsible for the uptake of fatty acids in mitochondria, a key initial step in the β -oxidation process¹⁵⁶. These results confirm an up-regulation of fatty acid breakdown through β -oxidation and emphasize the saturation of oxidative fuel in the mitochondria, which might alter energy homeostasis. In particular, the accumulation of hexanoyl-CoA being a C6 fatty acid, suggests an up-regulation of the early steps of β -oxidation. Besides, the increase in several TCA cycle intermediates and NAD catabolism end-products (2PY and 4PY) in urine of HF-fed mice brings further support to the energy over-production hypothesis. In particular, increased NAD catabolism has been shown to correlate with activation of β -oxidation and peroxisome proliferation¹⁵⁹. The chronic increase in mitochondrial oxidative pathways has been thought to be deleterious for mitochondria leading to the impairment of oxidative pathways and energy metabolism¹⁵¹. In addition, excess free fatty acids can be stored as triglycerides in adipose tissue as well as lean tissue which promotes organ dysfunctions and metabolic diseases such as hepatic steatosis or cardiovascular diseases¹³. In this context, urinary increase of 2PY and 4PY in obese mice is suggestive of hepatic fatty acid accumulation¹⁵⁹. Hence,

high fat feeding results in over-activation of fatty acid breakdown and reprocessing, which might contribute to obese associated metabolic disorders and ectopic fat accumulation.

The rapid and sustained increase in isovaleryl-glycine, α -keto- β -methylvalerate, and α -ketoisovalerate in urinary metabolic profiles of HF-fed mice at 7 days and 60 days also illustrates a consistent dysregulation of BCAA catabolism in obese mice during this experiment. BCAAs (leucine, isoleucine, valine) are degraded into keto-acids *via* reversible transamination by branched chain aminotransferase (BCAT)¹⁵². These keto-acids are in turn decarboxylated by branched-chain keto acid dehydrogenase (BCKDH) to produce acetyl-CoA and succinyl-CoA which eventually enter TCA cycle to produce ATP (Figure 25)^{152,154,160}. Isovaleryl-glycine derives from the conjugation of glycine and isovaleryl-CoA, which is an intermediate of leucine catabolism. Similarly, α -keto- β -methylvalerate and α -ketoisovalerate have been shown to be intermediates of isoleucine and valine degradation pathways respectively^{152,161}. BCAA catabolism primarily occurs in skeletal muscle where it stimulates muscle protein synthesis and participates in energy production¹⁵⁴. To a lesser extent, BCAA catabolism takes place in other tissues such as liver, kidney, heart or brains to provide energy substrates^{154,162}.

In the present experiment, the amount of carbohydrates in HF diet is significantly lower than the LF diet (see Appendix 1 Table S1 for detailed composition of diets). Carbohydrates represent the primary energy supply for the organism and are essential nutrients for several tissues such as brains, central nervous system and red blood cells¹⁵². In case of low carbohydrate dietary intake triggering a drop in blood glucose, several metabolic pathways using non-carbohydrate sources are activated to synthesize glucose^{152,163}. In particular, BCAAs stimulate hepatic gluconeogenesis *via* alanine production and contribute to maintain glucose recycling¹⁶³. Therefore, BCAA catabolism may be activated in HF-fed mice to induce gluconeogenesis and compensate the relative lack of carbohydrate. Several studies have highlighted the activation of gluconeogenesis in T2DM patients contributing to fasting hyperglycemia^{164,165}. In obese individuals or with T2DM, up-regulation of gluconeogenesis might be associated with increased fat mass and lipolysis, which favours the production of gluconeogenesis substrates such as glycerol¹⁶⁵. In addition, the accumulation of isovaleryl-CoA, α -ketoisovalerate, and α -keto- β -methylvalerate suggests that the activity of BCAT and BCKDH enzymes in the early steps of BCAA catabolism exceed the capacity of the downstream enzymes in processing these keto-acids and incorporating acetyl-CoA in TCA cycle. α -ketoisocaproate, α -keto- β -methylvalerate and α -ketoisovalerate formed by the transamination of BCAAs (Figure 24, Figure 25) are thought to be potent activator of BCKDH complex which promote BCAA catabolism¹⁶⁶. In the present study, it is most likely that HF diet feeding associated with high

excretion of keto-acids, up-regulates BCAT/BCKDH expression and provokes an activation of the initial steps of BCAA catabolism. Stimulation of fatty acid oxidation and particularly the activation of peroxisome proliferator-activated receptor α (PPAR α) has been also suggested to down-regulate BCKDH kinase expression in rat liver leading to an activation of the BCKDH complex and to the promotion of the second step of BCAA catabolism¹⁶⁶.

Additionally, increased protein degradation may also occur to provide enough essential amino acids such as BCAAs to maintain vital processes leading to a higher excretion of leucine, valine and isoleucine in urine of HF-fed mice. BCAAs are thought to have therapeutic potential¹⁶², in particular, leucine improves diet-induced thermogenesis, increases muscle protein synthesis, enhancing satiety and stabilizing glycemic regulations^{153,163,167}. However, BCAA supplementation has shown controversial biochemical effects. Zhang *et al* demonstrated that leucine-supplemented HF diet improves glucose and cholesterol metabolism and stimulates insulin secretion in obese rats¹⁶⁷. On the contrary, Newgard *et al* observed an exhibited impaired phosphorylation of insulin signalling intermediate kinases (akt/protein kinase B) in skeletal muscle and liver when rats were fed with HF diet enriched with BCAAs (leucine, isoleucine, valine)¹⁵³. In particular, they explained that BCAA associated impaired insulin action occurs only in the context of HF feeding¹⁵³. From these results they suggested that an increase in BCAA catabolism combined with fatty acid overflow rather than the up-regulation of BCAA catabolism alone is responsible for the development of metabolic disorders. The cross-talk between BCAA, carbohydrate and lipid metabolism needs to be better understood, however, BCAA catabolism in the context of high lipid intake might contribute in the deregulations of energy metabolism and in the development of metabolic disorders. It is important to note that HF and LF-fed mice had a different food and cumulative energy intake (see Appendix 1, Table S2). Therefore, variation in mitochondrial oxidative pathways might be partly driven by difference in food/energy intake between LF- and HF-fed mice. Indeed BCAA intermediates (α -keto- β -methylvalerate and α -ketoisovalerate) and leucine were positively correlated with cumulative energy intake throughout the experiment while 2PY, 4PY, nicotinic acid, and valine were positively correlated with energy intake 60 days after diet switch (see Appendices Figure S1).

To summarise, in response to fatty acid overflow, increased protein content and reduced carbohydrates, the HF-fed mice might switch from glucose to other substrates for acetyl-CoA synthesis and incorporation into TCA cycle. Consequently, there is an up-regulation of the early steps of fatty acid catabolism and BCAA catabolism. However, the influx of acetyl-CoA substrates seems to exceed the capacity for incorporation in TCA cycle and as a result there is an accumulation of β -oxidation (hexanoyl-CoA) and BCAA (isovaleryl-CoA, α -ketoisovalerate, and α -keto- β -methylvalerate)

intermediates, which can be exported out of the mitochondria. The activated intermediates (hexanoyl-CoA and isovaleryl-CoA) are then conjugated with glycine. Altogether, our results suggest that HF-fed mice tend to rely on fatty acid and BCAA catabolism to generate acetyl-CoA to be incorporated into TCA, in response to the reduced proportion of carbohydrates in the diet. Yet, an additional study with a better control of mouse energy intake and specifically focused on the balance between fat, carbohydrate and protein would be crucial to clearly establish the effect of low carbohydrate, high fat and high protein diets into obesity development.

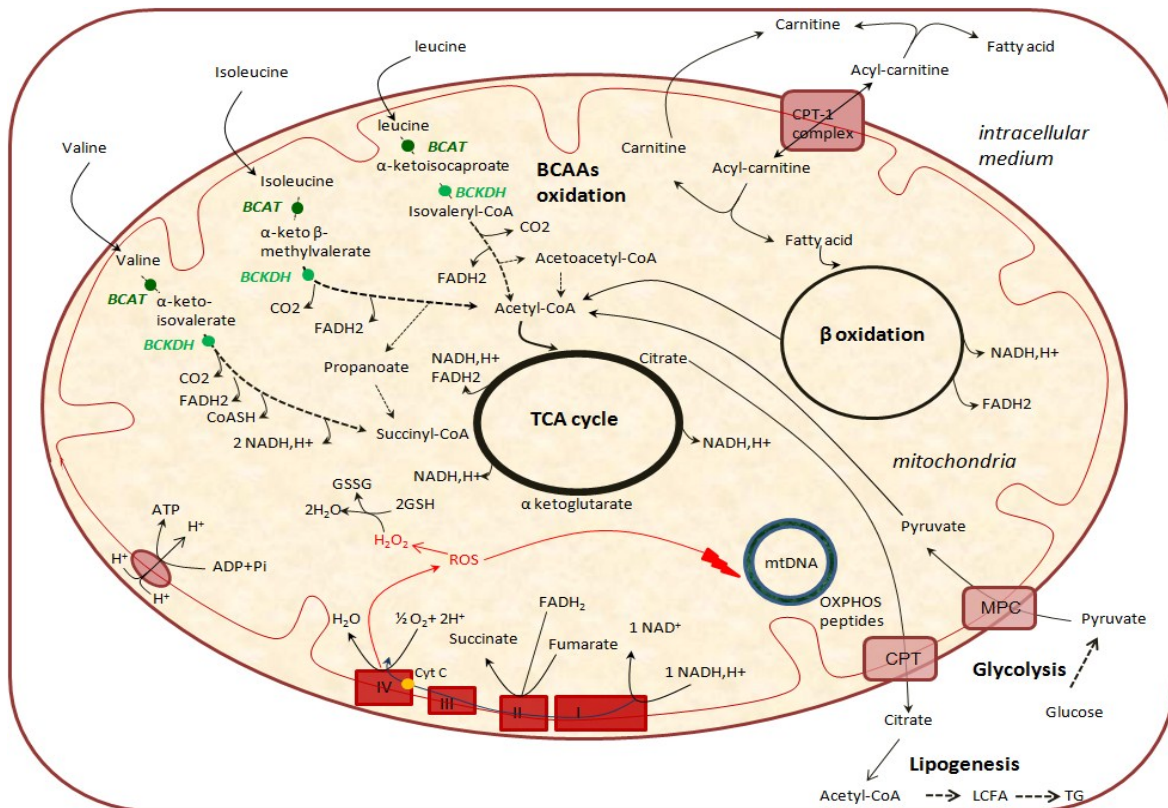


Figure 25: Summary of mitochondrial oxidative pathways and energy production. Glycolysis, β -oxidation, and BCAAs oxidation enable the production of reduced co-enzymes, which in turn are used to synthesize ATP. These pathways are also interconnected with TCA cycle, which contributes in energy production. Mitochondria nutrient overload leading to an up-regulation of oxidative pathway might increase the level of mitochondrial reactive oxygen species (ROS) formation, which triggers damage in mitochondrial (mt) DNA, proteins and lipids. Alternatively ROS can be detoxified *via* the glutathione pathway. This figure highlights in a simplified way, the general metabolic pathways occurring in the mitochondria and does not reflect any tissue-specific mitochondrial activity. GSSG: oxidized glutathione, GSH: reduced glutathione, LCFA: long chain fatty acid, TG; triglyceride. Cyt: cytochrome, CPT: citrate protein transporter, MPC: mitochondrial pyruvate carrier, BCAT: branched-chain aminotransferase, BCKDH: branched-chain keto acid dehydrogenase

4.2. SR and NR mice develop specific metabolic adaptations to HF diet based on BCAA catabolism and β -oxidation.

An increase in hexanoylglycine, and isovalerylglycine was observed in NR mice compared to SR mice which indicate an up-regulation of β -oxidation and leucine catabolism¹⁵². By contrast, TCA cycle seemed to be down regulated in NR mice after a long-term period of HF diet indicating a regulation of energy metabolism. Even though leucine degradation can produce a small amount of

acetyl-CoA, this pathway is exclusively ketogenic and cannot form glucose *via* gluconeogenesis¹⁵². These results suggest that the rapid activation of alternative pathways favouring ketogenesis may alleviate the over activation of TCA cycle and gluconeogenesis in NR mice and may maintain energy homeostasis¹⁵⁴. Moreover, Adams *et al* hypothesised that a reduction in BCKDH activity in high fatty acid containing tissues would alter the flux between BCAA catabolism and TCA cycle leading to anaplerotic stress¹⁶⁸. Therefore increasing BCKDH activity in a DIO context could improve TCA cycle and mitochondrial functions. Interestingly, the activation of several mitochondrial oxidative pathways associated with the obesity resistant phenotype under HF diet was observed in the comparison between LF and HF diet model. In the NR vs SR model, a conditional metabolic phenotype which is the consequence of HF diet was identified. This metabolic phenotype determines the individual adaption to the diet, eventually affecting the obesity phenotype⁷⁵. To summarise, HF-fed mice segregate into a spectrum of obesity phenotypes, which is suggestive of various adaptations to the HF diet challenge. In response to fatty acid overflow, NR mice present a relative up-regulation of the initial β -oxidation and leucine catabolism steps, leading to an increase in hexanoyl-CoA and isovaleryl-CoA production, which can form acetyl-CoA for energy purposes. Hexanoyl-CoA and isovaleryl-CoA are also ketogenic substrates, which can lead to the formation of acetoacetyl-CoA and ketone bodies. In the NR group, ketogenic pathways rather than TCA cycle seemed to be up-regulated which may maintain energy homeostasis. Overproduction of hexanoyl-CoA, and isovaleryl-CoA can then be exported out of the mitochondria for conjugation with glycine. In the SR group, this ketogenic response was not observed; on the contrary, there is an increase in succinate, which could be suggestive of substrate availability for gluconeogenesis. These results highlight that specific mitochondrial activity under HF diet has a determinant role in weight gain management.

4.3. Influence of gut microbiota on host energy metabolism

Other environmental factors such as gut microbial activity might encourage obesity development and modify mouse physiology/metabolism. The variation in urinary excretion of microbial-mammalian co-metabolites between NR and SR mice supports this hypothesis, as described by an increase in hippurate and hydroxyphenylacetyl glycine in SR mice compared to NR mice 60 days after diet switch^{31,89,102,169}. Interestingly, the differences in urinary microbial co-metabolites between NR and SR mice were observed at 60 days but not at 7 days after diet switch. This may reflect a divergence of gut microbial functions between NR and SR mice after long-term HF

challenge. Nutritional intervention-based experiments on germ free and conventional mice have highlighted the influence of gut microbiota on host energy homeostasis and metabolism. In particular Backhed *et al* have shown that gut bacterial colonization in germ free mice down-regulates the expression of genes involved in adipose tissue fat storage in the intestine (suppression of fasting induced adipose factors (FIAF)) and mitochondrial fatty acids oxidation in the liver and skeletal muscles (suppression of adenosine monophosphate activated protein kinase (APMK))⁷⁷. In our experiment, the variation in gut microbiota activity may contribute to the differential adaptation of mitochondrial oxidative pathways in NR and SR mice.

4.4. Fatty acid overflow is associated with saturation of mitochondrial oxidative pathways which might be involved in obesity development.

Mitochondria play a crucial role in maintaining energy homeostasis particularly through ATP synthesis by oxidative phosphorylation. In this process, nutrients such as fatty acid are oxidized to generate electron chemical gradient across the mitochondrial inner membrane. This gradient is then used for energy and heat production or for the transport of substrates and ions¹⁷⁰⁻¹⁷². Overflow of intracellular fatty acid generated by an imbalanced diet has been shown to saturate the mitochondria in fuel oxidation and in turn lead to dysfunction in mitochondrial oxidative pathways¹⁷⁰. In particular, incomplete β -oxidation has been associated with mitochondrial nutrients overload resulting in accumulation of incomplete oxidized lipid derivatives metabolites and acylcarnitines¹⁵¹. BCAA catabolism may also be strongly affected by mitochondrial nutrient overload, which would contribute in the dysregulation of BCAA metabolism in obese mice.

Impaired mitochondrial activity has been closely associated with a range of metabolic disorders such as IR, obesity, T2DM^{151,171,172}. In addition, incomplete mitochondrial β -oxidation was observed in patients with NAFLD¹⁷³. Several hypotheses have been proposed to explain the relation between a reduced mitochondrial respiration and development of obesity and IR. In particular, the increase of oxidative activity in mitochondria has been associated with a raise of reactive oxygen species (ROS) level which in turn may interfere with mitochondria themselves resulting in DNA mutation, lipid peroxidation and mitochondrial protein damage^{172,173}. Hence, saturation of mitochondrial oxidative pathways associated with HF diet may be a contributing factor of mitochondrial dysfunction leading to disorders in energy homeostasis. By contrast, NR mice under

HF diet may lower the production of oxidized molecules and maintain normal mitochondrial respiratory capacities by activating specific mitochondrial metabolic pathways. Interestingly, NAD⁺ is a key regulator of stress resistance, and cell longevity¹⁷⁴. Several mouse studies have reported a change in NAD metabolism in liver, heart, and kidney associated with mitochondrial oxidative stress^{175,176}. In the present experiment, NAD catabolism was up-regulated in HF-fed mice compared to LF-fed mice and tends to be down-regulated in NR mice compared to SR mice. These observation supports the hypothesis that fatty acid overflow may saturate mitochondrial oxidative pathways leading to the production of oxidative species whereas NR mice may alleviate chronic oxidative damage by developing specific metabolic adaptations.

Other studies have revealed that mitochondria health influence the activity of adipose tissue which regulates food intake, energy harvest and energy expenditure *via* multiple endocrine and non-endocrine processes¹⁷². Alteration of adipose tissue functions not only affects lipid processes in adipocytes but also overall lipid metabolism contributing in obesity development and T2DM¹⁷². However, it remains unclear whether mitochondrial dysfunction is a cause or a consequence of obesity and IR. Finally, despite its limitations, the current pre-clinical study provided novel molecular insights with which to re-evaluate under human clinical conditions the short-and long-term implications of significant changes in diets for disease management, as for instance with the Atkins diet including high-protein, high fat and low carbohydrate consumption. Future studies focusing on the balance and type of carbohydrate, proteins and fat would be required to further establish the competitive and complementary role of proteins, fat and carbohydrates in the development and nutritional management of obesity.

5. CONCLUSIONS

This study provides explanations on physiological mechanisms related to HF induced obesity development and particularly highlighting the specific metabolic adaptations associated to obese phenotype variability. High-fat diet provokes a rapid and consistent up-regulation of BCAA catabolism, β -oxidation, and TCA cycle, which might lead to a saturation of mitochondrial oxidative pathways. Upon HF-fed mice, obesity-resistant metabotype, was characterised by activated leucine catabolism, β -oxidation to produce substrates for ketogenesis and TCA cycle. Our results suggest that under HF challenge, ketogenesis tends to be preferably activated rather than TCA cycle in NR mice thus maintaining energy homeostasis and protecting mitochondria against fuel overloading. Additionally, the overproduction of β -oxidation and leucine catabolism intermediates is diverted towards glycine conjugation. Variation in gut microbial co-metabolites between NR and SR mice after long-term HF challenge also suggest that gut microbial activity participate in the differential physiological and metabolic adaptation to HF diet. This study further highlights the role of mitochondrial homeostasis in obesity development and its associated metabolic disorders.

CHAPTER IV: THE PREBIOTIC STUDY

A TOP-DOWN METABONOMIC APPROACH TO INVESTIGATE THE LONG-TERM EFFECTS OF PREBIOTICS ON C57BL/6 MOUSE PHYSIOLOGY AND METABOLISM DURING DIO.

1. INTRODUCTION

A substantial number of studies have reported that the composition of gut microbiota changes significantly in response to weight gain and obesity. The Gordon group first demonstrated this by showing variations in several bacterial phyla in the gut of obese humans and genetically obese mice^{33,177}. Gastric bypass surgery which consists in reducing stomach volume is an effective treatment to induce weight loss and has been observed to be accompanied by a modulation of gut microbial population^{178,179}. In the chapter III (section 3 and 4), it has been showed that mice resistant to HF-induced obesity specifically modulated BCAAs and lipid metabolism. In addition the results point out specific aspects of the regulation of gut bacterial metabolism in C57BL/6 mice predisposed to obesity, which supports the hypothesis that gut microbial activity may be a contributing factor of weight gain. As a result, re-shaping the gut microbiota may be a promising strategy to prevent obesity development.

The supplementation of prebiotics and/or probiotics in the diet is the most common approach to modify the functional interactions between the gut microbiota and host metabolism¹⁸⁰. Depending on the physiological context (healthy/unhealthy subjects), and the administrated dose, prebiotics and probiotics have been shown to confer immunomodulation, anticarcinogenic, antidiabetic and hypocholesterolemic effects (see Chapter I, section 2)¹⁸¹. The use of prebiotics are often advocated over probiotics for several reasons: As the complexity and variability of the gut ecosystem depends on its location in the gut, the selection of beneficial bugs by feeding the bacteria with suitable nutrients may be more effective than supplementing external strains in the diet. Prebiotics are also naturally stable compounds with a long shelf-life and are resistant to the digestion process before they reach the colon where they are degraded by bacteria⁹⁵. On the other hand, living microorganisms such as probiotics are more likely to be affected during manufacturing process (e.g. heating), and gastrointestinal process (e.g. gastric acids, bile salts) leading to a loss of

functionality and/or efficacy¹⁸¹. From an industrial point of view, probiotics might be more expensive to produce and to manufacture than prebiotics.

In a second study, we aimed at establishing the health implication of gut microbiota modulation by supplementation of several prebiotics and monitoring the gradual development of diet-induced obesity in C57BL/6 mice. This experiment investigates the physiological and metabolic effects of several prebiotic blends composed of galactosyl-oligosaccharides (GOS), a mixture of GOS and cow milk oligosaccharides containing glucose, lactose and galactose in addition to oligosaccharides (GOS_CMO) or a mix of inulin (long-chain fructosyl oligosaccharides, $n \geq 20$) and short-chain fructo-oligosaccharides ($n < 20$) (FOS_IN) during HF challenge. FOS, inulin and GOS have already proven to benefit circulating lipids and glucose-induced insulin secretion^{95,100}. On the other hand, GOS_CMO is a specific prebiotic blend developed by Nestlé; its impact on the physiology and metabolism of rodents is investigated hereafter.

It is important to note that C57BL/6 mice like many rodents and other mammalian may suffer from lactose mal-absorption as they exhibit a deficiency in lactase activity/expression, enabling lactose hydrolysis into galactose and glucose in the small intestine¹⁰⁴. Hence, lactose ingestion may cause gastrointestinal disorders and bowel inflammation to these mice¹⁸². A portion of undigested lactose reaching the colon can also influence mouse gut microbiota activity and alter host metabolism¹⁸². In the present study, GOS_CMO blend containing lactose and GOS allow us to investigate prebiotic effect on mouse physiology and metabolism suffering from lactose malabsorption.

Male mice aged 6 weeks were fed a low fat (LF) diet for a pre-intervention period of 3 weeks. Then, the animals were separated into 6 groups and each fed with different isocaloric diets for 70 days. First, the low fat (LF) group constituted the negative control group and enabled us to establish the normal physiological and metabolic status of C57BL/6 mouse strain. Second, those mice fed high fat (HF) or high fat containing the free sugars: glucose, lactose and galactose (HF_S) constituted the positive control groups for the development of obesity and glucose intolerance. HF_S diet having the same amount of free sugars as GOS_CMO was added in the experimental design to provide an appropriate positive control group for GOS_CMO intervention. In the 3 other prebiotic test groups, mice were fed with high fat and galactosyl-oligosaccharides (HF + GOS), or high fat and a mix of GOS and cow milk oligosaccharides (HF + GOS_CMO), or high fat and a mix of short-chain fructo-oligosaccharides and Inulin (HF + FOS_IN), to investigate whether prebiotics under HF diet modulate the obese phenotype. The physiological status of mice was assessed at several time

points in the experiment. For each mouse, body weight (BW) and food intake were recorded weekly and fasting glycaemia and fasting insulinemia were measured 7 days before the diet switch (D-7), plus 35 (D35) and 56 (D56) days after the diet switch. An oral glucose tolerance test (OGTT) was performed 56 days after the diet switch. Plasma inflammatory markers were measured at the end of the experiment. Urine and feces were sampled every week in order to monitor metabolic variations across time. Plasma samples were collected from the tail at D7, D35 and D56 to perform the OGTT test as well as fasting insulinemia and glycaemia measurements. At sacrifice (D70) plasma samples were collected from the portal vein.

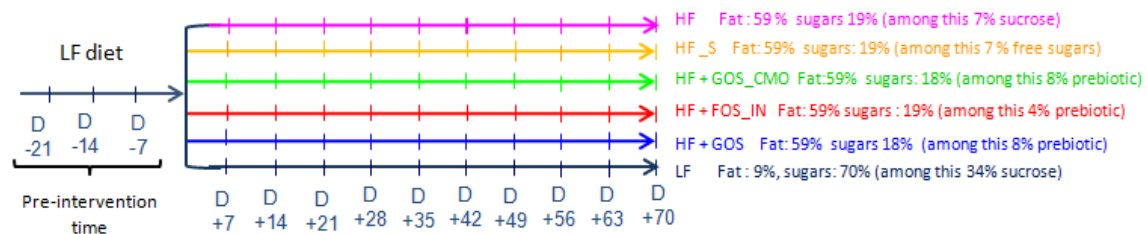


Figure 26: Experimental design to investigate the effect of prebiotic supplementation on physiological and metabolic adaptation of C57BL/6 mice during HF challenge. LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin).

Table 4: Treatment and sample collection during the experiment

Days of study	D-21	D-14	D-7	D0*	D7	D14	D21	D28	D35	D42	D49	D56	D63	D70
Arrival	X													
Randomization				X										
Body composition												X		
Weight	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Fasting glucose & insulin measure			X						X					
Oral glucose/insulin tolerance test												X		
Blood postprandial measures						X								
Faecal sample collection		X	X	X	X	X	X	X	X	X	X	X	X	X
Urine collection		X	X	X	X	X	X	X	X	X	X	X	X	X
Blood plasma collection			X			X			X			X		X
Tissue sampling														X
Sacrifice														X

In this chapter, phenotypic states of mice fed LF, HF or HF + prebiotics at different time points was established through several physiological measurements (Table 4). A top-down metabonomic approach using ^1H NMR was performed on urine, feces (from D7 days to D70 after

diet switch) and plasma (D70 after diet switch) samples to establish the metabolic adaptation associated with prebiotics supplementation during HF challenge. Plasma, urinary and fecal metabolic fingerprints capture essential information associated with host energy metabolism and trans-genomic co-metabolism. To further investigate variations in amino acid and lipid metabolism in the context of HF + prebiotics diets, plasma phospholipids and amino acid profiles of the different diet groups were measured using targeted MS approach.

2. MATERIALS AND METHODS.

2.1. Animal handling procedure and sample preparation

The experiment was performed at the NRC (Switzerland) following the national guidelines. A total of 90 C57BL/6 male mice aged of 6 weeks were housed in individual cage in 12h-12h of light-dark regime and fed ad libitum for the entire course of the experiment. Due to the high number of animals and the frequency of the intervention, the study was performed on two sub groups of animal treated one day delay. During a pre-intervention time of 3 weeks, all mice were fed with normal LF diet (see Appendix 2, Table S1 for diet composition). Then, 6 groups of 15 mice with a similar weight and glycemia distribution were selected to be submitted to different dietary treatment for 70 days:

- LF group: mice are kept under the standard LF diet
- HF group: mice received a standard HF diet (see diet composition in Appendix 2, Table S1).
- HF_S group: mice were fed with HF diet and glucose, lactose and galactose. This group constitutes the positive control group for HF + GOS_CMO diet which also contains free sugars (see diet composition in Appendix 2, Table S1).
- HF + GOS group: mice were fed with HF and galactosyl-oligosaccharides (see diet composition in Appendix 2, Table S1).
- HF + GOS_CMO group: mice were fed with HF and a blend of galactosyl-oligosaccharides and cow milk oligosaccharides containing glucose, lactose and galactose (see diet composition in Appendix 2, Table S1).
- HF + FOS_IN group: mice were fed with HF diet and a blend of fructosyl-oligosaccharides and inulin (see diet composition in Appendix 2, Table S1).

All diets were iso-caloric (diet compositions in Appendix 2, Table S1 and Table S2). In particular; HF diet contained the same proportion of proteins, fat, carbohydrate and fibres as HF + prebiotic diets. LF diet contains the same amount of fibres and proteins as the other diets, but less fat and more carbohydrate than HF and HF + prebiotic diet to maintain the same calorie supply. Urine and feces samples were collected weekly (n=1260 for each biological matrix) for the entire

course of the experiment (D-21, D-14, D-7, D0 before the diet and from 7 days (D7) to 70 days (D70) after the diet switch). 2 feces pellets were collected at each time point for metabolic profiling and gut bacterial profiling. Blood samples were also collected at 7 days before the diet switch (D-7), 14 days (D14), 35 days (D35), 56 days (D56) and 70 days (D70) after the diet switch by gently cutting on the side of the mouse tail vein (n=450). Plasma sample was extracted from the blood samples after centrifugation. All the samples were snap-frozen at -80 °C until analysis. Only the plasma samples at sacrifice (D70) were used for ¹H NMR metabonomic and MS targeted profiling. After 70 days of dietary intervention, mice were finally euthanized by anesthesia with isoflurane. Organs (kidney, liver, caecum), epididymal and mesenteric fat were also collected and weighted at sacrifice (n=90 for each tissue).

2.2. Measurement of body weight, body weight gain, fat mass, food intake and food efficiency

Mouse body weight and food consumption were weekly measured during the experiment. Mouse cumulative food intake at a given time point ($CFI_{(t)}$) was calculating by adding the amount of weekly consumed food from day 0 before diet switch up to the given time point. $CFI_{(t)}$ before diet intervention was calculated by adding the amount of consumed food from the first week of the experiment (D-21) up to day 0 before diet switch. Body weight gain ($BWG_{(t)}$) was calculating by subtracting mouse weight at a given time-point from mouse weight just before diet switch (day 0). Body weight gain during the pre-intervention period was calculating by subtracting mouse weight at a given time point to mouse weight at the beginning of the experiment (21 days before the diet switch). Finally, cumulative food efficiency at each time point ($CFE_{(t)}$) was determined by dividing the $BWG_{(t)}$ by $CFI_{(t)}$.

$$BWG_{(t)} = BW_{(t)} - BW_{(day\ 0)}$$

$$CFI_{(t)} = FI_{(day0)} + FI_{(t-1)} + \dots + FI_{(t)}$$

$$CFE_{(t)} = BWG_{(t)} / CFI_{(t)}$$

Differences in body weight and body weight gain, food intake and food efficiency between LF, HF and HF + prebiotics groups were assessed by non-parametric Wilcoxon-Mann-Whitney tests.

2.3. Measurement of body composition

Body fat composition was measured 56 days after the diet switch by using magnetic resonance spectroscopy (MRS). This non-invasive technique detects the signal of ^1H proton from methyl groups of lipids, excited by a magnetic field. The MRS signal produces a spectrum of resonances that corresponds to the level of different metabolite in the body tissues. It is often used to assess full body composition as well as to investigate metabolic changes in malign cells such as brain tumours, strokes or Alzheimer's disease^{183,184}. *In vivo* MRS spectrum can be obtained by placing the patient into a whole body magnetic resonance tomography unit. Depending on the machine design, small animals such as mice or even humans can be analysed. The scanner emits a strong magnetic field of 1.5 T, which aligns the nucleic spin orientation of hydrogen at a lower energy state in a patient. To change the direction of the nuclear spin to a higher energy state, a radiofrequency signal is emitted into the body. Similar to an NMR spectrometer, the resonance frequency receiver coil of the scanner detects the energy released by the hydrogen-bounded molecules returning to a lower energy state. The strength of the energy emission from hydrogen atoms which produce the signal is proportional to the magnetic field strength of the scanner¹⁸³. Therefore, this technique suffers from the inherent low sensitivity of NMR. More sophisticated devices with increased magnetic strength at 3 T and 7T are currently tested to improve sensitivity. Although the spectrum quality of these new generation scanners is generally improved, safety and patient health outcomes are still under evaluation¹⁸³. Additionally, contrast agents based on gadolinium complexes or iron oxides nanoparticles are sometimes administered to the patient to lower the relaxation times of biological water, which improves the quality of MRS signal¹⁸⁴.

In the present experiment, mice were placed in the instrument for 30 seconds. The recorded data provided information about total body fat, total body water and lean mass.

2.4. Glucose tolerance and insulin secretion test

Oral glucose tolerance was performed in overnight fasted mice, 56 days after diet switch. Mice were subjected to a gavage with a glucose solution (30%W/V) on the stomach. Blood samples (5 μl) were collected from the till of the tail before the intervention as well as 15, 30 and 60 minutes after intervention. Plasma samples were kept in -80°C until prepared for immunoreactive insulin test. The blood samples collected before intervention provides information about blood glucose and circulating insulin level at baseline. Cumulative glycemia (CumG) and cumulative insulinemia (CumI)

were calculated as an increment of the glucose and insulin concentration respectively, during the course of the experiment. The maximal glucose or insulin concentration (C_{max}I or C_{max}G) corresponds to the highest peak value during the OGTT test. Importantly, very low values of insulin concentration in the different diet groups during the OGTT test were noticed which may be explained by inefficient immunoreactive insulin tests. These values were below the normal range of postprandial insulin level for C57BL/6 mice and therefore CumI and C_{max}I were discarded for the analysis. Mouse fasting glycemia were also measured by quantifying the plasma glucose with ¹H NMR spectroscopy at D-7, D7 and D35.

2.5. Measurement of circulating cytokines with Multiplexed Elisa assay (Meso Scale platform)

Circulating excretion patterns of several pro- and anti-inflammatory cytokines in the different diet groups 70 days after the diet switch were measured in mouse plasma samples (n=90) using Meso Scale platform (MSD). This multiplex Elisa kit use electrolumescence technology where specific antibodies are coated at different spots on an electronic wired microplate to measure multiple inflammatory mediators simultaneously. In this experiment, 7 mediators (IL1-β, IL-12 p70, IFN-γ IL-6, KcGRO, IL-10, TFN-α) were measured in duplicate using the mouse pro-inflammatory 7-Plex ultra-sensitive MSD Kit. A volume of 50 μl per sample was required to perform all the analysis. The sample preparation and analysis were carried out as previously detailed¹⁸⁵. Several calibrators prepared from the dilutions series of a standard cytokine mixture were run on each plate in duplicates. The standard curve calculated from the calibrators was used to measure the inflammatory mediator concentrations in mouse plasma. The final cytokine concentrations in each sample correspond to the average value of the duplicate signals. All signals below the limit of quantification were discarded from the analysis.

2.6. Sample preparation and ¹H NMR spectroscopy

Because the volume of urine samples collected during the analysis was very low (<20ul), the samples preparation and ¹H NMR analysis was carried out at NRC (Switzerland) using a 600 MHz Avance III Bruker NMR spectrometer with an adapted 1.7 mm TXI NMR probe. On the other hand,

Plasma and feces samples were prepared and run in at Imperial College (London) using an Avance III Bruker NMR spectrometer and 5 mm NMR TXI probe.

2.6.1. Urine

A volume of 10 μ l of urine were homogenized in 50 μ l of phosphate buffer solution (NaHPO_4 , 0.6M pH=7.0, 100% D_2O) containing sodium azide (3mM) and TSP (0.5 mM). After centrifugation, samples were transferred in 1.7 mm diameter NMR tubes by using a syringe. A ^1H NMR sequence with water suppression (noesypr1d) was performed on 600.13 MHz spectrometer using a relaxation delay (RD) of 2 s, a mixing time (t_m) of 100 ms and a 90° pulse set at 10.75 μ s ((RD)- 90° - t_1 - 90° - t_m - 90° - acquisition)). ^1H NMR spectra were recorded with 64 free induction decays (FIDs) of and 98K data-points. The temperature of the NMR experiments was maintained at 300 K.

2.6.2. Plasma

A volume of 30 μ l of plasma samples were diluted into 30 μ l of phosphate buffer solution solution (NaHPO_4 , pH=7.4, 80% D_2O , 3mM NaN_3) and then transferred in 1.7 mm NMR tubes. No TSP was added in the phosphate buffer solution as it can interact with proteins in plasma. ^1H NMR sequences with water suppression (noesypr1d) with a mixing time of 100 ms were acquired. Spin echo Carr-Purcell-Meiboom-Gill (CPMG) sequences (RD- 90° -(D- 180° -D) $_n$ -acquisition) attenuating the signal of macromolecules were also recorded using a RD of 2s, a delay (D) of 30 ms and 90° pulse set at 10.50 μ s. Both ^1H NMR spectra were generated with 256 FID and 64K data points at a temperature of 300 K.

2.6.3. Feces

Feces samples were weighted and homogenized in 700 μ l of phosphate buffer solution (NaHPO_4 , pH=7.4, 0.01% TSP, 80% D_2O , 3mM NaN_3) by using a tissue lyser (7 minutes, frequency: 1/25). After centrifugation (15minutes at 13 000 rpm), 500 μ l of supernatant were transferred into 5 mm diameter NMR tubes. ^1H NMR spectra were recorded using a ^1H NMR sequence with water suppression (noesypr1d) which applied the following acquisition parameters: RD of 2 s, a mixing time (t_m) of 100 ms and a 90° pulse of 9.50 μ s. All the experiments were achieved at a constant temperature of 300K using 256 FIDs and 32K data points.

2.7. ^1H NMR spectra pre-processing

Processing of ^1H NMR spectra was carried out using TOPSPIN 2.1 software package (Bruker Biospin, Rheinstetten, Germany). For each spectrum, the FID was multiplied by an exponential function corresponding to a size of 1 Hz for urine samples or 0.3 Hz for plasma and feces samples. The FIDs were then transformed into spectrum by a Fourier transformation. The spectra were manually phased, baseline corrected and calibrated using the TSP signal at δ 0 for the urine and feces spectra and the glucose signal at δ 5.23 for plasma spectra.

2.8. ^1H NMR Data processing

The spectral data (from δ 0.2 to δ 9.5) were finally imported into Matlab software (version R2012b, the mathworks Inc, Natwick MA) and were transformed into 22K data-points. Resonance of water peak (δ 4.7-5.05) was also removed from each spectrum. ^1H NMR spectra were aligned using an in-house algorithm and normalized in order to remove variation in metabolite concentration linked to osmolarity and/or dilution error. Total area normalisation was suited to correct ^1H NMR spectra of urine and feces samples. On the other hand, ^1H NMR spectra from plasma samples containing broad peaks from lipid and proteins was corrected using median full change normalisation. Poor quality or highly diluted spectra were discarded from the subsequent analysis.

2.9. MS targeted profiling using the Biocrates kit

A Multiplex targeted MS/MS assay was carried out in mouse plasma samples collected at sacrifice (n=90) using the Absolute *IDQ*, P180 kit (BIOCRATES Life Science AG, Innsbruck, Austria). This analysis was conducted at NRC (Switzerland). As previously mentioned in Chapter II (section 2.3), this technique allows identifying 180 metabolites by using reverse phase liquid chromatography (Zorbax Eclipse XDB C18 column, 3.0x100mm 3.5micron) and direct flow injection with tandem mass spectrometry method. The analysis was carried out on Qtrap ABSciex 5500 using the protocol published previously¹²⁸. Several Quality controls and reference samples were introduced to monitor the analytical reproducibility. Data processing and metabolite quantification were achieved by using MetIQ software package provided with the Absolute *IDQ* kit as previously described¹²⁸. After the cleaning procedure, 173 metabolites including amino acids, biogenic amines, acylacnitines, sphingomyelins (SMs), lysophosphocholines, diacylphosphocholines (PCs) and one hexose were used for statistical analysis. Because of the large number of samples, the experiment was run in two different plates

2.10. ^1H NMR metabolic data and MS targeted data analysis

Multivariate statistical models (PCA, OPLS, OPLS-DA) were performed in Matlab software (version R2012b, the mathworks Inc, Natwick MA) and SIMCA-P 12.1 software (Umetrics, Umea, Sweden) using UV scaling. Q^2Y value was determined by plotting the score (T) against the cross validating scores (Tcv) to estimate the goodness of fit and level of overfitting of the OPLS and OPLS DA models. All models were subjected to a 7-fold cross validation and were validated by multiple permutation tests (permutation number =1000). Pair-wise OPLS-DA models with ^1H NMR metabolic profiles or MS targeted profiles were also performed between the different diet groups to identify the specific metabolic signatures related to each dietary intervention. OPLS coefficient plot from pair-wise models was used identify the relevant ^1H NMR peaks in metabolic profiles of the different diet groups as described by Trygg *et al* ¹¹⁷. The most relevant metabolites in pair-wise models with MS data were flagged by using S plot (see Chapter II, section 3.2 for more details on the S plot). Correlation heatmaps were generated by compiling the Pearson correlation coefficient values of the relevant metabolites from ^1H NMR metabolic profiles or MS targeted profiles. An arbitrary cut off values ($R^2>0.35$) was selected to facilitate data interpretation.

3. RESULTS

3.1. Physiological data

Physiological measurement data were acquired for the duration of the experiment, and at specific time points to determine whether prebiotic supplementation during HF diet challenge had a beneficial impact on obesity development and impaired insulin metabolism.

3.1.1. Body weight gain, food intake, cumulative food efficiency and fat mass

Body weight gain (BWG) of mice was calculated by subtracting the baseline body weight (BW) at D0 (before diet switch) from each mouse from BW measured at each time point after the diet switch (from D7 to D70). BWG of mice during the pre-intervention period was calculated by

subtracting mouse BW at the beginning of the experiment (D-21) from the mouse BW measured from D-14 to D0. Mean BWG of mice (Figure 27.A) fed with LF diet (negative control group), HF, HF_S diets (positive control groups) and HF + prebiotic diets (test groups) at each time point are presented in Figure 27.A. Significant differences in mouse BW and BWG between the diet groups were evaluated using non parametric Wilcoxon-Mann-Withney tests.

Both HF and HF_S groups showed significantly higher BWG than LF and HF + prebiotics groups. All HF + prebiotics groups had similar BWG growth curves overtime. In addition, no significant difference between BWG of HF + prebiotics and LF group was observed between D0 and D28. These results confirm that both positive control groups (HF and HF_S groups) quickly become overweight compared to negative control group (LF group) and GOS, GOS_CMO or FOS_IN supplementation during the HF challenge limited BWG. These statistical analyses also highlighted the different group trajectory in BWG curves. HF + GOS_CMO and HF + FOS_IN fed mice had significantly higher BWG than LF-fed mice from D35 after diet switch while HF + GOS fed mice had significantly higher BWG after 42 days of diet switch. As a result, mice fed HF + prebiotics for a long-term period became overweight compared to the negative control group (LF group). These results showed that prebiotic supplementation during HF challenge limited but did not prevent the development of obesity.

Statistical analyses on weekly food intake (Figure 27. B) also indicated that mean food intake at each time point was significantly higher in the LF group (3 g difference per week in average) than in all the other groups and significantly higher in both HF groups (1.7 g difference per week in average) compared to the HF + GOS and HF + GOS_CMO groups, during the overall course of the experiment. However, the food intake of HF + FOS_IN was only significantly lower from both HF groups on D49, D63 and D70 (with an average of 0.9 g difference per week). Hence, the ingestion of a prebiotic in addition to HF diet may increase satiety which may partly explain the lower BWG in HF + prebiotic groups over time. Noticeably, weekly food intake for each diet group plotted in the Figure 27.B was consistent along the study except at D21 after the diet switch. During this week, a dietary challenge with postprandial blood collection was performed which may have led to an increase of food intake for all animals.

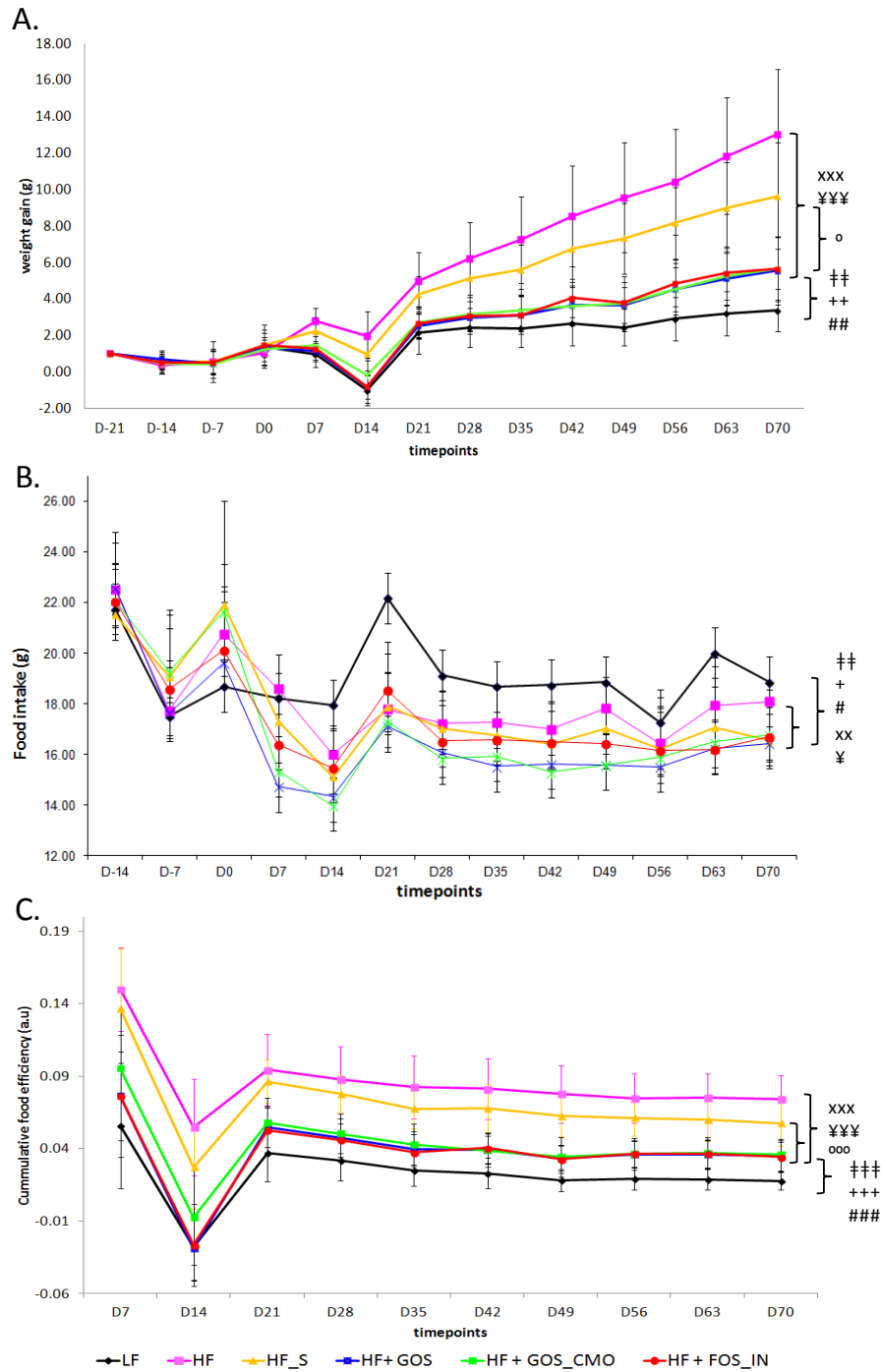


Figure 27: Impact of different diet interventions on (A.) mouse weight gain, (B.) food intake and (C.) cumulative food efficiency during the overall course of the experiment. Cumulative food intake (CFE) = body weight gain (BWG) /cumulative food intake (CFI). Wilcoxon-Mann-Whitney tests are presented at the last timepoint. 1 star for p -value<0.05, 2 stars for p -value<0.01, 3 stars for p -value<0.001 (x: HF vs HF+GOS, y: HF vs HF +FOS_IN, o: HF_S vs HF+GOS_CMO, ‡: LF vs HF+GOS, †: LF vs HF+FOS_IN, #:LF vs HF+GOS_CMO). LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin).

To determine the physiological effect of prebiotic supplementation on BWG during the HF challenge, cumulative food efficiency (CFE) was calculated for each diet group at each time point by dividing the BWG by the cumulative food intake (see formula material and methods). This parameter estimates the mice's efficiency at storing calories, from the first week after diet switch (D0). The mean CFE of each diet group calculated from D0 until the end of the experiment (D70) is summarised in Figure 27.C. Non parametric Wilcoxon-Mann-Whitney tests on the CFE indicated that all prebiotic supplementations during HF challenge induced significantly lower calorie storage than both HF groups during the course of the experiment except for HF + GOS_CMO group, which was only significantly lower than HF_S from D21. Interestingly, the different prebiotic groups had similar CFE across the study, and no prebiotic group showed a significant difference in CFE with LF group during the first 4 weeks after diet switch. A significant difference between HF + GOS_CMO and LF groups appeared at D35 after the diet switch, whereas it appeared at D42 after the diet switch for HF + FOS_IN and HF + GOS groups. These results highlighted that each prebiotic supplementation was unable to maintain normal calorie storage during a short-term period of HF challenge. However, prebiotic benefits were not sustainable after a month of HF challenge, which resulted in divergence in CFE between HF + prebiotic and LF groups. Noticeably, CFE values took 2 weeks to reach a stable pattern: CFE was significantly higher in all HF containing diet groups at D7 compared to the other time points, indicating that all mice challenged with HF had a boost in calorie storage 1 week after the diet switch. CFE values also dropped in all the animals at D14 due to a longer fasting period before the dietary challenge.

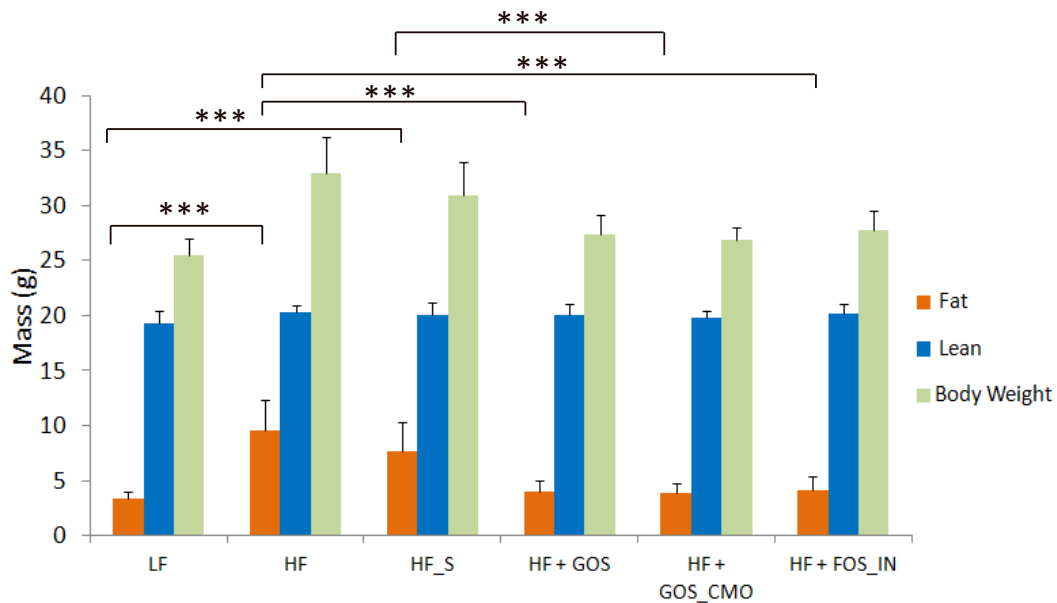


Figure 28: Body fat composition of mice fed with different diets at 56 days (D56). Fat mass (red), lean mass (blue) and total mass (green) levels of different diet groups are shown. Values are expressed as mean $\pm$ SEM. Differences between diet groups were assessed by non parametric Wilcoxon-Mann-Whitney tests . $P < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$. LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin).

Assessment of fat and lean mass was also carried out for each diet group 2 weeks (D56) before the sacrifice (Figure 28). An analysis of variance showed no significant difference for lean mass between all groups of mice. However, fat mass was significantly higher in HF and HF_S groups compared to LF and all prebiotic groups. In addition, mice fed with HF + prebiotics had the same amount of fat mass as LF fed mice. These results showed that supplementation of prebiotics during HF challenge induced a reduction of calorie storage, which is physiologically characterised by a restriction of fat mass accumulation and overall limited obesity development.

3.1.2. Systemic insulin and glucose homeostasis.

The regulation of glucose homeostasis and insulin secretion in mice fed with different diets was assessed 8 weeks after the diet switch (D56) by oral glucose tolerance test (OGTT). All HF + prebiotic groups presented significantly lower fasting glycaemia than HF and HF_S groups (5.97 ± 0.43 mM for LF group, 6.69 ± 0.70 mM for HF group, 6.82 ± 0.75 mM for HF_S group,

6.38±0.63mM for HF + GOS group 6.16±0.63mM for HF + GOS_CMO group, 6.01±0.70mM for HF + FOS_IN group). Fasting insulinemia was also significantly lower in prebiotic groups than in both HF groups and not significantly different from the LF group indicating that prebiotics improved glucose associated insulin secretion at systemic level. Analysis of glycaemia pattern during OGTT showed that HF + GOS and HF + FOS_IN groups had lower cumulative glycaemia (CumG) and lower maximal glucose concentration (CmaxG) than HF group, but not HF + GOS_CMO group (CumG of 571±80.1 mM min⁻¹, CmaxG of 14.6±2.4 mM; for LF group, CumG of 906± 129.9 mM min⁻¹ and CmaxG of 18.6±2.5 mM for HF group, CumG of 847.4± 98.1 mM min⁻¹ and CmaxG of 17.7±2.33 mM for HF_S group, CumG of 783.7±77.2 mM min⁻¹ and CmaxG of 16.5±1.9 mM for HF + GOS group, CumG of 821.0± 158.8 mM min⁻¹ and CmaxG of 17.9±2.5 mM for HF + GOS_CMO group, CumG of 787.7 ±102.5 mM min⁻¹ and CmaxG of 16.8±2.3 mM for HF + FOS_IN). These differences were essentially due to variation of glycaemia at baseline. Indeed when the effect of the baseline value was removed, no difference in response to glucose was observed between HF + prebiotics groups and both HF groups. However, HF challenge with or without prebiotic supplementation induced an increase in fasting glycaemia, CumG and Cmax compared to LF group indicating relative glucose intolerance.

Fasting glycemia was also measured before the diet switch (D-7), and 5 weeks (D35) and 8 weeks (D56) after the diet switch for each group. As shown on the histogram (Figure 29), no significant increase in blood glucose was observed in LF-fed mice indicating that glycaemia was maintained at normal values between D-7 and D56. On the other hand, blood glucose significantly increased in both HF and HF_S groups, in HF + GOS and HF + GOS_CMO overtime with a maximum glucose level reached at D35. The values of fasting glycaemia at D35 and D56 for these groups were characteristic of impaired glucose tolerance (glucose concentration between 7.8 and 11 mM) but not diabetes (glucose concentration > 11mM). Only mice fed with HF + FOS_IN had stable glycaemia between D-7 and D56 with values of blood glucose similar to those found in LF group. Even though, mice fed with HF + prebiotic showed a lower fasting insulinemia compared to HF and HF_S groups, HF ingestion induced a profound deregulation of glucose homeostasis overtime generating impaired glucose tolerance in all HF diet groups. However, amongst all prebiotics, FOS_IN exhibited the greatest capacity to attenuate glucose intolerance as suggested by limited fasting hyperglycemia.

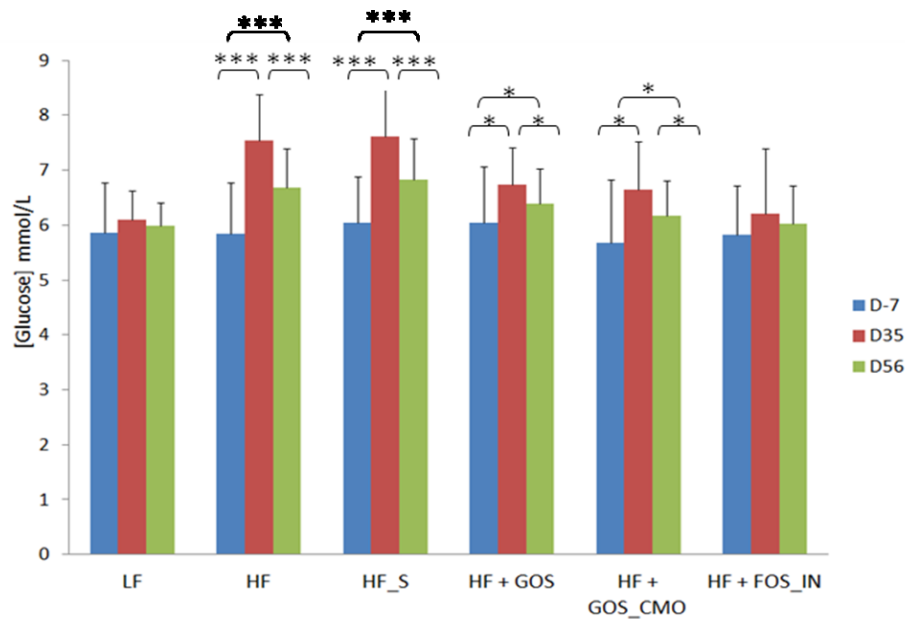


Figure 29: Time-dependent effect of different dietary interventions on mouse fasting glycaemia. Concentration of glucose was obtained 7 days (D-7) before the diet switch, 35 (D35) and 49 (D49) days after the diet switch and the values are expressed as mean $\pm$ SEM. Differences between groups were assessed by non parametric Wilcoxon-Mann-Whitney tests, $P < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$. LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF + GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin).

3.1.3. Inflammatory markers

Using multiplexed Elisa essays, the concentrations (pg/ml) of 7 inflammatory cytokines (IFN- γ , IL-1 β , IL-6, IL-10, IL-12, KcGRO, TNF- α) in plasma of each diet group 70 days (D70) after the diet switch, were measured in order to establish the long-term effect of HF and prebiotic supplementation on systemic inflammation. As displayed in the following histogram (Figure 30), HF and HF_S feeding tended to raise the level of pro-inflammatory (IFN- γ , IL-1 β , IL-6, IL-12, KcGRO, TNF- α) and anti-inflammatory (IL-10) markers in the circulation compared to LF feeding. However, due to a low plasma concentration of cytokines in all mice, the recorded signals of each cytokine were close to the limit of quantification inducing high variations into the measurements (as indicated by a large error bars). Significant differences among the cytokine concentrations between diet groups were calculated using non-parametric Wilcoxon-Mann-Whitney tests. No difference in the plasma concentrations of IFN- γ , IL6 and IL12 were observed between LF, HF and HF_S groups indicating that HF ingestions did not significantly affect the circulating pattern of these cytokines. However, IL-1 β

level was higher in HF and HF_S groups than LF group and IL10, KcGRO, TNF- α levels significantly increased in HF_S group compared to LF group. Prebiotic supplementation also triggered significant variations in the circulating cytokine pattern. In particular, HF + GOS group was associated with a reduction in IFN- γ , IL-1 β , IL-10 and IL-12 compared to HF and HF_S groups. HF + GOS_CMO supplementation induced a drop of IFN- γ and IL-1 β compared to HF_S group whereas HF + FOS_IN supplementation decreased plasma concentration of KcGRO and IL-1 β compared to HF and HF_S groups. By contrast the level of TNF- α in circulation was significantly higher in HF + GOS_CMO and HF + FOS_IN groups compared to LF group which pointed out a specific prebiotic induced inflammatory response. Prebiotic supplementation tended to decrease the global inflammatory status of mice as plasma concentration of all cytokines except TNF- α decreased in HF + prebiotic groups compared to HF and HF_S groups even though variations between groups were not all significant.

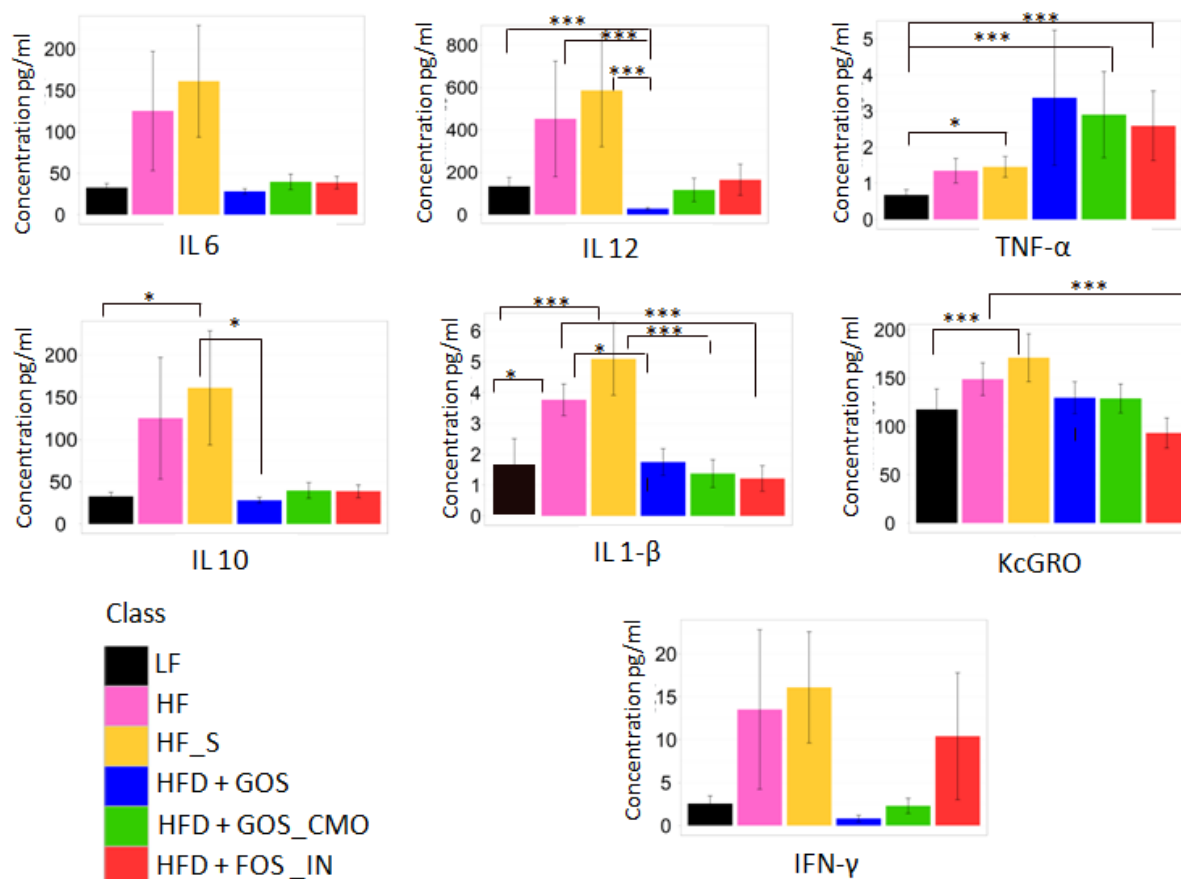


Figure 30: Circulating inflammatory status of mice fed different diets 70 days (D70) after diet switch. Plasma level of IFN- γ , IL-1 β , IL-6, IL-10, IL-12, KcGRO, TNF- α were measured in each dietary group at D70 after diet switch with the MesoScale Discovery platform. Results are expressed as mean concentration (pg/ml) $\pm$ SEM. For each cytokine, the differences between groups were assessed by non parametric Wilcoxon-Mann-Whitney tests, $P < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$. LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF + GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin).

3.2. Metabonomic data

3.2.1. Characterisation of ^1H NMR urinary, fecal and plasma metabolic profiles of C57BL/6 mice

Mean ^1H NMR spectrum of urinary, fecal or plasma from LF, HF, HF_S and HF + prebiotic fed mice were assigned with the help of 2D ^1H - ^1H and ^1H - ^{13}C NMR experiments and appropriate database (^1H NMR metabolites assignment in Table 5). Urinary metabolic profiles (Figure 31) present

a numerous signals from organic acids (acetate, butyrate, citrate, hippurate, α -keto- β -methylvalerate, isovalerate), gut microbial co-metabolites³² (phenylacetylglycine (PAG), *p*-cresol sulfate (PCS), *p*-cresol glucuronide (PCG), indoxylsulfate) and other metabolites such as aliphatic amines (dimethylamine (DMA), trimethylamine (TMA), trimethylamine-N-oxide (TMAO)). Amino acids (leucine, isoleucine, alanine) sugars (D-glucose, D-galactose), creatine and creatinine were also observed in urine of C57BL/6 mice.

In fecal metabolic profiles (Figure 32) resonances of organic acids (acetate, 3-D-hydroxybutyrate, formate, succinate, glycerate, butyrate, propionate), and sugars (α/β -D-glucose, xylose) were identified. ¹H NMR fecal spectra were also characterised by signals of amino acids (such as leucine, isoleucine, valine, threonine, methionine, asparagines, phenylalanine, glutamate, and lysine) and nucleic acids derivatives (hypoxanthine, xanthine and uracil).

Plasma CPMG ¹H NMR spectra (Figure 33.A) showed small molecule resonances such as acetate, pyruvate, and amino acids (glutamate, leucine, isoleucine, valine). On the other hand, plasma standard ¹H NMR spectra (Figure 33.B) mainly displayed lipid signals from unsaturated and saturated compounds, lipoproteins (overlapped resonances of very low density lipoproteins (VLDLs) and low density lipoproteins (LDLs)) and sugars (D-glucose).

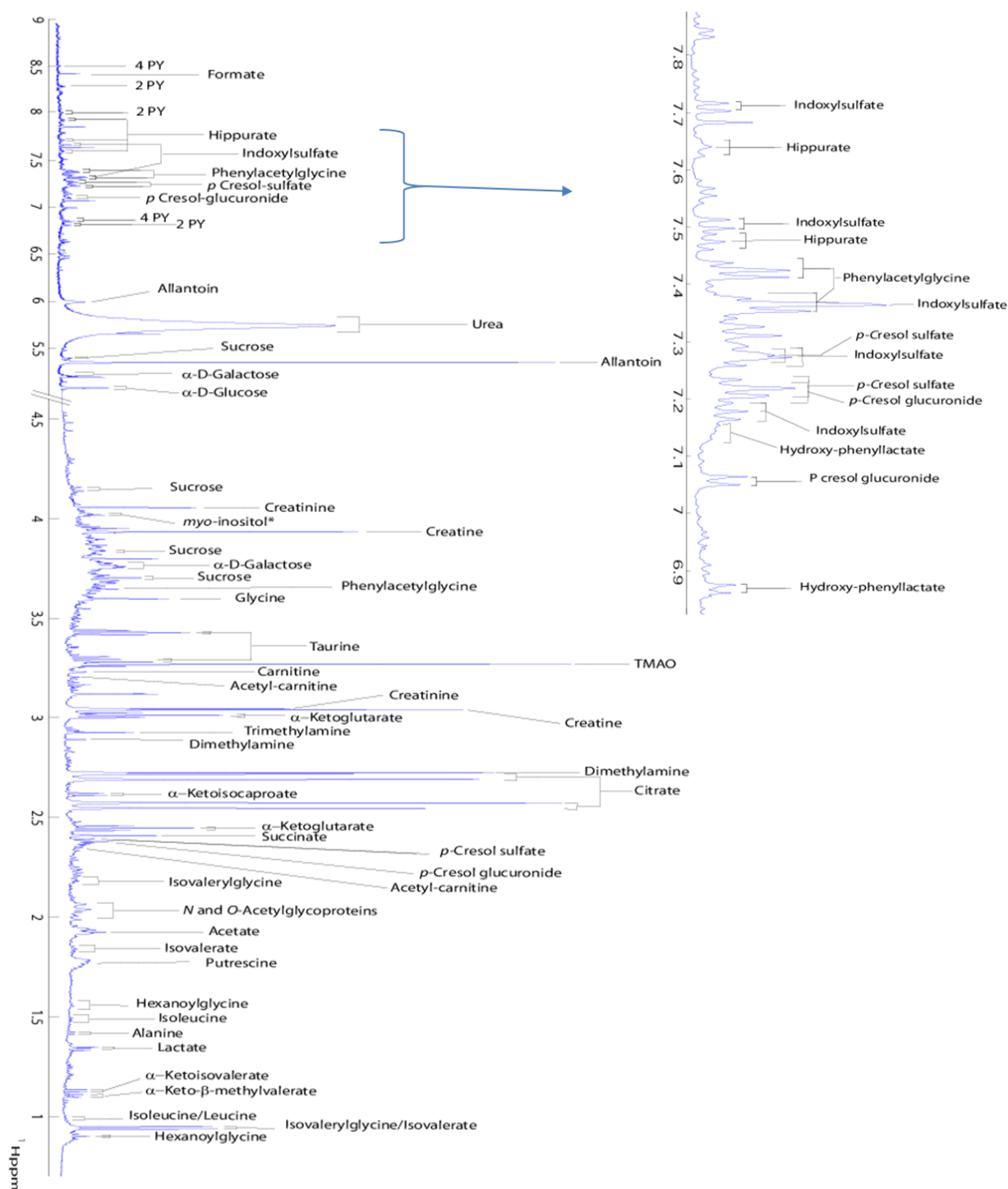


Figure 31: Mean of ^1H NMR spectra of urine from LF, HF and HF + prebiotic fed mice from D7 to D70. The aromatic region of the spectrum (6.8-7.8ppm) has been zoomed to improve the spectral assignment. 4PY : *N*-methyl-4-pyridone-3-carboxamide, 2PY: *N*-methyl-2-pyridone-5-carboxamide.

*: tentative assignment.

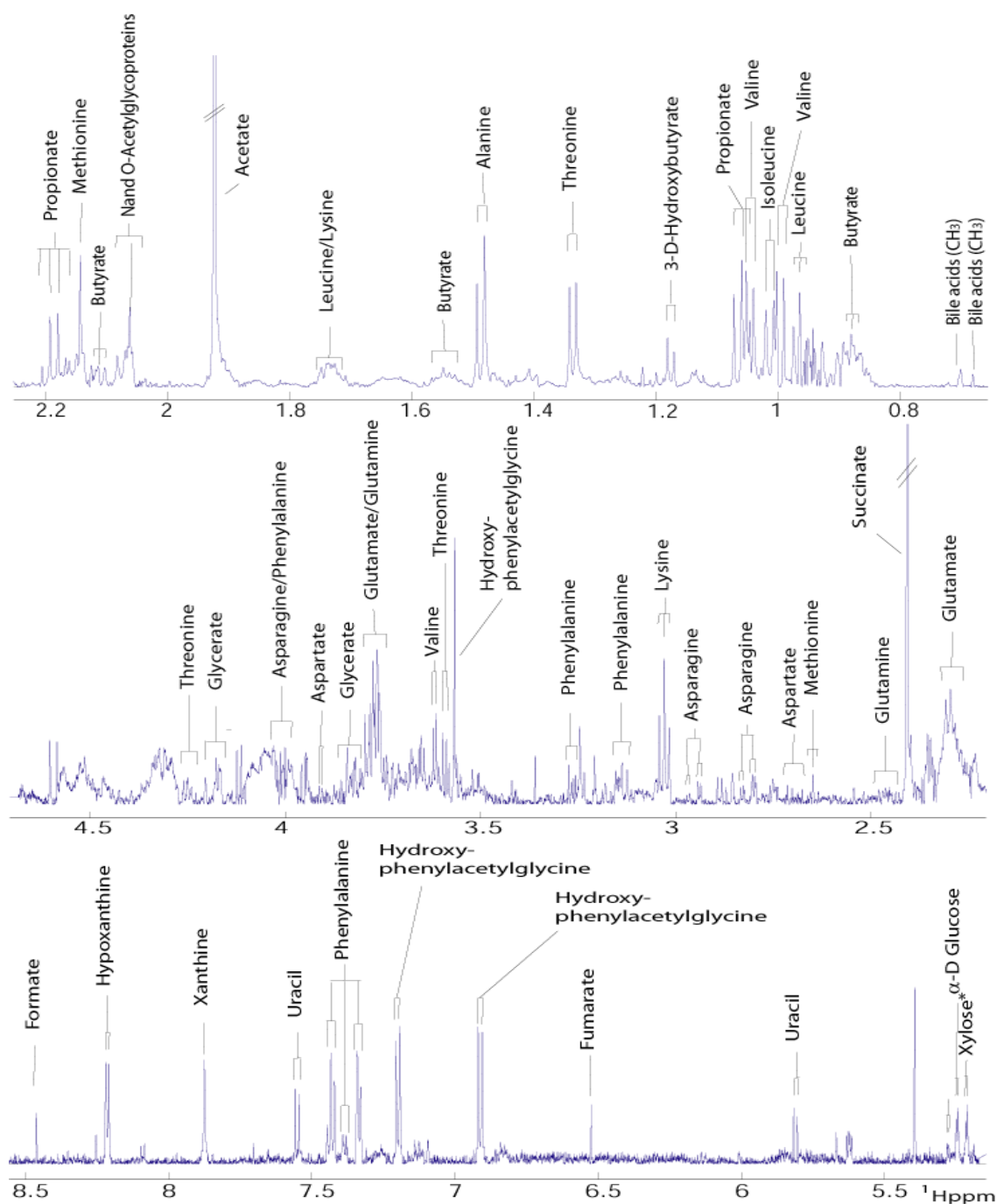


Figure 32: Mean of ^1H NMR spectra of feces from LF, HF and HF + prebiotic fed mice from D7 to D70. The spectrum has been zoomed in three different regions (0-2.2, 2.3-4.5 and 4.7-8.55ppm) to identify peak multiplicity and improve the spectral assignment. *: tentative assignment

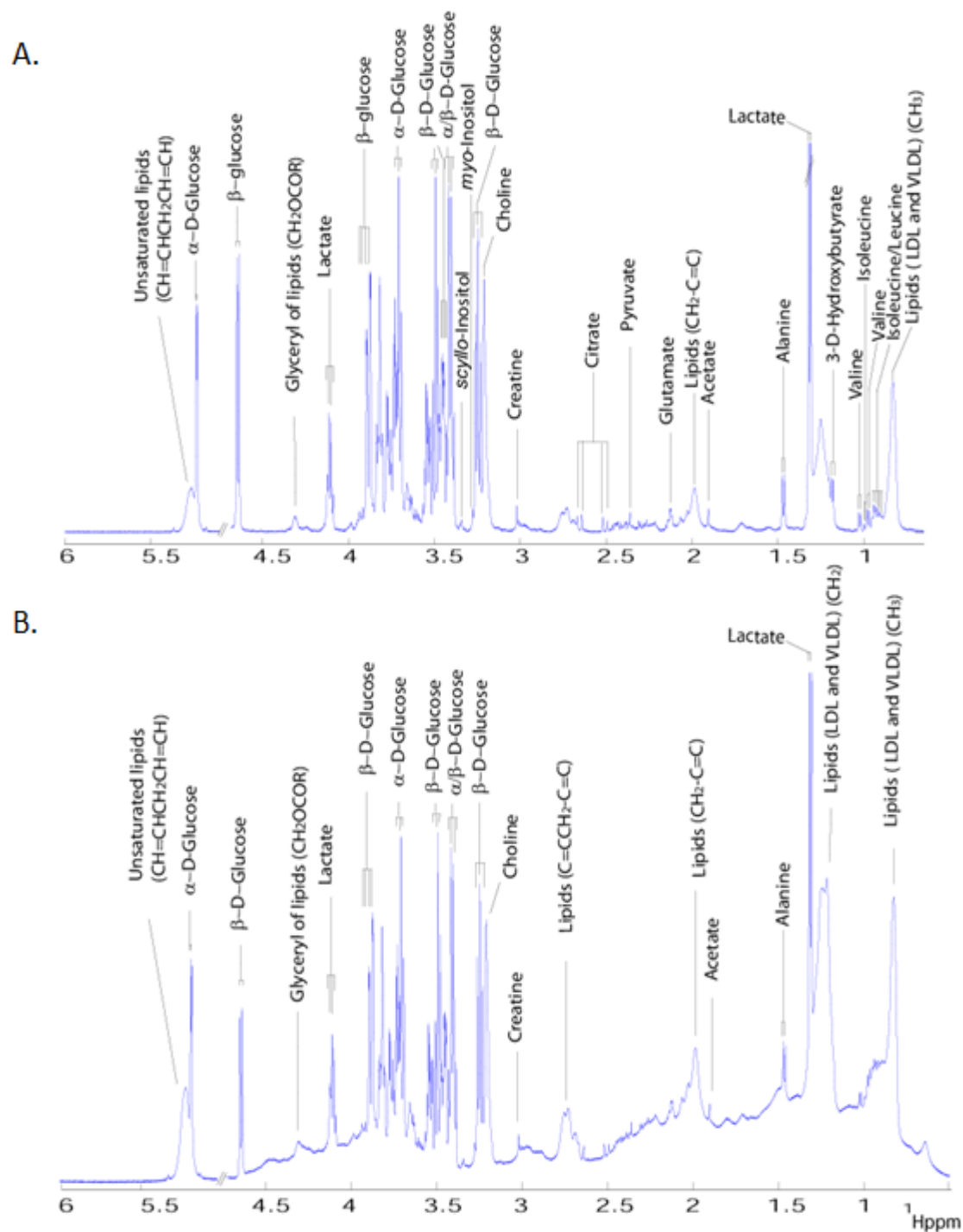


Figure 33: Mean of ^1H NMR spectra of plasma from LF, HF and HF + prebiotic fed mice from D7 to D70. (A) ^1H NMR CPMG spectra (B) ^1H NMR standard spectra.

*: tentative assignment

Table 5: ^1H NMR chemical shifts from metabolites assigned in urinary, fecal or plasma metabolic profiles. (Only resonances identified in the spectra are reported and signals of unassigned metabolites are not included in the table).

Metabolite	^1H NMR ppm multiplicity group	Biological matrix observed
<i>N</i> -methyl-2-pyridone-5-carboxamide	6.66 (d) C3H, 7.83 (dd), C4H, 8.32 (d) C5H,	U
3-D-Hydroxybutyrate	1.20 (d) CH ₃ , , 4,16 (Dt) CH	P/F
<i>N</i> -methyl-4-pyridone-3-carboxamide	6.69 (d) C3H, 7.97 (dd) C4H, 8.54 (d) C5H	U
Acetate	1, 92 (s) βCH_3	U/P/F
Acetyl-Carnitine	3.183 (s) (CH ₃) ₃ , 2.31 (s) CH ₃	U
Alanine	1.46 (d) (βCH_3)	U/P/F
Allantoin	5.38 (s) CH, 6.02 9 (s) (NH)	U
Asparagine	2.86 (dd) (1/2 βCH_2), (dd) 2.96 (1/2 βCH_2), (dd) 4.00(αCH)	F
Aspartate	2.68 (dd) (1/2 βCH_2), 3.91 (dd) (αCH)	F
Bile acids	0.68 (m) CH ₃ , 0.70(m) CH ₃	F
Butyrate	0.88 (s) γCH_3 , 1.55 (m) βCH_2 , 2.14 (m) αCH_2	F
Carnitine	3.23 (s) CH ₃	U
Choline	3.22 (s) N-(CH ₃) ₃	U/P
Citrate	2.55 (d) 1/2 γCH 2.7 (d) 1/2 γCH ,	U/P
Creatine	3.03 (s) N-CH ₃ , 3.94 (s) N-CH ₂	U/P
Creatinine	3.05 (s) N-CH ₃ , 4.06 (s) N-CH ₂	U
Dimethylamine	2.72 (s) CH ₃	U
Formate	8.46 (s) HCOOH	U/F
Fumarate	6.51 (s) CH	F
Glutamate	2.02(M) βCH_2 2.34 γCH_2 3.76 (dd) αCH	P/F
Glutamine	2.15 (m) βCH_2 2.44 (m) γCH_2 3.77 (t) αCH	F
Glycerate	3.81 (m) CH, 4.14 (m) CH ₂	F
Glycerol of lipids	4.06 (m) CH ₂ OCOR	P
Glycine	3.55 (s), αCH_2	U
Hexanoylglycine	0.88 (t) CH ₃ , 1.62 (m) CH ₂	U
Hippurate	7.55 (t) <i>m</i> CH, 7.64(t) <i>p</i> CH, 7.84 (d) <i>o</i> CH	U
Hydroxy-phenylacteylglycine	3.58 (s) CH ₂ , 6.88 (d) C1H & C4H, 7.21 (d) C3H & C3H	F

Hydroxy-phenyllactate	3.04 (dd) CH ₂ , 4.35 (dd) CH, 6.85 (d) C1H & C4H, 7.17 (d) C2H & C3H	U
Hypoxanthine	8.18 (s) CH, 8.20 (s) CH	F
Indoxylsulfate	7.27 (m) C4H, 7.36 (s) CH, 7.50 (d) C5H, 7.70 (d) C6H	U
Isoleucine	0.94 (t) γCH ₃ , 1.02 (t) δCH ₃ ,	U/P/F
Isovalerate	0.94, (CH ₃) ₂ , 1.94 (m) CH	U
Isovalerylglycine	0.94 (d) εCH ₃ , 2.00 (d) δCH ₂ , 2.20 (d) γCH ₂ ,	U
Lactate	1.33 (d) βCH ₃ , 4.12 (q) αCH	U/P
Leucine	0.93 (d) δCH ₃ 0.94 (d) βCH ₂ 1.71 (m) γCH	U/P/F
Lysine	1.71 (m) δCH ₂ , 1.84 (m) βCH ₂ , 3.01(t) εCH ₂	F
Methionine	2.13 (s) δCH ₃ , 2.14 (m) βCH, 2.6 (t) γCH ₂	F
N and O-Acetylglycoproteins	2.04 (s) N/OHCOCH ₃	U/F
<i>p</i> -Cresol glucuronide	2.3 CH ₃ , 7.06 (m) C2H & C6H, 7.23 (m) C3H & C5H	U
<i>p</i> -Cresol sulphate	2.34 (s) CH ₃ , 7.11 (m) C2H & C6H, 6.80 (m) C3H & C5H	U
Phenylacetylglutamine	3.68 (s) CH ₂ , 3.75 (d) CH ₂ , 7.37 (m) C2H & C6H, 7.43 (m) C3H & C5H	U
Phenylalanine	3.12 (dd) 1/2-βCH ₂ , 3.26 (dd) 1/2-βCH ₂ , 7.33 (m) C3H & C5H, 7.35 (m) C4H, 7.4 (m) C3H & C6H	F
Propionate	1.05 (t) βCH ₃ , 2.183 (q) α CH ₂	F
Putrescine	1.80 (m broad) βCH ₂	U
Pyruvate	2.35 (s) CH ₃	P
Succinate	2.41 (s) CH ₃	U
Sucrose	5.4 (d) C1H, 3.87 (dd) C5H (glucose), 3.89 (dd) C1H, 4.04 (dd) C2H, 3.67 (m) CH ₂ (fructose)	U
Taurine	3.26 (t) N-CH ₂ , 3.43 (t) S-CH ₂	U
Threonine	1.32 (d) γCH ₃ , 3.6 (d) αCH, 4.25 (m) βCH	F
Trimethylamine-N-oxide	3.27 (s) N-(CH ₃) ₃	U
Trimethylamine	2.88 (s) CH ₃	U
Uracil	5.80 (d)C5H 7.54 (d) C6H,	F
Urea	3.4 (s) α,β NH ₂	U
Valine	0.98 (d) γCH ₃ 1.04 (d) γCH ₃ , 3.62 (d) αCH	P/F
Xanthine	7.89 (s) CH	F
Xylose *	5.19 (d) C1H	F
α-D-Galactose	5.26 (d) C1H, 3.72 (m) C2H & C5H, 3.72 (m) CH ₂	U
α-D-Glucose	3.42 (m) C4H, 3.72 (m) C3H, 3.73 (m) ½-C6H, 3.77 (m) ½-C6H, 5.24 (d) C1H	U/P/F
α-Ketoglutarate	2.44 (t) βCH ₂ 3.01 (t) γCH ₂	U

α -Ketoisocaproate	0.94 (d) (CH ₃) ₂ , 2.61 (d) CH ₂	U
α -Ketoisovalerate	1.11 (d) (CH ₃) ₂	U
α -Keto- β -methylvalerate	1.10 (d) CH ₃	U
β -D-Glucose	3.25 (m) C ₂ H, 3.49 (m) C ₄ H, 3.49 (m) C ₅ H, 3.5 (m) C ₃ H, 3.91 (d) $\frac{1}{2}$ -C ₆ H, 4.66 (d) C ₁ H	P
myo-inotisol*	3.29 (t) C ₅ H, 4.06 (t) C ₂ H	U/P
scyllo-inotisol	3.35 (s) CH	P
Lipid fractions	0.90 (m) CH ₃ , 1.20 (m) (CH ₂) _n , 1.97 (m) & 2.0 (m) CH ₂ C=C, 2.23 (m) CH ₂ CO, 2.72 (m) C=CCH ₂ C=C, 5.29 (m) CH=CH	P

U: Urine, P: Plasma, F: Feces. * Tentative assignment

3.2.2. Effect of prebiotics supplemented HF diet at D70 after the diet switch in C57BL/6 mice

Physiological data showed that prebiotic supplementation had beneficial impacts on HF induced obesity. To understand biological processes inducing phenotypic changes in mice fed HF + prebiotics, a fecal, urinary, and plasma metabonomic analysis was performed. Firstly, urinary and fecal metabolic profiles of all diet groups from D0 to D70 were compared to identify specific patterns of metabolic adaptation to different isocaloric diets over time. Secondly, the impact of HF and prebiotic in urinary, fecal and plasma metabolic variations at D70 after the diet switch were identified. To assess the prebiotic-induced improvement of the metabolic phenotype towards a “healthy metabolic profile”, a metric of the metabolic signatures was calculated in plasma, urine and feces, using the metabolic phenotypes of the negative (LF) and positive (HF or HF_S) control groups. The position of the metabolic phenotypes of HF + prebiotic fed mice onto these metrics estimates the “metabolic rescue” induced by prebiotics supplementation during HF challenge in each biological matrix. Thirdly, pair-wise OPLS-DA models comparing the different diet groups were performed to establish the urinary, fecal and plasma metabolic signatures associated to HF diets and different prebiotics supplementation during HF challenge at D70 after the diet switch. Finally pair-wise comparison of lipid and amino acid profiles of different diet groups acquired by a MS targeted assay provided additional information on prebiotic associated variations of lipid and amino acid metabolism.

3.2.3. Global OPLS-DA models of urine and feces samples describe metabolic adaptations associated with nutritional interventions.

Global OPLS-DA models were performed on urine and fecal samples to assess overall differences related to urinary or fecal metabolic profiles of mice fed various diets. For each biological matrix, the model grouped together all ^1H NMR spectra of each group between D7 and D70. The Y matrix was built with 5 dummy variables identifying the diet groups and 1 continuous variable defining the time-points. Therefore, the models categorise the different diet groups as separate classes of samples and look for linear variations linked to time effect. Numbers of components and goodness of fit statistics corresponding to each model are summarised in the Table 6. In both models, the first three components separated the different diet groups whereas the time trajectory was modeled from the fourth component which demonstrates that nutritional intervention induced greater variability in the mouse urinary and fecal metabolic profiles than time effect. The OPLS-DA score plots obtained from urinary metabolic profiles (Figure 34.A) showed that HF and HF_S groups were separated from LF group along the first predictive component. This highlights that HF feeding introduced the strongest source of variability in the metabolic profiles. The second and the third predictive components described the metabolic variation related to the different prebiotic supplementation. By contrast, the OPLS-DA scores plots of fecal metabolic profiles showed a separation between HF + GOS_CMO, HF + FOS_IN groups and the other diet groups along the first component (Figure 34.B). Such observations suggest that prebiotic supplementation induced a greater metabolic variation than HF diet. The second predictive components discriminated mice fed with HF, HF_S and HF + GOS_CMO from mice fed with LF, HF + FOS_IN and HF + GOS. Both urinary and fecal metabolic profiles of HF + FOS_IN and HF + GOS fed mice approached LF-fed mouse metabolic profiles. On the other hand, HF + GOS_CMO fed mice had specific metabolic pattern and were closer to HF and HF_S mouse metabolic profiles. Hence, ingestion of the prebiotic FOS_IN and GOS during HF challenge tended to shift urinary and fecal metabolic profiles of mice towards an healthy metabolic profile. In addition, HF + GOS_CMO diet had lower fibre contents than HF + GOS or HF + FOS_IN diets. Therefore differences in fibre percentage may lead to dose dependent metabolic responses in urine and feces.

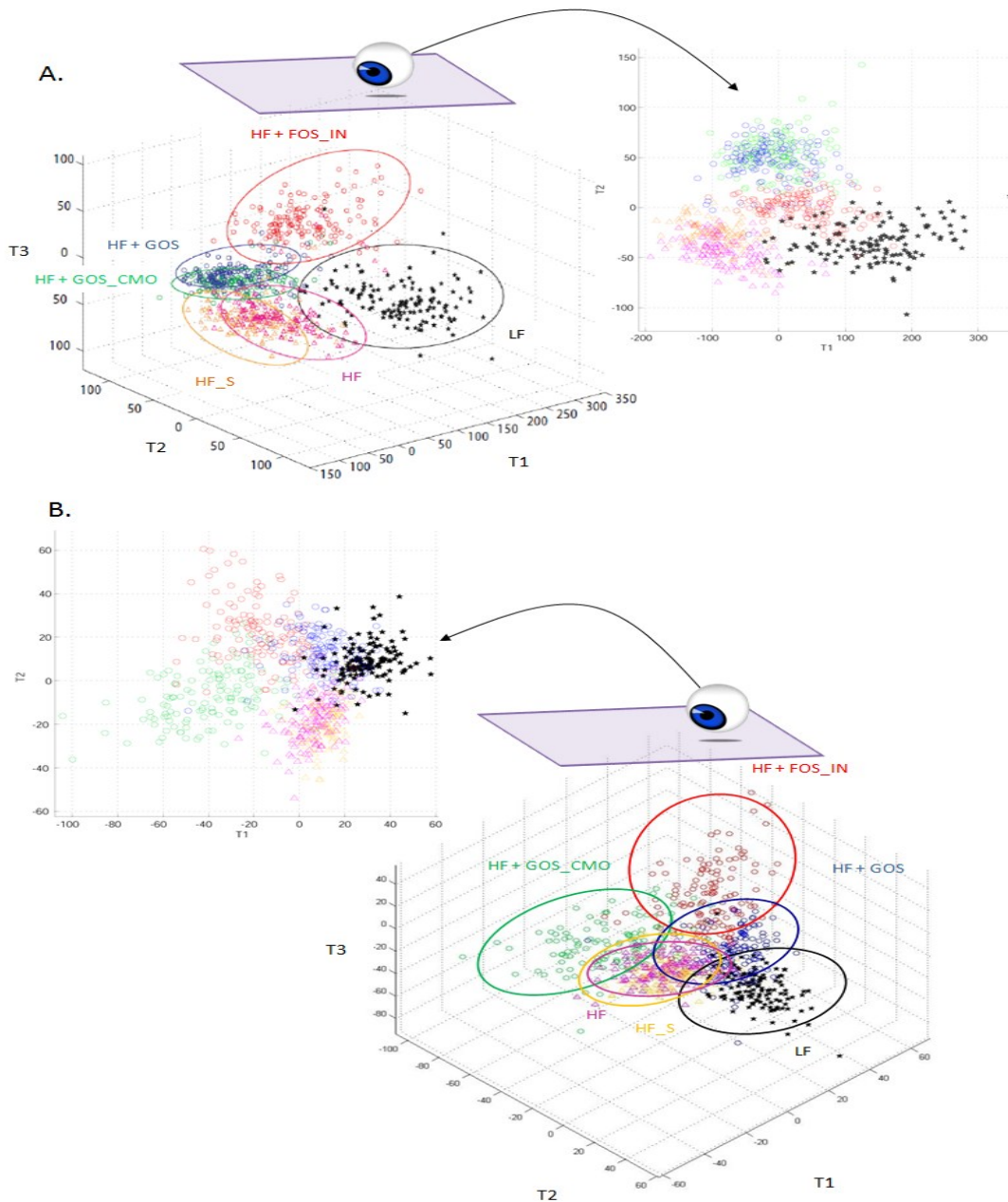


Figure 34: Global urinary (A) and fecal (B) metabolic profile of each diet group. All urine (n=826) or fecal (n=785) samples acquired from 7 days (D7) to 70 days (D70) after the diet switch were used to calculate OPLS-DA models. For each matrix, 3D scores plots showed the three dimensional dispersion of the scores of each diet group across time. The 2D projections indicate the biggest variability in the dataset along the first and the second component. Both models were built with 6 predictive and 2 orthogonal components. $Q^2Y=0.46$ and $R^2Y=0.49$ for urinary metabolic profile. $Q^2Y=0.24$, $R^2Y=0.46$ for fecal metabolic profile: LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF + GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin)

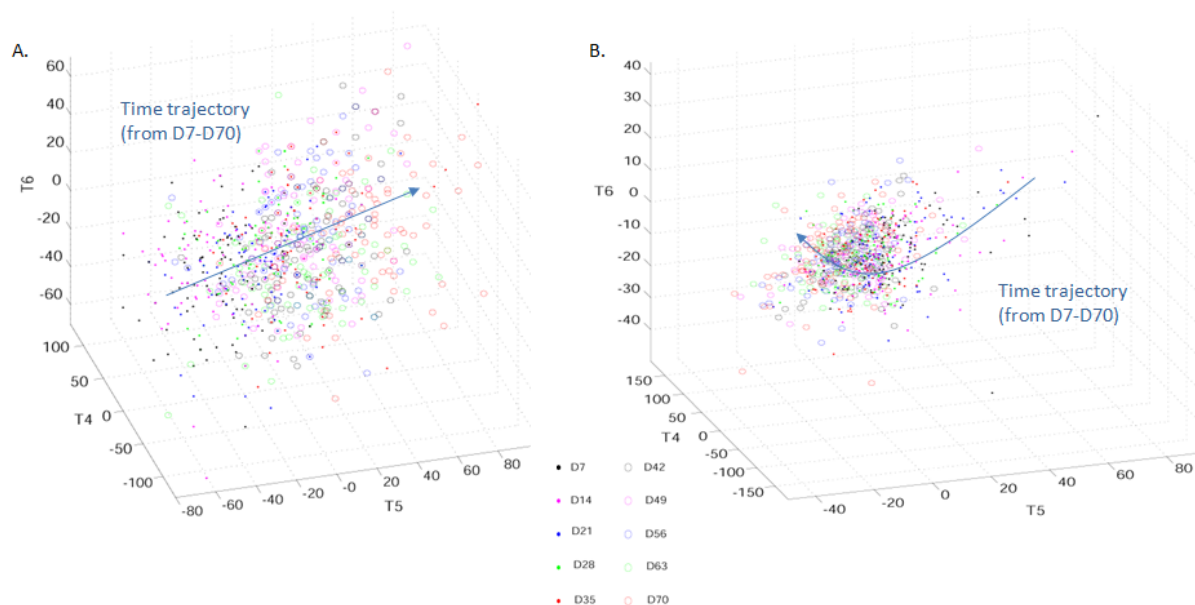


Figure 35: OPLS-DA score plot from global urinary (A) and fecal (B) metabolic profile highlighting the time-dependent trajectories: Urine (n=826) or fecal (n=785) samples acquired from 7 days (D7) to 70 days (D70) after the diet switch were used to calculate OPLS-DA models. For each matrix, 3D scores plots showed the three dimensional dispersion of the scores on the 4th, 5th and 6th component which highlights the time trajectory.

3.2.4. Comparison of urinary, fecal and plasma metabolic profiles of LF, HF and each HF + prebiotic groups using OPLS-DA emphasize prebiotics induced metabolic rescue.

Long-term effects of each prebiotic on mouse metabotype were further investigated by using OPLS-DA models at D70 after the diet switch. OPLS-DA scores plots (Figure 36) exhibited the various group comparisons for each biofluid and enabled to visually decompose the metabolic adaptation associated to HF, the partial normalisation of the metabolic profile induced by the prebiotic supplementation and the specific metabolic responses associated to prebiotic supplementation (statistics of the models in Table 6). The range of metabolic adaptations to each diet group varies according to the biofluid. In fact, on the OPLS-DA scores plot built from urinary metabolic profile (Figure 36.A), the first component discriminated LF-fed mice and HF-fed mice (with or without prebiotics supplementation). The second component highlighted the secondary effects, which were mainly characterised by the specific effects of prebiotics. The urinary metabolic profiles of mice fed with HF + prebiotics and particularly HF + FOS_IN were also partially normalised

compared to the negative control group as their scores got closer to those of LF-fed mice. On the contrary, the main source of variation in fecal metabolic profiles was associated with specific effects of prebiotics supplementation, as the biggest separation was observed between HF and HF + prebiotic along the first component (Figure 36.B). The separation of LF and both HF groups was mainly noticeable on the second component. The comparison of plasma metabolic profiles between the different groups (Figure 35.C) showed a combination of diet and prebiotic effects influencing the dataset. HF ingestion during a long time period triggered the main variation in the systemic metabolic profile as LF and HF group were discriminated along the first component. However, the long-term supplementation of prebiotic during HF challenge lead to a significant improvement of the metabolic profiles as the score plots of all HF + prebiotics groups were significantly closer to those of LF group in each score plot. The second component mainly highlighted the separation between LF and HF + prebiotics, which was associated with prebiotic specific effects. The decomposition of the effect involved in metabolic variations pointed out that HF ingestion strongly disturbed circulating metabolic flux and excretion of C57BL/6 mice which was characterised by a modulation of urinary and plasma metabolic profiles. On the other hand, prebiotic supplementation tended to specifically affect nutrient ingestion and gut metabolism as it triggered the greatest variability in fecal metabolic profiles.

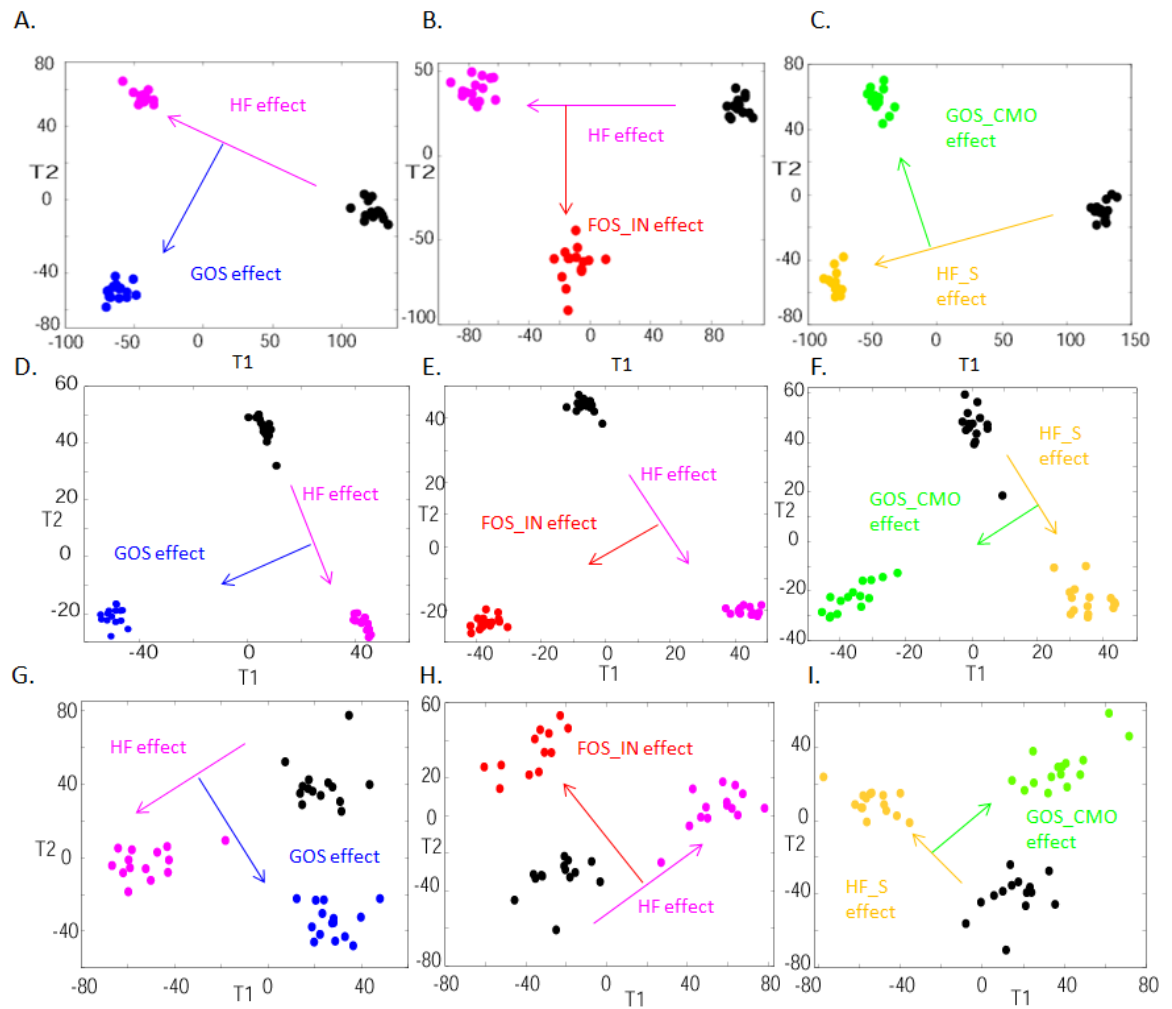


Figure 36: Identification of specific metabolic effects and partial normalisation of metabolic profiles by prebiotic supplementation in urine (A, B, C), feces (D, E, F) and plasma (G, H, I) after 70 days of HF diet. For each biological matrix, OPLS-DAs were calculated with LF, HF and HF + GOS groups (A, D, G), or LF, HF and HF + FOS_IN (B, E, H) or LF, HF_S and HF + GOS_CMO (C, F, I). Q^2Y and R^2Y for each model are given in Table 6.. LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin)

Table 6: Statistics of global OPLS-DAs models and OPLS-DAs models with three groups generated with ¹H NMR urinary, fecal or plasma metabolic signatures. (PC: predictive components, OC: orthogonal components)

OPLS/OPLS-DAs	Class/continuous variables (Y)	time points	components (PC+OC)	R ² Y	Q ² Y	<i>p value</i>
Urine	LF, HF, HF_S, HF+GOS, HF+GOS_CMO, HF+FOS_IN, time	from D7 to D70	6+2	0.49	0.46	0.01
	LF, HF, HF + GOS	D70	2+4	0.97	0.88	1 10 ⁻³
	LF, HF, HF + FOS_IN	D70	2+4	0.9682	0.8519	1 10 ⁻³
	LF, HF_S, HF + GOS_CMO	D70	2+5	0.97	0.8922	1 10 ⁻³
Feces	LF, HF, HF_S, HF+GOS, HF+GOS_CMO, HF+FOS_IN, time	from D7 to D70	6+2	0.46	0.24	0.03
	LF, HF, HF + GOS	D70	2+3	0.96	0.63	1 10 ⁻³
	LF, HF, HF + FOS_IN	D70	2+2	0.95	0.5301	1 10 ⁻³
	LF, HF_S, HF + GOS_CMO	D70	2+2	0.96	0.37	1 10 ⁻³
Plasma ¹ H NMR cmpg spectra	LF, HF, HF + GOS	D70	2+3	0.91	0.38	0.03
	LF, HF, HF + FOS_IN	D70	2+3	0.94	0.35	0.02
	LF, HF_S, HF + GOS_CMO	D70	2+4	0.92	0.49	0.02

To confirm these observations, we aimed at estimating the percentage of “metabolic rescue” of urinary, fecal and plasma metabolic profiles induced by the supplementation of prebiotic during HF feeding (Figure 37). This percentage was estimated by predicting the scores of HF + GOS, HF + FOS_IN and HF + GOS_CMO onto OPLS-DA models generated with the negative control group (LF) and the positive control group (HF or HF_S). Therefore, a metric of the metabolic adaptation is built based on the scores of LF and HF groups. The projections of the scores of the HF + prebiotic groups onto this metric are exclusively driven by changes in metabolic pathways affected during HF challenge and not by prebiotics derived metabolites. Significant differences between the predicted scores and the score of the negative control group (LF) or the positive control group (HF or HF_S) were estimated by non parametric Wilcoxon-Mann-Whitney tests (Table 7). Importantly, FOS_IN but not GOS and GOS_CMO supplementation induced significant “metabolic rescue” in urine. (HF + GOS: 0.5% *p value*=0.09, HF + FOS_IN: 16.9 %, *p value*= 9.110⁻⁴, HF + GOS_CMO: 7.3% *p value*=0.11) However, all prebiotics triggered significant “metabolic rescue” in feces and plasma (Table 7). As previously observed, the “metabolic rescue” induced by prebiotics was more prominent in plasma (HF + GOS: 56.6%, HF + FOS_IN: 58.8%, HF + GOS_CMO: 51.7%) than feces (HF + GOS: 13.5%, HF + FOS_IN: 38.5%, HF + GOS_CMO: 49.5 %). This indicates that FOS_IN, GOS and GOS_CMO enhanced circulating metabolite fluxes, which are crucial to maintain homeostasis in the organism. FOS_IN

triggered a greater “metabolic rescue” of urinary and plasma metabolic profiles suggesting that it was the most effective prebiotic for the maintenance of homeostasis. On the other hand, GOS_CMO had higher percentage of metabolic rescue in fecal metabolic profiles, which may indicate a greater ability to rescue the pattern of nutrient absorption.

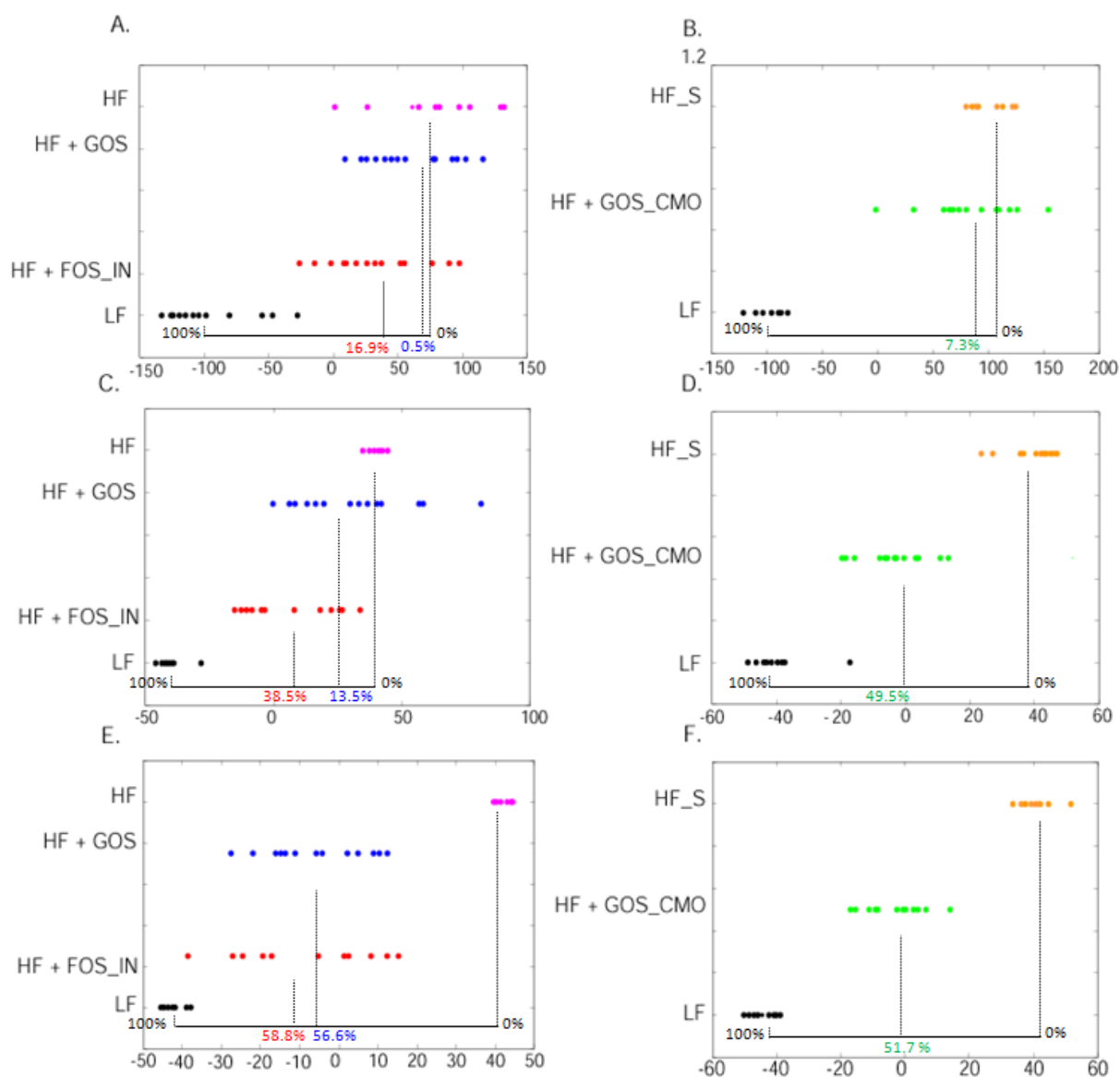


Figure 37: Evaluation of the “metabolic rescue” of different prebiotics during HF challenge in urine, feces and plasma. The metabolic rescue of FOS_IN and GOS was estimated (in percentage) by predicting the score of HF + GOS and HF + FOS_IN groups onto the first component of OPLS-DA model built with urinary (A), fecal (C) or plasma (E) metabolic profile of HF and LF groups. The “metabolic rescue” of GOS_CMO was calculated by predicting HF + GOS_CMO scores onto the first predictive component of the OPLS-DA models built with urinary (B), fecal (D) or plasma (F) metabolic profile of LF and HF groups. Negative control group (LF), positive control group (HF , or HF_S) and test groups (HF + FOS_IN , HF + GOS or HF + GOS_CMO) LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin).

Table 7: Significance (*p values*) of the differences between the predicted scores (FOS_IN, GOS, or GOS_CMO) and the negative control group (LF) or the positive control group (HF or HF_S), *p values* were calculated by non parametric Wilcoxon-Mann-Whitney tests

<i>p values</i>	Urine	feces	plasma
LF vs GOS	3.65 10 ⁻¹²	1.35 10 ⁻¹²	1.04 10 ⁻¹¹
HF vs GOS	0.09	0.04	1.88 10 ⁻¹⁴
LF vs FOS_IN	2.11 10 ⁻⁰⁹	5.56 10 ⁻¹²	5.03 10 ⁻⁰⁸
HF vs FOS_IN	9.18 10 ⁻⁴	7.29 10 ⁻⁰⁸	1.02 10 ⁻¹⁰
GOS vs FOS_IN	0.01	5.00 10 ⁻³	0.41
LF vs GOS_CMO	6.69 10 ⁻¹⁵	2.14 10 ⁻⁰⁹	7.93 10 ⁻¹⁶
HF_S vs GOS_CMO	0.11	2.96 10 ⁻⁰⁹	7.03 10 ⁻¹⁷

3.2.5. ¹H NMR spectral data of urine, feces and plasma samples at D70 were compared between the diet groups using pair-wise OPLS-DA models

In each biological matrix, metabolic variations associated with different HF feeding were derived by comparing metabolic profiles of LF group with either the metabolic profiles of HF, HF_S or each HF + prebiotic groups at D70 after the diet switch. To identify the individual metabolic response of each prebiotics, metabolic profiles of HF + GOS or HF +FOS_IN group were compared with those of HF group. Similarly, metabolic profiles of HF + GOS_CMO group were compared with those of HF_S group. Metabolic variation between mice fed with HF or HF_S diets were also investigated to evaluate the impact of free sugars and particularly lactose supplementation on the mouse metabolome during HF challenge. The heatmaps (Figure 38) showing metabolites with highest correlation coefficient values provide clearer information about the metabolic variations associated to the ingestion of HF, to the supplementation of prebiotics or lactose and the variation in the level of common metabolites between the different groups.

High-fat ingestion strongly disturbs mouse fecal, plasma and urinary metabolic profile.

Consumption of HF diet for 70 days led to a disruption in nutrient digestion process. In particular, the bile acid pool responsible for the emulsion and digestion of lipids increased in feces of HF-fed mice (Figure 38.D)¹⁵². Fecal glycerate level was also higher in HF and HF_S fed mice whereas fecal valine level exclusively increased in HF group (Figure 38.D).

Long-term HF feeding markedly affected plasma metabolic profiles which were characterised by an increase in D-glucose, LDL and unsaturated lipids in ^1H NMR standard spectra of the HF and HF_S groups (Figure 38.A, Figure 38.B). Leucine, valine and citrate levels were also similarly reduced in plasma of the same groups. In addition, resonances of *scyllo*-inositol and *myo*-inositol were significantly lower in all HF containing diet groups excepted for the HF + GOS group in which *myo*-inositol level was comparable to those of the LF group.

HF diet with or without prebiotic supplementation was remarkably associated with a urinary metabolic signature involving an increase in branched chain amino acids (BCAAs) catabolism intermediates (ketoleucine, α -keto- β methylvalerate and α -ketoisovalerate) and an increase in putrescine (Figure 38.C). TMA is produced by gut microbial degradation of choline, and TMAO is formed in the liver by TMA detoxification process. Both metabolites significantly decreased in urinary profiles of all HF groups compared to the LF group with the exception of the HF + FOS_IN group which had urinary TMAO level comparable with those of the LF group. With the exception of HF + FOS_IN group, urinary metabolic profiles of HF, HF_S and HF + prebiotics groups were characterised by an increase in glucose and unassigned *o*-acetyl-glycan. This result confirms the physiological data which indicated that FOS_IN supplementation during HF challenge enabled to maintain normal fasting glycaemia over time. HF, HF_S, HF + GOS, HF + GOS_CMO but not HF + FOS_IN groups were associated with an increase in 2 PY and 4 PY which are end-products of NAD/NADP pathways and an increase in carnitine known as an essential transporter allowing fatty acid (FAs) uptake in mitochondria for β -oxidation. Sucrose resonances were also decreased in HF and HF + prebiotic groups compared to LF group. This is explained by the fact that LF diet, rich in carbohydrate, contained more sucrose than all HF diets (see Appendix 2, Table S1). However a urinary increase in sucrose was noticed in the HF + GOS_CMO group compared to the HF_S group even though none of the diets were supplemented in sucrose. The presence of sucrose in HF + GOS_CMO diet may arise from the commercial fibres or CMO blends.

Supplementation with GOS, GOS_CMO and FOS_IN triggered various metabolic rescue effects.

The estimation of “metabolic rescue” of prebiotics in each matrix indicated that prebiotics consumption during HF challenge improved the metabolic profiles by reducing the range of metabolic variations observed between mice susceptible to develop obesity (HF and HF_S groups) and healthy mice (LF group). OPLS-loadings plots interpretation from pair-wise OPLS-DA models enabled us to identify which metabolites are significantly recovered by prebiotic supplementation in each matrix. In particular, prebiotic supplementation rescued lipid levels and circulating LDL as no

difference in standard ^1H NMR spectra from plasma was noticed between LF and HF + prebiotics groups with the exception of HF + GOS_CMO group, which had higher LDL level than LF group. GOS and FOS_IN supplementation specifically enabled to maintain plasma *scyllo*-inositol and citrate levels similar to those of the LF group. As previously mentioned, FOS_IN supplementation improved the urinary metabolic signatures, as it maintained the urinary concentration of D-glucose, unassigned *o*-acetyl-glycan, TMAO, indoxylsulfate, carnitine, 2 PY and 4 PY similar to those observed in the LF group.

Prebiotic supplementation triggered common metabolic regulations

Prebiotic supplementation during HF challenge initiated a common urinary metabolic signature involving gut microbial activity. The excretion of PCS, PCG, phenylacetyl-glycine (PAG) and hydroxyphenylacetic acid, which are end-products of gut bacterial amino acid metabolism, were found in significantly lower levels in urinary metabolic profiles of all HF + prebiotics compared to HF, HF_S and LF groups. Common changes in fecal metabolic profiles were observed as all prebiotics induced an up-regulation of 3-D-hydroxybutyrate, butyrate and succinate compared to LF and HF and HF_S groups.

The differential metabolic signature associated with prebiotics supplementation results from a complex interaction between diet composition and secondary metabolism.

Each prebiotic supplementation induced a specific metabolic signature, which resulted from a complex interaction between diet composition and secondary metabolism. The increased urinary galactose and glucose excretion in HF + GOS fed mice or HF + GOS_CMO fed mice was exclusively due to a higher galactose and glucose supply from the diet as GOS fibres were made from polymers of galactose and glucose. On the other hand, GOS and FOS_IN groups were associated with a urinary increase in α -ketoisovalerate and ketoleucine respectively, characterising a modulation of BCAAs metabolism. FOS_IN supplementation also induced higher circulating levels of pyruvate, acetate and 3-D-hydroxybutyrate whereas GOS_CMO supplementation was positively correlated with urinary carnitine and acylcarnitine excretion. These results suggest prebiotics specific variations of mitochondrial oxidative pathways and energy metabolism. In addition, fecal metabolic profiles of HF + FOS_IN and HF + GOS_CMO fed mice were marked by a higher excretion of xanthine and uracil, which are respectively purine and pyrimidine base.

Free sugars supplementation effects during HF challenge

There was no significant difference in fecal, and plasma metabolic profile (CPMG and standard ^1H NMR spectra) between HF and HF_S groups indicating that lactose ingestion does not induce significant variation in plasma and fecal metabolism as detected by ^1H NMR analysis. However, urinary metabolic profile exhibited an increased concentration of galactose and glucose in HF_S fed mice and an increased concentration of sucrose in HF-fed mice. This is explained by the fact that sucrose was the only carbohydrate source in HF diet. This disaccharide is predominantly digested by sucrase in the small intestine to form glucose and fructose which are in turn absorbed by the mucosal barrier and further metabolized in the liver¹⁵². However, a small amount of undigested sucrose can directly cross the intestinal barrier and reach the blood or being bypassed in urine¹⁸⁶. In case of inadequate nutrition, stress, or critical illness, sucrase activity can be altered and/or mucosal barrier integrity can be compromised leading to increased absorption of undigested sucrose¹⁸⁷. In the present experiment, long-term ingestion of HF diet may modify mucosal permeability and brush border enzymes activity such as sucrase, favouring sucrose absorption.

On the other hand, HF_S diet was exclusively supplemented by free sugars i.e: glucose, galactose and lactose (glucose+galactose). The increased urinary glucose level in HF_S fed mice might arise from dextrose absorption or from lactose digestion into glucose and galactose followed by the absorption of these free sugars. HF_S diet also induced a decrease in PAG and PCS in comparison to HF diet. This suggested that free sugars supplementation during HF challenge affects gut bacterial activity and/or host conjugation metabolism compared to sucrose supplementation.

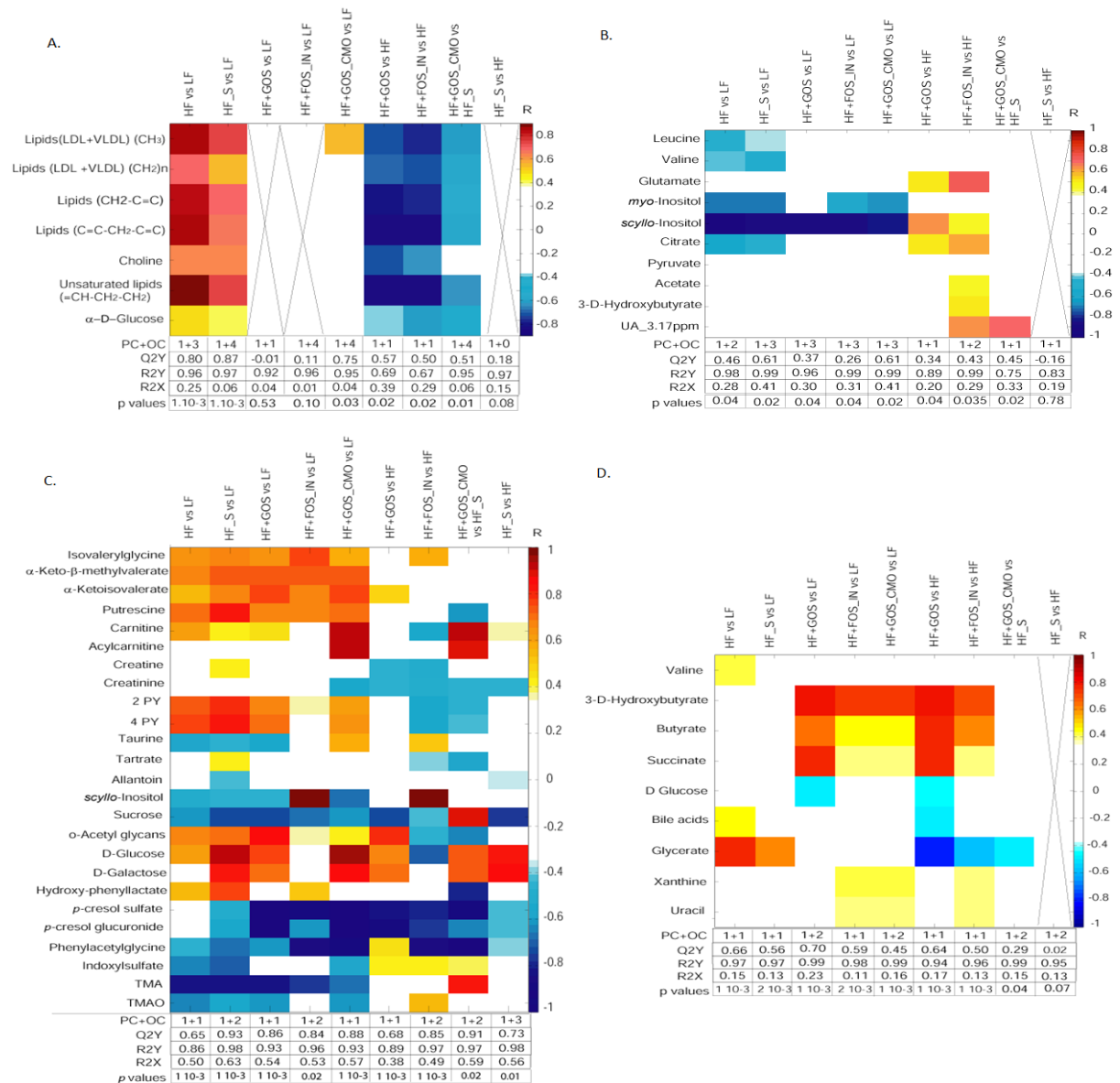


Figure 38: Comparison of metabolic signatures associated to different diet groups. (A) Standard ^1H NMR plasma metabolic profiles (B) CPMG ^1H NMR plasma metabolic profiles (C) Standard ^1H NMR urinary metabolic profiles (D) Standard ^1H NMR faecal metabolic profiles. Correlation values (R) of the metabolites are displayed by color code. Gradient of red color are for metabolites positively correlated with HF or HF_S (in LF vs HF or LF vs HF_S models) or HF + prebiotics (in LF vs HF+prebiotics or HF vs HF + prebiotic models). The gradient of blue colors are for metabolites negatively correlated with the same diet groups LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin) (PC: predictive components, OC: orthogonal components).

3.2.6. Targeted LC/direct infusion MS analysis of plasma samples reveals strong variations in lipid and amino acid metabolism with prebiotic supplementation after 70 days of HF challenge.

Targeted MS analysis of plasma samples using internal isotopically labeled standards was performed to further investigate the regulation of lipid and amino acid metabolism in different diet groups. A global OPLS-DA model comparing all diet groups together was performed to identify the general difference in lipid and amino acid profile between groups. The Y matrix of the models was built with 5 dummy variables to categorise the different diet groups in separate classes (statistics of the model in Table 8). The OPLS-DA score plot (Figure 39.A) showed a good separation between the negative control group (LF) the positive control groups (HF and HF_S groups) and the prebiotic groups (HF + GOS, HF + GOS_CMO, HF + FOS_IN). The corresponding loadings plot (Figure 39.B) highlighted HF associated increase in phosphatidylcholines (PCs and PC-Os), sphingomyelins (SMs) and lysophosphatidylcholines (LPCs), which is in accordance with ¹H NMR plasma metabolic profiles indicating higher lipid content in circulation with HF diets. On the other hand, several lysophospholipids (LPCs), acylcarnitines and amino acids followed an opposite trend as they were positively correlated with LF group or prebiotics groups. Additionally, pair-wise OPLS models were calculated to obtain correlations between metabolites and group separation (statistics of the models in Table 8). For each model, significant correlations were used in a correlation matrix to determine common patterns of regulation of circulating lipids and amino acids between groups. The correlation matrix emphasised several metabolic clusters (Figure 40) indicating significant trends in lipids and amino acids variations. The cluster grouping PC and PC-O indicated that HF ingestion with or without prebiotic supplementation triggered an increase in long chain fatty acid (LCFAs) containing phosphatidylcholines with various levels of unsaturations. In addition, SM C16, SM C16:1, SM-OH C16:0, SM C18:0, SM C18:1 and LPC C17:0 and C18:0 were positively correlated with HF containing diets. On the other hand, LPC a C18:1 and LPC a C16:1 increased in LF group compared to all HF diet groups as observed on LPC cluster (Figure 40). Interestingly, the comparison of correlation coefficients between OPLS-DA model built with LF and HF groups or LF and HF_S groups showed that ingestion of HF_S tended to increase the level of circulating LCFAs and very long chain fatty acids (VLCFAs) containing PCs with several insaturations. However, some of these lipids (such as PC C30:0, C30:2, C40:6 and PC-O C30:0 and PC-O C38:4) were negatively correlated with HF + GOS_CMO compared to HF_S (as indicated in the 8th column in the correlation matrix). This result highlighted that GOS and CMO supplementation during HF challenge improved circulating LCFAs containing lipids. It is also important to note that no significant difference in plasma lipid and amino acids

profile between HF and HF_S groups was identified by OPLS-DA model suggesting that lactose and free sugars supplementation in HF diet had a subtle effect on lipid metabolism. Lipid profiles of HF and HF_S fed mice were also marked by a reduction in short chain acetyl-carnitines (C2), propionyl-carnitine (C3), butyryl-carnitine (C4), isovaleryl-carnitine (C5), palmitoyl-carnitine(C16), palmitoleyl-carnitine C16:1 oleyl-carnitine (C18:1) (Figure 40). Prebiotic supplementation during HF challenge allowed to recover acylcarnitine profiles as C2, C3, C4, C5 increased in HF + prebiotic diets compared to HF or HF_S diets and were not significantly different between the HF + prebiotics and the LF group. In addition, the linoleic acid (C18:2) which is an essential FA exclusively increased in mice fed with HF + prebiotic.

Circulating amino acid profile was not significantly modulated by HF ingestion and prebiotic supplementation with the exception of FOS_IN supplementation that triggered an increase in several amino acids during HF challenge as observed in the “amino acids” cluster. In addition, circulating kynurenine, which is the end-product of tryptophan degradation, was found in higher levels in LF group compared to all HF containing diet groups excepted for HF + FOS_IN. All together, these results indicated a specific regulation of amino acid metabolism in HF + FOS_IN fed mice.

Overall, targeted MS method are corroborating the results obtained by untargeted ^1H NMR profiling as significant increase in circulating lipids in HF-fed mice compared to LF-fed mice were observed. Targeted MS analysis gives also additional information in lipid class differences between the diet groups. Interestingly, it showed that HF + prebiotics fed mice exhibit an increase in several lipid classes compared to LF-fed mice whereas no significant difference in circulating lipids content was found by ^1H NMR between these groups. In addition, the targeted MS method, which offers a better sensitivity (see Chapter II, section 2.3 for more details on the MS targeted analysis) enabled us to spot difference in several amino acids between HF + FOS_IN and HF-fed mice not detectable by the ^1H NMR untargeted metabolic explanation approach. Therefore, MS targeted method provide highly reliable results on semi-quantitative metabolite differences and improve the level of information on specific metabolic pathways already selected by ^1H NMR untargeted profiling.

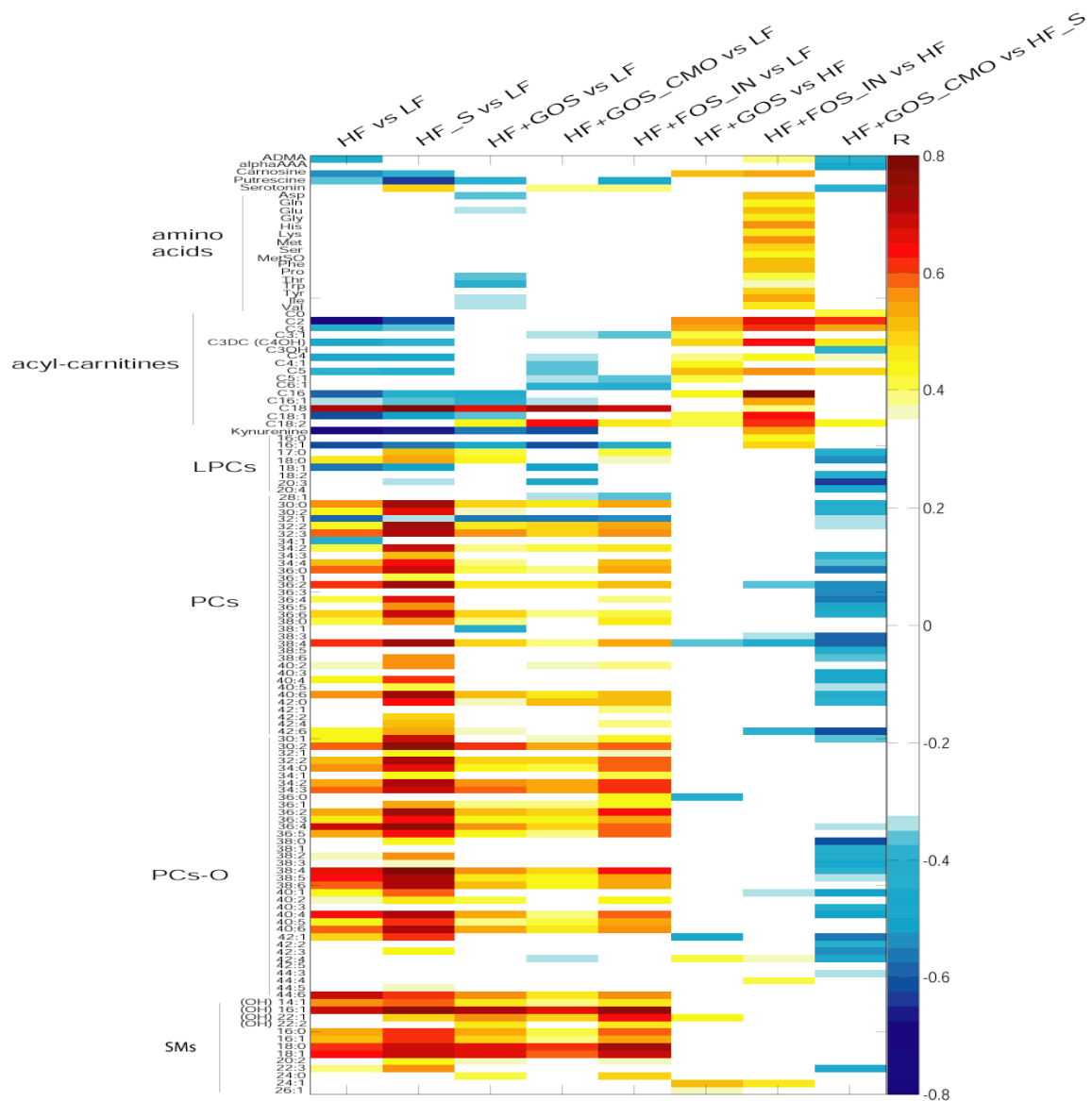


Figure 40: Comparison of the plasma metabolic signature acquired by MS targeted profiling analysis among the different diet group at D70 after the diet switch. The correlation matrix summarises the pair-wise OPLS-DA models between the different diet groups. Each column represent an OPLS-DA model comparing the two groups groups indicated above each column, and illustrates the variation in circulating levels of lipids, amino acids, acylcarnitines and other metabolites. For each model, correlation coefficients were obtained from the OPLS-DA coefficient plot. Correlation values (R) of the metabolites are displayed by color code. Gradient of red colors is for metabolites positively correlated with HF or HF_S (in LF vs HF or LF vs HF_S models) or HF + prebiotics (in LF vs HF+prebiotics or HF vs HF + prebiotic models). The gradient of blue colors is for metabolites negatively correlated with the same diet groups. Cx:y is the abbreviation of lipid chain (x :the number of carbons in the side chains, Y: number of insaturations). LF (low fat), HF (high fat), HF_S (HF + glucose-galactose-lactose), HF + GOS (HF + galactosyl-oligosaccharides), HF+ GOS_CMO (HF + GOS and cow milk oligosaccharides), HF + FOS_IN (HF + fructosyl-oligosaccharides and inulin). LPC (lysophosphocholine) PC/PC-Os (phosphocholines) SMs (sphingomyelins).

Table 8: Statistics of the OPLS-DAs models calculated with MS targeted metabolic profiles of plasma samples at D70 after the diet switch (PC: predictive components, OC: orthogonal components).

Groups	components (PC+OC)	R ² Y	Q ² Y	<i>p value</i>
LF, HF, HF_S, HF+GOS, HF+FOS_IN, HF+GOS_CMO	4+1	0.48	0.23	0.04
LF vs HF	1+2	0.99	0.95	1 10 ⁻³
LF vs HF_S	1+2	0.98	0.95	1 10 ⁻³
LF vs HF+GOS	1+4	0.99	0.91	1 10 ⁻³
LF vs HF+GOS_CMO	1+2	0.98	0.94	1 10 ⁻³
LF vs HF+FOS_IN	1+2	0.98	0.88	1 10 ⁻³
HF vs HF+GOS	1+3	0.97	0.67	1 10 ⁻³
HF vs HF+FOS_IN	1+1	0.92	0.86	1 10 ⁻³
HF_S vs HF+GOS_CMO	1+2	0.95	0.73	1 10 ⁻³
HF vs HF_S	1+1	0.8	-0.51	0.8

4. DISCUSSION

After a pre-intervention period of 3 weeks (where all mice were fed a LF diet), C57BL/6 mice were divided among 6 groups of similar weight and glycemia and fed with either LF, HF, HF_S, HF+GOS, HF+GOS_CMO or HF+ FOS_IN diets for 70 days. Physiological measurement (BWG, food intake, glycemia, insulinemia, fat mass) were performed across the experiment and circulating cytokines were measured at D70 after the diet switch. ¹H NMR metabolomic analysis (urine, plasma, feces) and targeted MS analysis (plasma) were carried out across the experiment and particularly at D70 after the diet switch.

The present study showed that long-term HF challenge triggered progressive development of obesity, disturbed lipoprotein profiles and caused fasting hyperinsulinemia and hyperglycaemia. After 70 days, the physiological status of HF and HF_S fed mice met enough criteria (hyperinsulinemia, hyperglycaemia, and obesity) for the metabolic syndrome which is known to raise the occurrence of metabolic diseases such as T2DM NAFLD and CVDs¹⁸⁸. Our results also demonstrated that supplementation of different prebiotics during HF challenge led to a strong and similar preventive effect on BWG as well as a comparable reduction in fat mass. Inflammatory responses associated with long-term HF diets were also partly reduced with prebiotic supplementation. In addition, by using a ¹H NMR and targeted MS profiling of urine, plasma and feces, specific disruption of energy metabolism and gut microbial–host metabolites interactions were identified during HF challenge which are partly prevented with prebiotic supplementation. This study highlighted the role of prebiotics in the improvement of mouse phenotype and metabolic homeostasis under diet induced obesity (DIO).

4.1. HF ingestion induce a saturation of hepatic lipid metabolism

¹H NMR and MS profiling both showed an increase in serum lipids and PCs content for HF and HF_S fed mice. In particular, an increase in LDL in HF and HF_S fed mice compared to LF-fed mice was associated with an increase in LCFAs and VLCFAs containing phosphocholines in both HF groups. Importantly, plasma collections were carried out at fast which provides insight about endogenous lipid recirculation with the different diet interventions. During a fasting period, triglycerides (TGs) are broken down into FAs in the adipose tissues which are in turn released in the

peripheral tissues (see introduction). FAs in peripheral tissues (such as kidney, muscle, liver) are finally degraded *via* mitochondrial β -oxidation to produce ATP. However, in the context of prolonged HF feeding leading to high calorie supply, lipids in the liver can be redirected toward peripheral tissues in the fasting period¹⁸⁹. Hepatic TG, PCs or SMs are compartmented in VLDL and secreted in the blood. TGs packed in VLDL reach capillaries in organs and tissues and are degraded into FAs by lipoprotein lipase (LPL). FAs are finally absorbed in tissues and organs¹⁵². Therefore, high fat ingestion is often associated with increased FAs in circulation and dyslipidemia^{49,189}.

In this experiment, it is most likely that the increase in several circulating PCs and lipoproteins during a long-term HF challenge was triggered by a saturation of hepatic lipid storage. The overproduction of hepatic TGs associated with HF ingestion and impaired glucose metabolism might promote lipid exportation from the liver to peripheral tissues where they can be used to produce energy or being transformed and stocked into TGs. This ectopic fat accumulation is commonly observed in obese subjects and contributes to host metabolic dysregulation (see introduction)¹⁸⁹.

HF diet was also associated with an increase in LPCs and SMs. Alteration of lipid composition is believed to play a role in chronic inflammatory and metabolic disorders *via* various signalling pathways. LPCs are one of the most important oxidation products derived from phosphatidylcholine in oxidized LDL. This biologically active class of lipids has been thought to promote the release of cytokines by activating the TLR-4, affects the permeability of endothelial cells and raise oxidative stress. LPCs may also impair insulin secretion *via* the activation of protein kinases (IK β and JNK) (see Chapter I, section 1.4)¹⁹⁰. SMs obtained *via* the transfer of phosphocholine head groups from phosphatidylcholine onto ceramides are also biologically active molecules. They interact with G-protein receptors that result in activation of downstream signalling pathways including the inhibition of phosphatidylinositol-3-kinase (PI3K)/Akt protein kinases pathways^{191,192}. Both LPCs and SM have been shown to increase in genetically obese rats or mice^{193,194}. Although physiological functions of these lipids are still under investigations, it seems clear that HF ingestion leading to an increase in circulating lipids in the form of SMs and LPCs and PCs contributes to the deregulation of glucose metabolism, inflammation and the development of metabolic diseases. To summarise, long-term HF feeding triggered dyslipidemia and increased several biologically active lipids in circulation probably due to an hepatic TG saturation. This might contribute to DIO associated metabolic and immunological disturbances.

4.2. HF challenge alter mitochondrial oxidation efficiency

Circulating acylcarnitines profiles at fast were also modulated with long-term HF challenge as indicated by an increase in C18, and a decrease in C2, C4, C18:1, C18:2, C16 and C16:1 in HF and HS_S mice compared to LF-fed mice. Carnitine is essential for the transport of FAs from the cytosol to the mitochondria for subsequent β -oxidation. Acylcarnitines are carnitine-esters derived from FAs or amino acids transferred into the mitochondria. While odd acylcarnitines exclusively result from the BCCA catabolism even acylcarnitines are produced from β -oxidation and C4 can be produced from both¹⁵².

Decreased in C16:1, C18:1, C18:2 in HF and HF_S mice suggests a greater mitochondrial β -oxidation of long chain mono or poly-unsaturated FAs with HF diet. In addition, a decrease in short chain fatty acylcarnitines (C2, C4) also support the hypothesis of an effective β -oxidation of SCFAs. On the other hand, higher circulating C18:0 in HF and HF_fed mice might reflect an incomplete oxidation of saturated FAs. Altered circulating acylcarnitines profiles have been already observed in obese and T2 DM subjects^{195,196}. Moreover, reduced skeletal muscle β -oxidation of LCFAs and MCFAs has been already associated with insulin resistance and T2DM¹⁵¹. Our results indicate a differential mitochondrial efficiency in FAs oxidation with HF challenge which might contribute to impaired glucose metabolism and metabolic disturbances¹⁵¹. This might be explained by a mitochondrial fat overloading leading to altered β -oxidation and generation of incomplete β -oxidation end-products (C18:0).

In the previous Chapter III (section 3 and 4), a urinary increase in BCAAs intermediates, TCA cycle intermediates and NAD pathways end-products (2 PY, 4 PY) were identified in HF-fed mice compared to LF-fed mice 7 days and 60 days after the diet switch. From these findings, HF diet has been suggested to provoke a rapid and consistent up-regulation of BCAAS, β -oxidation and TCA cycle which might lead to a saturation of mitochondrial oxidative pathways^{151,153,170}. In the present experiment, urinary metabolic profiles of HF and HF_S fed mice also exhibited a higher excretion of BCAA catabolism intermediates (isovalerylglycine, α -keto- β methylvalerate, α -ketoisovalerate), 2 PY and 4 PY than LF-fed mice which highlight a saturation of mitochondrial oxidative pathways and energy metabolism in the context of HF induced obesity. By contrast, the concentration of short-chain acylcarnitines (C2, C3, C4, C4-OH, C5) decreased in plasma of HF and HF_S fed mice. This suggests a reduced transport of BCAA catabolism end-products in the general circulation and increased clearance of intermediate metabolites of BCAA catabolism in urine. Altogether, these results highlight an altered activity of β -oxidation and BCAA catabolism in HF-fed mice which might

results from an mitochondrial oxidative fuel overloading. Mitochondrial activity, which play a crucial role in energy production have been found to affect insulin signalling¹⁵¹. Therefore, impaired mitochondrial metabolism may be the triggering factor of insulin resistance and host metabolic disturbances in DIO.

4.3. Prebiotic supplementation during HF diet limit dyslipidemia improves mitochondrial oxidative pathways.

This experiment showed that supplementation of different prebiotics during HF challenge led to a strong and similar limitation of BWG as well as a comparable restriction of fat mass. In addition, prebiotic supplementation induced specific metabolic regulations allowing a partial return of internal homeostasis as characterised by a significant proportion of “metabolic rescue” in feces, and plasma of HF + prebiotics fed mice. Interestingly GOS and GOS_CMO induced “metabolic rescue” was significant in feces and plasma but not in urine. In addition, the metabolic rescues of FOS_IN were significant in all biological matrices but more important in feces and plasma than in urine. In a perturbed system such as HF-fed mouse, plasma is strongly regulated to maintain effective biological processes but this requests the activation of specific metabolic pathways allowing compensation for the metabolic stress. Urinary metabolic profiles on the other hand, contain end-products of metabolic pathways and products of degradation from the organism; hence it reflects the “homeostatic effort” i.e the prebiotic-specific metabolic adaptation necessary for the organism to maintain “this biological robustness”.

¹H NMR and MS metabolomic data exhibited a partial rescue of lipid profiles toward a healthy status due to prebiotic supplementation. In particular, no difference in plasma lipoproteins were found between LF and HF + prebiotic fed mice at D70 after the diet switch was noticed. This highlights a prebiotic induced-reduction in mouse dyslipidemia during HF challenge. In addition, changes in several plasma acylcarnitines levels were observed in HF + prebiotic fed mice compared to HF and HF_S fed mice at D70 after the diet switch¹⁸⁹. All HF + prebiotic diets showed an increase in C2, and C4 compared to HF and HF_S fed mice and no difference of C2 and C4 level with LF-fed mice. These variations might be explained by a reduced utilization of short chain fatty acylcarnitines to from energy or by an activation of the last β -oxidation steps producing short chain fatty acids (SCFAs) which could then be transported out of the mitochondria. If the last scenario is true, this would indicate that prebiotic supplementation enhance a complete β -oxidation of FAs. Previous

studies have already shown positive correlations between increased muscle acylcarnitines and enhanced FA oxidation^{197,198}. HF + FOS_IN diet was also marked by an increase in C16, C16:1, C18:1, while HF + GOS diet was positively correlated with C16, C18:1, C3:1 and C4:1 compared with HF fed mice. On the other hand, C4, C4:1, and C16 levels were not different between LF and HF + FOS or HF + GOS fed mice. GOS or FOS supplementation during HF challenge may specifically improve β -oxidation of unsaturated FAs and increase mitochondrial oxidative efficiency toward those of LF-fed mice as indicated by similar acylcarnitine levels. Interestingly, C18:2 were also found to be elevated with HF + prebiotic compared to LF, HF or HF_S fed mice. This is also suggestive of a specific regulation in mitochondrial β -oxidation of poly-unsaturated FAs. In addition, urinary level of NAD/NADP pathways end-products (2PY and 4PY) in HF + FOS_IN fed mice were similar to those of LF-fed mice which displayed a normalization of mitochondrial oxidative activity with FOS_IN supplementation^{151,159,199}.

Interestingly, isovalerylglutamate and α -ketoglutarate which derived from leucine and valine catabolism respectively were found elevated in HF + FOS and HF + GOS fed mice compared to HF and HF_S fed mice¹⁵². BCAA catabolism also occurred within the mitochondria and its activation is affected by mitochondrial fuel overloading^{151,170}. Therefore, increased end-products of BCAA catabolism intermediates (isovaleryl-CoA and α -ketoglutarate) might reflect an activation of mitochondrial BCAA oxidation with prebiotics.

Altogether, our results suggest that prebiotic supplementation decreased ectopic fat storage, lowering dyslipidemia and improving mitochondrial oxidative pathways. Furthermore, by enhancing mitochondrial metabolism, prebiotic supplementation may favour the transport of mitochondrial oxidative end-products, (i.e acylcarnitines and BCAA catabolism intermediates), and reduced oxidative product accumulation which is deleterious for host cells^{172,173}.

4.4. FOS_IN activate amino acid metabolism involved in insulin signalling and mitochondrial metabolism

Based on the percentage of “metabolic rescue” in urine and plasma, FOS_IN was the most effective prebiotic in recovering partial internal homeostasis. In addition, only FOS_IN supplementation during HF challenge maintained normal fasting glycemia which is consistent with previous nutritional studies indicating that FOS improves glucose secretion^{95,200}. Interestingly, circulating amino acid concentration increased in response to FOS_IN supplementation (Asp, Gln,

Glu, His, Lys, Met, Ser, MetSo, Phe, Pro, Thr, Tyr, Ile, Val). Amino acids are known to regulate key metabolic pathways that are involved in cell signalling, inflammation and hormone secretion⁴⁴. In particular, leucine and arginine have been thought to stimulate insulin signalling pathways. Gunnerud *et al* have also demonstrated that drinking beverage containing 5 insulinogenic amino acids before any meal limits glucose postprandial responses²⁰¹. Therefore, increased circulating amino acids might trigger or at least contribute in the improvement of glucose homeostasis in HF + FOS_IN fed mice.

Additionally, FOS_IN like the other prebiotics was found to improve mitochondrial oxidative pathways during HF challenge. This might be explained by an activation of amino acid metabolism. Amino acids play a crucial role in energy homeostasis since they are precursors of TCA cycle intermediates and activate mitochondrial enzymes⁴⁴. BCAAs have been reported to up-regulate mTOR which promotes cells oxidative capacity and PGC-1 mediated mitochondrial gene expression and results in enhanced mitochondrial biogenesis²⁰². Higher mitochondrial biogenesis has been thought to favour amino acid catabolism. On the other hand, mTOR is also a key regulatory enzyme of protein synthesis⁴⁴. In line with this, BCAA transaminase KO mice displayed higher level of circulating BCAAs and were associated with increased muscle proteins synthesis, decreased fat mass and increased body energy expenditure²⁰³. This continuous synthesis and protein degradation is characterised as proteins turn over and consumes a large amount of ATP^{44,152}. Hence, FOS_IN supplementation during HF challenge probably elevates protein turnover rate resulting in increased energy expenditure and improved energy homeostasis.

Interestingly, FOS_IN supplementation were found to specifically increase urinary isovalerylglycine, which is a glycine conjugate of leucine catabolism intermediate: isovaleryl-CoA. Higher ketone body level (3-D-hydroxybutyrate) in plasma metabolic profiles of HF + FOS_IN compared to HF-fed mice also indicated an up-regulation of ketogenesis with FOS_IN supplementation. In the previous chapter (Chapter III, section 4), HF diet containing relatively low carbohydrate content induced an increase in BCAA catabolism which in turn might activate gluconeogenesis and exacerbate glucose intolerance in HF-fed mice. However, obesity resistant mice under HF challenge tend to preferably activate leucine catabolism which is a ketogenic pathway thus protecting mitochondria against fuel overloading and limiting the formation of glucose *via* gluconeogenesis. Hence, HF + FOS_IN fed mice exhibit a significant lower fasting glycemia than all HF containing diet groups. Hence, FOS_IN might specifically up-regulate leucine catabolism and ketogenic pathways which in turn might alleviate HF induced impaired mitochondrial oxidation. A

specific activation of ketogenic pathways rather than gluconeogenesis with FOS_IN supplementation might also protect the organism against impaired glucose homeostasis.

Nevertheless the exact reason why FOS_IN but not GOS or GOS_CMO activated amino acid metabolism remains to be explained. FOS_IN supplementation during HF diet challenge may increase dietary amino acid absorption by selecting specific gut microbial activity/composition which in turn may modulate the internal amino acid metabolism. However, it is important to note that these amino acid variations occur during a fasting period. Several amino acids such as phenylalanine, tryptophan, lysine and methionine are mainly absorbed intact in the organism but recent studies have shown that up to 30% of the extracted dietary amino acids are oxidized in the small intestine⁴⁴. Of note, Kong *et al* demonstrated that herbal powder supplemented diet improves dietary amino acid extraction and absorption which results in elevated amino acid in circulation in post weaning pigs²⁰⁴. In the present trial, all prebiotics triggered a similar down regulation of bacterial amino acids degradation in the large intestine. However, specific regulation of amino acids processing in the small intestine could not be monitored by our experimental design.

4.5. Prebiotics supplementation during HF challenge improves mouse health by modulating colonic bacterial fermentation.

The inflammatory responses triggered by ingestion of HF have also been partly balanced by prebiotic supplementation since the concentration of circulating cytokines tended to decrease in HF + prebiotic groups compared to HF and HF_S groups with the exception of TNF- α . Common metabolic signatures associated with various prebiotic supplementations have been identified at D70 after the diet switch and involved a specific gut microbial activity. Indeed, higher excretion of SCFAs was observed in feces of mice fed HF + prebiotics compared to mice fed a fibre content matching HF diets, which might indicate an increase production of SCFAs in the colon. Different types of nondigestible polysaccharides including resistant starch, non-digested oligosaccharides, undigested portions of oligosaccharides and sugars alcohols enter the large bowel where they are fermented by colonic bacteria which produce SCFAs mainly acetate, propionate and butyrate²⁰⁵. The proportion of fermentation of these carbohydrates varies according to host, nutritional, and dietary factors, which affect the yield and the type of SCFAs produced by the gut microbiota^{205,206}. To a

certain extent, SCFAs promote saccharolytic bacteria in the gut and induce various physiological impacts on host body²⁰⁶.

Many of health benefits conferred to prebiotics can be largely attributed to fermentation process and SCFAs production⁹⁵. In particular, SCFAs have been associated with a large range of anti-inflammatory properties in the literature. They interact with GTP-binding proteins (GPCR41, GPCR43) in the immune cells of human colons and promote chemokine expression in the epithelium^{207,208}. They also repress NF-κB and they differently affect the production of pro-inflammatory markers in the leukocytes such as IL-2 and IL-10²⁰⁹. Production of SCFAs has been also reported to reduce food intake and hunger feeling by various physiological processes. Higher SCFAs concentration in the lumen stimulates upper gut motility and decreases transit time, resulting in the modulation of nutrient digestion and absorption. Butyrate enhances satiety by increasing peptide YY and proglucagon synthesis in epithelial cells and by inhibiting the expression of neuroendocrine factors such as leptin^{209,210}. For instance, Delzenne, *et al* highlighted that oligo-fructoses consumption reduces food intake and reverses diet-induced insulin resistance in mice. These phenomena are likely to be caused by increased SCFA production such as butyrate²⁰⁰. Elevated concentrations of succinate in fecal content of HF + prebiotic groups suggest an increased fermentation in the gut. However this metabolite may be an intermediate product of fermentation from carbohydrates as well as proteins, endogenous and exogenous glycoproteins²⁰⁶. The higher level of ketone body (3-D-hydroxybutyrate) in feces of HF + prebiotics groups also pointed out an increased butyrate oxidation in the mucosa of the distal colon. All SCFAs are metabolised to provide energy to epithelium, however butyrate is the main source of fuel for colonocytes and its oxidative product can enter TCA cycle or being oxidised by mammalian enzymes into ketones bodies. Indeed, butyrate is absorbed by colonic epithelial cells and transformed into butyryl-CoA by CoA transeferase. Butyryl-CoA can be then oxidised into crotonyl-CoA by the butanoyl-CoA dehydrogenase which in turn induce the formation of acetoacetate. Finally acetoacetate can trigger the formation of ketone bodies or being transformed into acetyl-CoA before entering TCA cycle¹⁵². It is acknowledged that higher utilisation of butyrate as energy source for the colonocytes stimulates cell growth and differentiation which is essential in maintaining gut health²⁰⁶. In addition, De Vadder *et al*, have recently demonstrated that SCFAs stimulate intestinal gluconeogenesis by complementary mechanisms which contribute to the metabolic benefits of fibre enriched diets²¹¹. In particular, propionate acts as an agonist of the fatty acid receptor FFA3 and initiates the gut brain neural circuit in the peripheral afferent neural system which triggers the activation of intestinal gluconeogenesis gene expression. On the other hand, butyrate directly activates intestinal

gluconeogenesis gene expression in the enterocytes by increasing cAMP²¹¹. As previously mentioned in the introduction (section 3.2), intestinal gluconeogenesis is suggested to play an important role in satiety control and in the regulation of glucose homeostasis. Therefore, activation of intestinal gluconeogenesis by SCFAs may be among the underlying mechanisms contributing to the decreased energy storage and fasting glycaemia in HF + prebiotic fed mice in our experiment. However, no gene expression or enzyme activity of intestinal gluconeogenesis have been measured to support this hypothesis.

The modification of the balance of the intestinal ecosystem by different prebiotic supplementation has led to a regulation of other bacterial fermentation processes. In particular, the fecal content of HF + prebiotics groups was marked by a reduction of PCG and PCS which derived from end product of gut bacterial degradation of tyrosine^{31,102}. In healthy subjects, the normal population of bacteria produces a limited amount of these molecules. However, previous studies have shown that a modulation of gut microbial composition leading to an increased enzyme activity involved in bacterial proteolysis, triggers an increased level of phenol derivatives in the colons (*i.e.* nitrosamines, heterocyclic amino acid, indoles, phenol, *p*-cresol) which may have carcinogenic effects^{87,212}. By contrast, the diminution of pH associated with increased ingestion of fermentable carbohydrates and SCFA production has been thought to decrease bacterial proteolysis¹⁸¹. GOS, FOS and GOS_CMO may therefore, promote the growth of commensal bacteria, which in turn may promote a more favourable micro-environment in the intestine (ideal pH, reduction of potentially harmful bacteria) and inhibit gut bacterial amino acid degradation.

4.6. GOS supplementation alleviated inflammatory responses induced by the combination of HF and lactose mal-absorption.

C57BL/6 mice as many other rodents suffered from lactose mal-absorption. Lactose is a disaccharide composed of galactose linked by β , 1-4 glycosidic bond to α or β -glucose and are present in various dietary foods such as milk and dairies. Dietary lactose is digested in the small intestine by lactase releasing glucose and galactose which are then absorbed in the organism^{152,213}. A small proportion of lactose that escapes digestion enters the large intestine where it can be digested by bacterial enzymes such as β galactosidases¹⁰⁵. Individuals suffering from lactose mal-absorption are deficient in lactase enzymes and cannot breakdown lactose in the small intestine¹⁰⁴. As a result, a

significant amount of lactose reaches the large intestine and is degraded by colonic bacteria, which induces the production of gases (H_2 , CO_2 , CH_4) and fermentation end-products (formate, SCFAs) and causes gastrointestinal discomfort such as bloating, rumbling, diarrhea and abdominal pain. Therefore lactose mal-absorption is accompanied with lactose intolerance symptoms. However, the causes of these symptoms are not well understood. Recent studies have suggested that colonic bacterial fermentation of lactose, galactose and glucose participate in the onset of lactose intolerance²¹⁴.

In the present experiment, C57BL/6 mice fed with HF_S diet containing HF, free lactose, glucose and galactose, showed subtle variations in phenotype and metabotype compared to HF-fed mice. Although all diets were isocaloric, HF_S diet tended to lower energy storage compared to HF diet. However, the difference in CFE between these groups was not consistent along the experiment. In addition HF and HF_S diets induced similar increases in body fat mass. Lactose ingestion did not seem to affect lipid metabolism and energy homeostasis as ^1H NMR plasma and fecal metabolic profiles and plasma lipid profiles were not different between HF and HF_S groups at D70 after the diet switch. On the other hand, lactose and other free sugars supplementation seemed to exacerbate inflammatory responses under HF diet as the production of cytokines in circulation tended to increase in HF_S group compared to HF group. However, none of the measured cytokines were significantly different between HF and HF_S groups probably due to the large within group variability. Ingestion of HF_S diet also induced a significant increase of KcGRO compared to LF diet which was not observed with HF diet. Gut inflammatory responses are often observed in lactose intolerant individuals^{104,215}. In severe allergy reaction, it can also result in the development of autoimmune diseases such as inflammatory bowel diseases²¹⁵.

Urinary metabolic profiles of HF_S group exhibited higher concentration of galactose and glucose than HF group. In addition, lactose resonances were not observed in ^1H NMR fecal metabolic profiles of HF_S fed mice which suggested that lactose was mainly digested into galactose and glucose in the intestine, absorbed in the organism before being by-passed to the urine. Interestingly, the excretion of bacterial saccharolytic fermentation end-products (succinate, lactate, acetate, SCFAs) in urine, feces and plasma did not increase with HF_S diet suggesting that monosaccharides were rapidly absorbed in the organism limiting the colonic fermentation of carbohydrate by gut bacteria^{206,214}. Altogether, these results tend to indicate that in C57BL/6 mice, lactose is still digested in the small intestine. The lactose digestion end-products (galactose and glucose) may in turn be rapidly absorbed in the host which limits their fermentation in colon. However, decreased PCS and PAG in urinary metabolic profiles of HF_S group compared to HF group pointed out that lactose and

free sugar supplementation during HF diet lower bacterial amino acid fermentation^{102,212,214}. Lactose digestion in the small intestine may trigger changes in the gut environment (pH, transit time, motility, gut bacterial composition and gut bacterial cross-feeding) which in turn may reduce colonic amino acid fermentation and affect physiological status of mice. In particular, it may lower calorie extraction from the diet resulting in reduced calorie storage²¹⁶.

GOS_CMO supplementation containing lactose and galactose alleviated inflammatory responses induced by the combination of HF and lactose mal-absorption. In this experiment, GOS_CMO as well as other prebiotics were proved to enhance mouse health by modulating bacterial colonic fermentation. Since the disruption of colonic lactose fermentation may be a contributing factor of lactose intolerance, GOS supplementation is likely to beneficially affect gut lactose processing, gut microenvironment and reduce symptoms of lactose intolerance¹⁰⁵. In particular, milk products consumption containing probiotics have been found to improve the physiological status of lactose intolerant individuals by modifying pH, increasing β -galactosidase activity and reducing colonic time transit²¹⁷. However, probiotics supplementation alone showed inconsistent effects on lactose intolerance^{217,218}. Prebiotics such as GOS favour the growth of *Bifidobacteria* and *Lactobacilli* species containing specific galactosidase activity and provide laxative effect which increased the availability of carbohydrate in distal colon for fermentation and reduce pH⁹⁵. Thus, these physiological processes may underlie the beneficial effect of GOS_CMO on inflammatory status of mice suffering from lactose mal-absorption. However, it is important to note that HF diet ingestion triggered a larger disruption of physiological and metabolic status than lactose ingestion in C57BL/6 mice. Hence, to clearly identify the role of GOS on lactose mal-absorption in C57BL/6 mice and to get rid of confounding factors associated with HF diet, it is necessary to investigate the effect of diets containing GOS and lactose under LF diet condition.

5. CONCLUSION

Prebiotic supplementation during HF challenge improved mouse phenotype by reducing BW gain, energy storage and the circulating inflammatory status, although it did not prevent obesity development. High fat diet significantly increased circulating lipid levels, and altered mitochondrial oxidative pathways compared to low fat diet. On the other hand, prebiotic supplementation reduced plasma lipoproteins and modifies plasma acylcarnitines profiles which might reflect a lower dyslipemia and an improved mitochondrial oxidative capacity. In addition, prebiotic

supplementation activated colonic carbohydrate fermentation and reduced bacterial amino acid fermentation which might beneficially affects the mouse inflammatory status. FOS_IN was the most effective prebiotic in recovering partial internal homeostasis and it was enabled to maintained normal fasting glycemia during HF challenge. ¹H NMR and MS metabonomics indicated that FOS_IN specifically activate amino acids metabolism and ketogenesis which might contribute to improve mitochondrial metabolism and maintain glucose homeostasis. Our results also highlighted that lactose and free sugar supplementation affect gut bacterial amino acid fermentation and may increase mouse circulating inflammatory responses to HF diet. On the other hand, GOS and CMO supplementation (containing lactose) lower mouse inflammatory status. However, HF diet ingestion triggered a greater disruption of mouse metabolic status than lactose ingestion which might hide lactose specific effect on mouse metabolome and inflammation.

CHAPTER V: THE PREBIOTIC STUDY

CORRELATIVE ANALYSIS OF FECAL MICROBIOTA, ^1H METABOLIC PROFILES AND CIRCULATING CYTOKINE LEVELS.

1. INTRODUCTION

Changes in the composition of the colonic gut microbiota can be either the triggering factor or the protective agent in intestinal and systemic disorders. Repeated utilization of antibiotics, infection by intestinal pathogens (e.g. *Clostridium difficile* or *Escherichia coli*) and/or reductions in overall species diversity contribute to the etiology of intestinal pathologies such as ulcerative colitis, antibiotic-associated colitis, diarrhea, and Crohn's disease^{219,220}. At the systemic level, altered activity of the gut microbiota has been associated with gut-origin septicemia, pancreatitis and multiple organ failure syndrome^{179,221}. Alteration of the diversity/activity of the gut microbiome by high-fat (HF) diet has been associated with a range of metabolic disorders and may contribute to obesity, type 2 diabetes mellitus (T2DM), and non-alcoholic fatty liver disease (NAFLD)^{72,77,89}. However, modulation of the gut microbiota and its activity by ingestion of functional foods (i.e. probiotics and prebiotics) has been effective in reducing colonic inflammation, which is a potent factor in gut microbiota-induced intestinal and systemic diseases^{88,95}. Prebiotics (non-digestible fermentable carbohydrates), in particular, have been shown to reduce serum triglycerides and cholesterol in healthy and T2DM individuals; as a result these functional foods may help prevent or attenuate the development of obesity and obesity-associated metabolic disorders^{94,97,222}. There is no evidence to suggest that prebiotic supplementation of infant formulae improves growth or clinical outcomes, or causes adverse effects in term infants. Prebiotic effects are able to reduce the incidence of atopic disease in children; however, confirmation of effectiveness is needed to support available data²²³. Studies in adults and the elderly vary in quality and outcomes, but prebiotics have been shown to modulate the fecal microbiota and immune function in elderly individuals, and to reduce markers of metabolic syndrome in overweight adults^{224,225}.

In the previous chapter (Chapter IV), the effects of dietary supplementation with different prebiotic blends (fructo-oligosaccharides–inulin (FOS_IN), galactosyl-oligosaccharides (GOS), and galactosyl-oligosaccharides–cow milk oligosaccharides (GOS_CMO)) for 70 days (after 2 weeks on a low-fat (LF) diet) on the development of obesity and metabolic profiles in C57BL/6 mice fed a HF diet

were tested. The results highlighted that all prebiotics induced a significant reduction of weight gain and fat mass during HF challenge, while FOS_IN specifically prevented HF-diet-induced fasting hyperglycemia. A systemic inflammatory response, indicated by an increase in plasma IL-1 β levels was noticed in HF-fed mice. This inflammatory response was exacerbated when mice were fed with HF + glucose–lactose–galactose (HF_S), as increases in plasma IL-1 β , KcGRo and TNF- α were observed in HF_S-fed mice compared with LF-fed mice. Conversely, prebiotic supplementation during HF challenge and HF_S challenge tended to alleviate this central inflammatory response mainly by reducing levels of circulating IL-1 β . Ingestion of prebiotics during HF-feeding also increased microbial carbohydrate fermentation and decreased microbial amino acid fermentation in the colon, and affected host mitochondrial oxidative pathways. Significant variations in metabolic profiles associated with each dietary intervention are summarised in Table 9.

In this chapter, the work presented previously on the short-term (7 days, (D7)) and long-term (70 days, (D70)) effects of LF, HF, HF_S and HF + prebiotic (FOS_IN, GOS and GOS_CMO) diets on our mouse model was extended to specifically examine the effects of these diets on the gut microbiota of the isogenic C57BL/6 mice. Community diversity and abundance of the fecal microbiota in the different feeding groups were examined using high-throughput 16S rRNA gene sequence analysis (pyrosequencing) at D-7 before the diet switch, D7 and D70 after the switch from the LF maintenance diet. To highlight the contribution of the gut microbiota to the modulation of host metabolism, correlation analyses between microbial profiles and relevant metabolites from urinary, fecal and plasma metabolic profiles at D70 were performed using the total variance of each dataset ($n = 90$). In addition, the relationships among gut microbiota composition, host metabolism and central inflammation were investigated using multiple correlations testing analysis on plasma inflammatory markers, fecal microbiota profiles and metabolic profiles of urine, feces and plasma samples collected at D70.

Table 9: Metabolites that varied significantly between feeding groups after a 70-day dietary intervention.

Effect with:	Comparison*	Metabolic variations†	Biological interpretation
HF diet/HF diet + sugars	LF vs HF LF vs HF_S	<p>↑ Urinary BCAA intermediates (α-keto-β-methylvalerate, isovaleryl-glycine, α-ketoisovalerate)</p> <p>↓ Plasma acetyl-carnitines (C_2), butyryl-carnitine (C_4), palmitoyl-carnitine (C_{16}), palmitoleyl-carnitine ($C_{16:1}$), oleyl-carnitine ($C_{18:1}$), linoleic acid ($C_{18:2}$)</p> <p>↑ Plasma stearoyl-carnitine ($C_{18:0}$)</p> <p>↑ Urinary 2PY, 4PY</p> <p>↑ Plasma lipoproteins (VLDL/LDLs)</p> <p>↑ Plasma SMs, LPCs, PCs</p>	<p>Up-regulation of mitochondrial oxidative pathways^{151,152}</p> <p>Increased fasting dyslipidemia^{49,152,189}</p>
HF diet + sugars	HF vs HF_S	↓ Urinary <i>p</i> -cresol sulfate, phenylacetyl-glycine	Decreased fermentation of amino acids by gut bacteria ²²⁶
Prebiotics	HF vs HF + GOS	<p>↓ Plasma lipoproteins (VLDL/LDLs)</p> <p>↑ Plasma acetyl-carnitines (C_2), butyryl-carnitine (C_4), palmitoyl-carnitine (C_{16}), palmitoleyl-carnitine ($C_{16:1}$), oleyl-carnitine ($C_{18:1}$), linoleic acid ($C_{18:2}$)</p> <p>↑ Plasma propionyl-carnitine (C_3), isovaleryl-carnitine (C_5)</p> <p>↑ Urinary α-keto-β-methylvalerate, isovaleryl-glycine for HF + FOS_IN and HF + GOS groups exclusively</p>	<p>Reduced dyslipidemia^{49,152}</p> <p>Increased complete mitochondrial β-oxidation^{151,152}</p> <p>Enhanced mitochondrial oxidative pathways¹⁵²</p>
	HF vs HF + FOS_IN	↑ Fecal butyrate, 3-D-hydroxybutyrate, succinate	Increased carbohydrate fermentation by gut bacteria ^{205,210}
	HF vs HF + GOS_CMO	↓ Urinary <i>p</i> -cresol sulfate, <i>p</i> -cresol glucuronide	Decreased fermentation of amino acids by gut bacteria ²²⁶
HF diet + FOS_IN	HF vs HF + FOS_IN	<p>↑ Plasma amino acids</p> <p>↑ Plasma 3-D-hydroxybutyrate</p> <p>↓ Urinary 2PY, 4PY</p>	<p>Increased protein degradation^{44,152}</p> <p>Activation of ketogenic pathways</p> <p>Reduction of mitochondrial oxidative pathways^{152,227}</p>

*Diets: LF, low fat (negative control); HF, high fat; HF_S, high fat + glucose–lactose–galactose (positive control for GOS_CMO); HF + GOS, high fat + galactosyl-oligosaccharides; HF + FOS_IN, high fat + fructo-oligosaccharides–inulin; HF + GOS_CMO, high fat + galactosyl-oligosaccharides–cow milk oligosaccharides (glucose–lactose–galactose).

†BCAA, branched-chain amino acid; 2PY, *N*-methyl-2-pyridone-5-carboxamide; 4PY, *N*-methyl-4-pyridone-3-carboxamide; VLDLs, very-low-density lipoproteins; LDLs, low-density lipoproteins; SMs, sphingomyelins; PCs, phosphatidylcholines; LPCs, lysophosphatidylcholines.

2. MATERIALS AND METHODS

2.1. Animal handling procedure and sample preparation.

See Chapter IV, section 2.1 (Materials and Methods).

2.2. Measurement of circulating cytokines with Multiplexed Elisa assay (Meso Scale platform).

See Chapter IV, section 2.5 (Materials and Methods).

2.3. Sample preparation and ^1H NMR spectroscopy.

See Chapter IV, section 2.6 (Materials and Methods).

2.4. ^1H NMR spectra pre-processing and ^1H NMR data processing.

See Chapter IV, section 2.7 (Materials and Methods) for ^1H NMR spectra pre-processing and Chapter IV, section 2.8 (Materials and Methods) for ^1H NMR data processing.

2.5. ^1H NMR data binning using statistical recoupling of variables.

Prior doing correlative analyses between ^1H NMR metabolic profiling and other multivariate data (fecal microbiota profiles or cytokines profiles), ^1H NMR spectra were reduced using statistical recoupling of variables (SRV)²²⁸. This method employs the degree of correlation to covariance profiles of consecutive variables along the chemical shift axis to identify the spectral dependency

and group all the consecutive variables corresponding to the same biological and chemical entities in a cluster. This method allows discarding unstructured noise and reducing ^1H NMR data dimensionality to facilitate correlative analysis between multivariate dataset. A default resolution of 0.00055 ppm was used to perform the data reduction. Full resolution ^1H NMR (44K data-points) spectra were then reduced to 296 clusters for fecal metabolic profiles, 420 clusters for urinary metabolic profiles and 86 clusters for plasma metabolic profiles (^1H NMR CPMG spectra).

2.6. 16S rRNA gene sequencing of fecal microbiota.

This DNA extraction and pyrosequencing were carried out at NRC (Switzerland). DNA was extracted from fecal samples at baseline (D0) D7 and D70 after the diet switch (n=180) using the QiAamp Stool DNA mini kit (Qiagen, Hilden, Germany) as previously described by the manufacturer. Additional mechanical disruption steps (11X45sec) using a FastPrep apparatus and Lysing Matrix B tubes (MP Biochemicals, USA) (Junick 2012 AEM) were also performed to help the extraction. PCR amplification was carried out using two sets of primers matching the hypervariable regions (V) 1 to 3 (V123), and 4 to 6 (V456) of the 16S rRNA gene. In particular, The V123 region was amplified by combining a mixture of forward primers in order to ensure optimal coverage of bacterial phylogenetic diversity, as previously described²²⁹. The primers were designed according to the reference publication of Hamady *et al*²³⁰. V123 forward primer 1, 5'-CTATGCGCCTTGCCAGCCCGCTCAGTCAAGAGTTTGATYMTGGCTCAG-3'; V123 forward primers 2, 5'-CTATGCGCCTTGCCAGCCCGCTCAGTCAAGGGTTCGATTCTGGCTCAG-3'; V123 forward primer 3, 5'-CTATGCGCCTTGCCAGCCCGCTCAGTCAAGAGTTTGATCCTGGCTTAG-3'; V123 forward primer 4, 5'-CTATGCGCCTTGCCAGCCCGCTCAGTCAGAATTTGATCTTGGTTTCAG-3'; V123 reverse primer, 5'-CGTATCGCCTCCCTCGCGCCATCAGNNNNNNNNNGGTTACCGCGGCTGCTGGCAC-3'; V456 forward primer, 5'-CTATGCGCCTTGCCAGCCCGCTCAGGCCRRRCACGAGCTGACGAC-3'; V456 reverse primer, CGTATCGCCTCCCTCGCGCCATCAGNNNNNNNNAGGCCAGCAGCCGCGGTAA-3'.(where the adapter sequences for Roche 454 FLX Titanium sequencing are italicized, the linkers are underlined, NNNNNNNN sequences designate the sample-specific 8-base barcodes used to tag each PCR product, and bold sequences correspond to broadly conserved 16S rRNA gene regions). V123 forward primers 1, 2, 3 and 4 were mixed in 4:1:1:1 ratios. For each PCR amplification reaction, we used 2 μl of DNA extract, 50 μM dNTPs, 200 nM forward primer (mix of forward primers for V123 region), 200 nM reverse primers, 1x Expand High Fidelity Reaction Buffer and 2.5 U of Expand High Fidelity enzyme blend (Roche Applied Science, Switzerland). The PCR experiments followed specific

conditions: 94°C for 2 minutes followed by 25 cycles of 94°C for 30 sec, 49°C for 30 sec, and 72°C for 1 min, and end with a last step of 72°C for 7 min. After purification, the PCR products were pooled in equimolar amount, and sequenced using the 454 FLX Titanium technology (Microsynth AG, Balgach, Switzerland). Low readers were excluded according to the following criteria (adapted from Huse et al).

- The reads could not be unambiguously assigned to any of the PCR samples due to undefined sequence key.
- 5' primer sequence could not be recognised.
- 5' primer sequence contained more than one error.
- Ambiguous characters (N') were noticed.
- The reads were not matching the expected length. First, primer sequences were removed using the vectorstrip software (-besthits 1, - mismatch 30, -besthits parameters). The reads with less than 100 bp were discarded. Subsequently, the median length and the Median Absolute Deviation (MAD) of the remaining reads were calculated separately for each of the 8 different primer pairs. All reads shorter than the median -5 * Mad were discarded.
- The reads were not matching any BLAST (1) hit to the ARB²⁹ 16S DNA database (Evaluate cut-off:10⁻¹⁰). These reads are likely to not represent regions of 16S DNA genes.

All high-quality reads were classified into Bergey's taxonomy using the RDP-Classifer (80% confidence cut-off). The sequences unassigned to any taxonomic group at a certain rank (phylum, class, order, family or genus) were classified as "Unknown" at that rank. Conversely, the "Unknown" category was defined for each rank. Reads were also grouped into Operational Taxonomic Groups (OTUs) based on their best BLAST hit to full-length reference 16S DNA using the RDP database using BLAST. The reads were iteratively grouped into OTUs according to their sequence identity matching. The perfect matching corresponded to a sequence-identity of 100%, the remaining reads were then attributed using different sequence-identity cut-offs of 98%, 95%, 90%, 80% and 70%. However, in this experiment, we focused our analysis at the phylum and family like taxonomic levels.

2.7. Multiplatform data analysis

A multivariate factor analysis integrating NMR metabolic profiles from urine feces and plasma, MS targeted profiles and cytokines profiles of all mice was conducted in SIMCA-P 12 software (Umetrics, Umea, Sweden). This method consists in performing a PCA on a merged dataset

integrating several matrices with the same number of individuals, $X_{\text{microbiota}}$, X_{NMR} , $X_{\text{cytokines}}$. For each matrix, an individual PCA was built and the largest eigenvalue (γ) was determined by calculated the standard deviation of the score T1. The X matrix was then weighted by $1/\gamma$ to correct for individual matrix dimensionality. The MFA finally was conducted with the merged dataset using mean-centering and UV scaling.

A functional correlative analysis was also carried out to investigate transgenomic relationship between host metabolism and gut microbiota. The hierarchical clustering and the Spearman correlation heatmaps between fecal microbial profiles and ^1H NMR SRV clusters (feces, urine or plasma) of all mice 70 days after diet switch were performed in Matlab software using in-house scripts developed by JTM Pearce.

A bipartite graph was also used to highlight a correlation network between host inflammatory responses, host metabolism and fecal microbiota. Multiple Spearman correlations were computed between plasma cytokines profiles, fecal bacteria composition and ^1H NMR SRV clusters (feces, urine or plasma) of all mice at D70 after the diet switch. All correlation coefficients with an absolute value $|r| > 0.3$ were selected and tested against the null hypothesis H_0 . Noticeably, none of the absolute correlation coefficients values among the entities were above 0.5.

3. RESULTS

3.1. HF diet and prebiotic supplementation during HF challenge induce significant changes in the composition of the fecal microbiota

Analysis of microbiota data at D-7 (baseline) showed that all animals had essentially the same fecal microbiota at the start of the dietary intervention (Figure 41) although subtle differences were observed in *Bacteroidetes* and *Proteobacteria* levels among the groups. After 7 days of dietary intervention, gut microbiota composition differed from that of the pre-intervention period in all diet groups. In particular, levels of unclassified bacteria, *Actinobacteria* and *Verrucomicrobia* were elevated and the level of *Proteobacteria* was reduced in HF, HF_S and HF + prebiotic-fed mice at D7 compared to D-7. 16S rRNA gene sequence analysis of the fecal microbiota also disclosed significant

changes in composition between the different dietary groups at D7 and D70 after the switch from the maintenance diet. At the phylum level at D7 (Figure 41.B), increases were observed in the proportions of the *Bacteroidetes* and *Verrucomicrobia* and a decrease was observed in the proportion of *Firmicutes* in mice fed HF, HF_S and HF + prebiotic mice compared with the LF-fed mice. Prebiotic supplementation, particularly with FOS_IN – during the HF challenge also induced a proliferation of *Actinobacteria* at D7. At D70 (C), the general microbiota profiles of each diet group were conserved and early trends were amplified: in particular, the *Bacteroidetes*-to-*Firmicutes* ratio at D70 was higher than those at D7 in all HF-containing diets. The proportions of *Actinobacteria* in HF + prebiotic-fed mice also increased at D70 by comparison with those at D7.

Compositional differences occurred at the phylum level, but more subtle variation was observed at lower taxonomic levels (data not shown). Analyses presented herein are focused at the family level (which, similar to the phylum-level analysis, contained a limited number of unclassified species) and enable investigation of subtle diet-specific modulation of the gut microbiota (Figure 42). HF challenge with or without prebiotic supplementation led to a significant increase in representation of the *Porphyromonadaceae* and *Bacteroidaceae* (*Bacteroidetes*) and *Lachnospiraceae* (*Firmicutes*), and a decrease in the *Erysipelotrichaceae* (*Firmicutes*) compared to the LF-fed mice at D7 and D70. The decrease in abundance of members of the phylum *Firmicutes* in HF- and HF_S-fed mice was due to the decrease in *Erysipelotrichaceae*, which was the most abundant family of bacteria in the feces of the mice examined in this study. Interestingly, the abundance of the *Lachnospiraceae* was similar in the LF- and the HF + FOS_IN-fed mice at D7 and D70, which suggests that FOS_IN and/or its by-products were not a preferred nutrient source for this group of bacteria. In addition, abundance of the family *Coriobacteriaceae* (*Actinobacteria*) was significantly higher in the HF- and HF + prebiotic-fed groups compared to the LF group at D7 and D70. The abundance of *Coriobacteriaceae* in the HF_GOS-fed animals was notably higher than in the other diet groups at D7, but dropped to levels similar to the HF + GOS_CMO group at D70. Abundance of the *Coriobacteriaceae* in the fecal microbiota of the HF + FOS_IN-fed animals fell at D70, but was still significantly higher than that in the LF-fed mice at the same time point. The representation of *Enterobacteriaceae* were also found to increase in HF and HF_S fed mice compared to HF + GOS and HF + GOS_CMO fed mice at D7. However, it important to note the large intra-group variability in the relative abundance of this bacterial family as indicated by a large standard deviation in HF, HF_S, HF + GOS and HF + GOS_CMO groups. In addition, no read corresponding to *Enterobacteriaceae* was measured in LF and HF + FOS_IN fed mice.

Prebiotic supplementation during HF challenge increased the abundance of several families of bacteria within the fecal microbiota of the C57BL/6 mice (Figure 42). In particular, HF + GOS-fed mice had significantly higher levels of the *Bacteroidaceae* (*Bacteroidetes*) than HF- and HF_S-fed mice at D7 and D70. Similarly, *Lachnospiraceae* (*Firmicutes*) were more abundant in the HF + GOS_CMO group than the other HF groups at D7 (representation was lower at D70, but still higher than in the other HF groups). Specific families within the phyla *Firmicutes* (*Streptococcaceae*, *Ruminococcaceae* and *Peptostreptococcaceae*) and *Bacteroidetes* (*Rikenellaceae*) were present at significantly higher levels in the HF and HF_S groups than in the HF + prebiotic-fed animals at D7 and D70. These results showed that the HF diet induced consistent changes in the composition of the gut microbiota, with prebiotic supplementation able to attenuate or contribute to these family-dependent compositional changes. Prebiotics also triggered specific variations in the gut microbiota. HF + prebiotic-fed mice showed an increase in the abundance of the *Verrucomicrobiaceae* (*Verrucomicrobia*) compared with the LF-, HF- and HF_S-fed mice at D7 and D70. FOS_IN supplementation during HF challenge specifically increased the abundance of the *Bifidobacteriaceae* (*Actinobacteria*) at D7 and D70. GOS supplementation also led to an increase in the abundance of the *Bifidobacteriaceae*, but this was only noticeable at D70. GOS_CMO supplementation did not have a significant effect on the *Bifidobacteriaceae*, but did induce an increase in the abundance of the *Lactobacillaceae* observed at D70.

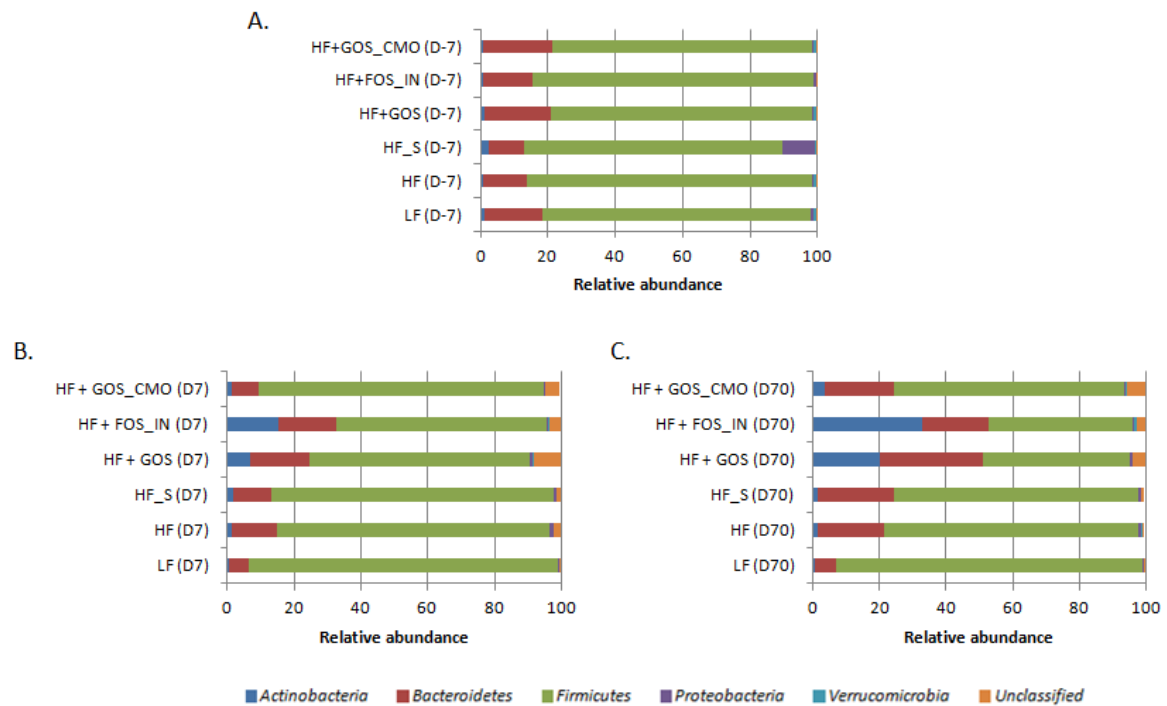


Figure 41: Phylum-level analysis of the fecal microbiota of the isogenic C57BL/6 mice at (A) D-7, (B) D7 and (C) D70 of the different dietary interventions. Prior to D-7, animals were fed the LF diet for 2 weeks. Diets: LF, low fat; HF, high fat; HF_S, high fat + glucose–lactose–galactose (positive control for GOS_CMO); HF + GOS, high fat + galactosyl-oligosaccharides; HF + FOS_IN, high fat + fructo-oligosaccharides–inulin; HF + GOS_CMO, high fat + galactosyl-oligosaccharides–cow milk oligosaccharides (glucose–lactose–galactose). The stacked bar graphs show the mean percentage of the major phyla contributing to the total fecal microbiota of the mice at each time point.

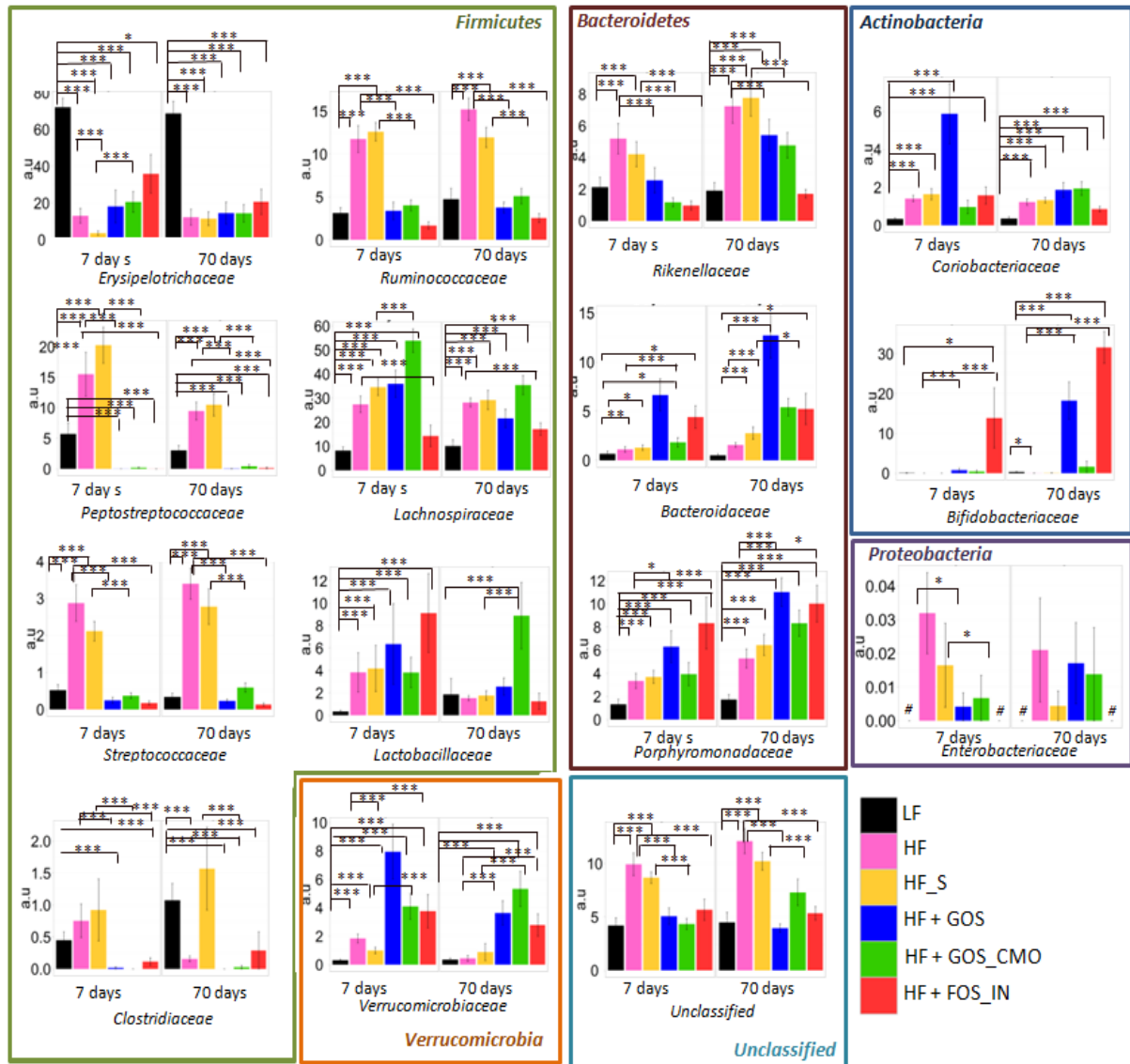


Figure 42: Family-level analysis of the fecal microbiota of the isogenic C57BL/6 mice at D7 and D70 of the different dietary interventions. Diets: LF, low fat; HF, high fat; HF_S, high fat + glucose–lactose–galactose (positive control for GOS_CMO); HF + GOS, high fat + galactosyl-oligosaccharides; HF + FOS_IN, high fat + fructo-oligosaccharides–inulin; HF + GOS_CMO, high fat + galactosyl-oligosaccharides–cow milk oligosaccharides (glucose–lactose–galactose). Bar plot intensities for each family of bacteria (in au : arbitrary unit) correspond to the mean percentage of the total detected bacteria at family level. Significant differences in the relative abundances of detected taxa between different diet groups were evaluated using the non-parametric Mann–Whitney tests (p value: * <0.05 , ** <0.001 , *** <0.0001). #: NaN

3.2. Integration of D70 family-level fecal microbiota, metabolomic (¹H NMR urine, plasma and feces) and plasma cytokine data

Family-level analyses of the fecal microbiota of the mice showed that HF and HF + prebiotic diets triggered significant changes in the composition of the gut microbiota. In the Chapter IV (section 3.1), it was demonstrated that long-term prebiotic supplementation during HF challenge lowered gains in body weight, reduced cumulative food efficiency (CFE), the levels of circulating inflammatory markers and triggered a strong modulation of mouse metabolism. To investigate the contribution of the gut microbiota to metabolic variation and inflammatory status in the mice in the different diet groups, a multivariate factor analysis (MFA) integrating data derived from the host's fecal microbiota, metabolism and immune system at D70 was carried out (Figure 43). First, common variation patterns in the different datasets were captured using a principal component analysis (PCA) model with the combined dataset comprising microbiota profiles, circulating inflammatory markers, and plasma, urinary and fecal metabolic profiles. Each dataset was mean-centered and individually scaled to remove all variations exclusively associated with individual matrix dimensionality. In this case, the PCA was built from common characteristics among all the matrices and no particular matrix dominated the first component(s). The scaling method was described by Dumas *et al.* The variance-covariance associated with each dataset (X) was modeled by PCA which allowed calculation of the largest eigenvalue (γ)²³¹. The X matrix was then corrected by $1/\gamma$. Secondly, relationships among gut microbiota, metabolic variations and circulating inflammatory markers were confirmed by correlation analyses using the total variance of each dataset.

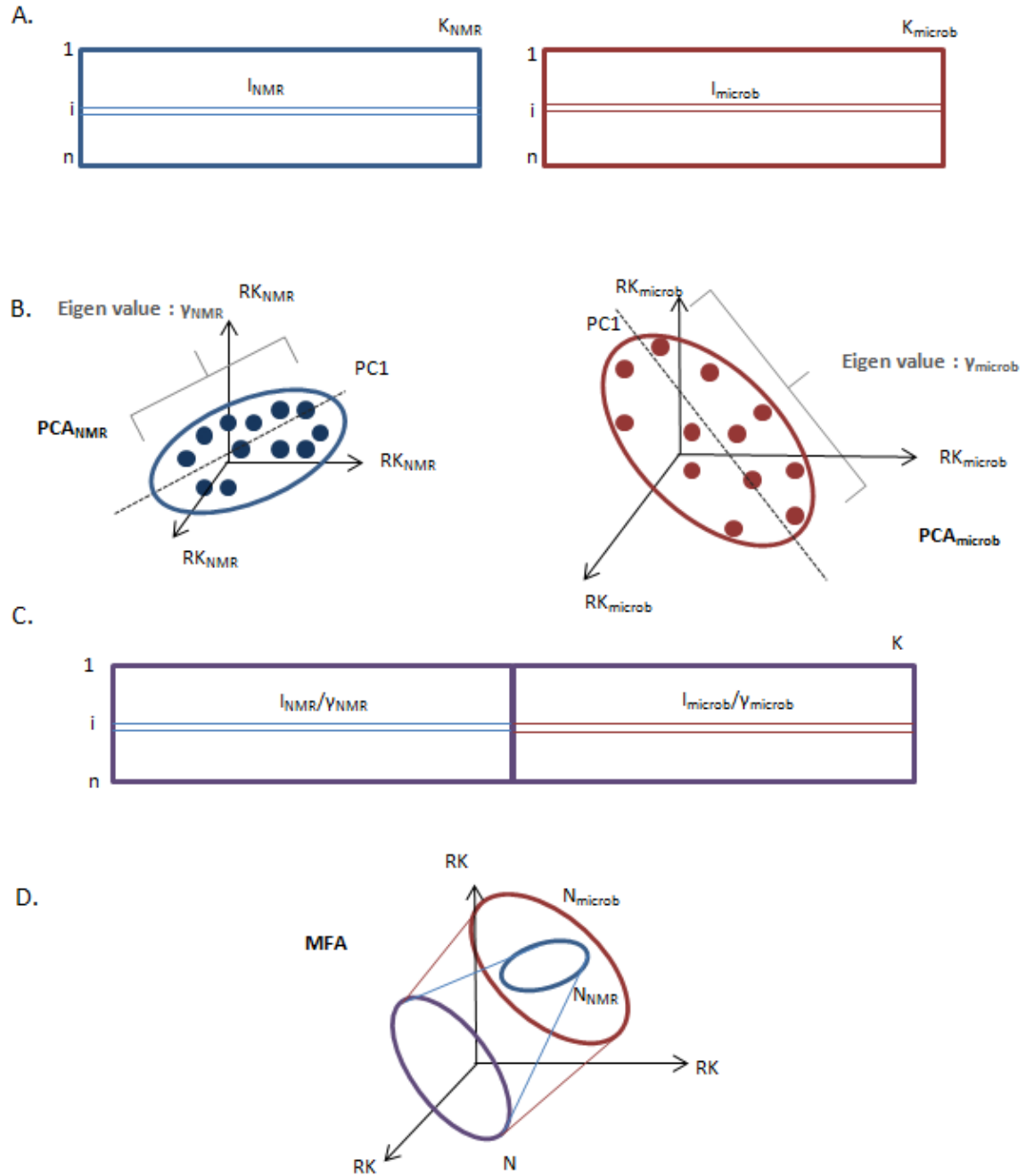


Figure 43: Individual matrix scaling in MFA, adapted from Dumas *et al.* (A) X_{NMR} and X_{microb} correspond to NMR and microbiota datasets with K_{NMR} and K_{microb} variables, respectively. (B) PCA of each dataset, which enables calculation of the largest eigenvalue (γ). (C) All datasets were individually scaled with $1/\gamma$ and merged in one block-dataset. (D) Global PCA using the new block-dataset.

3.2.1. MFA highlights similar patterns of variation in the composition of the fecal microbiota, metabolic profiles and circulating inflammatory markers in the different diet groups

MFA of combined metabolic (^1H NMR urine, plasma and feces), fecal microbiota and circulating inflammatory marker data at D70 captured the main variations responsible for the separation of the dietary groups in these datasets. The PCA score plot (Figure 44.A) showed that the LF- and HF + prebiotic-fed groups were separated from the HF- and HF_S-fed groups along the first principal component (PC1). This showed that supplementation of the HF diet with prebiotics for 70 days maintained metabolic profiles, circulating inflammatory markers and fecal-microbiota composition in the HF + prebiotic-fed mice so that they were more similar to the combined data of the LF-fed mice than to those of the HF- and HF_S-fed mice. The second principal component (PC2) tended to cluster the HF + prebiotic-fed groups of mice together and separate from the LF-fed group. The PCA loadings plot (Figure 44.B) revealed similar behavior between the variables of the different datasets and highlighted which variables were responsible for diet-group discrimination. A similar increase in urinary *p*-cresol sulfate (PCS) and *p*-cresol glucuronide (PCG), fecal bile acids, plasma lipids and IL-1 β , and fecal *Streptococcaceae*, *Ruminococcaceae* and *Peptostreptococcaceae* was identified in the positive control (HF, HF_S) groups. The LF-fed group was separated from the other feeding groups because of its increased urinary excretion of taurine, inositol and indoxylsulfate, an increase in plasma *scyllo*-inositol and a higher abundance of *Erysipelotrichaceae* in the fecal microbiota. Clustering of the HF + prebiotic-fed groups was because of their high abundances of *Verrucomicrobiaceae*, *Bacteroidaceae* and *Bifidobacteriaceae* in the fecal microbiota and higher levels of plasma pyruvate, 3-D-hydroxybutyrate and fecal butyrate compared with the other feeding groups. Within the HF + prebiotic-fed groups, the HF + FOS_IN group was separated from the other prebiotic-fed groups by its higher abundance of fecal *Bifidobacteriaceae* and higher levels of plasma citrate, acetate and glutamate, while the HF + GOS group tended to cluster separately because of its higher abundances of fecal *Bacteroidaceae* and *Porphyromonadaceae* and higher level of urinary galactose. LF-fed mice were also anti-correlated with *Lachnospiraceae*, *Rikenellaceae* and *Coriobacteriaceae* in the fecal microbiota and urinary excretion of *N*-methyl-4-pyridone-3-carboxamide (4PY), α -keto- β -methylvalerate, isovaleryl-glycine, isovalerate, α -ketoisovalerate, choline and *o*-acetyl-glycoproteins. This highlights a reduction in levels of these metabolites and bacterial families in LF-fed mice compared to the other diet groups. With the exception of IL-1 β , circulating cytokines tended to cluster in the center of the PCA loadings plot, indicating that they did not strongly influence the separation of the diet groups.

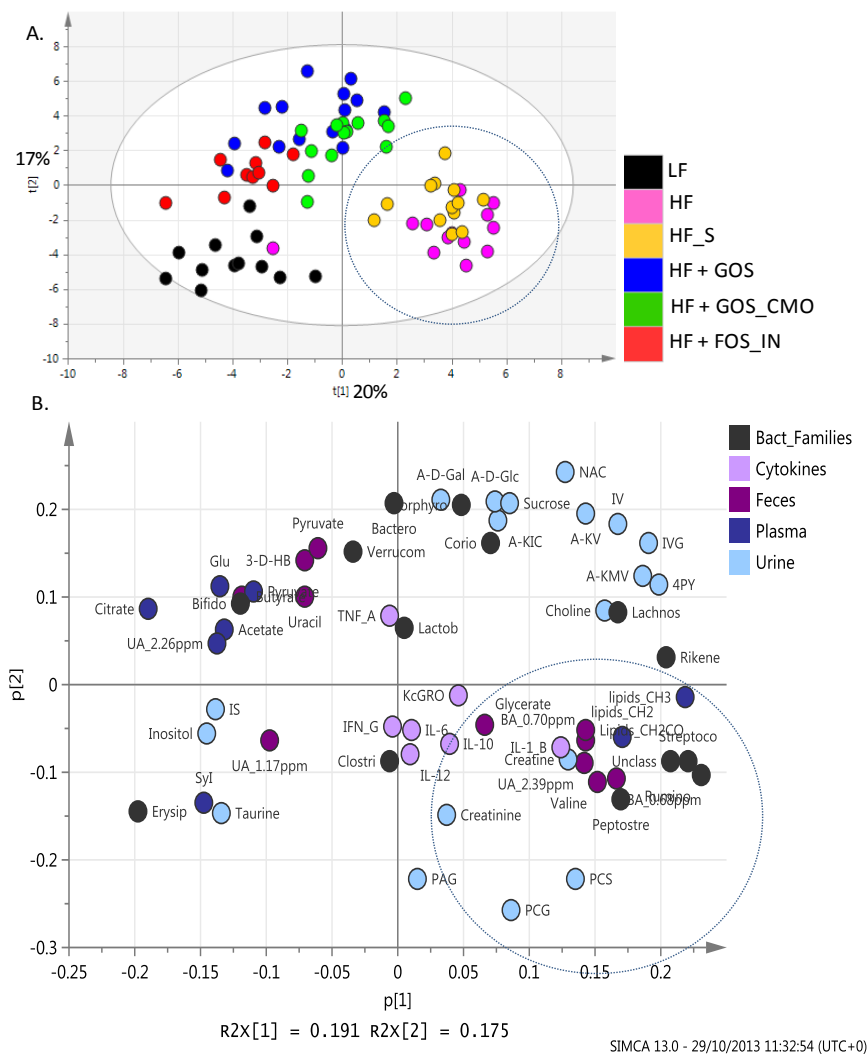


Figure 44: PCA model of the combined data from the fecal microbiota (family level), relevant metabolites (^1H NMR urine, plasma and feces) and circulating inflammatory markers at D70. (A) The PCA scores plot shows the dispersion of scores for each diet group: 20 % of the variance in the merged matrix (X) is explained by the first component, while 17 % of the variance in X is explained by the second component. (B) The PCA loadings plot indicated the contribution of variables from the different datasets in the separation of the diet groups. For instance, HF and HF_S groups present in the dotted circle in the scores plot showed an increase in several metabolites, cytokines and bacterial families highlighted by the dotted circle in the loadings plot. BA, bile acids; 3-D-HB, 3-D-hydroxybutyrate; A-KIC, α -ketoisocaproate; A-KMV, α -keto- β -methylvalerate; A-KV, α -ketoisovalerate; IV, isovalerate; IVG, isovalerylglutamine; NAC, N -acetylglucosamine; A-D-Glc, α -D-glucose; A-D-Gal, α -D-galactose; 4PY, N -methyl-4-pyridone-3-carboxamide; PCS, p -cresol sulfate; PCG, p -cresol glucuronide; PAG, phenylacetylglutamine; IS, indoxylsulfate, Syl, scyllo-inositol; Bacterio, *Bacteroidaceae*; Bifido, *Bifidobacteriaceae*; Clostri, *Clostridiaceae*; Corio, *Coriobacteriaceae*; Erysip, *Erysipelotrichaceae*; Lachnos, *Lachnospiraceae*; Lactob, *Lactobacillaceae*; Peptostre, *Peptostreptococcaceae*; Porphyro, *Porphyromonadaceae*; Rikene, *Rikenellaceae*; Ruminoc, *Ruminococcaceae*; Strepto, *Streptococcaceae*; Unclass, unclassified bacteria; Verrucom, *Verrucomicrobiaceae*; IFN-G, IFN- γ ; IL-1B, IL-1 β ; TNF-A, TNF- α ; Bact_families, families of bacteria.

3.2.2. Correlation analyses of the family-level microbiota and metabolic (¹H NMR urine, plasma and feces) data from D70 confirm microbiome-metabolome associations.

Correlation matrices were built (from which bidirectionally clustered heatmaps were generated) to confirm the associations between the diet-specific fecal microbiota and host metabolic plasma (Figure 45.A), fecal (Figure 45.B) and urinary (Figure 45.C) variations at D70. For each biofluid, different correlation patterns were found between metabolites and families of bacteria. For the plasma metabolites (Figure 45.A), the data appeared to separate the fecal microbiota into two main blocks. The first 'block' represented fecal *Erysipelotrichaceae*, *Verrucomicrobiaceae* and *Clostridiaceae*, which were negatively correlated with lipids and positively or not correlated with the metabolites grouping TCA cycle intermediates (pyruvate, citrate), acetate, unidentified (UA) metabolites, *scyllo*-inositol and glutamate. The second 'block' of bacterial families contained the *Lachnospiraceae*, *Rikenellaceae*, *Streptococcaceae*, *Ruminococcaceae*, *Peptostreptococcaceae* and unclassified bacteria, which were positively correlated with lipids and negatively correlated with the other metabolites. *Lactobacillaceae*, *Bifidobacteriaceae* and *Porphyromonadaceae* were negatively correlated with *scyllo*-inositol, while the *Coriobacteriaceae* were positively correlated with this metabolite. The *Enterobacteriaceae* were also negatively correlated with *scyllo*-inositol, but positively correlated with lipid_CH₃.

For the fecal metabolites (Figure 45.B), the *Erysipelotrichaceae* were negatively correlated with lipids_CH₂CO and positively correlated with lipids_CH₂C=C and UA_1.17ppm. *Lachnospiraceae* and *Lactobacillaceae* were negatively correlated with UA_1.17ppm. *Coriobacteriaceae* and *Lachnospiraceae* were negatively correlated with lipids_CH₂C=C, with the former family positively correlated with glutamine and the latter family also negatively correlated with butyrate. The *Verrucomicrobiaceae*, *Bifidobacteriaceae*, *Enterobacteriaceae*, *Porphyromonadaceae* and *Bacteroidaceae* were positively correlated with two or more of the following metabolites: butyrate, 3-D-hydroxybutyrate, uracil, pyruvate and UA_1.24ppm. The *Bacteroidetes* (*Porphyromonadaceae*, *Bacteroidaceae* and *Rikenellaceae*) were also negatively correlated with lipids_CH₂C=C, while the *Bifidobacteriaceae* were negatively correlated with bile acids, glycerate, glutamine and two UA metabolites. Unlike the other members of the phylum *Bacteroidetes*, the *Rikenellaceae* were positively correlated with glutamine, UA_3.73ppm, bile acids and lipids_CH₂CO. The *Clostridiaceae*, *Peptostreptococcaceae*, *Streptococcaceae*, *Ruminococcaceae* and unclassified bacteria were negatively correlated with four or more of the following metabolites: butyrate, 3-D-hydroxybutyrate,

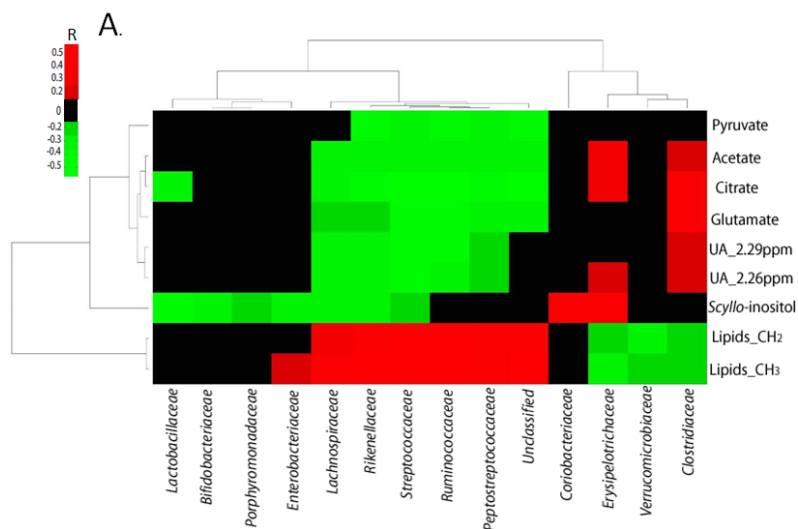
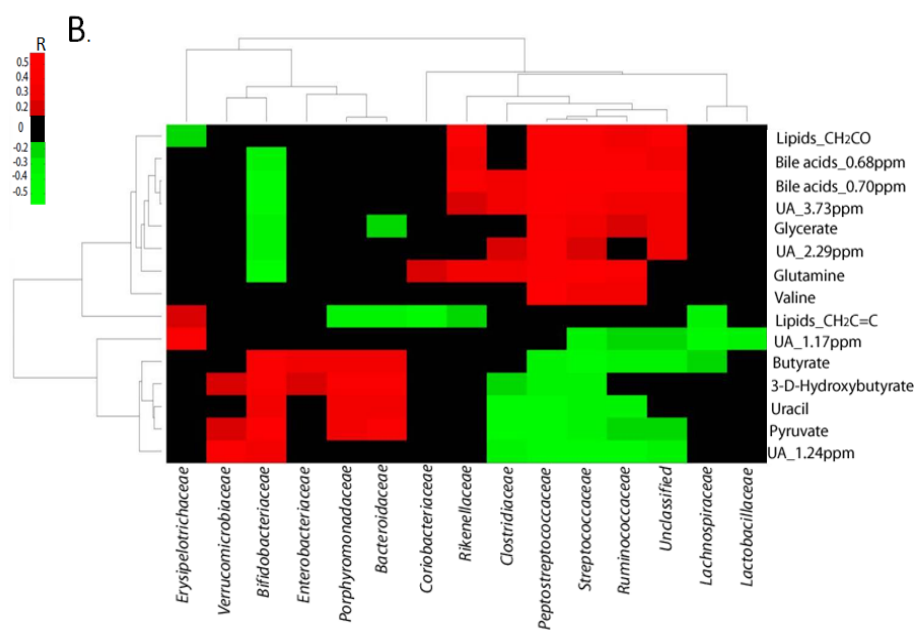
uracil, pyruvate, UA_1.24ppm and UA_1.17ppm. In addition, these families were positively correlated with four or more of the following metabolites: valine, glutamine, bile acids, lipids_CH₂CO, glycerate, UA_2.29ppm and UA_3.73ppm.

Bidirectional hierarchical clustering of the urinary metabolites with family-level microbiota data clustered the metabolites in two 'blocks' (Figure 45.C). However, no obvious patterns of clustering were observed for the families of bacteria. The first 'block' of metabolites grouped small molecules (taurine, creatine, creatinine, inositol) and bacterial co-metabolites (PCS, PCG, phenylacetylglutamine, indoxylsulfate) together. With respect to the bacterial co-metabolites, *Peptostreptococcaceae*, unclassified bacteria, *Ruminococcaceae*, *Streptococcaceae*, *Rikenellaceae*, *Lachnospiraceae* and *Coriobacteriaceae* were negatively correlated with indoxylsulfate, while the *Bifidobacteriaceae* were positively correlated with this co-metabolite. Several families of the phylum *Firmicutes* (*Clostridiaceae*, *Peptostreptococcaceae*, *Ruminococcaceae*, and *Streptococcaceae*) and the family *Rikenellaceae* were positively correlated with PCS, while *Porphyromonadaceae*, *Bacteroidaceae*, *Lactobacillaceae*, *Verrucomicrobiaceae* and *Bifidobacteriaceae* were negatively correlated with this co-metabolite. Correlation results observed for PCG and phenylacetylglutamine broadly reflected those presented for PCS. The second 'block' of metabolites classified intermediates of BCAA catabolism (α -keto- β -methylvalerate, isovaleric acid, isovalerylglutamine, α -ketoisovalerate, ketoleucine), choline, 4PY and sugars (glucose, galactose, sucrose, O-acetylglucoproteins) together. *Bacteroidaceae*, *Porphyromonadaceae* and *Coriobacteriaceae* were positively correlated with all metabolites in block 2, while the *Erysipelotrichaceae* were negatively correlated with all metabolites in this block. *Clostridiaceae* were negatively correlated with seven of the 11 metabolites in this block; *Peptostreptococcaceae* were negatively correlated with ketoleucine, galactose and O-acetylglucoproteins; *Bifidobacteriaceae* were negatively correlated with choline and 4PY. *Enterobacteriaceae* were positively correlated with galactose and sucrose; the remaining families of bacteria were positively correlated with three or more of the metabolites in block 2.

Some of the trends identified in the global PCA model (Figure 44) were verified in the different correlation matrices. For instance, the family *Erysipelotrichaceae* was found to be positively correlated with urinary inositol and taurine and plasma scyllo-inositol, and negatively correlated with intermediates of BCAA catabolism (α -keto- β -methylvalerate, isovalerylglutamine, isovalerate, α -ketoisovalerate) and the NAD/NADP pathway end-product 4PY. *Verrucomicrobiaceae* was positively correlated with fecal levels of 3-D-hydroxybutyrate and pyruvate, while *Bacteroidaceae*, *Bifidobacteriaceae* and *Porphyromonadaceae* were positively correlated with fecal butyrate, uracil, pyruvate, and 3-D-hydroxybutyrate. Correlation analyses also confirmed the positive association

between *Lachnospiraceae*, *Rikenellaceae*, *Coriobacteriaceae*, *Porphyromonadaceae* and *Bacteroidaceae* and the urinary intermediates of BCAA catabolism, the NAD/NADP pathway end-product 4PY and choline, as already described in the PCA loadings plot (Figure 44.B). Noticeably, *Streptococcaceae*, *Ruminococcaceae* and *Peptostreptococcaceae* were clustered together in the heatmaps for the fecal and plasma data (Figure 45, B). These families of bacteria were all positively correlated with fecal bile acids and glycerate and negatively correlated with fecal butyrate and pyruvate; they were also negatively correlated with plasma pyruvate, citrate and acetate. However, *Peptostreptococcaceae* were not clustered with *Streptococcaceae* and *Ruminococcaceae* in the heatmaps with urinary metabolites. *Bacteroidaceae*, *Bifidobacteriaceae* and *Porphyromonadaceae* were found to be negatively correlated with urinary phenylacetylglutamine, PCS and PCG, previously noticed in the PCA loadings plot (Figure 44.B). Finally, it is important to note that the families *Bacteroidaceae* and *Lactobacillaceae* were not significantly correlated with any plasma and fecal metabolites, respectively.

A correlation analysis between circulating markers of inflammation, the (family level) fecal microbiota and metabolites from urine, plasma and fecal samples at D70 was carried out. Of the circulating cytokines measured with a multiplex ELISA assay (as described in Chapter IV, section 2.5 (Materials and Methods) and section 3.1.3 (Results)), only IL-1 β showed significant correlations with the fecal microbiota and metabolites. The correlation network around IL-1 β was summarised in a bipartite graph (Figure 46). With a correlation cut-off IRI >0.3 and the corresponding *p* value <0.05, IL-1 β was connected with nine entities (three families of bacteria and six metabolites), which were in turn correlated with each other. In particular positive correlations were found between circulating IL-1 β , fecal *Streptococcaceae* ($R=0.34$), *Peptostreptococcaceae* ($R=0.40$), fecal bile acids ($R=0.34$), urinary PCG ($R=0.42$) or PCS ($R=0.39$), the last two metabolites being associated with bacterial fermentation of amino acids.²²⁶ In addition, fecal *Bifidobacteriaceae*, fecal levels of butyrate and plasma levels of citrate, which were positively correlated with each other, were all negatively correlated with IL-1 β ($R=-0.33$ for correlation between IL-1 β and butyrate, and $R=-0.33$ for correlation between IL-1 β and citrate). These results highlight the link between specific members of the gut microbiota, microbial fermentation of amino acids and levels of circulating markers of inflammation. However, it is important to note that no correlations between IL-1 β and other entities were found when the correlation cut-off was set at 0.5, which indicates that this inflammatory marker was not strongly correlated with the fecal microbiota and host/bacterial metabolites.



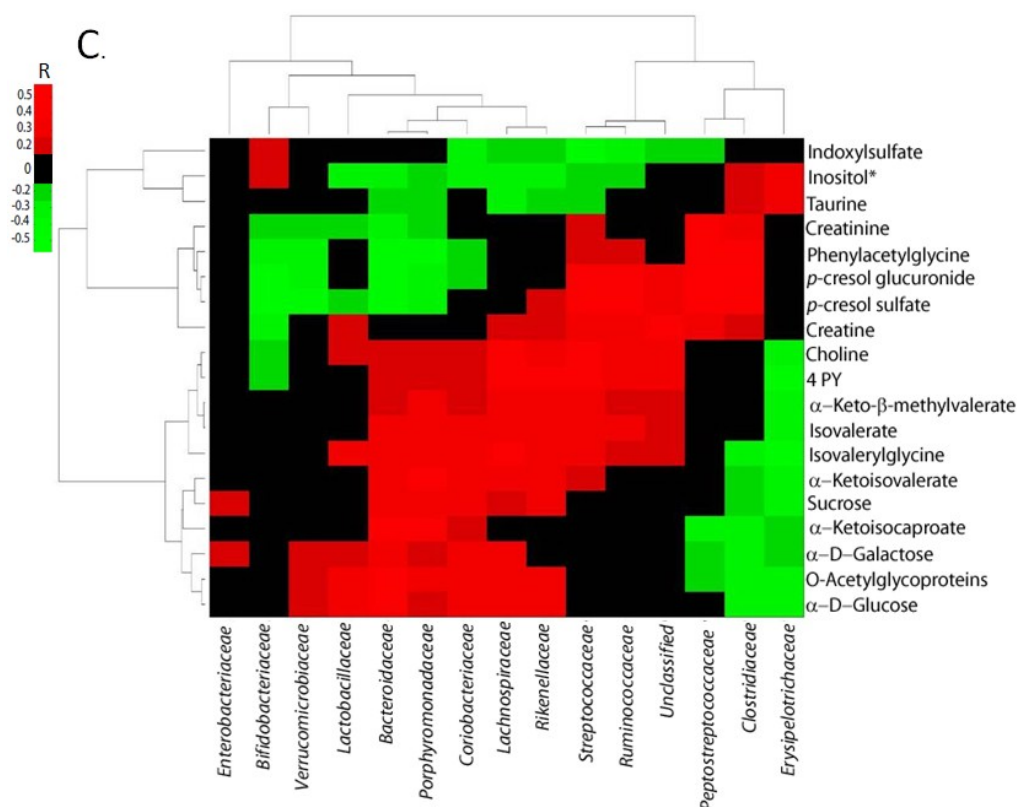


Figure 45: Heatmaps showing bidirectional clustering of Spearman correlation data for families of fecal bacteria and (A) plasma, (B) fecal and (C) urinary metabolites at D70. Heatmaps were produced using Spearman correlation values generated from family-level microbiota data and statistical recoupling of variables (SRV) clusters of ^1H NMR peak intensities²²⁸ (see Material and Methods). Each SRV cluster was in turn assigned as a metabolite, but several clusters remained unidentified (UA metabolites). Sign of correlation: red, positive correlations; green, negative correlations. The p values were determined for Spearman correlations; p value <0.05 was taken as representing statistically significant correlations



Figure 46: Bipartite graph showing the correlation network for IL-1 β , metabolites (urine, plasma and feces) and members of the fecal microbiota. The correlation network was built using significant Spearman correlation results using an arbitrary cut-off of IRI >0.3 and *p* value <0.05. Each node corresponds to an entity (i.e. a metabolite, family or cytokine). The nodes colors are associated with the type of entity: brown, fecal metabolites; yellow, urinary metabolites; pink, plasma metabolites; blue, fecal bacteria at family level; white, circulating cytokine. The edge between two nodes represents a significant Spearman correlation (*R*) between the two entities the nodes represent. The colors of the edges are dependent on the sign of *R*: green, positive correlation; red, negative correlation. The thickness of the lines indicates the absolute value of *R*: thinnest lines for IRI $\leq$ 0.49; medium thickness lines for $0.5 \leq$ IRI $\leq$ 0.599; and thickest lines for IRI > 0.6.

4. DISCUSSION

Ninety C57BL/6 mice were fed a LF diet for 3 weeks. The mice were then divided among six groups of animals with similar distributions of weight and glycemia. One group of animals was kept on the LF diet, while the animals in the other groups were fed HF, HF_S, HF + FOS_IN, HF + GOS or

HF + GOS_CMO. At D-7, D7 and D70, the composition of the fecal microbiota of the animals was determined using 16S rRNA gene sequence analysis. Metabolites (¹H NMR urine, plasma and feces) and circulating markers of inflammation were also examined for the D70 samples.

4.1. HF diet induced rapid changes in the composition of the gut microbiota

Analysis of 16S rRNA gene sequence data obtained from the fecal microbiota of the mice at D7 of the dietary intervention showed that the HF diet with or without a sugar blend or prebiotics modulated the gut microbiota. In particular, supplementation of the HF diet with various prebiotics (FOS_IN, GOS, and GOS_CMO) affected the population levels of a number of different phyla and families of bacteria (Figure 41 and Figure 42). A marked prebiotic effect, as evidenced by statistically significant increases in the abundance of fecal *Bifidobacteriaceae*, was observed for the HF + prebiotic-fed animals at D7 and D70 of the study. This effect was most obvious with HF + FOS_IN or HF + GOS at D70). Correlation analyses done using the total variance of the fecal microbial profile (at the family level), ¹H NMR metabolite (urine, plasma and feces) data and levels of circulating markers of inflammation at D70 pointed out the substantial influence of the gut microbiota on the regulation of host inflammatory status and host metabolism.

It was found that *Firmicutes* and *Bacteroidetes* dominated the fecal microbiota of the mice (Figure 41). However, a lower ratio of *Firmicutes*-to-*Bacteroidetes* was observed in mice fed HF and HF_S compared with the LF-fed mice at D7 and D70. These results do not support the work of those who have demonstrated a higher *Firmicutes*-to-*Bacteroidetes* ratio in the cecal microbiota of genetically obese *ob/ob* mice, and the feces of obese humans and pigs^{77,177,232,233}. This is not the first time the findings of studies have been contradicted with respect to the *Bacteroidetes*-to-*Firmicutes* ratio and its association with obesity: studies in humans have shown a higher *Bacteroidetes*-to-*Firmicutes* ratio in overweight individuals or no significant difference between obese and lean individuals²³⁴⁻²³⁶. At the phylum level, the composition of the fecal microbiota in the different diet groups was similar at D7 and D70, but the difference in the relative abundances of *Bacteroidetes* and *Firmicutes* in the HF-fed groups compared with the LF-fed group observed at D7 was pronounced at D70.

At the family level, more subtle variations in the composition of the fecal microbiota occurred (Figure 42), which illustrated the need to study the gut microbiota at lower taxonomic

levels. In particular, HF- and HF_S-fed mice were associated with increases in *Ruminococcaceae*, *Peptostreptococcaceae*, *Streptococcaceae* and *Lachnospiraceae*, and a decrease in *Erysipelotrichaceae* (all phylum *Firmicutes*); in addition, they had increased levels of *Rikenellaceae* and *Porphyromonadaceae* (both phylum *Bacteroidetes*) compared with the LF-fed mice. Variations in the composition of the fecal microbiota were obvious at D7 of the dietary intervention, with these variations consistent with those observed at D70. These results indicated a rapid remodelling of the gut microbial environment, which persists over time. However, time-dependent changes in the fecal community profiles were not monitored: these would have given substantial information about the stability of the fecal microbiota in the different diet groups. Dietary changes have been proven to induce strong and rapid changes in the composition of the gut microbiota^{237,238}. Wu *et al.* pointed out variations in the human gut microbiota 1 day after the initiation of controlled feeding. However, gut microbial enterotypes are altered only after long-term dietary changes²³⁷. To summarise, in our experiment, ingestion of a HF diet induced a rapid remodelling of the intestinal bacteria belonging to several families within the phyla *Firmicutes* and *Bacteroidetes* and long-term dietary intervention contributed to the maintenance of this modulated ecosystem.

4.2. HF-induced modulation of the gut microbiota accompanies changes in host lipid storage and metabolism

The families *Streptococcaceae*, *Ruminococcaceae* and *Peptostreptococcaceae* (all phylum *Firmicutes*) and *Rikenellaceae* (*Bacteroidetes*) were positively correlated with circulating lipids and bile acids excreted in feces of the C57BL/6 mice when D70 data from all feeding groups were combined (Figure 45). Examination of the abundance data for these families demonstrated that all four were present at significantly higher levels in the feces of the HF- and HF_S-fed mice compared with the LF- and HF + prebiotic-fed mice (Figure 42). In the Chapter IV (section 3.2.5), it was demonstrated that the HF and HF_S diets led to increased production of BCAA and circulating lipids compared with the LF diet, while the HF + prebiotic diets led to increased butyrate, 3-D-hydroxybutyrate and succinate (a precursor for microbially produced propionate) production and decreased levels of circulating lipoproteins (Table 10). A *Firmicutes*-rich gut microbiota in obese people has been suggested to increase the capacity of the microbiota for energy harvesting from the diet as many of these bacteria harbor genes that encode enzymes which enable the extraction of energy from undigested polysaccharides and proteins³³. However, these observations must be interpreted with caution given the importance of some of these bacteria in the production of the

short-chain fatty acids (SCFAs) butyrate (e.g. *Roseburia* and *Faecalibacterium* spp.), propionate and acetate in the human intestine either directly or *via* cross-feeding on acetate, lactate and succinate and which confer beneficial effects on human health²³⁹. In addition, genome analysis of *Bacteroides thetaoitaomicron*, a member of the *Bacteroidetes*, has shown this gut bacterium has a capacity to utilize undigested dietary polysaccharides and host-derived glycans that exceeds that of *Clostridium perfringens*, *Bifidobacterium longum* and *Escherichia coli*⁷¹. It has been shown here that at least one family of the *Bacteroidetes*, *Rikenellaceae*, is positively correlated with bile acids and lipids in the mouse model, implicating members of both the *Firmicutes* and *Bacteroidetes* in host lipid metabolism in the presence of a HF or HF_S diet. Therefore, the role of *Rikenellaceae* in host lipid metabolism warrants further attention.

Increased microbial fermentation of polysaccharides, such as prebiotics, in the colon results in higher production of SCFAs, which activate signal transduction pathways through GPR41 and GPR43^{232,240-242}. GPR41 liberates peptide YY, which reduces appetite by reducing motility and gastric emptying and thereby increases efficiency of digestion and improves nutrient absorption^{210,241,243}. GPR43 is involved in anti-inflammatory processes and the secretion of peptides (including peptide YY) in the colon, and participates in adipocyte differentiation and inhibits lipolysis in adipocytes.²⁴⁴ Activity of these receptors in the intestinal tract was not monitored in the current study but it was found that prebiotic supplementation of the HF diet led to significant decreases in weight gain and fat mass during HF challenge (see Chapter IV, section 3.1.1), with increases in fecal butyrate and succinate detected in the prebiotic-fed animals (Table 10). This suggests that appetite suppression caused by SCFAs produced by the gut microbiota from fermentation of prebiotics in the intestine may contribute for decreases in weight gain and fat mass. This hypothesis is supported by the fact that HF + prebiotic fed mice were found to decrease their weekly food intake compared to HF and HF_S fed mice (see Chapter IV, section 3.1.1). *Peptostreptococcaceae*, *Streptococcaceae* and *Ruminococcaceae* were negatively correlated with fecal butyrate, while *Bifidobacteriaceae*, *Bacteroidaceae*, *Porphyromonadaceae* and *Enterobacteriaceae* were positively correlated with this metabolite.(Figure 45.B). Abundance data for these families in the different diets demonstrated that *Peptostreptococcaceae*, *Streptococcaceae* and *Ruminococcaceae* were present at significantly reduced levels in the HF + prebiotic groups, while the *Bifidobacteriaceae*, *Porphyromonadaceae* and *Bacteroidaceae* were present at significantly higher levels in these feeding groups (Figure 42); *Enterobacteriaceae* were also present at lower levels in the HF + prebiotic groups compared with the HF and HF_S groups at D7 and not measurable in LF and HF + FOS_IN fed mice at D7 and D70. However, the variation in the representation of *Enterobacteriaceae* in the diet groups as well as

correlations between *Enterobacteriaceae* and metabolites needs to be interpreted with caution given the large within-group variability in the measurement of this bacterial family. A positive correlation between the *Bifidobacteriaceae*, *Poryphyromonadaceae* and *Bacteroidaceae* and fecal butyrate does not indicate these bacteria are responsible for butyrate production, particularly as data from human gut-derived isolates demonstrate members of *Clostridium* clusters XIVa and IV are responsible for butyrate production in humans and not the aforementioned families (though butyrate-producing *Butyricimonas* spp. (family *Porphyromonadaceae*) have been isolated from rat faeces and detected in human faeces)^{239,245}. However, increase in the abundance of fecal bifidobacteria during intervention studies is considered indicative of the beneficial effect of prebiotics. The bacterial remodelling by prebiotic enhancing gut ecosystem might indirectly favour the production of SCFAs.

To summarise, HF diets in the presence or absence of small sugars such as glucose, lactose and galactose cause a change in the gut microbiota that leads to a proliferation of seemingly 'undesirable' members of the gut microbiota (*Peptostreptococcaceae*, *Streptococcaceae* and *Ruminococcaceae*), at the expense of those known to be of benefit to host health. This modulation of the microbiota is correlated with changes in circulating lipids in the host. HF + prebiotic feeding appears to overcome some of the undesirable effects associated with HF-feeding. Much more work is required to determine the exact effect of the gut microbiota on host lipid metabolism, and whether members of the families specifically mentioned here play a role in this metabolism or merely indicate perturbation of the system by a HF diet.

4.3. Prebiotic supplementation during HF diet leads to increased representation of *Bifidobacteriaceae* or *Lactobacillaceae* in the fecal microbiota

Prebiotic supplementation during HF challenge increased the proliferation of health-promoting bacteria in the current study, which is in accordance with the literature⁹⁵. FOS_IN promoted a rapid and consistent increase in representation of *Bifidobacteriaceae*, detectable at D7 and D70 after the diet switch (Figure 42). By contrast, GOS supplementation induced an increase in representation of *Bifidobacteriaceae* only at D70 after the diet switch, which suggests the microbiota took more time to adapt to utilization of this prebiotic. In addition, there were transitory increases in the representation of *Lactobacillaceae* in the HF, HF + GOS and HF + FOS_IN groups at D7 after the

diet switch. GOS_CMO supplementation triggered a sustained increase in representation of the family *Lactobacillaceae*. This is unsurprising given that GOS_CMO contained lactose, a preferred substrate for lactobacilli, in addition to the prebiotic GOS. A recent study identified that GOS supplementation specifically triggers expression of a cluster of genes in *Lactobacillus acidophilus* encoding intracellular proteins involved in galactose and lactose metabolism²⁴⁶. These findings, along with results presented herein, suggest that lactose and galactose metabolism in members of the family *Lactobacillaceae* is boosted by GOS supplementation, allowing better utilization of the prebiotic and the glucose–lactose–galactose in GOS_CMO, which in turn might increase the representation of these bacteria in the gut^{105,246}. In summary, it appears that the prebiotic effect elicited by GOS and FOS_IN was bifidogenic in nature, whereas that elicited by GOS_CMO favoured the lactobacilli.

In vitro studies have pointed out that prebiotic effects on the gut microbiota depend on monosaccharide composition, glycosidic bond between monosaccharides and finally the length of the resulting polysaccharide chain^{181,247-249}. For instance, Roberfroid *et al* demonstrated that the ability to grow bifidobacteria was almost directly proportional to the degree of polymerization of the prebiotic²⁴⁸. *In vitro* studies have demonstrated that galactose-containing oligosaccharides are more efficient than FOS or inulin in promoting growth of bifidobacteria, which results presented here do not support. However, it is important to note that *in vitro* experiments do not take into account the time-dependent adaptation of gut microbiome to prebiotics. The bacterial ecology of the intestinal tract is much more complex than a controlled culture medium, which may affect the fermentation activity of specific strains^{181,250}. In addition, the activity of rodent bifidobacteria with respect of their ability to digest prebiotic have been less characterised *in vitro*, so their prebiotic preference is not known. Oligosaccharide fermentation in the human colon results from a sequence of enzymatic reactions performed by numerous bacteria that are not necessarily present *in vitro*²⁵⁰. A cross-feeding phenomenon occurs *in vivo* where some bacteria digest complex carbohydrates and release degradation products that are used as substrates by other bacteria. These bacteria might also have dietary preferences for a type of fibre and release different bacterial products according to the carbohydrate availability, which in turn preferably stimulates the growth of other bacteria. For instance, Duncan *et al* found that *Roseburia hominis* A2-183^T was able to growth better on complex carbohydrates in pure culture than *Roseburia inulinivorans* A2-194^T, although A2-183^T was unable to digest complex sugars. Growth of *Roseburia inulinivorans* A2-194^T, which can digest carbohydrates, was significantly increased when inulin was added to the carbohydrates cultures²⁵¹.

Impurities in prebiotic formulations, dosage, and additional nutritional interventions also influence the fermentative process of prebiotics and the gut microbiota composition *in vivo*²⁴⁹. Unfortunately, no information about the degree of polymerization and the type of glycosidic bonds of our commercial prebiotic blends was provided. However, it can be suggested that FOS_IN is more easily fermented by colonic bacteria than GOS, leading to greater and more rapid increases in representation of bifidobacteria at D7 (Figure 42). This might be explained by the fact that these anaerobic bacteria have a substrate preference for FOS_IN over GOS.

4.4. Prebiotic supplementation during HF challenge changes the proportions of families belonging to the phyla *Bacteroidetes* and *Firmicutes*

Prebiotic supplementation during HF challenge was found to change the relative abundances of all taxa from the phylum to family level. All prebiotics increased the representation of the families *Porphyromonadaceae* and *Bacteroidaceae* (both phylum *Bacteroidetes*). These bacteria are among the most abundant saccharolytic microorganisms in the mammalian gut and play a major role in polysaccharide digestion in the human large intestine¹⁸¹. *Bacteroidetes* spp. colonize mainly the ileum and the colon; therefore, increased polysaccharide availability in the colon due to prebiotic supplementation triggered the proliferation of this family of bacteria above levels seen in the HF- and HF_S-fed animals (Figure 42). The *Porphyromonadaceae* and *Bacteroidaceae* were positively correlated with urinary excretion of galactose, glucose and sucrose, which supports the evidence of increased FOS_IN and GOS fermentation with *Bacteroidetes* proliferation. These results are in line with the literature, highlighting a higher representation of several members of phylum *Bacteroidetes* with prebiotic supplementation^{181,252}.

HF + prebiotic diets also led to a decrease in representation of four families belonging to the *Firmicutes* (*Streptococcaceae*, *Clostridiaceae*, *Ruminococcaceae* and *Peptostreptococcaceae*) compared with the HF- and HF_S-fed groups and an increase in *Verrucomicrobiaceae* (phylum *Verrucomicrobia*) at D7 after the diet switch (Figure 42). This is consistent with results published with Everard *et al*, who identified in prebiotic-fed genetically obese C57BL/6 mice, a decrease in six genera of the phylum *Firmicutes* such as *Ruminococcaceae* as well as an increase in *Verrucomicrobiaceae*²⁵². *Verrucomicrobiaceae* have been associated with decreased obesity in animals and human models^{236,252}. *Akkermansia muciniphila*, a member of the *Verrucomicrobiaceae*

family, has also received particular attention since it has been inversely correlated with type 1 diabetes and body weight in humans and mice^{252,253}. This mucin-degrading bacteria living in the mucus layer represents 3 to 5% of the microbial ecosystem in healthy individuals and is the dominant colonizer of nutrient-rich environments in human gut²⁵⁴. It has been found to increase around 100-fold in genetically obese mice after oligofructose administration and may participate in the beneficial role of prebiotic on obesity²⁵². Everard *et al* have recently demonstrated reduced *A. muciniphila* abundance in obese C57BL/6 mice, and the restoration of *A. muciniphila* abundance with prebiotics improves systemic inflammatory status, and lowers diet-induced body weight gain, fat mass and fasting hyperglycaemia independently of food intake. This reduced energy storage is explained by an activation of adipose differentiation, lipogenesis and fatty acid oxidation²⁵⁵. Everard and co-workers also provided the evidence that *A. muciniphila* activates the endocannabinoid system, which is implicated in the control of gut barrier and inflammation²⁵⁵. In particular, 2-oleoylglycerol, which was found elevated with *A. muciniphila* supplementation, is involved in the stimulation of glucagon-like peptides secretion from the intestinal cells, regulating gut barrier function. 2-arachidonoylglycerol, also increased with *A. muciniphila* supplementation, was associated with reduced endotoxemia and circulating inflammatory cytokines²⁵⁵. High circulating LPS level has been previously found to inhibit adipogenesis (adipose differentiation and lipogenesis), disturbing adipose metabolism and promoting obesity²⁵⁶. Therefore, their findings suggest that *A. muciniphila* restores gut function *via* a stimulation of the endocannabinoid system limiting metabolic endotoxemia, improving adipocyte physiology and lowering host inflammatory status. In the present experiment, *Verrucomicrobiaceae* was negatively correlated with circulating lipid levels and urinary PAG, PCS and PCG levels. Therefore, prebiotic supplementation during HF challenge may increase the representation of *Verrucomicrobiaceae* and *A. muciniphila* species particularly, which improves gut barrier function and adipose metabolism, and lowers dyslipidemia by the above mentioned mechanisms. In addition, reduced urinary PCS and PCG in HF +prebiotic fed mice may result from *A. muciniphila* proliferation and improved gut permeability, which lowers the transfer of microbial co-metabolites into systemic circulation. However, additional experiments evaluating *A. muciniphila* abundance in each diet group and its impact on circulating PCS, PCG and lipoprotein levels are needed to confirm these hypotheses.

Interestingly, significant changes in *Firmicutes* and *Verrucomicrobiaceae* abundance associated with HF + GOS and HF + GOS_CMO supplementation occurred before the proliferation of the *Bifidobacteriaceae* and *Lactobacillaceae*, respectively (Figure 42). These results suggest that prebiotics modify the gut ecosystem independently of the increase in representation of beneficial

bacteria during the initial stages of dietary supplementation. Several studies have shown that prebiotics, and particularly FOS, are able to stimulate many other groups of bacteria²⁵¹. In this context, bifidobacteria may not be the most responsive bacteria to prebiotics^{251,257}. For instance, several *Clostridium* and *Roseburia* strains were found to be stimulated by inulin supplementation in a complex culture of fecal bacteria²⁵¹. Prebiotic preferences of bacteria are not completely understood as researchers have tended to focus on characterization of 'beneficial bacteria' in pure and mixed cultures, but utilization of prebiotics by the mixed microbiota involves bacterial cross-feeding and changes in gut environment (pH, hydrogen release, nutrient availability), factors that influence the viability of different bacteria in different ways²⁵⁷. In addition, possible mechanisms by which prebiotic receptors could competitively bind pathogenic bacteria and reduce their colonization have been envisaged²⁵⁸.

To summarise, GOS, FOS_IN and GOS_CMO supplementation during HF diet differently affect gut environment and bacterial cross-feeding, leading to a remodelling of the gut microbiota that is independent of increases in representation of the *Bifidobacteriaceae* or *Lactobacillaceae*. The development of this new ecosystem might also participate in the overall improvement of host physiology during prebiotic intervention. It is important to better understand the mechanisms by which prebiotics favour the growth of some bacteria over others, as it would enable us to develop more selective prebiotics targeting the inhibition and/or the proliferation of specific bacteria. It would also enable us to identify the primary prebiotic utilizers rather than those bacteria that are used to measure a prebiotic effect (i.e. the bifidobacteria and lactic acid bacteria). Finally, it is necessary to further investigate the effects of these other members of the gut microbiota on host physiology as they may hold probiotic properties.

4.5. Correlation analysis highlights associations between *Lachnospiraceae*, *Bacteroidaceae*, *Porphyromonadaceae*, *Rikenellaceae*, *Coriobacteriaceae*, or *Erysipelotrichaceae* and host mitochondrial oxidative pathways.

Data integration between fecal microbiota profiles and urinary, plasma or fecal metabolic profiles acquired by ¹H NMR were performed using the total variance of the dataset at D70.

Lachnospiraceae, *Bacteroidaceae*, *Porphyromonadaceae*, *Coriobacteriaceae* and *Rikenellaceae*, which increased in all HF-fed mice (Figure 42), were positively correlated with a urinary increase in BCAA catabolism intermediates (α -keto- β -methylvalerate, α -ketoisovalerate, isovalerate, isovalerylglycine) and 4PY (Figure 45.C). *Erysipelotrichaceae*, which decreased in HF-fed mice (Figure 42), were negatively correlated with the same metabolites (Figure 45.C). The β -oxidation and BCAA catabolism provide substrate for TCA cycle and energy production in the mitochondria. Therefore, these results suggest that HF-diet-induced remodelling of the gut microbiota is linked with host mitochondrial activity. Relationships between gut microbiota and host mitochondrial activity have been already evidenced. Backhed and co-workers showed that GF mice re-conventionalized with gut bacteria (mainly *Bacteroidetes* and *Clostridium* spp.) produced 60 % more fat mass, and had increased hepatic triglycerides and fasting blood glucose compared to GF mice despite comparable energy expenditure and less food consumption⁷⁷. They also highlighted that the gut microbiota promoted fat storage by suppressing the expression of angiopoietin-like protein 4 (anglp4) in the ileum, an enzyme which controls fatty acid oxidation and storage in skeletal muscle cell and adipocytes through the regulation of peroxisomal proliferator activated receptor coactivator (PGC-1) α expression²⁵⁹. A recent publication has reported correlation between *Lachnospiraceae* and the expression of mitochondrial transporter *Slc25a25*, which is responsible for the shuttle of ATP-Mg²⁺ and inorganic phosphate across the mitochondrial membrane. The suppression of this transporter modulates mouse metabolic efficiency²⁶⁰⁻²⁶². However, the mechanisms involving the interactions between gut microbiota and host mitochondrial activity has not been clearly defined in the literature. Mitochondrial oxidative pathways as well as gut microbial activity are strongly affected by nutritional intervention: this relationship requires further investigation.

4.6. Correlation analysis highlighted an association between circulating inflammatory status of mice and gut microbial activity/composition

On the one hand, the HF diet raised the levels of pro- and anti-inflammatory markers in C57BL/6 mice. On the other hand, prebiotic supplementation during HF challenge tended to alleviate this inflammatory response, particularly by limiting the levels of circulating IL-1 β (see Chapter IV, section 3.1.3). *Bifidobacteriaceae* was negatively correlated with circulating levels of IL-1 β , PCG, PCS (Figure 46), suggesting that prebiotic supplementation during HF-feeding can lessen inflammation in

obese individuals and fermentation of proteins by the gut microbiota. Conversely, *Streptococcaceae* and *Peptostreptococceae* were positively correlated with IL-1 β levels and those of products of microbial amino acid fermentation. In the previous chapter (Chapter IV, section 4.1), the link between adipose tissue expansion, hepatic triglycerides saturation, ectopic fatty acid accumulation and the development of sub-clinical inflammatory status during a prolonged weight gain, HF-feeding period was discussed. It is suggested in this chapter that the activity of specific members of the gut microbiota and/or their metabolites may have an effect on the inflammatory status of mice fed a HF diet.

It is well documented that bacteria or by-products of bacterial metabolism can enter host circulation, provoking an inflammatory response and in some cases metabolic dysfunction^{263,264}. For example, the endotoxin lipopolysaccharide (LPS) derived from the outer cell membrane of the Gram-negative bacteria. LPS has been shown to activate Toll-like receptors (TLRs) *via* several processes, leading to intracellular activation of NF κ B and in turn the stimulation of the innate immune response^{263,265,266}. LPS contains a lipid structure that is able to cross the gastrointestinal mucosa by infiltrating chylomicrons in the intestine or *via* damaged intestinal tight junctions²⁶⁵⁻²⁶⁷. Once it reaches systemic circulation, LPS infiltrates tissues such as liver or adipocytes enhancing the production of pro-inflammatory cytokines and metabolic dysfunction²⁶⁶. The metabolic endotoxemia phenomenon has been previously correlated with obesity and other metabolic disorders in mouse and human models^{78,264,266,267}. Interestingly, an increase in gram-negative bacteria of the family *Rikenellaceae* (phylum *Bacteroidetes*) was observed during HF and HF_S feeding (Figure 42), with these bacteria also positively correlated with urinary PCS, and plasma lipids (Figure 45.A, Figure 45.C). In addition, it is interesting to mention that *Enterobacteriaceae* (phylum *Proteobacteria*) was increased in HF and HF_S fed mice compared to LF and HF + prebiotics fed mice at D7 although a large within-group variability was observed in the abundance of this bacterial family. Several gram-negative bacteria belonging to *Enterobacteriaceae* family including *Salmonella spp* and *Shigella spp* have been responsible for multiple intestinal and systemic infections by endotoxic activity of LPS^{268,269}. Other gram-negative bacteria, *Porphyromonadaceae* and *Bacteroidaceae* (both phylum *Bacteroidetes*) were found at significantly higher levels in the HF + prebiotic-fed mice than in the HF-fed mice (Figure 42). These families were positively correlated with faecal butyrate (Figure 45.B), negatively correlated with products of microbial amino acid fermentation (Figure 45.C); and were not correlated with plasma lipids. Therefore, it is clear that the role of gram-negative bacteria in LPS-induced inflammation is not fully understood. It should be noted that none of these families

belonging to the *Bacteroidetes* were significantly correlated with circulating cytokines in the present study.

A negative correlation with IL-1 β and the family *Bifidobacteriaceae*, which itself was positively correlated with faecal butyrate, was found. SCFAs interact with different cell types involved in inflammatory responses in the gut^{206,270}. They modulate the production and the release of chemokines which are responsible for the recruitment of neutrophils and macrophages to the infection site.²⁷¹ They change the expression of adhesion molecules in neutrophils and endothelial cells which disturb the interaction between these leukocytes and the gut membranes²⁷⁰. Finally SCFAs, and butyrate in particular, suppress the production of pro-inflammatory cytokines in neutrophils and macrophages while they may activate the release of the anti-inflammatory cytokine IL-10²⁷⁰⁻²⁷². For instance butyrate supplementation has been found to reduce IL-1 β levels in patients with sepsis, which diminishes tissues injury and multiple organ dysfunctions in rats²⁷³. Bifidobacteria were found to reduce endotoxemia in mice and rats^{88,274}. Cani and co-workers tested the effects of prebiotic-stimulated growth of *Bifidobacterium* spp. on intestinal endotoxin level and metabolic disorders in HF-fed mice. They found an increase in intestinal endotoxemia and plasma levels of cytokines IL-1 α , IL-1 β and IL-6 in HF-fed mice. However, prebiotic supplementation during HF challenge reduced intestinal LPS levels and prevented increases in levels of circulating pro-inflammatory markers. Representation of *Bifidobacterium* spp. was also anti-correlated with adipose tissues, glucose intolerance, body weight gain, increased fasted insulinemia and inflammatory markers, highlighting the strong relationship between gut microbiota composition/activity and the development of metabolic disorders⁸⁸. Therefore, prebiotic supplementation during HF challenge might restore host metabolism–gut microbial interactions and reduce inflammatory status of mice by promoting growth of *Bifidobacteriaceae* and SCFA production in the intestine. Prebiotics may lower inflammatory responses to HF diet by reducing LPS infiltration; however, additional measurements of intestinal and plasma LPS levels and membrane permeability would be necessary to confirm this hypothesis.

4.7. Increased gut bacterial proteolysis contribute to inflammation in HF-fed mice

In the present experiment, HF + prebiotic diets were associated with a decrease in urine PCS and PCG compared to the HF, HF_S or LF diet (Table 10). *p*-Cresol is produced by gut bacteria from

tyrosine fermentation and a large proportion of *p*-cresol can cross the mucosal barrier to reach blood circulation. Approximately 95 % of *p*-cresol in mice is transformed by *o*-sulfation into PCS in the colonic epithelial cells and in the liver. A remaining proportion of *p*-cresol (around 4 %) is phase II glucuronidated into PCG exclusively in the liver while 1 % stays in a free form. *p*-Cresol has been thought to have carcinogenic properties and has been found in high concentrations in the intestine of patient suffering of inflammatory bowel diseases (IBD) and colorectal cancer. *p*-Cresol and PCS also participate in the pathogenesis of uremic syndrome through inflammatory mechanisms²⁷⁵. In particular, *in vitro* studies have reported that *p*-cresol inhibits cytokines-stimulated expression of endothelial adhesion molecules which are involved in the recruitment of leukocytes in endothelial cells, while PCS increases the proportion of free radical leukocytes which may contribute to the occurrence of vascular damage in uremic populations^{275,276}.

Urinary PCS and PCG were positively correlated with *Peptostreptococcaceae*, *Streptococcaceae* and IL-1 β and negatively correlated with *Bifidobacteriaceae* in our experiment. Several publications have demonstrated that *p*-cresol is mainly produced in the distal colon by anaerobic bacteria. *Peptostreptococcaceae* are defined as strict anaerobes while *Streptococcaceae* are facultative anaerobes, and members of both families are proteolytic²⁷⁷. It can be speculated that modulation of gut microbiota composition toward anaerobic bacteria in HF-fed mice enhances proteolytic activity and the production of toxic molecules such as *p*-cresol. In this context, elevated *p*-cresol and its conjugated derivatives may contribute to intestinal permeability and inflammatory responses in the gut. The proportion of *p*-cresol reaching the circulation and organs such as the kidneys may also trigger immune responses and promote the chronic inflammation associated with a HF diet. However, modulation of the gut microbiota towards an increase in *Bifidobacteriaceae* and a decrease in *Peptostreptococcaceae* and *Streptococcaceae* in HF + prebiotic-fed mice might lower bacterial proteolysis and *p*-cresol production. As previously mentioned in Chapter IV (section 4.5), prebiotic-associated growth of *Bifidobacteria* in the gut has been found to increase SCFA production, which reduces intestinal pH and down-regulates bacterial enzymes associated with fermentation of amino acids⁹⁵. Taken together, results presented here demonstrate that prebiotic supplementation during HF challenge modulates gut microbial composition and activity, resulting in lower production of toxic and carcinogenic metabolites such as *p*-cresol, which in turn might limit the inflammatory response to a HF diet. In addition, IL-1 β is a crucial key regulator in multiple inflammatory processes; as a result, it may have a specific impact in the onset of diet-induced obesity (DIO). Recently, several studies have shown compelling evidence for the role of IL-1 β in the development of type 2 diabetes mellitus (T2DM) and atherosclerosis^{62,278}. For instance, anakinra (IL-1 β antagonist) treatment

commonly used to treat rheumatoid arthritis has been found to reduce glycated hemoglobin levels and increase insulin production in patients with T2DM²⁷⁹. To conclude, the reduction of IL-1 β mediated inflammation with prebiotics might contribute to lower HF induced metabolic disturbances and improves mouse physiology.

5. CONCLUSION

HF diet modulated *Firmicutes* and *Bacteroidetes* populations in C57BL/6 mice, and this modulation may enhance lipid digestion and contribute to the regulation of host lipid metabolism. In particular, *Lachnospiraceae*, *Porphyromonadaceae*, *Rikenellaceae*, *Bacteroidetes*, *Coriobacteriaceae* and *Erysipelotrichaceae* in HF-fed mice have been suggested to influence host mitochondrial oxidative pathways. Changes in gut microbial composition with HF diet toward more *Peptostreptococcaceae*, *Streptococcaceae* may also promote gut bacterial proteolysis and contribute to the HF-diet-induced inflammatory response. In particular *p*-cresol produced by gut microbial fermentation of amino acids in HF-fed mice is thought to increase circulating inflammatory status of mice probably through IL-1 β -related pathways. Prebiotic supplementation during HF challenge promotes the growth of beneficial bacteria but in a qualitative manner: the proliferation of *Bifidobacteriaceae* and *Lactobacillaceae* is strongly dependent on prebiotic composition (e.g. purity, degree of polymerization, type of carbohydrates) and nutrients brought by the diet. HF + prebiotic diets reduced the abundance of several families belonging to the phylum *Firmicutes* and increased the abundance of several families belonging to the phylum *Bacteroidetes*. This suggests that other members of the gut microbiota are responsive to prebiotics and may have a beneficial effect on host metabolism. Finally, changes in gut microbial composition toward fewer *Peptostreptococcaceae* and *Streptococcaceae* with prebiotic supplementation might reduce bacterial proteolysis and in turn lower circulating inflammatory cytokine levels of HF-fed mice.

CHAPTER VI: THE PREBIOTIC STUDY

IDENTIFICATION OF CANDIDATE MARKERS PREDICTING THE LONG-TERM EFFECT OF PREBIOTICS ON MOUSE PHYSIOLOGY DURING DIET-INDUCED OBESITY

1. INTRODUCTION

Individual dietary selection resulting from the combination of biological, physiological, behavioural and economical factors is a major determinant in individual health and particularly in obesity development⁶⁹. Obesity is also a strong predictor for cardiovascular diseases (CVDs), non alcoholic fatty liver diseases (NAFLD) and type 2 diabetes mellitus (T2 DM) risks⁹. For instance, long-term ingestion of high saturated fat or carbohydrate diets is strongly correlated with cardio-metabolic diseases^{9,69}. Furthermore, the impact of food selection on host health depends on the complex interplay between lifestyle, genetics, and symbiotic gut microbial activity leading to various individual predispositions. Dietary modulation as well as obesity, has been proven to impact individual metabolism in several studies including ours^{199,280}. In particular, high fat (HF) induced obesity was associated with a high urinary excretion of fatty acid β -oxidation intermediate (hexanoylglycine), BCAA catabolism intermediates (isovalerylglycine, α -keto- β -methylvalerate, and α -ketosisovalerate) and end-products of NAD/NADP metabolism (*N*-methyl-2-pyridone-5-carboxamide: 2PY, *N*-methyl-4-pyridone-3-carboxamide : 4PY) in C57BL/6 mice in the chapter IV (section 4.2)¹⁹⁹. In addition, since metabolic phenotypes are influenced by a combination of genetic and environmental factors such as dietary habit and gut microbial activity, one can hypothesise that differences in basal metabolic phenotype (not modified by any drug or dietary stimuli) can be used to evaluate individual predisposition to metabolic diseases^{69,115,281}. This concept has been previously developed in pharmacometabonomic studies by Nicholson and co-workers who reported that individual metabolic phenotype (metabotypes) could be used to predict individual responses to a drug treatment and therefore assess individual predisposition²⁸². The authors proposed this approach as a population screening tool to select individuals that could appropriately react to particular drugs and drug doses^{68,282}. In the context of nutrition, assessing the long-term individual risks could also provide the basis for adjusting dietary habits and offering suitable nutritional intervention. This principle has been termed “nutrimetabonomics”⁶⁹. In addition, when individuals are subjected to a specific nutritional intervention, it is also of key importance to develop statistical

models based on metabolic information and to find early markers allowing the follow-up of this intervention in order to assess the improvement of individual physiological status²⁸¹.

In the previous chapters (Chapter IV and Chapter V), the results demonstrated that galactosyl-oligosaccharides (GOS), GOS and cow milk oligosaccharides (GOS_CMO) or fructosyl-oligosaccharides and inulin (FOS_IN) supplementation during HF challenge in C57BL/6 mice limit obesity development and improve mouse physiology. Indeed, HF + prebiotic fed mice had significantly lower body weight gain (BWG), calorie storage, fat mass, fasting glycemia and circulating pro-inflammatory interleukin-1 β (IL-1 β) levels compared to their positive control group: HF and HF_S fed mice 70 days (D70) after the diet switch. In particular, during the first 35 days after the diet switch, HF + prebiotic fed mice had a similar BWG to LF-fed mice highlighting a prevention of obesity development during the early phase of dietary intervention (Figure 47). However, after 35 days (D35), a divergence in weight trajectory between HF + prebiotic fed mice and LF-fed mice was observed which indicates that prebiotic supplementation no longer prevented obesity development after a long-term period of HF challenge (Figure 47). Importantly, mice encounter a weight loss at D14 because they were subjected to a 12 hours fasting followed by a challenge with ¹³C linoleic acids. This intervention explains the drop in BWG and CFE dynamic curve observed at this time point. Our previous results also demonstrated that prebiotic supplementation during HF diet quickly modify gut microbiota composition leading to profound changes in gut microbial amino acid degradation, host lipid metabolism and host mitochondrial metabolic pathways. Interesting correlations between plasma, fecal, urinary metabolic profiles, fecal microbial profiles, and circulating cytokine profiles, at D70 after the diet switch highlight the importance of the host mammalian-gut microbial interactions in the development of metabolic disorders and obesity.

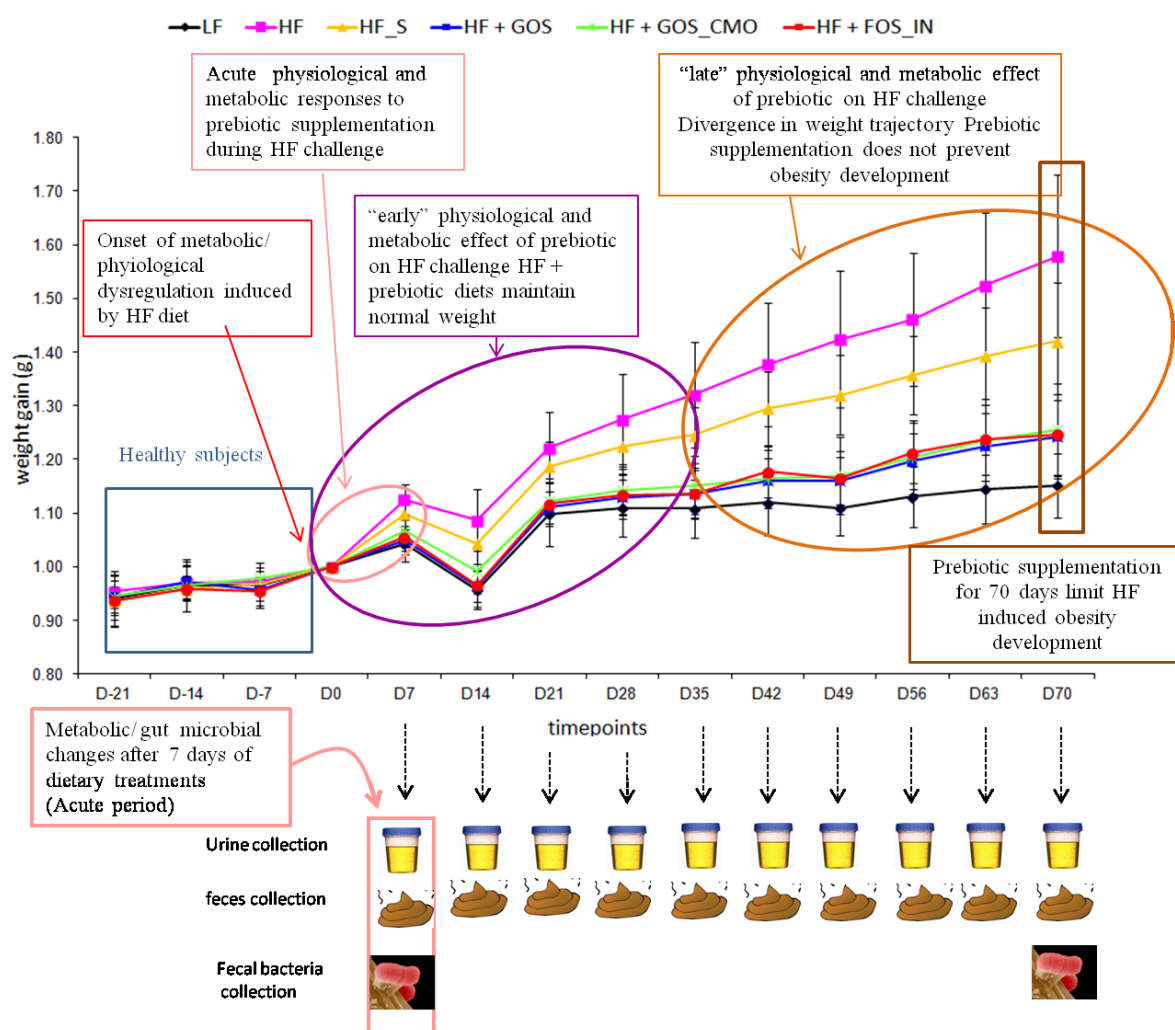


Figure 47: Identification of metabolic/biological information from urine and feces samples predicting the long-term prebiotic effect on obesity development during HF challenge. BWG monitoring of negative control group (LF), positive control groups (HF and HF_S) and prebiotic test groups (HF + GOS, HF + GOS_CMO, HF + FOS_IN) indicated 2 phases in the physiological adaptation to prebiotic supplementation. Time-dependant metabolic and gut microbial adaptation to each diet intervention underlying the mouse physiological variations was also suggested. Therefore, ^1H NMR metabolic profiles for urine and feces at each time-point, and fecal microbiota profiles at D7 and D70 after the diet switch were used to describe the long-term effect of prebiotic on obesity development and metabolic disorders. LF: low fat, HF: high fat, HF_S: HF and glucose–lactose–galactose (positive control of HF + GOS_CMO group,) GOS : galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose).

In the present chapter, we aimed to evaluate whether GOS, GOS_CMO or FOS_IN efficiency in limiting obesity development could be predicted from any metabolic information and/or specific bacterial composition during the early phase of dietary intervention. This would allow the

identification of candidate markers providing an early assessment of prebiotic interventions on mouse phenotype during HF challenge. Hence, a set of OPLS regression models predicting cumulative food efficiency at D70 after the diet switch (CFE_D70) in the positive control group (HF or HF_S fed mice) and the prebiotic test groups (HF + GOS, HF + GOS_CMO or HF + FOS_IN fed mice) were calculated using feces or urine ^1H NMR metabolic profiles at each time point. OPLS regression models predicting CFE_D70 from fecal microbiota at D7 and D70 after the diet switch were also generated. Finally, from the identification of early candidate markers of prebiotic efficiency, additional mathematical models predicting the long-term effect of prebiotics on circulating IL-1 β , fat mass, fasting hyperinsulinemia, fasting hyperglycemia, and organ weights during HF challenge were tested. Importantly, low fat (LF) diet containing different proportions of fat and carbohydrates do not induce obesity development (low mouse BWG, fasting insulinemia, fasting glycemia and circulating IL-1 β concentration compared to HF diet). However, here, the data analysis was strictly focused on identifying biological markers related to prebiotic effect during HF challenge. Therefore, LF-fed mice were excluded from all the predicting models.

2. MATERIALS AND METHODS

2.1. Animal handling procedure and sample preparation

See Chapter IV section 2.1 (Materials and Methods).

2.2. Measurement of body weight, body weight gain, fat mass, food intake and food efficiency

See Chapter IV, section 2.2 (Materials and Methods).

2.3. Measurement of organ weights

After 70 days of dietary intervention, mice were euthanized by anesthesia with isoflurane. Organs (kidney, liver and caecum), epididymal and mesenteric fat were also collected directly after

the sacrifice weighted and snap-frozen at -80 °C. (n=90 for each tissue).The mouse caecum were emptied of undigested nutrients before being weighted.

2.4. Measurement of body composition

See Chapter IV, section 2.3 (Materials and Methods).

2.5. Glucose tolerance and insulin secretion test

See Chapter IV, section 2.4 (Materials and Methods).

2.6. Measurement of circulating cytokines with Multiplexed Elisa assay (Meso Scale platform)

See Chapter IV, section 2.5 (Materials and Methods).

2.7. Sample preparation and ^1H NMR spectroscopy

See Chapter IV section 2.6 (Materials and Methods).

2.8. ^1H NMR spectra pre-processing and ^1H NMR data processing.

See Chapter IV section 2.7 (Materials and Methods) for ^1H NMR spectra pre-processing and Chapter IV, section 2.8 (Materials and Methods) for ^1H NMR data processing.

2.9. 16S rRNA gene sequencing of fecal microbiota

See Chapter V section 2.6 (Materials and Methods).

2.10. Prediction of physiological outcomes of mice fed HF and HF + prebiotics by OPLS regression models

Several OPLS models predicting CFE, fat mass, fasting insulinemia, and organ weights (caecum, liver, kidney, epididymal fat and mesenteric fat weight) of HF and HF+ prebiotics at D70 after the diet switch were generated using the relative abundance of *p*-cresol sulfate, *p*-cresol glucuronide, phenylacetylglycine and *Streptococcaceae* at 7 days after diet switch. Each model enables the calculation of a general equation predicting the physiological outcome. The significance of models was assessed by multiple permutation testing.

3. RESULTS

3.1. Prebiotic supplementation during HF diet limits energy storage and adipose accumulation, and affects organ weights.

Cumulative food efficiency (CFE) defined by BWG: sum of food intake ratio, is a suitable parameter to estimate mouse calorie storage in the different diet groups and overcome the variability induced by food intake. In other words, CFE provide a good estimation of obesity development independently from “satiety-driven” energy intake. As previously mentioned in chapter IV (section 3.1.1), all HF + prebiotic fed mice had lower CFE than HF and HF_S groups during the overall course of the experiment except for HF + GOS_CMO group, which was only significantly lower than HF_S group from D21 onward. Interestingly, the different prebiotic groups had comparable CFE along the study and exhibited similar energy expenditure as LF-fed mice for 4 weeks after the diet switch (Figure 48). This indicates that each prebiotic supplementation during HF challenge was associated with an increase in energy expenditure lowering CFE values throughout the experiment. However, prebiotic benefits were not sustainable after a month of HF challenge, which

resulted in divergence in CFE between HF + prebiotic and LF groups. Noticeably, CFE was significantly higher in all HF containing diet groups at D7 compared to the other time points, and CFE became stable 2 weeks after the diet switch. This indicates that all mice challenged with HF had a boost in calorie storage 1 week after the diet switch whereas a second phase in metabolic adaptations occurs 3 weeks after the diet switch causing an increase in energy expenditure. CFE values also dropped in all the animals at D14 because mice were subjected to a 12 hours fasting (see Chapter IV, section 2.1 (Materials and methods)). It is also important to remind that GOS, FOS_IN and GOS_CMO supplementation during HF diet significantly reduced fat mass, fasting hyperinsulinemia and hyperglycemia at D56 after the diet switch as well as circulating IL-1 β concentration at sacrifice (Table 10). Therefore, as already emphasized in the Chapter IV (section 3.1.1 and section 4), prebiotic supplementation during HF challenge limits obesity development, improves glucose metabolism and reduces HF associated inflammatory responses.

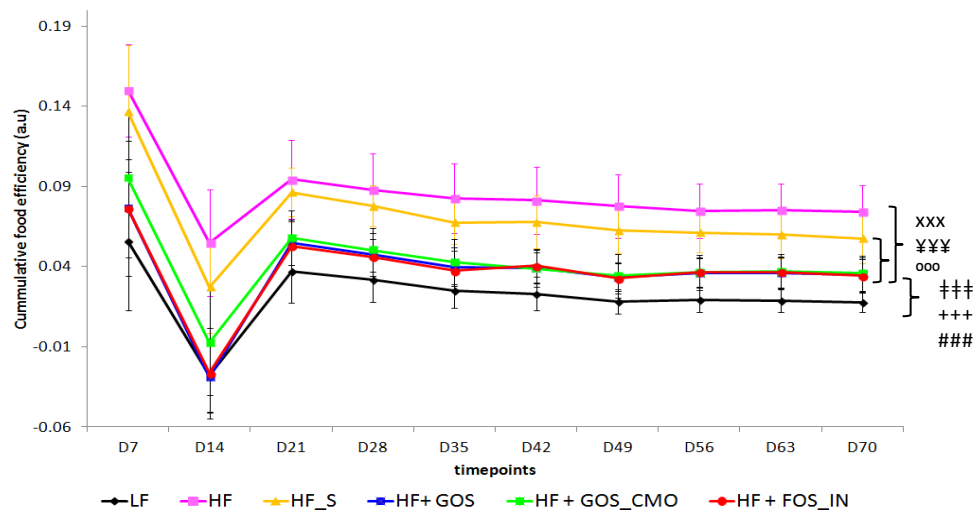


Figure 48: Cumulative food efficiency of negative control group Wilcoxon-Mann-Whitney tests are presented at the last timepoint. 1 star for p -value<0.05, 2 stars for p -value<0.01, 3 stars for p -value<0.001 (x: HF vs HF+GOS, ¥ HF vs HF +FOS_IN, °: HF_S vs HF+GOS_CMO, ‡: LF vs HF+GOS, +: LF vs HF+FOS_IN, #:LF vs HF+GOS_CMO) (LF), positive control groups (HF, HF_S) and prebiotic test groups (HF + GOS, HF + GOS_CMO and HF + FOS_IN) overtime. LF: low fat, HF : high fat, HF_S : HF and glucose–lactose–galactose (positive control of HF + GOS_CMO group,) GOS : galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose).

At sacrifice (D70 after the diet switch), liver, kidney, caecum, mesenteric and epididymal fat weights were also recorded for each mouse. Non parametric Wilcoxon-Mann-Whitney tests (Table 10), indicated that HF + GOS, FOS_IN or GOS_CMO fed mice had a significantly lower mesenteric and epididymal fat compared to their positive control groups (HF and HF_S fed mice). Liver and kidney weight of HF + GOS and HF + FOS_IN fed mice were also significantly lower than HF-fed mice. However, no significant difference in these organ weights was observed between HF + GOS_CMO and HF_S fed mice. This can be explained by the fact that liver and kidney weight tend to be lower in HF_S fed mice than those of HF-fed mice. In addition, a significant decrease in these organ weights were noticed, when HF + GOS_CMO fed mice were compared with HF-fed mice. Interestingly, caecum weight significantly increased in all HF + prebiotic fed mice compared to HF and HF_S fed mice. Consequently, prebiotic supplementation during HF challenge affects mouse physiology by modulating organ weight and reducing adipose accumulation.

Table 10: Effect of long-term dietary interventions on physiological outcomes (*p values* calculated with non parametric Wilcoxon-Mann-Whitney tests)

	56 days (D56) after the diet switch			70 days (D70) after the diet switch					
mean ± sd	Fat mass (g)	fast glycemia (mmol/L)	fast insulinemia (ng/mL)	circulating IL-1β (pg/ml)	Epididymal fat (g)	Mesenteric fat (g)	liver (g)	Kidney (g)	Caceum (g)
LF	3.34 ±0.60	5.97 ±0.43	0.35 ±0.22	1.31 ±0.99	0.13 ±0.036	0.21 ±0.10	0.9 ±0.11	0.15 ±0.025	0.24 ±0.095
HF	9.52 ±2.86	6.69 ±0.70	0.93 ±0.24	3.764 ±1.84	0.51 ±0.20	0.49 ±0.19	1.04 ±0.11	0.21 ±0.06	0.21 ±0.08
HF_S	7.71 ±2.54	6.829 ±0.75	0.48 ±0.12	5.09 ±3.71	0.40 ±0.13	0.42 ±0.20	0.96 ±0.20	0.17 ±0.05	0.21 ±0.04
HF + GOS	3.97 ±0.98	6.387 ±0.64	0.21 ±0.12	1.74 ±0.75	0.22 ±0.09	0.26 ±0.15	0.91 ±0.10	0.18 ±0.06	0.48 ±0.10
HF +GOS_CMO	3.82 ±0.93	6.166 ±0.64	0.30 ±0.23	1.37 ±1.34	0.23 ±0.087	0.23 ±0.10	0.90 ±0.10	0.17 ±0.04	0.34 ±0.09
HF + FOS_IN	4.07 ±1.27	6.014 ±0.70	0.44 ±0.29	1.21 ±0.82	0.24 ±0.09	0.28 ±0.10	0.89 ±0.10	0.15 ±0.05	0.46 ±0.15
p value									
HF vs HF_GOS	8.25 E-13	0.01	0.02	0.05	0.87 E-04	0.26 E-02	0.13 E-02	0.11 E-02	0.76 E-05
HFvs HF +FOS_IN	8.55 E-13	0.00	0.03	0.02	0.68 E-04	0.13 E-02	0.51 E-03	0.62 E-03	0.78 E-04
HF_S vs HF +GOS_CMO	5.85 E-08	0.01	0.01	0.02	0.11 E-03	0.16 E-02	0.25	0.43	0.31 E-06
HF vs HF + GOS_CMO	3.3 E-13	0.1 E-3	0.01	0.32 E-02	0.11 E-03	0.27 E-03	0.16 E-02	0.37 E-02	0.61 E-03

sd: standard deviation LF: low fat, HF : high fat, HF_S : HF and glucose–lactose–galactose (positive control of HF + GOS_CMO group,) GOS : galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose).

3.2. Multivariate prediction of the long-term (D70) prebiotic efficiency in limiting HF induced obesity and metabolic disorders.

Following this observation, we aimed to test whether urinary and fecal metabolic profiles at different time point could predict prebiotic efficiency to reduce calorie storage at sacrifice. Therefore, ¹H NMR spectra from a positive control group (HF or HF_S fed mice) and a prebiotic test group (HF + GOS, HF + GOS_CMO or HF + FOS_IN fed mice) at different time points was correlated with CFE at D70 after the diet switch by using an OPLS (Orthogonal Partial Least Squares)–based pattern recognition method. Using the same method, supervised statistical methods were also applied to investigate whether mouse fecal microbiota at D7 and D70 were a suitable matrix to

predict the long-term prebiotic effect on calorie storage. Because, the LF diet contains a higher carbohydrate/fat ratio compared to HF and HF + prebiotic diets, LF-fed group was purposely excluded from the OPLS regression models to avoid identifying diet-composition related biomarkers. Furthermore, this strategy would enable the identification of early and stable biological markers predicting the long-term prebiotic efficiency in limiting HF associated obesity development and metabolic disorders.

3.2.1. ^1H NMR urinary metabolic profiles from D7 after the diet switch predict prebiotic efficiency in reducing long-term calorie storage during HF challenge.

In order to identify early and specific urinary markers of GOS, GOS_CMO and FOS_IN efficiency in reducing mouse calorie storage during HF challenge, 3 OPLS regressions were performed using urinary metabolic data of HF and HF + GOS, HF_S and HF + GOS_CMO or HF and HF + FOS_IN at D7 and CFE at D70 (CFE_D70). For each model, OPLS scores plots showed a good discrimination between the positive control group (HF or HF_S) and the prebiotic test group (HF + GOS, HF + GOS_CMO or HF + FOS_IN) along the first component (T1). In addition, the good correlation between the T1 scores and CFE_D70 indicates that early metabolic profiles allow the prediction of long-term mouse calorie storage. (Figure 49.A, Figure 49.C, Figure 49.E). The OPLS-loadings plot allowed identifying early discriminatory metabolites being positively or negatively correlated with CFE values for each model. (Figure 49.B, Figure 49.D, Figure 49.F). Furthermore, in order to evaluate the stability in the relationship between urinary metabolic profiles and CFE at D70 after the diet switch, OPLS regression models predicting CFE_D70 from urinary metabolic profiles of HF and HF + GOS, HF and HF + FOS_IN or HF_S and HF + GOS_CMO groups at each time point (from D7 to D70) were calculated. The optimal number of components was determined by R^2Y and Q^2Y statistics (Figure 50). All models were validated by re-sampling the Y variable in permutation tests which showed that urine is a good matrix for energy storage prediction. Noticeably, Q^2Y value under 0.3 was observed in the predictive model with urinary metabolic profiles of HF_S and HF + GOS_CMO at D35 after the diet switch highlighting a lower goodness of prediction at this time point (Figure 50.B). Interestingly, Q^2Y values for models derived from HF and HF_GOS urinary metabolic profiles (Figure 49A) or HF and HF + FOS_IN urinary metabolic profiles (Figure 49.C) dropped at D21 after the diet switch. This is probably due to the fact that at D14 after the diet switch, all mice were subjected to 12 hours fasting followed by gavages with ^{13}C linoleic acids inducing strong metabolic perturbations. Mouse basal urinary metabolic profile might be still affected one week after the

intervention resulting in a loss of matrix predictability. However, this was not observed in the predictive model calculated with HF_S and HF + GOS_CMO urinary metabolic profiles.

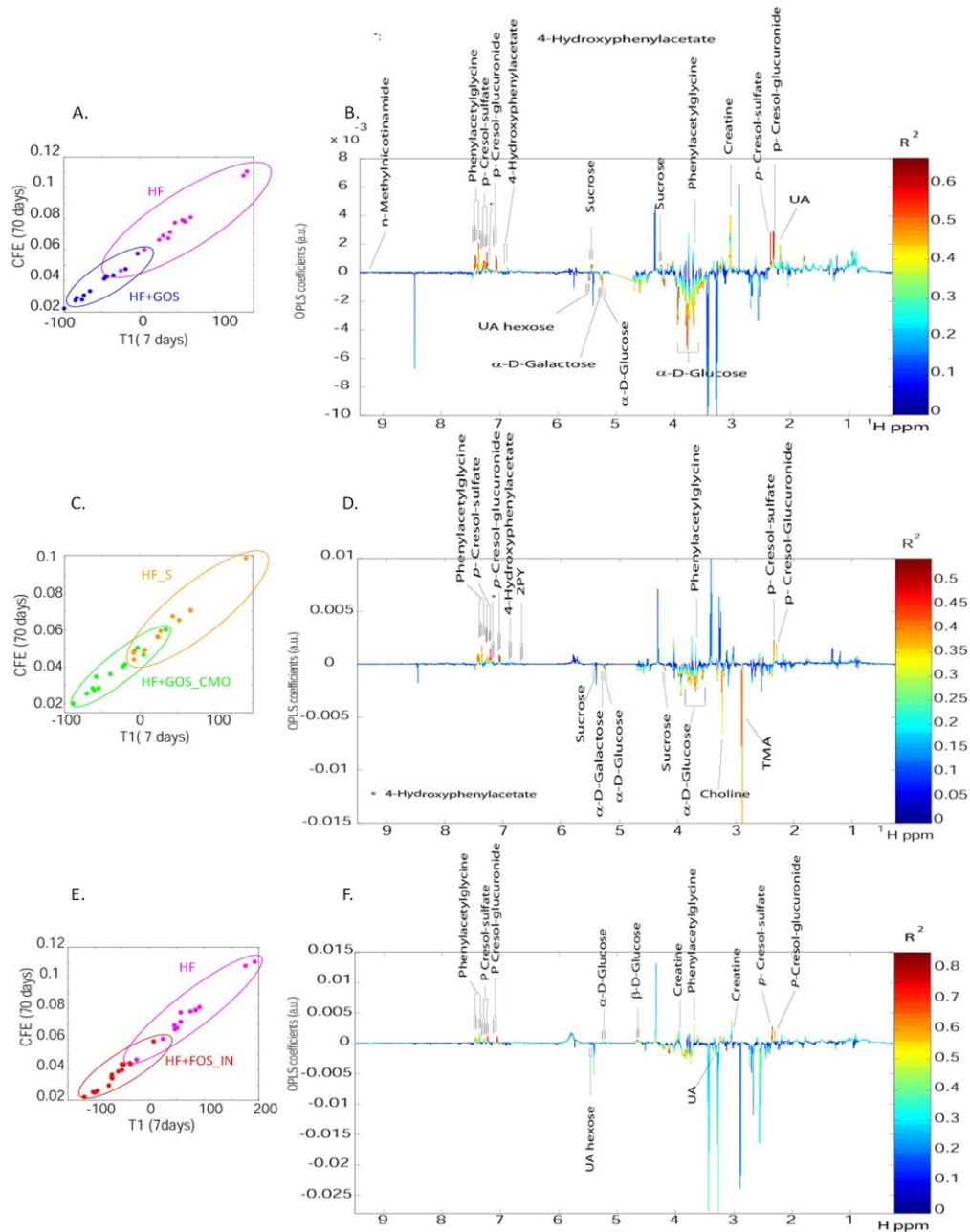


Figure 49: OPLS regression models predicting mouse calorie storage at D70 after the diet switch from D7 urinary metabolic profiles of HF and HF + GOS fed mice (A, B), HF_S and HF + GOS_CMO fed mice (C, D), or HF and HF + FOS_IN fed mice (E, F). For each model, the score plots indicate a good relationship between early metabolic profiles and CFE_D70 (A, C, E). The OPLS-loadings plot highlights metabolites that are positively or negatively correlated with CFE_D70 (B, D, F). LF: low fat, HF: high fat, HF_S: HF and glucose–lactose–galactose (positive control of HF + GOS_CMO group,) GOS: galactosyl-oligosaccharides; FOS_IN: fructosyl-

oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose).

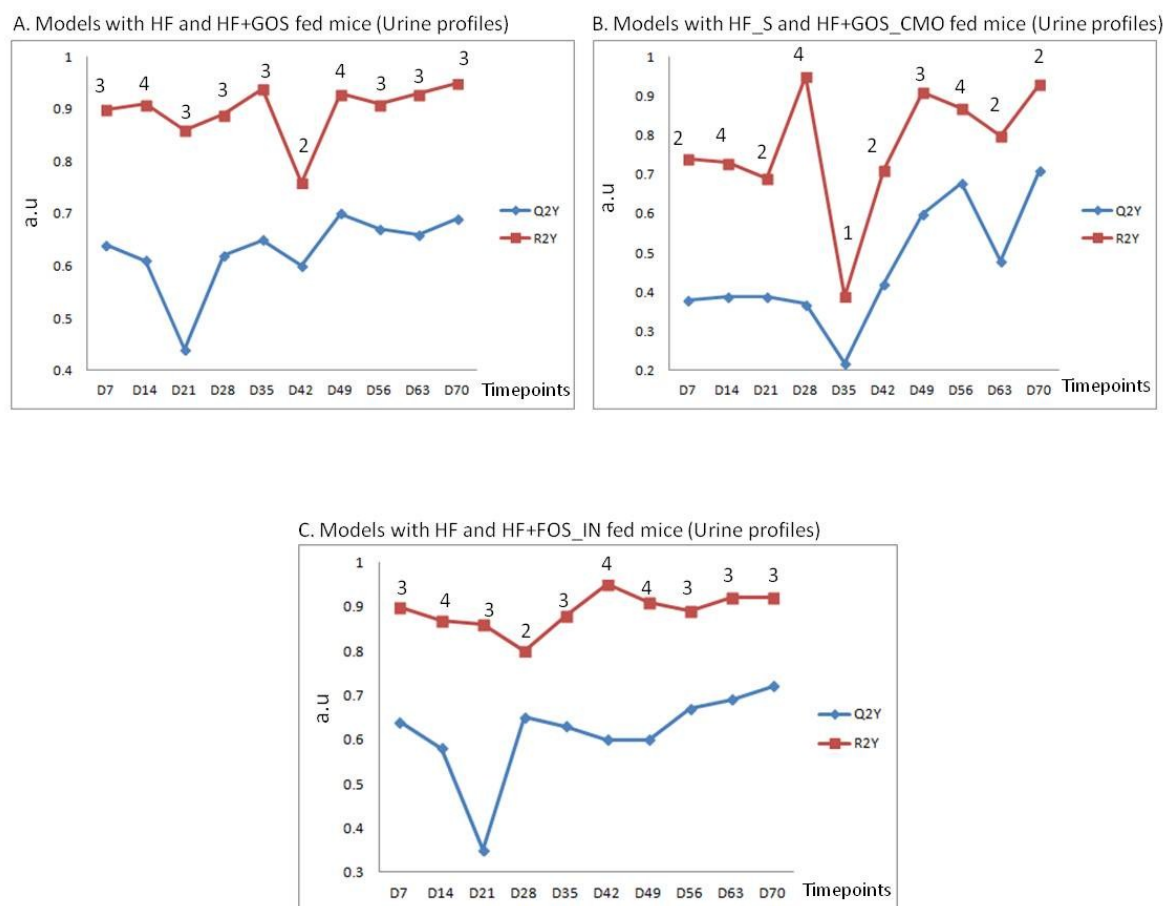


Figure 50: Goodness of fit (R^2Y) and goodness of prediction (Q^2Y) of OPLS-regression models predicting CFE_D70 with urinary metabolic profiles of HF and HF + GOS groups (A), HF_S and HF + GOS_CMO groups (B) or HF and HF + FOS_IN groups (C) at each time point. The models were calculated with 1 predictive component and various numbers of orthogonal components. For each model, the numbers of component are indicated at each time point on top of the R^2Y value. All models were validated with multiple permutation testing.(see Appendix 2, Table S6) LF: low fat, HF : high fat, HF_S : HF and glucose–lactose–galactose (positive control of HF + GOS_CMO group,) GOS : galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose).

The heatmaps summarised correlation values of metabolites being positively or negatively associated with CFE_D70 at each time-point, in the models with HF and HF + GOS groups (Figure 51.A), HF_S and HF + GOS_CMO groups (Figure 51.B) or HF and HF + FOS_IN groups (Figure 51.C) .

Interestingly, early and stable urinary markers positively correlated with CFE in each model were identified. These markers were assigned as microbial co-metabolites *p*-cresol sulfate (PCS), *p*-cresol glucuronide (PCG) and phenylacetylglutamine (PAG)³¹. Therefore a drop of these urinary metabolites was consistently associated with the long-term GOS, GOS_CMO or FOS_IN efficiency in reducing calorie storage during HF challenge. 2 PY, 4 PY and *N*-methylnicotinamide were also late predictors of the prebiotic efficiency in reducing calorie storage since they became significantly correlated with GOS_CMO and FOS_IN efficiency at D42 and appeared discriminatory markers at D56 in the models calculating GOS efficiency. Specific markers of prebiotic efficiency were also identified as listed in the following paragraphs.

Specific markers of GOS efficiency

OPLS regression models calculated with urinary metabolic profiles of HF and HF + GOS fed mice showed that urinary D-glucose, D-galactose and an unassigned (UA) hexose were negatively correlated with CFE_D70 from D7 to D70 (Figure 51.A). On the contrary, sucrose was positively correlated with CFE_D70 throughout the experiment. As previously mentioned GOS fibres are made from glucose and galactose polymerisation, therefore, the relationship between CFE_D70 and urinary excretion of D-glucose and D-galactose was most probably due to an increase in glucose and galactose supply from GOS digestion. 4-hydroxyphenylacetate and creatine were also positively correlated with CFE_D70 but the relationships between these metabolites and CFE_D70 were not stable over time.

Specific markers of GOS_CMO efficiency

OPLS models predicting CFE_D70 from urinary metabolic profiles of HF and HF + GOS_CMO fed mice also pointed out negative correlations between D-glucose, D-galactose and CFE_D70 which can be explained by the GOS containing prebiotic digestion (Figure 51.B). On the other hand, urinary sucrose was negatively correlated with CFE_D70 while UA hexose was positively correlated which is not consistent with previous observations (sucrose was positively correlated with HF diet in OPLS-DA model discriminating HF and HF + GOS groups at D70 (Chapter IV, section 3.2.5). Interestingly, TMA and choline were found to be specific markers of GOS_CMO efficiency. As TMA is produced from choline degradation by the gut microbiota, our results suggest that GOS and cow milk oligosaccharide supplementation during HF challenge may also alter energy efficiency *via* a regulation of gut microbial choline metabolism¹⁶⁹. However, only sucrose and choline were stable and specific predictors of GOS_CMO efficiency over time as indicated in the heatmap by high correlations coefficients at each time point (Figure 51.B).

Specific markers of FOS_IN efficiency

Unlike the models predicting GOS and GOS_CMO efficiency, α/β -D glucose was found to be positively correlated with CFE_D70 in the models calculated with urinary metabolic profiles of HF and HF + FOS_IN fed mice (Figure 51. C). In addition, creatine and sucrose were positively correlated with CFE_D70 while UA hexose and UA metabolite (3.35ppm) were negatively correlated with this physiological outcome at several time points. However, only α -D glucose and UA metabolite constitute early, stable and specific markers of FOS_IN efficiency in reducing CFE over time.

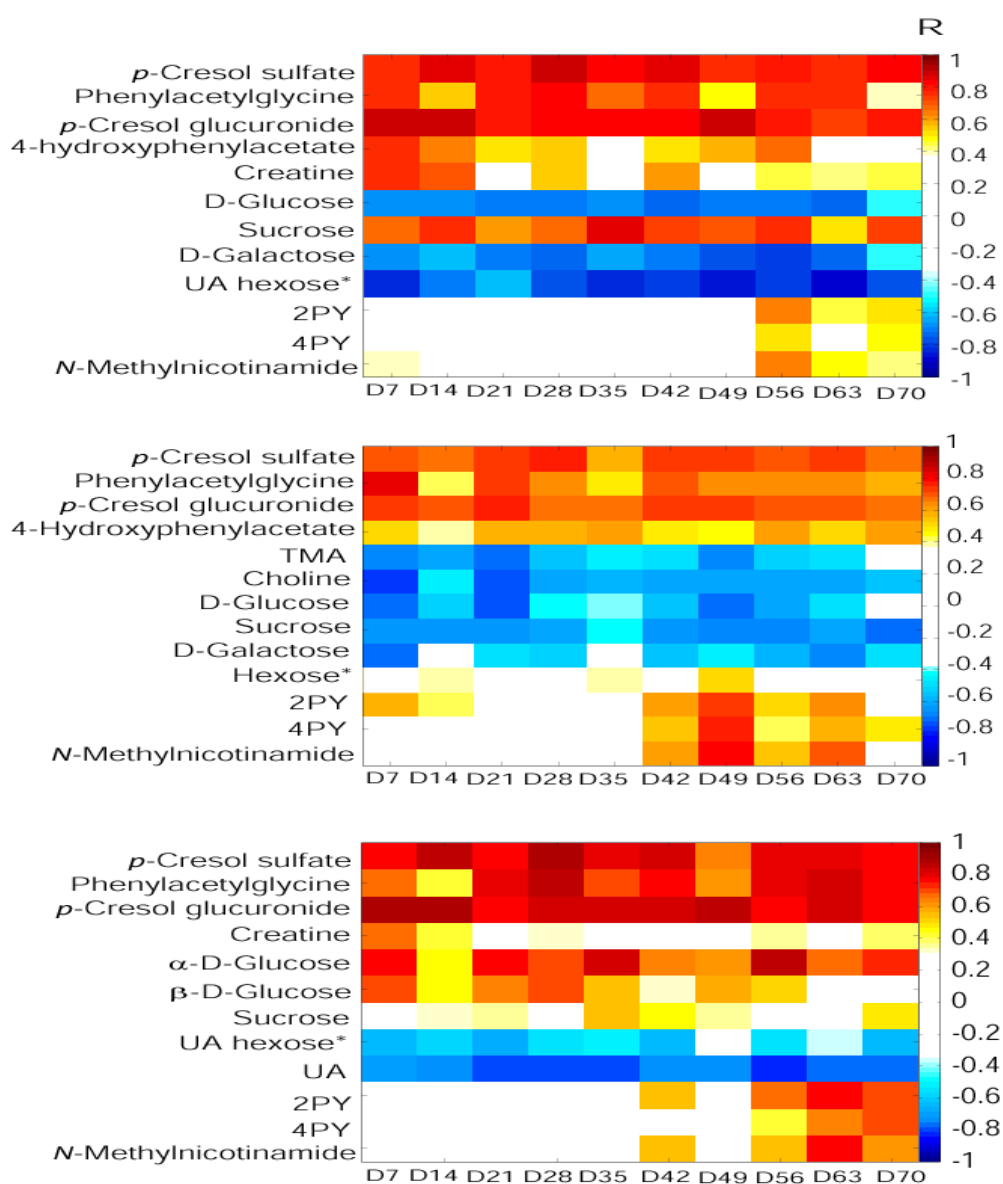


Figure 51: Heatmaps highlighting discriminatory metabolites at each timepoint being positively or negatively correlated with mouse calorie storage at D70 after the diet switch. Correlation coefficients were obtained from the OPLS-DA coefficient plot of predicting models from urinary metabolic profiles of HF and HF + GOS fed mice (A), HF_S and HF + GOS_CMO fed mice (B) or HF and HF + FOS_IN fed mice (C) at each timepoint. Positive values are color-coded in red shades while negative values are color-coded in blue shades. *, tentative assignment LF: low fat, HF: high fat, HF_S: HF and glucose–lactose–galactose (positive control of HF + GOS_CMO group,) GOS : galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose).

3.2.2. ^1H NMR fecal metabolic profiles do not consistently predict the long-term prebiotic efficiency in reducing calorie storage during HF challenge.

Using the same strategy as for urinary metabolic profiles, we aimed to predict the long-term prebiotic efficiency in reducing calorie with mouse fecal metabolic profiles at different time points. In particular, OPLS regression models predicting CFE_D70 using fecal metabolic profiles of HF and HF + GOS, HF_S and HF + GOS_CMO or HF and HF + FOS_IN fed mice were built at each time point. In order to evaluate the goodness of fit and prediction of each model, R^2Y and Q^2Y were displayed in Figure 52. In general, the goodness of prediction (Q^2Y) of the models generating with faecal metabolic profiles were lower than those generating with urinary metabolic profiles. Only the models predicting CFE_D70 of HF and HF + GOS fed mice were validated at D7, D14, D35, D42, and D56 with permutation tests (Figure 52.A). The OPLS regression models performed with HF and HF + FOS_IN fed mice were validated at D14, D28, D35, D42, D49, and D56 (Figure 52.B) while the OPLS regression models with HF_S and HF +GOS_CMO fed mice were significant from D63 (Figure 52.C). Therefore, the correlation between fecal metabolic profiles and CFE_D70 was not stable over time which did not allow the identification of early and stable fecal biomarkers of prebiotic efficiency. To conclude, these results point out that urinary metabolic profile is a more suitable matrix than the fecal metabolic profile to consistently predict long-term prebiotic efficiency in reducing calorie storage during HF challenge in the present experiment.

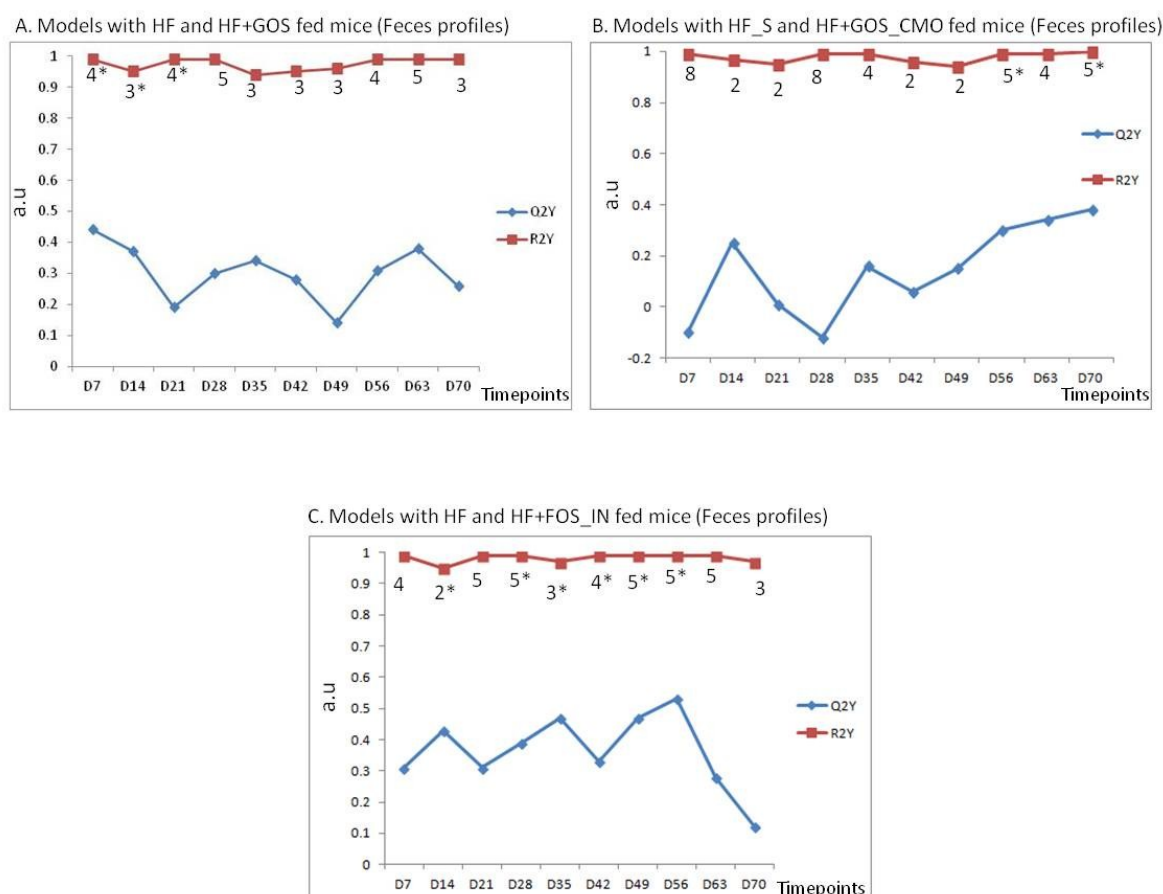


Figure 52: Goodness of fit (R^2Y) and goodness of prediction (Q^2Y) of OPLS-regression models predicting CFE_D70 with fecal metabolic profiles of HF and HF + GOS groups (A), HF_S and HF + GOS_CMO groups (B) or HF and HF + FOS_IN groups (C) at each time point. The models were calculated with 1 predictive component and various numbers of orthogonal components. For each model, the numbers of component are indicated at each time point on top of the R^2Y value. *: indicates the models validated with multiple permutation testing. (see Appendix 2, Table S7). LF: low fat, HF: high fat, HF_S : HF and glucose–lactose–galactose (positive control of HF + GOS_CMO group,) GOS: galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose).

3.2.3. Fecal microbial profiles at D7 and D70 after the diet switch predict the long-term prebiotic efficiency in reducing calorie storage during HF challenge

Fecal microbiota were analysed using high throughput 16s rRNA gene sequencing at D7 and D70 after the diet switch. As previously indicated in Chapter V (section 3.1), the data analysis was focused at family level since it provides us detailed information about functional/compositional

variability among the diet groups and limit intra-individual variability. After data processing (see Materials and methods), 15 bacterial families were unequivocally detected in the mouse fecal microbiota. Here, the experiment aimed to investigate whether bacterial profiles at family level D7 or D70 after the diet switch, could predict the long-term GOS, GOS_CMO or FOS_IN efficiency in reducing calorie storage. Therefore, OPLS regression models predicting CFE_D70 from fecal bacteria profiles of HF and HF + GOS fed mice, HF_S and HF + GOS_CMO fed mice, or HF and HF + FOS_IN fed mice at D7 and D70 after the diet switch were performed and validated. For each model, the goodness of fit (R^2Y) and the goodness of prediction (Q^2Y) are displayed in legend Figure 53. The loadings plots generated from predicting models with HF and HF + GOS groups (Figure 53.A, Figure 53.B), HF_S and HF + GOS_CMO groups (Figure 53.C, Figure 53.D), or HF and HF + FOS_IN groups (Figure 53.E, Figure 53.F) highlighted discriminatory families of bacteria being positively or negative correlated with CFE_D70. Interestingly, fecal abundance of *Streptococcaceae* at D7 and D70 after the diet switch, were highly correlated with CFE_D70 in each model. This shows that a rapid drop in fecal *Streptococcaceae* abundance with prebiotic supplementation can predict GOS, FOS_IN or GOS_CMO efficiency in reducing calorie storage. Hence, it constitutes a stable and common biological marker of prebiotic efficiency. It is important to note that the correlation coefficient (R^2) between *Streptococcaceae* and FOS_IN or GOS efficiency in reducing CFE_D70 rise from 0.4 to 0.6 after 70 days of diet intervention. On the contrary, correlation coefficient between *Streptococcaceae* and GOS_CMO efficiency drop from 0.4 to 0.25. Other bacteria families were also found to be specific markers of GOS, FOS_IN or GOS_CMO efficiency:

Specific markers of GOS efficiency

Peptostreptococcaceae were found to be highly correlated with CFE_D70 in models with HF and HF + GOS fed mice at D7 ($R^2=0.45$) and D70 ($R^2=0.46$) after the diet switch (Figure 53.A, Figure 53.B). As a result, this family of bacteria constitutes an early and stable biological marker of GOS efficiency. Interestingly, the correlation coefficient between *Peptostreptococcaceae* and CFE_D70 was similar at both time points which highlight the consistency of this marker in predicting CFE_D70. Fecal abundances of unclassified family and *Ruminococcaceae* at D70 after the diet switch were also highly correlated with CFE_D70 (Figure 53.B). Therefore, they are late predictors of GOS efficiency in reducing calorie storage.

Specific markers of GOS_CMO efficiency

Ruminococcaceae and unclassified family at D7 after the diet switch were highly correlated with CFE_D70 ($R^2=0.4$, $R^2=0.38$ respectively) in models with HF_S and HF + GOS_CMO fed mice.

Therefore they constitute early and specific predictors of GOS_CMO efficiency (Figure 53.C). However, at D70 after the diet switch, only *Ruminococcaceae* was found to be highly correlated ($R^2=0.55$) with CFE_D70 in our model (Figure 53.D).

Specific markers of FOS_IN efficiency

The model predicting CFE_D70 from fecal microbiota of HF and HF + FOS_IN fed mice at D7 after the diet switch indicated that *Peptostreptococcaceae* ($R^2=0.30$) and *Rikinellaceae* ($R^2=0.38$) were early and significant predictors of FOS_IN efficiency (Figure 53.E). These bacteria families were also significantly correlated ($R^2=0.30$ for *Peptostreptococcaceae* and $R^2=0.39$ for *Rikinellaceae*) with CFE_D70, at D70 after the diet switch (Figure 53.F). Therefore, they constitute early, stable and specific markers of long-term FOS_IN efficiency in reducing calorie storage. Interestingly, the correlation values of these markers were consistent between D7 and D70 which confirm their stability in predicting CFE_D70. At D70 after the diet switch other bacteria families became good predictors of FOS_IN efficiency. *Bifidobacteriaceae* were negatively correlated ($R^2=0.62$) with CFE_D70 while *Ruminococcaceae* ($R^2=0.46$) and unclassified bacteria ($R^2=0.44$) were positively correlated with this physiological outcome.

To summarise, in each model, fecal microbiota profiles at early time point (D7) or at sacrifice (D70) were good predictors of final mouse calorie storage. Several families of bacteria were found to specifically predict long-term GOS, GOS_CMO and FOS_IN efficiency in reducing calorie storage. *Streptococcaceae* constitutes the only stable and common candidate marker of prebiotic efficiency. However, additional sampling of mouse fecal microbiota at other time-points is needed to confirm the stability of fecal microbiota profiles and particularly *Streptococcaceae* in predicting CFE_D70.

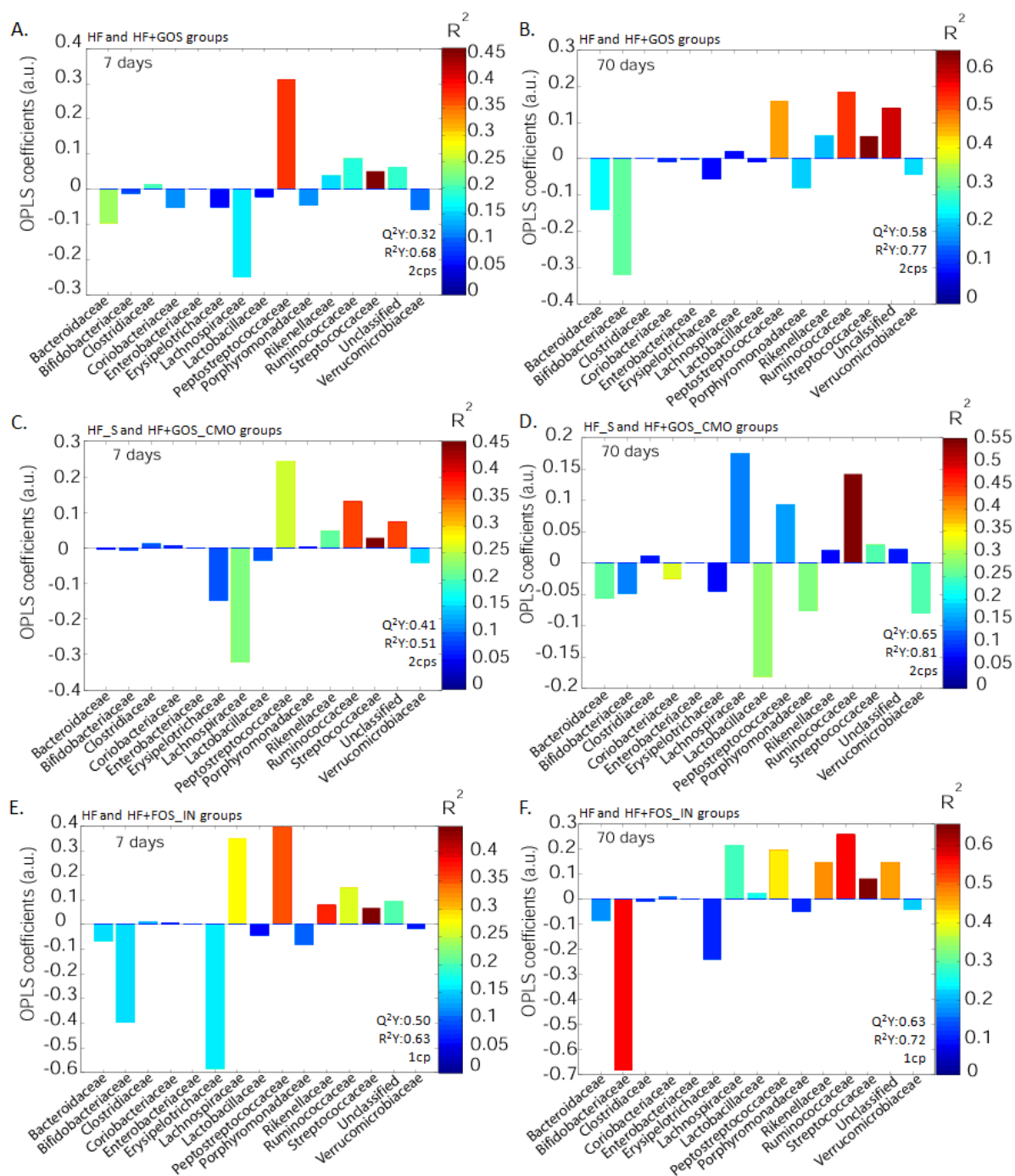


Figure 53: OPLS-coefficient plots indicating correlation coefficients between bacteria families at D7 and D70 and GOS (A, B), GOS_CMO (C, D) or FOS_IN (E, F) efficiency in reducing mouse calorie storage at D70 after the diet switch. OPLS-DA coefficient plots were obtained from models with HF and HF + GOS fed mice (A, B), HF_S and HF + GOS_CMO fed mice (C, D) or HF and HF + FOS_IN fed mice (E, F) at D7 and D70 after the diet switch. Correlation values (R^2) color-coded in red shades are close to 1 while those color-coded in blue shades are close to 0.

3.2.4. Early, stable candidate markers commonly predict the long-term efficiency of GOS, GOS_CMO and FOS_IN in reducing calorie storage.

A set of urinary and microbial candidate biomarkers allowing an early and consistent prediction of prebiotic efficiency were identified. The Venn diagram (Figure 54) illustrates the proportion of specific and common candidate markers of CFE_D70 among the different prebiotics. For each prebiotic, 8 candidate markers were found to be early and stable predictors of prebiotic efficiency. Among these, 4 stable candidate markers predicting GOS efficiency, 3 stable candidate markers predicting FOS_IN efficiency and 3 candidate markers predicting GOS_CMO efficiency were found. In addition, 4 candidate markers (PAG, PCG, PCS, and *Streptococcaceae*) were commonly identified as early and stable predictors of GOS, GOS_CMO and FOS_IN efficiency. PAG, PCG, PCS are well known end product metabolites of gut microbial amino acid degradation, therefore, it can be suggested that a reduction of gut bacterial amino acid fermentation by prebiotic supplementation during HF challenge lower calorie storage.

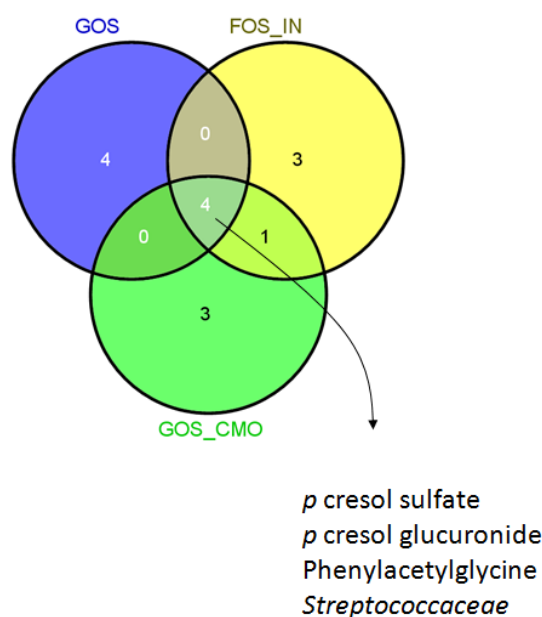


Figure 54: Venn diagram indicating the number of specific and common markers of calorie storage at D70 after the diet switch among the different prebiotics. GOS: galactosyl-oligosaccharides; FOS_IN: fructosyl-oligosaccharides and inulin; GOS_CMO, galactosyl-oligosaccharides and cow milk oligosaccharides (containing glucose–lactose–galactose).

3.2.5. Urinary abundance of PAG, PCG, PCS and fecal *Streptococcaceae* at D7 after the diet switch predicts long-term beneficial effect of prebiotic on calorie storage, organ weights and other physiological disorders during HF challenge.

Here, prebiotic supplementation during HF challenge had beneficial impact on obesity development since it reduced the mouse calorie storage, fat mass and several organ weights. The relative abundances of PAG, PCG, PCS were calculated by integrating their peaks in ^1H NMR urine spectra. Using the relative abundance of PAG, PCG, PCS and fecal *Streptococcaceae* at D7 after the diet switch, robust OPLS regression models predicting CFE_D70 of the positive control groups (HF and HF_S) and the prebiotic test groups (HF + GOS, HF + GOS_CMO and HF + FOS_IN) were generated and validated. The scores plot highlighted the significant association between CFE_D70 with this combination of markers (T1) (Figure 55.A). The most important variable underlying the model predictability are PAG and *Streptococcaceae* (Figure 55.B.). A general equation was calculated based on this model, which estimates at early time point, the long-term prebiotic efficiency in reducing calorie storage (Figure 55.B).

Additionally, the combination of 4 candidate markers was tested to potentially predict other physiological outcomes associated with prebiotic induced improvement of obesity phenotype (Figure 55, Figure 56). A set of OPLS regression models was performed using the relative abundance of PAG, PCG, PCS and fecal *Streptococcaceae* at D7 to predict fat mass, fasting hyperinsulinemia, fasting hyperglycemia, circulating IL-1 β abundance and organ weights of positive control groups (HF and HF_S mice) and prebiotic test groups (all HF + prebiotics fed mice). The robustness and the predictability of these models were tested using multiple permutations method. The models predicting liver and kidney weight at D70 as well as fasting glycemia at D56 from the same combination of markers were not significant. However, specific patterns of urinary PAG, PCG, PCS and fecal *Streptococcaceae* at D7 after the diet switch were able to predict fat mass and fasting hyperinsulinemia at D56, circulating IL-1 β abundance, mesenteric and epididymal fat and caecum weight at sacrifice (Figure 55, Figure 56). The scores plot pointed out the significant relationship between the marker abundances and the various physiological outcomes (Figure 55.A, Figure 54.D, Figure 54.G, and Figure 54.J). It is noteworthy that some overlaps among diet groups along the mesenteric and epididymal fat axis were observed. In particular, HF_S fed mice tend to have reduced mesenteric and epididymal fat compared to HF diet and comparable amount of fat to HF + prebiotic fed mice (Table 1).

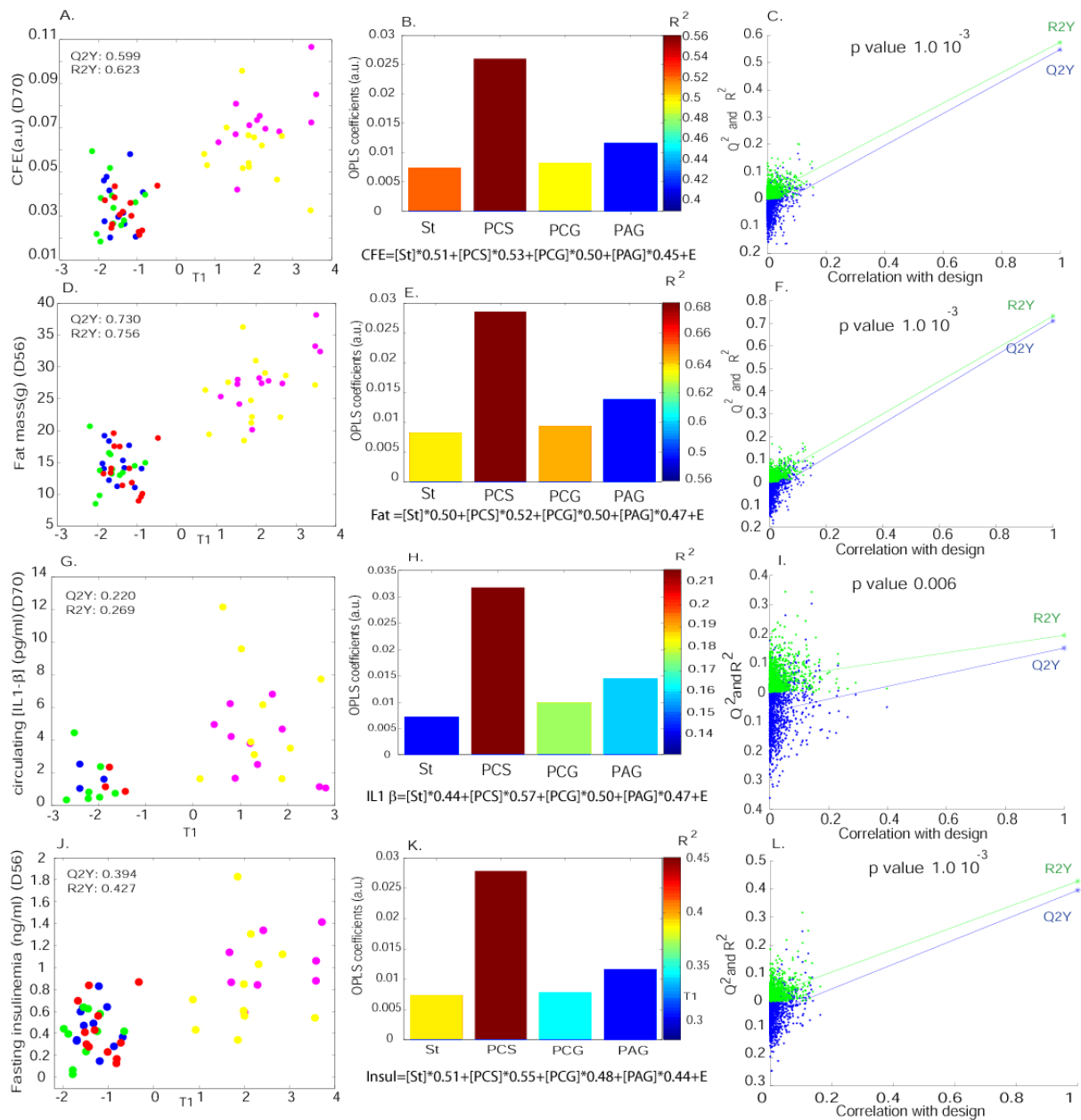
The loadings plots highlighted that PCG and PCS were the most important variables underlying the fat mass-predicting model (Figure 55.E). On the other hand, *Streptococcaceae*, and PCS were the most significant variables predicting fasting insulinemia in the models (Figure 55.H) while the main factors predicting circulating IL-1 β concentration were PCG and PCS (Figure 55.K). Finally, *Streptococcaceae*, and PCG were the main variables predicting mesenteric fat and epididymal fat in the OPLS regression models (Figure 56. B, Figure 56.E). The models were validated with multiple permutation testing (Figure 55.C, Figure 55.F, Figure 55.I, Figure 55.L, Figure 56.C, and Figure 56.F).

Physiological data have shown that prebiotic supplementation during HF diet also induced a significant increase in caecum weight compared to the positive control groups. Interestingly, urinary abundance of PAG, PCG, PCS and fecal abundance *Streptococcaceae* at D7 after the diet switch could predict the caecum weight of positive control groups (HF and HF_S fed mice) and prebiotic test groups (all HF + prebiotic fed mice) at D70 after the diet switch. The OPLS scores plot indicated a relationship between caecum weight and the excretion pattern of these markers (Figure 56.G). The loadings plot showed that PAG, PCG, PCS and fecal *Streptococcaceae* were negatively correlated with the caecum weight. In particular, PCG, PCS were the most correlated variables in the predicting model (Figure 56.H). The model was also validated under multiple permutations testing (Figure 56.I).

Importantly, although all models were validated, the scores distribution along fasting hyperglycemia, circulating IL-1 β , epididymal fat, mesenteric fat or caecum weight axis was not linear and significant within-group variability was noticed. This tends to indicate a group-driven stratification of the data rather than a linear prediction of these physiological outcomes. PCG, PCS, PAG and *Streptococcaceae* are qualitative candidate markers discriminating the prebiotic effects from the HF effect in these models. Therefore, the best predictive models were those calculated with CFE and fat mass at D70 as indicated by high goodness of fit ($Q^2Y > 0.5$) and well-distributed scores plot.

To conclude, in the present experiment several physiological outcomes indicative of the beneficial prebiotic effects on mouse physiology during HF challenge were predicted by using a combination of biological markers from feces and urine samples at D7 after the diet switch. Based on each model, generic linear equations predicting the different physiological outcomes were calculated. As these markers were not specific of any prebiotic, these mathematical models could be used as a general basis to assess the long-term mouse phenotype during the early phase of any

prebiotic intervention. However, non-linear fitting strategy may provide better predictive models of these physiological outcomes.



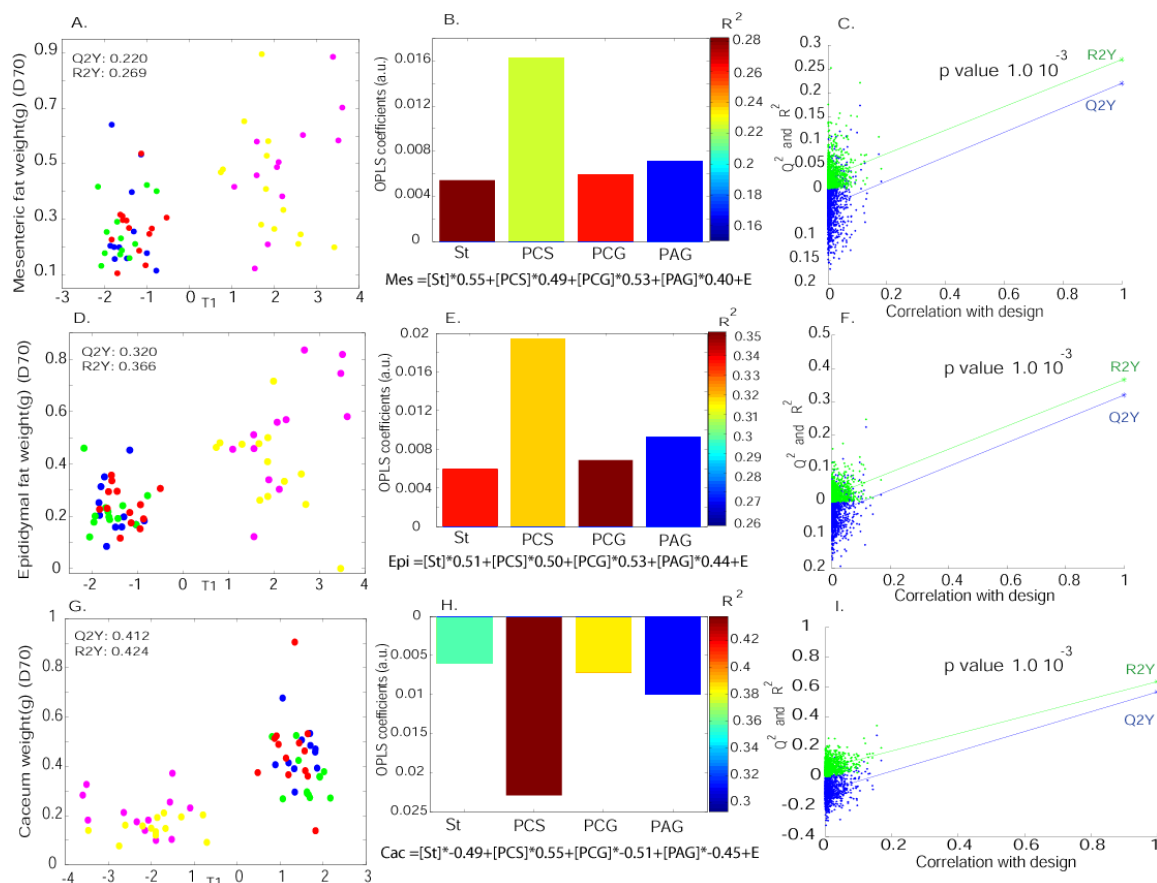


Figure 56: OPLS-regression models predicting organ weight at sacrifice based on the abundance of *Streptococcaceae* (St), *p*-cresol sulfate (PCS), *p*-cresol glucuronide (PCG) and phenylacetyl glycine (PAG) at D7 after the diet switch. For each model, the scores plots indicate a significant relationship between a specific pattern of excretion of these markers and organ weights (Figure 56.A for mesenteric fat, Figure 56.D for epididymal fat, Figure 56.G for caecum weight,). The OPLS-loadings plot indicated the correlation coefficients between the variables and the physiological outcomes (Figure 56.B for mesenteric fat, Figure 56.E for epididymal fat, Figure 56.H for caecum weight). All the models were validated with multiple permutations testing (p value < 0.05) outcomes (Figure 56.C for mesenteric fat, Figure 56.F for epididymal fat, Figure 56.I for caecum weight)

4. DISCUSSION

The prebiotic study findings show the ability to predict at early stage from urinary metabolic profile or fecal microbial profiles the long-term prebiotic efficiency to reduce calorie storage. More precisely, urinary PCS, PCG, PAG were common and stable markers of GOS, GOS_CMO and FOS_IN efficiency to reduce calorie storage overtime. Fecal *Streptococcaceae* were also found to be

consistently correlated with calorie storage at D7 and D70 in each predictive model. From the identification of these non specific markers, a general model predicting the prebiotic efficiency to reduce calorie storage was calculated based on the abundance of urinary PCS, PCG, PAG and fecal *Streptococcaceae* at D7 after HF the diet switch. Furthermore using the same combination of non-specific markers, robust models predicting at early stage (D7 after the diet switch), the long-term beneficial effect of any prebiotic on fat mass, fasting insulin level and adiposity were also calculated. Hence, a combination of urinary and fecal markers at an early time point can estimate the prebiotic efficiency in the delay of obesity development and the associated physiological disorders. Such a biomarker combination may be a first step toward defining early generic biological signatures to assess the optimum effect of prebiotic intervention on obesity. These data also support the hypothesis of a functional interaction between gut microbial amino acid fermentation and host physiology. Therefore, potential pharmacological effects of PCS, PCG and PAG on host immunological/metabolic pathways were discussed in this chapter. Finally renal dysfunction which is commonly observed in long-term obesity may contribute to increase circulating PCS and PCG which may promote metabolic disturbances and inflammation during diet induced obesity (DIO).

4.1. Rapid changes in colonic bacterial amino acid fermentation influence mouse physiology during DIO.

Early (D7) urinary excretion of PAG, PCG, PCS and fecal excretion of *Streptococcaceae* was positively correlated with fat mass, CFE, circulating IL-1 β , fasting insulinemia, epididymal and mesenteric adipose tissues at D70 in HF and HF + prebiotic fed mice. Indeed, PAG, PCS, and PCG are phase II detoxification products of microbial amino acid degradation. Tyrosine fermentation by colonic bacteria triggers the formation of *p*-cresol and phenylacetate. These colonic compounds are in turn excreted in the feces or absorbed by colonic bacteria and detoxified by sulfation, glucuronidation or glycation into PCS, PCG and PAG in the colonic mucosa or liver²²⁶. Subsequently, these detoxification products are shuttled in the kidney to undergo elimination in urine²²⁶. In addition *Streptococcaceae* are anaerobic bacteria which confer high proteolytic activities²⁷⁷.

Interestingly, fecal *Streptococcaceae* was the most important variable underlying the prediction of mesenteric adiposity sites. Fecal *Streptococcaceae* was already found to increase in HF-fed mice compared to the other diet groups at D7 after the diet switch and were positively correlated with fecal bile acids, fecal and plasma lipids at D70 after the diet switch (chapter V,

section 3.2). Therefore, HF diet associated gut microbiota remodelling may help extracting lipid from the diet and promote lipid storage^{241,283}. In our predictive models, urinary PCS at D7 was also strongly correlated with insulin level, fat mass and circulating IL-1 β of HF and HF + prebiotics fed mice. On the other hand, PAG was the least correlated variable in all the models. Amino acid fermentation end-products i.e. *p*-cresol, PAG, PCG and PCS may cross host physiological barriers, enter systemic circulation and biochemically affect host metabolic/immunological pathways. In particular, PCS may promote a pro-inflammatory response toward IL-1 β related pathways which contributes to DIO associated low grade inflammation and physiological disorders. This will be further developed in this discussion.

It has been previously demonstrated in this PhD thesis that GOS, GOS_CMO or FOS_IN supplementation during HF challenge for 70 days significantly lowers gut bacterial tyrosine fermentation through a reduced urinary level of PCS, PAG, PCG. In addition, prebiotic supplementation induced a significant reduction in CFE, fat mass, epididymal and mesenteric adipose tissue, circulating IL-1 β , fasting insulinemia at D70 after the diet switch. This supports the hypothesis that reduced colonic amino acids fermentation by dietary intervention (i.e prebiotics) lowering *p*-cresol, PAG, PCG and PCS limits HF-induced ectopic fat accumulation, low grade inflammation and impaired insulin action. A significant increase in caecum weight was also observed in HF + prebiotics fed mice compared to HF-fed mice at D70 after the diet switch. This caecum hypertrophy highlights an activation of bacterial fermentation in colon probably through SCFAs production²⁸⁴. Interestingly, the same combination of markers at D7 after the diet switch was negatively correlated with caecum weight. These results suggest that prebiotic in the context of HF induced obesity rapidly modify gut microbial activity toward a decrease in amino acid fermentation and a concomitant increase in carbohydrate fermentation which influences host metabolism to improve host physiology. In the present chapter, our results raise the hypothesis of causal relationships among protein fermentation by colonic bacteria and host adiposity growth, low grade inflammation and impaired insulin action during HF diet induced obesity. This might be due to the fact that protein fermentation end-products confer specific pharmacological effects on host metabolic and inflammatory pathways.

4.2. PCS, PCG and PAG may confer pharmacological effects on host fat storage and insulin signalling pathways

Phenol and *p*-cresol derivatives have been suggested to have toxic properties²²⁶. In particular, PCS is the most abundant derivative product of *p*-cresol in human and mouse^{285,286}. It has

recently received particular attention, since it has been identified as prognostic and diagnostic markers of several physiological outcomes in pharmacometabonomic and epidemiological studies^{277,282,286,287}. For example, individuals producing high PCS from gut microbiota mediated *p*-cresol in pre-dose were found to be less efficient in detoxifying acetaminophen by *o*-sulfation²⁸². Hence, gut microbial activity might have an impact in determining the individual responses to drug treatment. PCS is also considered as one of the major uremic toxins contributing to the propensity for metabolic disorders and vascular diseases in uremic patients^{285,286}. Because PCS is highly protein bound, it cannot be properly cleared with classic hemodialysis. As a result, it is commonly found in high concentration in blood of patients with kidney failure²⁸⁶. Serum PCS has been found to predict CVDs and all cause of mortality in the elderly hemodialysis patients²⁸⁷. A mechanistic study investigating the causal relationship between serum PCS and the development of metabolic disorders, has demonstrated PCS-induced insulin resistance *in vitro* and *in vivo* in patient suffering chronic kidney disorders (CKDs). In particular, PCS alters glucose uptake in C2C12 myotubule cells by activating ERK1/2 phosphorylation which inhibits the insulin induced PKB/AKT phosphorylation and triggers insulin resistance²⁸⁸. PCS also promoted lipolysis and fat redistribution towards ectopic sites in mice²⁸⁸. Additionally, urinary PAG and PCG have been positively correlated with obesity in mice although their mechanisms of actions are not well understood²⁸⁹. Hence, in the context of obesity, PAG and particularly PCS and PCG produced from the detoxification of gut microbial tyrosine fermentation end-products reaching blood circulation and organs (liver, kidney) might have pharmacological effects contributing to the development of ectopic fat accumulation and hyperinsulinemia in our experiment. Additional *in vitro* experiments studying the biochemical effects of PCS, PCG and PAG on triglyceride (TG) synthesis, lipoprotein formation and circulation and insulin signalling pathways are needed to provide new insights about their functionalities on host lipid and glucose metabolism.

4.3. PCS, PCG and PAG may contribute to host IL-1 β related inflammatory pathways

In the Chapter V (section 3.2), positive correlations between plasma IL-1 β , urinary PCS, PCG and *Streptococcaceae* D70 after the diet switch were identified. This suggested that a modulation of gut microbiota composition and activation of microbial amino acid fermentation in HF-fed mice contribute to HF induced low grade inflammation probably through IL-1 β pathway^{226,290}. Here, urinary abundance of PCS, PCG, PAG and fecal abundance of *Streptococcaceae* at D7 after the diet switch also allowed the prediction of long-term circulating IL-1 β production. These additional results

raise the hypothesis that compounds derived from gut microbial tyrosine degradation end-products (PCS, PCG) have a functional relationship with IL-1 β production and the development of circulating inflammation. During the detoxification process, PCG and PCS are produced in the liver, reach the kidney, and may be transferred to other peripheral tissues, directly triggering local inflammatory responses²⁹¹. Noticeably, patients with uremic syndrome and kidney failure are subjected to an impaired immunity, with an excessive cytokine excretion^{286,292,293}. Higuchi *et al* showed that peripheral blood mononuclear cells cultured with the serum of uremic patients rich in protein bound solutes such as PCS and PCG induced the production of IL-1 β ²⁹². Lim *et al* also presented results that myeloid dendritic cells from healthy individuals treated with uremic serum had altered expression of co-stimulatory molecules and decreased IL-12 production when stimulated with LPS²⁹³.

4.4. HF associated renal dysfunction may contribute to increase PCS, PCG or PAG in systemic circulation

Interestingly, potential links among obesity, metabolic syndrome and renal damage were recently shown²⁹⁴. Increased body mass index and energy intake are associated with an excess excretory load leading to nephromegaly and renal dysfunction²⁹⁴. For example, Na excretion capacity has been found to be reduced in obese individuals²⁹⁵. Lipotoxicity associated with obesity is also recognized as afflicting renal cells and triggering inflammation^{294,296}. Finally, recent evidence suggest adverse affects of hyperinsulinemia and IR on renal function²⁹⁴. In the present experiment, HF and HF_S fed mice may gradually develop renal dysfunction which raises the proportion of waste products in circulation including PCS, PCG and PAG. These compounds could then easily reach the peripheral organs directly contributing to metabolic deregulations, ectopic fat accumulation, inflammation and hyperinsulinemia by the abovementioned mechanisms. Prebiotic supplementation during HF diet on the other hand, may alleviate HF associated kidney damage by limiting energy intake, body weight and the production of toxic metabolites which in turn may improve mouse phenotype. However, PAG, PCS, PCG were not observed in ¹H NMR plasma metabolic profiles in all diet groups. Because these compounds are highly bound with plasma proteins, it is most likely that their signals appear highly broadened in standard spectra or are suppressed in CPMG spectra. To verify this hypothesis, it would be necessary to evaluate kidney metabolism and functions in HF and HF + prebiotic fed mice as well as measuring plasma PCS, PCG, PAG levels by a more suitable method.

4.5. Variability in fecal metabolic profiles does not allow predicting the physiological outcomes

Unlike urinary metabolic profiles, the food efficiency predicting models calculated with fecal metabolic profiles were not valid at each time point highlighting time related instability of metabolic profiles over the duration of the study. Saric *et al*, studying interspecies and temporal variations in fecal metabolic profiles have demonstrated significant variations associated to time/age in female NMRI mice and female Wistar rats fed the same diet²⁹⁷. Therefore, it can be assumed that time associated perturbation of fecal metabolic profiles generating within group variability alters the predictive power of this matrix.

4.6. The predictive models present some limitations

The effects of prebiotic supplementation in HF-fed mice 7 days after the beginning of the intervention, were already noticed since body weight gain and calories storage were significantly lower in all HF + prebiotic fed mice compared to HF-fed mice. The early prebiotic associated reduction of bacterial proteolysis may be an early consequence of lower BWG and CFE reduction. Therefore, the causal effects of bacterial proteolysis regulation on host metabolism remain to be proven. In addition, it is important to note that PCS, PCG, PAG are diet-conditioned markers of prebiotic efficiency as they derived from protein fermentation by colonic bacteria. It can be assumed that prebiotic supplementation in the context of low protein diets would not induce any changes in PCG, PCS and PAG production. Finally although all models were significant, PCS, PCG, PAG and *Streptococcaceae* do not provide a linear prediction of the fasting hyperglycemia, circulating IL-1 β , epididymal fat, mesenteric fat or caecum weight of HF and HF+ prebiotic fed mice (as indicated by a large within group variability in the OPLS model scores plots). These metabolites can be considered as qualitative markers of the mouse physiological improvement by prebiotics rather than quantitative markers of these physiological outcomes in our experiment. Further studies are needed to evaluate the links between these metabolites and fasting hyperglycemia, circulating IL-1 β , epididymal fat, mesenteric fat, and caecum weight.

5. CONCLUSION

This project has provided the evidences that prebiotic supplementation during HF challenge significantly limits obesity development, influencing host metabolism by modulating gut microbiota composition/activity. In the present chapter, the abundance of 4 markers (*Streptococcaceae*, PCS, PCG, and PAG) measured at D7 after the diet switch with ¹H NMR spectroscopy and 16S rRNA gene sequencing allowed to model the long-term prebiotics efficiency to limit calorie storage, fat mass, adiposity, hyperinsulinemia, and circulating IL-1 β production. These results have also supported the hypothesis of the causal relationship between *Streptococcaceae*, the production of bacterial amino acid fermentation end-products, metabolic disturbances, and inflammation during diet induced obesity (DIO). Because these markers were successfully predicting GOS, GOS_CMO and FOS_IN efficiency in the experiment, the calculated models may provide an early stage evaluation of the beneficial effect of any prebiotic on obesity development and related metabolic disorders. However, there is a clear need to further test the reliability of these markers in other contexts because various diets, containing structurally different prebiotics, and different animal models might strongly influence the ability to predict prebiotic effect on host physiology^{181,249}. In addition, transferring these mathematical models from a single strain laboratory to human subjects presenting greater genetic and environmental variability might be challenging²⁸¹. However, it is worth investigating as the concept of monitoring the relevance of prebiotic intervention offers potential applications in relation to personalised human healthcare. Indeed in principle, this early diagnostic would allow healthcare professionals to quickly estimate the efficiency of a prebiotic intervention and adapt the prebiotic dosages according to the characteristics and needs of a specific population^{111,281}. Pharmacometabonomic principles which would be used to predict BWG and host physiological status from pre-dose baseline samples should also be considered in nutritional studies⁶⁸. Hence by taking into account individual predispositions which are influenced by both genetic and environmental factors, suitable nutritional intervention (i.e prebiotics) targeting individual needs could be selected before any intervention¹¹¹. Altogether, the measurement of prognostic and diagnostic makers of individual responses, respectively before and during the early phase of any nutritional intervention, would allow the monitoring of its impacts on individual health.

CHAPTER VII: GENERAL DISCUSSION

The wide range of gut microbiota-mammalian host interactions associated with the development of obesity and metabolic disorders were widely explored in this PhD thesis *via* 2 experiments. The first experiment aimed to identify the metabolic signature differentiating obesity-prone and obesity-resistant C57BL/6 mice after 7 days and 60 days of high-fat (HF) diet feeding. The second experiment evaluated the impact of dietary interventions involving different prebiotics such as galactosyl-oligosaccharides, (GOS), fructosyl-oligosaccharides and inulin (FOS_IN), or galactosyl-oligosaccharides with cow milk oligosaccharides (GOS_CMO) on the modulation of host metabolism and immune system as well as on gut microbiota composition, in C57BL/6 mice during the gradual development of diet induced obesity (DIO).

1. KEY FINDINGS

It has been demonstrated that host metabolism is profoundly altered by HF feeding as indicated by a rapid saturation of host mitochondrial oxidative pathways (β -oxidation, branched chain amino acid (BCCA) catabolism, Chapter III, section 3.2 and Chapter IV, section 3.2.5). In addition, obesity-resistant mice fed a HF diet differentially activate ketogenic pathways and TCA cycle, thus maintaining energy homeostasis and protecting mitochondria against fuel overloading. Hence, specific regulations of host mitochondrial metabolism might play crucial role in the onset of obesity during HF challenge.

The results presented in this PhD thesis also demonstrated that gut microbiota are involved in the onset of obesity and related metabolic disorders. In the first experiment (Chapter III), the heterogeneous adaptation to HF diet in a population of genetically homogenous C57BL/6 mice was associated with a differential excretion of microbial co-metabolites suggesting that gut microbial activity plays a role in the adaptation of DIO-associated traits. In the second experiment (Chapter IV, V and VI), gut microbial remodelling by different prebiotics (GOS, GOS_CMO, FOS_IN) during DIO was associated with lower weight gain and fat mass, an improvement of the host immune status and variations in host metabolism. In particular, various long-term (70 days) prebiotic supplementations during a HF challenge improved host mitochondrial metabolism and reduced dyslipidemia.

Interestingly, FOS_IN supplementation specifically triggered protein degradation and ketogenesis, and allowed the maintenance of normo-glycemia (Chapter IV, section 4.4).

Variations in gut bacterial composition and metabolic activity were observed with different diets supplemented with prebiotics. Prebiotic supplementations increased gut bacterial carbohydrate fermentation and decreased gut bacterial tyrosine fermentation (Chapter IV, section 4.5). All prebiotics triggered commensal bacteria growth (Chapter V, section 4.3). However, FOS_IN induced a faster increase in the representation of *Bifidobacteriaceae* compared to GOS supplementation, while GOS_CMO preferably triggered an increase in the representation of *Lactobacillaceae* rather than *Bifidobacteriaceae*. Moreover, other bacterial families were modified with prebiotics. *Bacteroidaceae* representation increased while several families of the *Firmicutes* phylum (such as *Peptostreptococceae*, *Ruminocacceae*, and *Streptococcaceae*) decreased with prebiotic supplementation.

Correlative analysis between ^1H NMR metabolic profiles (urine, feces, plasma), fecal microbiota (at family level) and plasma cytokines at 70 days after the diet switch using the total variance of the dataset (all diet groups) have highlighted new associations among gut microbiota, host immune system and host metabolism:

- *Lachnospiraceae*, *Bacteroidetes*, *Porphyromonadaceae*, *Coriobacteriaceae*, and *Rikenellaceae* (increasing with HF diet) were positively correlated with urinary BCAA catabolism intermediates (α -keto- β -methylvalerate, α -ketoisovalerate, isovalerate, isovaleryl-glycine) and 4PY. On the other hand, *Erysipelotrichaceae* (decreasing with HF diet) were negatively correlated with these metabolites.
- Circulating interleukin-1 β (IL-1 β), was positively correlated with urinary *p*-cresol sulfate (PCS) and *p*-cresol glucuronide (PCG), *Peptostreptococceae* and *Streptococcaceae* and was negatively correlated with *Bifidobacteriaceae* and fecal butyrate (Chapter V, section 3.2).

Finally, urinary metabolic signatures at each time point (from 7 days to 70 days) and fecal bacterial profiles at 7 days and 70 days (at family level) predicted the long-term (70 days) cumulative food efficiency (CFE) of HF and prebiotic groups. These statistical models allowed the identification of early and stable biomarkers of GOS, FOS_IN and GOS_CMO efficiency in reducing calorie storage during HF challenge. These markers are urinary PCS, PCG, phenylacetyl-glycine (PAG) and fecal *Streptococcaceae*. Early reductions of these markers in feces and urine were associated with the long-term reduction of CFE by prebiotics. PCS, PCG PAG and *Streptococcaceae* at 7 days were also

used to calculate general models predicting the long-term efficiency of prebiotic in reducing IL-1 β , fat mass, fasting insulinemia caecum weight, epididymal and mesenteric fat.

2. STATEMENTS OF NOVELTY.

The studies carried out during this PhD allowed the identification of new candidate markers predicting the physiological adaptations of C57BL/6 mice to DIO under different dietary conditions. In particular, the study of the short-term and long-term urinary metabolic signatures of obesity-resistant and obesity-prone mice under HF diet indicated that hexanoylglycine, isovalerylglycine and isobutyrate were early and consistent candidate markers predicting the differential body weight gain (BWG) of HF-fed mice. Variations in isobutyrate and other short chain fatty acids (SCFAs) (butyrate and propionate) have been already observed during obesity development²³⁵. In particular, increased isobutyrate and butyrate have been found to improve lipid metabolism, inflammatory status and insulin sensitivity during DIO in human and mouse studies^{207,208}. Hexanoylglycine and isovalerylglycine are glycine conjugates of hexanoyl-CoA and Isovaleryl-CoA which are intermediates of β -oxidation and leucine catabolism respectively. Increased hexanoylglycine and isovalerylglycine have been previously associated with the disruption of mitochondrial oxidative pathways^{298,299}. However to the extent of my knowledge, these metabolites have never been reported as predictors of BW and obesity development.

PCS, PCG PAG and *Streptococcaceae* at early time point (7 days after the diet switch) were also found to predict GOS, GOS_CMO and FOS_IN efficiency to reduce long-term calorie storage and improve mouse physiology during DIO. Therefore, these metabolites and bacterial taxa constitute stable and generic candidate makers evaluating the beneficial effect of the prebiotics on obesity development. If these markers are validated in other biological models, they may be used as a generic biological signature to evaluate the individual predisposition to DIO or assess the impact of prebiotic intervention on obesity development.

In addition, the novelty of this work, based on the integration of complementary data from several analytical methodologies, has enabled me to identify new associations between gut bacterial families, and host physiological mechanisms. From these results, possible relationships between gut microbiota and host mitochondrial oxidative pathways have been suggested. In addition, significant correlations between metabolites from bacterial tyrosine degradation (PCS, PCG), IL-1 β , *Peptostreptococceae* and *Streptococcaceae* were evidenced which highlighted the interactions

between gut microbiota and host inflammatory responses during obesity development. Interestingly, a common pattern in gut microbial and host metabolic adaptation among prebiotics was noticed. All prebiotics were found to lower *Peptostreptococceae* and *Streptococcaceae* growth, urinary PCS, PCG and circulating IL-1 β level at 70 days after dietary intervention. Moreover, PCS, PCG, PAG and *Streptococcaceae* at early time point (7 days after the diet switch) were associated with the long-term prebiotic efficiency to reduce calorie storage during HF challenge. Altogether, these findings suggest that gut microbial changes and reduced bacterial amino acid fermentation reduces the production of PCS, PCG, and *p*-cresol which in turn might lower inflammatory response to HF diet and improve host physiology.

3. STRENGTHS AND WEAKNESSES OF THE EXPERIMENTS

In each experiment, the dietary interventions provided significant effects on mouse phenotypes and metatypes. Weight gain and food intake measurements allowed the characterisation of the impact of different diets on mouse obesity development. In particular, the second experiment provided a detailed description of mouse physiology and mouse inflammatory status by measuring fat distribution, organ weights, circulating cytokines, circulating glucose and insulin levels. Biofluid collections at several time points were useful to establish the short-term and long-term metabolic adaptation to the dietary interventions. In particular, the longitudinal study in the second experiment enabled the identification of early and consistent candidate biomarkers of prebiotic effects on host physiology. It is also important to note that isogenic C57BL/6 mouse is a suitable model to investigate microbial-host metabolic interactions during obesity development^{144,145}. This mouse strain also showed differential adaptation to HF diet, which allowed a better understanding of the role of environmental factors (i.e. gut microbiota) and the biochemical mechanisms underlying obese phenotype variability.

However, both experiments have drawbacks:

- In the first experiment, the mice were fed with a baseline diet (before the diet switch) containing different amounts of carbohydrates, proteins, vitamins and fat contents compared to both LF and HF diets. Hence, mouse metabolic profiles during the pre-intervention period were strongly different to those during HF and LF intervention due to variations in calorie supply. This may be the reason why urinary metabolic profiles of pre-

intervention time points (before the HF diet switch) could not efficiently predict the differential adaptation to HF diet during the post-intervention.

- In the second experiment, prebiotics supplemented LF diets (for instance LF + GOS, LF + GOS_CMO) were not provided. Therefore, it was impossible to differentiate the prebiotic-specific effects (independent from HF diet) and the effects associated with the interactions between HF and prebiotics on host physiology and host metabolism.

Our results ultimately showed that ^1H NMR and MS based metabolic profiling are robust and appropriate analytical methods to establish gradual metabolic changes linked to dietary intervention and disease development^{69,108}. In particular, numerous microbial co-metabolites such as SCFAs or bacterial amino acid degradation end-products have been measured by these analytical methods in plasma, urine and feces of mice fed with different diets. Metabonomic data, supported by 16s rRNA gene profiling and cytokine profiles, have contributed to our overall understanding of the onset of obesity and the metabolic syndrome (MetS). These techniques highlighting the contribution of metabolites involved in energy metabolism (TCA cycle, lipid and amino acid processing), microbial-co-metabolites, as well as gut microbial composition, and inflammatory markers for these pathological processes. Metabonomic-based analysis generated several hypotheses that will be further developed in this discussion. However, additional experiments would be useful to support our findings. For instance, genome-wide (microarrays or RNAseq) and targeted (qPCR) transcriptomic analyses of peripheral organs (liver, adipose tissue, muscle...) would be useful to identify variations in expression of genes encoding for proteins involved in lipid metabolism, mitochondrial oxidative or inflammatory pathways in the different diet groups and thus confirm these hypotheses.

16S rRNA gene sequencing is nowadays a relatively inexpensive method to specifically identify bacterial taxa at different taxonomic levels (down to species level). However, some bacterial taxa are not equally covered by the pyrosequencing technique. Full metagenomic analysis is an alternative and more expensive approach based on the sequencing of the entire gut bacterial genome. This technique is less prone to biases and could provide a broader overview of the impact of HF diet and prebiotic interventions on gut microbial remodelling in our experiment. In addition, it would allow establishing new potential functions of a set of microbial species, which are selected by HF or prebiotic interventions. Further investigation of gut bacterial functions may point out other bacterial species conferring probiotic properties.

4. OBESITY DEVELOPMENT OF C57BL/6 MICE UNDER NUTRITIONAL INTERVENTIONS.

Metabolic adaptations to obesity development in C57BL/6 mice have been investigated under several conditions in this PhD thesis. In the first experiment, (Chapter III) short-term (7 days) and long-term (60 days) urinary metabolic signatures to HF diet induced obesity were characterised. Several metabolites were found to be positively or negatively correlated with HF diet at both time points highlighting a consistent metabolic signature associated to DIO. A stratification of the mouse population under HF diet based on body weight gain (BWG) distribution (Chapter III, section 3.1) were performed in the same experiment and candidate markers discriminating obesity-prone (SR) and obesity-resistant (NR) mice under HF diet were identified. In the second experiment, the comparison of metabolic signatures of LF, HF and HF+ prebiotic fed mice pointed out specific metabolic adaptations to HF diet and different prebiotic interventions in lowering obesity (Chapter IV, section 3.1). The metabolic signatures associated with different dietary intervention are summarised in Table 11. This table aims to emphasize the metabolic adaptations associated with increasing obesity under different dietary interventions; therefore, it shows the increased or decreased levels of metabolites in most obese mice (HF or SR group) by comparison to the control group. (LF-fed mice), or the least obese mice (NR, or HF + prebiotics fed mice) in both experiments. Interestingly, several metabolites were consistently affected by the different diet interventions. In particular, HF induced obesity consistently affects lipid metabolism, nicotinamide-adenine dinucleotide (NAD) metabolism, and host mitochondrial oxidative pathways (β -oxidation, BCAA catabolism, and the tricarboxylic acid (TCA) cycle). Specific gut microbial-mammalian host metabolic interactions are affected by the development of obesity, as indicated by the modulation of bacterial methylamines metabolism, and bacterial amino acid degradation in HF diet compared to LF diet.

Table 11: Summary of the main metabolic adaptations to dietary interventions in each experiment

				Experiment 1		Experiment 2					
			Metabolites	HFvs LF	SR vs NR	HF vs LF	HF_S vs LF	HF vs HF+GOS	HF vs HF + FOS_IN	HF_S vs HF + GOS_CMO	
Energy metabolism	Mitochondrial metabolism	BCAAs metabolism	Leucine		↓						
			Leucine			↓	↓				
			Valine			↓	↓				
			Isovalerylglycine	↑	↓	↑	↑		↓		
			α-Ketoisovalerate	↑		↑	↑	↓			
			α-Keto-β-methylvalerate	↑		↑	↑				
		β-oxidation	Carnitine	↑		↑	↑			↓	
			Hexanoylglycine	↑	↓						
		TCA cycle	Citrate			↓	↓				
			Pyruvate			↓	↓				
		Energy metabolism	Ketogenesis	3-D-Hydroxybutyrate							↓
	NAD pathways			2PY	↑		↑			↑	↑
				4PY	↑		↑			↑	↑
	SCFAs		Acetate						↓		
			Butyrate					↓	↓		
			Isobutyrate		↓						
	Lipid metabolism		Bile acids mix			↑		↓			
			Lipids			↑	↑	↓	↑		
	Carbohydrate metabolism		Sucrose			↓				↓	
			D-Galactose				↑			↓	
			D-Glucose			↑	↑	↓	↑	↓	
			α-D-Glucose			↑	↑	↑	↑	↑	
	Muscle energy homeostasis		Creatine				↑	↑	↑		
			Creatinine					↑	↑	↑	
	Cell processes		Cell signalling	myo-inositol			↓				
				scyllo-inositol			↓	↓	↓	↓	
				scyllo-inositol			↓	↓		↑	
			Nucleic acid metabolism	Xanthine			↓			↑	
				Uracil						↑	
	Gut bacteria activity	Choline and methylamine metabolism	Choline			↑		↓	↓		
TMA			↓		↓				↑		
TMAO			↓		↓			↑			
Bacterial amino acid metabolism		Indoxylsulfate			↓	↓	↑	↑	↑		
		Phenylacetylglutamine	↓		↓	↓					
		Hydroxyphenylacetate									
		p-cresol glucuronide					↓	↓	↓		
		p-cresol sulfate		↓			↓	↓	↓		

LF: low fat, HF: high fat, NR: non-responder to HF diet, SR: strong responder to HF diet, HF_S: HF diet with glucose, galactose, and lactose, GOS: galactosyl-oligosaccharides, GOS_CMO: galactosyl-oligosaccharides and cow milk oligosaccharides, FOS_IN: fructosyl-oligosaccharides and inulin. 2PY: *N*-methyl-2-pyridone-5-carboxamide 4PY: *N*-methyl-4-pyridone-3-carboxamide. TMA: trimethylamine TMAO: trimethylamine-*N*-oxide. NAD: nicotinic adenine dinucleotide. TCA: tricarboxylic acid ↓ negatively correlated with HF diet or SR mice compared to the other group ↑ positively correlated with HF diet or SR mice compared to the other group. The metabolites found in plasma, urine or feces are color-coded as following:

Urine	Plasma	feces
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The decrease in obesity development by prebiotic supplementation was also associated with a regulation of mitochondrial metabolism (BCAA catabolism, or β -oxidation) and bacterial amino acid metabolism. This highlights the presence of key metabolic pathways being consistently regulated with obesity development irrespective of the diet. These metabolic pathways may influence the onset of obesity during HF challenge. The role of mitochondrial oxidative pathways and bacterial amino acids metabolism are further discussed in the following sections.

4.1. The role of mitochondrial oxidative pathways in obesity development.

As expected, HF diet led to modifications of several metabolic pathways in C57BL/6 mice in order to process the nutrient overflow and maintain energy homeostasis. High lipid absorption was associated with an activation of lipid metabolism characterised by a modulation of circulating lipid content and an adaptation of mitochondrial oxidative pathways (β -oxidation, BCAA catabolism). An excess of circulating fatty acids was also associated with an increase in lipoproteins, promoting ectopic fat accumulation in several organs. HF feeding was found to convey an increase in body fat as well as an increase in liver weight, and epididymal and mesenteric adipose tissues weights. Concomitantly, long-term HF feeding raised circulating glucose levels, and promoted a loss of insulin sensitivity and an inflammatory response to HF diet. Interestingly, several C57BL/6 mice were resistant to DIO and were found to have stimulated alternative mitochondrial oxidative pathways. In particular, a specific increase in the glycine conjugate of leucine catabolism end-product (isovaleryl-glycine) and a concomitant decrease in TCA cycle intermediate were observed in obesity-resistant mice compared to obesity-prone mice to HF diet¹⁹⁹. It has been suggested that an activation of ketogenic pathways in the mitochondria may limit the saturation of mitochondrial oxidative pathways and improve mouse physiology¹⁹⁹. These results provide the evidence that mitochondrial homeostasis, which regulates ATP synthesis and energy metabolism, plays a crucial role in obesity development and associated metabolic disorders³⁴.

4.2. Influence of gut microbiota on host mitochondrial metabolism.

It is valid to say that in isogenic C57BL/6 mice, the genetic variations among subjects have been cancelled. Therefore, the differential activation of mitochondrial oxidative pathways in obesity-resistant and obesity-prone mice was exclusively governed by environmental factors in our experiments. Epigenetic regulations linked to stress or dietary interventions as well as gut microbial activity have been found to influence host metabolism and host physiology irrespective of genetic background in previous studies^{27,28}. In this PhD thesis, differential urinary excretion of microbial co-metabolites (hippurate and hydroxyphenylacetyl glycine) in obesity-resistant and obesity-prone mice on a HF diet were observed, which indicates a specific gut microbial activity in these two groups. These results suggested that gut microbiota contributed to the differential adaptation to the HF diet and may participate in regulation of mitochondrial oxidative pathways.

This hypothesis was further investigated in this thesis by studying the effect of prebiotic on C57BL/6 mouse physiology during HF challenge. Different prebiotic blends (GOS, GOS_CMO and FOS_IN) were tested to selectively modify the gut microbiota in the context of DIO. Our results demonstrated that the different prebiotics induced profound changes in gut microbial composition and improve mouse physiology during HF diet. In addition, prebiotic supplementation during HF diet improved mouse metabolic homeostasis by partially recovering a healthy metabolic phenotype. Indeed, a significant shift in the global metabolic signature of HF+ prebiotic fed mice toward the metabolic pattern of LF-fed mice was observed in plasma and feces. This shift was characterised by a partial return of a normal, healthy profile of lipoproteins and metabolites involved in the mitochondrial oxidative pathways. In particular FOS_IN was the most efficient prebiotic in recovering healthy metabolic profiles as indicated by a significant reduction in circulating lipids, urinary 2PY, 4PY and glucose. These key findings support the hypothesis that gut microbiota influence host energy metabolism and play a significant role in the set of obesity.

In addition, FOS_IN supplementation during HF diet showed an activation of leucine catabolism and ketogenesis and maintained a normal glycemia during HF challenge. This supports the hypothesis that the activation of leucine catabolism and ketogenic pathways improves insulin signalling pathways and may contribute to mouse weight reduction in DIO.

In the second experiment, several correlative analyses between 16S rRNA sequencing data and ¹H NMR urinary, plasma and fecal metabolic profiles allowed the identification of correlations

between bacterial families and metabolites involved in host energy metabolism. In particular, *Streptococcaceae*, *Ruminococcaceae*, *Peptostreptococcaceae* and *Lachnospiraceae* families from the *Firmicutes* phylum were positively correlated with circulating lipoproteins and fecal bile acids. *Lachnospiraceae*, *Porphyromonadaceae*, *Rikinellaceae* families were also positively correlated with urinary α -keto- β -methylvalerate, isovalerylglycine, isovalerate, α -ketoisovalerate and 4PY which derived from BCAA catabolism and NAD pathways respectively (Table 11). All these bacterial families were found to be stimulated by HF feeding. Therefore, bacterial communities selected by HF diet may promote lipid digestion and absorption and the stimulation of host energy metabolism. However, the correlative analyses did not provide any proof of the causative links between gut microbiota and host mitochondrial oxidative pathways. Increased nutrient supply due to the HF diet may independently affect the gut bacterial ecosystem and host energy metabolism in this experiment. Therefore, additional experiments are needed to precisely identify the role of gut microbial activity and bacterial co-metabolites on host energy metabolism.

The influence of the gut microbiota on host energy homeostasis has been already highlighted in previous publications^{33,140,259}. Studies of germ free mice and re-conventionalised mice have shown that the presence of the gut microbiota helps harvesting energy and increase host fat storage^{33,259}. Gut microbiota also influence gut physiology and gut motility, which help extracting calories from the diet³⁰⁰. Finally the gut microbiota are involved in carbohydrate digestion and the formation of bacterial fermentation products (mainly SCFAs), which are used by the host^{140,300}. In addition, gut bacterial activity has been found to impact host energy metabolism^{77,301}. Several studies have shown dramatic differences in the host gene expression of germ-free mice and conventionally raised mice in the different segments of the gut as well as the liver and adipose tissues³⁰¹. Interestingly, BCAA catabolism related genes coding for branched chain amino transferase (BCAT1 and BCAT2) in subcutaneous fat were differently regulated in germ-free and colonized mice. BCKDH gene expressing α -ketoacids dehydrogenase was also up-regulated in the liver of germ-free mice compared to conventional mice³⁰¹ (<http://microbiota.wall.gu.se/>).

Recently, Ridaura *et al* have established causal links between gut microbial communities and obesity development by transplanting fecal samples from obese or lean discordant co-twins into separate group of germ free mice³⁰². They found that mice colonized with obese co-twin fecal microbiota had a greater increase in body weight and adipose tissues compared to the mice colonized with lean co-twin fecal microbiota. In addition, obese mouse co-housed with lean mice also exhibited a lower weight gain, and a shift in gut microbiota composition towards a lean-like status. In particular, a stimulation of *Bacteroidetes* growth occurred in obese mice co-housed with

lean mice, which was associated with an increased protein expressions involved in BCAA catabolism and increased circulating SCFAs³⁰². These results were in accordance with the abovementioned correlative analysis. Therefore, the work presented in this thesis along with these publications indicates that gut microbiota strongly contribute to a differential adaptation to DIO through a modulation of mitochondrial oxidative pathways and energy homeostasis. It can be suggested that specific gut microbiota remodelling by dietary interventions (such as prebiotics or probiotics) inducing a reduction of calories harvest and/or the activation of ketogenic pathways, may reduce obesity development and improve host physiological status. Nevertheless, further investigations are needed to define the precise interactions between gut microbiota, host mitochondrial oxidative pathways and energy homeostasis.

4.3. Interactions between gut microbial tyrosine fermentation, host inflammatory pathways and host metabolism during diet induced obesity

The work presented in this thesis demonstrated that, long-term (70 days) ingestion of GOS, GOS_CMO or FOS_IN during HF diet limits weight gain, fat mass production, food efficiency, IL-1 β mediated immune response and organ weights (liver, adipose tissues). Urinary decreases in PCG and PCS were also observed in mice fed HF + prebiotics compared to HF or LF-fed mice. These molecules are both derived from the colonic bacterial degradation of tyrosine²²⁶. *p*-Cresol and PCS have been associated with gut inflammatory diseases, kidney diseases in human and insulin resistance in mice^{226,288,303}. In particular, PCS has been found to activate inflammatory gene expressions (*Tgfb1*, *Fas*, *Il-6*, *Il-5*, *Csf1/3*, *Cxc,10*) in renal tubular cells *in vitro*²⁹¹. Therefore, their decrease indicated strong changes in gut microbial activity with DIO which might modify host physiology. The gut microbial remodelling by prebiotics was also characterised by the growth of commensal bacteria (*Bifidobacteriaceae* or *Lactobacillaceae*) and the reduction of other bacterial families such as *Peptostreptococaceae* and *Streptococaceae*. Interestingly, weak but significant correlations ($0.3 < \text{IRI} < 0.5$, *p* value < 0.05) were identified between PCG, PCS, IL-1 β and *Peptostreptococaceae*. These results suggest that in the context of obesity, the production of *p*-cresol, PCG and PCS by gut bacterial tyrosine degradation in the mouse colon contributes to the mouse inflammatory responses to HF diet and affects mouse physiology. On the other hand, decreases in *Peptostreptococaceae* and *Streptococaceae* counts and down-regulation of bacterial tyrosine degradation by prebiotic

supplementation may lower IL-1 β mediated inflammatory responses to HF challenge and improve host physiology.

IL-1 β is a key cytokine involved in multiple inflammatory processes. IL-1 β activity is dependent on the transcription of pro-IL-1 β genes, the maturation of pro-IL-1 β and the regulation of mature IL-1 β by the expression of antagonists such as siIL1-Ra²⁷⁹. IL-1 β is also processed and released in macrophages and neutrophils *via* two mechanisms. The first mechanism involves the activation of TLRs (see Chapter I, section 1.4) and enables the transcription and the maturation of pro-IL-1 β ²⁷⁹. The second mechanism requires the protein caspase 1 which is activated by a multimeric cytosolic protein complex called the inflammasome comprising NOD-like receptors (NLRs) amongst other proteins. These both mechanisms can be activated by bacterial and/or dietary components (see Chapter I, section 1.4.1). IL-1 β plays an essential role in the pathogenesis of auto-inflammatory diseases such as inflammatory bowel diseases (IBD) and non-hereditary inflammatory diseases²⁷⁹. For instance, increased IL-1 β production has been found in mononuclear cells from inflamed mucosa with ulcerative colitis or Crohn's disease *in vitro*³⁰⁴. In addition, Carvalho *et al* showed that TLR5 KO mice were predisposed of developing colitis due to an activation of IL-1 β -dependent inflammatory pathways³⁰⁵. In the intestine, the inflammasome pathways (NLRP3, ASC, TXNP and caspase-1) can be regulated by molecules derived from gram-positive and gram-negative bacteria⁴⁸. In particular, LPS levels in inflamed mucosa enhanced IL-1 β production in the activated macrophages³⁰⁴. Therefore, alterations of gut microbiota by dietary intervention such as HF diet may trigger IL-1 β mediated inflammatory responses in the gut. In addition, IL-1 β has been suggested to play a crucial role in impaired insulin signalling, lipid metabolism and T2DM²⁷⁹. In particular, this interleukin was found to alter β -cells in mice triggering hyperglycemia, insulin resistance in peripheral tissues and fat accumulation in adipocytes and hepatocytes^{61,279,306}. On the other hand, SiIL1-Ra treatment on adipocytes was shown to increase adipocytes differentiation and insulin sensitivity⁶¹. Increased inflammatory effectors including IL-1 β in renal cells of chronic kidney disease patients was associated with renal failure³⁰⁷. Kidney failure may also increase the amount of protein bound solutes in circulation such as PCS and PCG. Interestingly, the etiopathogenesis of ulcerative colitis, kidney diseases and T2DM is thought to be promoted by an increase in *p*-cresol and PCS and is also associated with IL-1 β mediated inflammatory responses^{226,279,288}. Altogether these findings support the hypothesis of a causal relationship between metabolites derived from gut microbial tyrosine degradation, host immune system and host physiology (Figure 57). However, the nature of the interactions between the gut microbiota and the host metabolism remain to be explained.

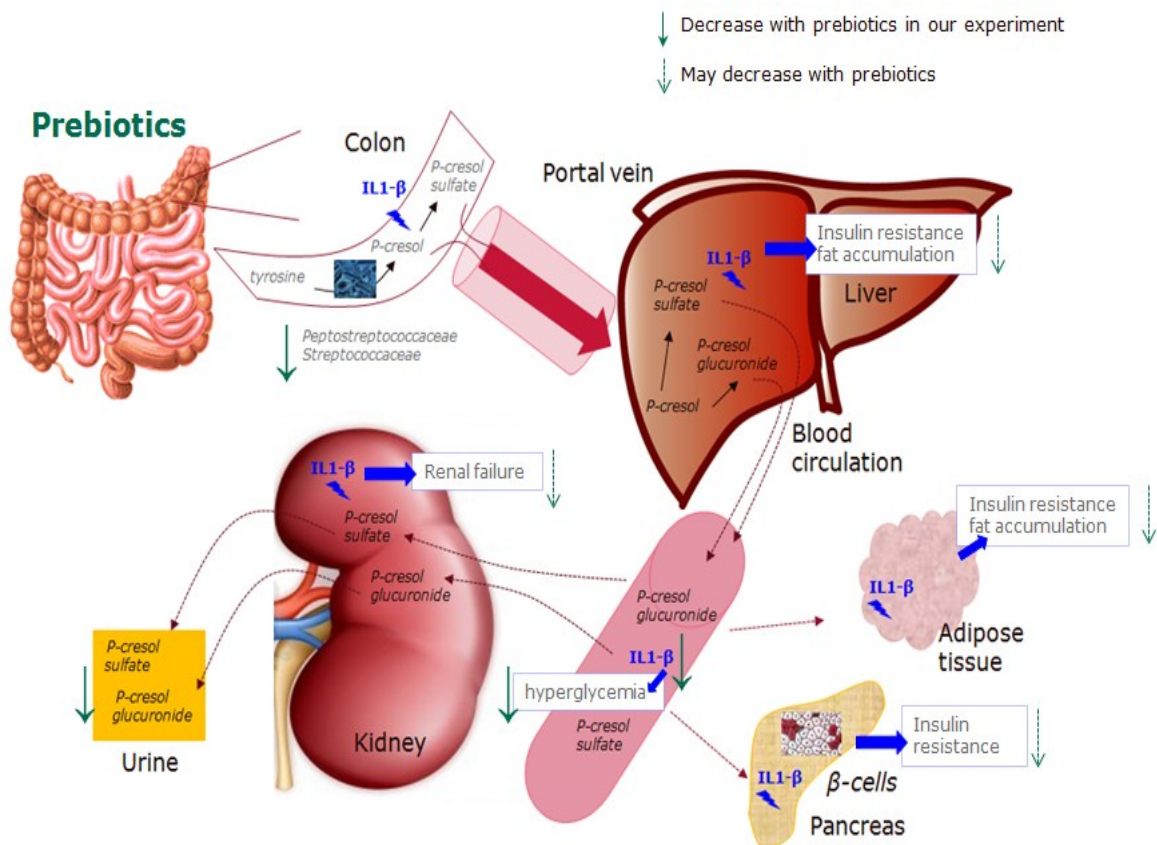


Figure 57: Possible mechanisms linking gut bacterial tyrosine degradation, host inflammatory pathways and host physiological disturbances in diet induced obesity and its regulation with prebiotics. The production of *p*-cresol and *p*-cresol sulfate from bacterial tyrosine degradation during HF challenge may promote interleukin-1 β (IL-1 β) related inflammatory pathways in the colon. Increased level of proteolytic bacteria induced by HF challenge (*Peptostreptococcaceae* and *Streptococcaceae*) may also contribute to amino acid degradation and inflammation in the gut. *p*-Cresol and *p*-cresol sulfate can cross mucosa and reach the liver via the portal vein. *p*-Cresol is further detoxified in *p*-cresol sulfate or *p*-cresol glucuronide in the liver. *p*-Cresol sulfate and *p*-cresol glucuronide can enter systemic circulation and reach peripheral tissues (i.e pancreas, adipose tissues, kidney, liver), triggering local inflammatory responses. *p*-Cresol sulfate and *p*-cresol glucuronide are ultimately transferred to the kidney and released in urine. IL-1 β production may alter β cell functions in the pancreas inducing insulin resistance, hyperglycemia and fat accumulation (green arrows for observed phenotypes in the prebiotic study and dashed green arrows for hypothetic phenotypes). IL-1 β associated inflammation may also affect various organ functions. In particular it may promote renal failure. Prebiotic supplementations during HF challenge lowers *Peptostreptococcaceae* and *Streptococcaceae* growth and urinary *p*-cresol sulfate and *p*-cresol glucuronide prebiotic supplementations also reduce hyperglycemia circulating IL-1 β , fat mass and organ weight. Hence, gut microbial remodelling by prebiotic inducing a decrease in anaerobic bacteria and bacterial tyrosine degradation may lower host inflammatory responses to HF diet and improve mouse physiology.

Firstly, it is important to determine whether *p*-cresol, or its derivatives (PCG, PCS) have a pharmacological effect on host metabolic/inflammatory pathways. They are differently processed by the host organism and therefore may affect different tissues. Indeed, *p*-cresol is rapidly detoxified by sulfation or glucuronidation in colonic mucosa or liver²²⁶. Hence, a large amount of *p*-cresol produced by colonic amino acid degradation may be deleterious for the colonocyte and hepatocyte. On the other hand, PCG and PCS are transferred in the kidney and released in urine²²⁶. They can also easily enter blood circulation and reach peripheral organs. Therefore PCG and PCS may be biologically active in renal cells as well as myotubules, hepatocytes and adipocytes. PCS has been already found to affect insulin signalling pathways in myotube cells *in vitro* by activating ERK1/2 kinase. However, the impact of *p*-cresol or PCG on host metabolic pathways and host immune system is far from being understood.

Further experiments are therefore needed to verify this hypothesis. Several *in vitro* experiments are in the pipeline to test the pharmacological effects of PCS, PCG or PAG on host metabolism. The preliminary experiments will consist in testing whether these molecules have a positive or negative effects on pharmacological targets in the host, such as G proteins coupled receptors (GPCR), intracellular kinases or toll-like receptors (TLRs). GPCRs are coupled with various enzymatic effectors regulating many physiological processes. Intracellular kinases are involved in several signalling cascades such as insulin signalling pathways or growth factor cascade. Finally TLRs are key receptors of innate immune systems (see Chapter I, section 1.4.1). This high-throughput screening will allow the identification of physiological pathways which are affected by PCS, PCG and *p*-cresol. Additionally, it will be also important to test the dose-dependent effect of these molecules on adipocyte, colonocyte and hepatocyte metabolism (glucose uptake, insulin secretion, ROS production) to assess the impact of PCS, PCG and *p*-cresol on energy homeostasis and oxidative pathways. Finally, to test the pro- or anti-inflammatory properties of PCS, PCG and *p*-cresol, dose dependent experiments on macrophage will be carried out *in vitro*. Altogether these experiments will provide the evidence for a causative link between amino acid degradation end-products and the deregulation of the host energy metabolism and host immune system.

Secondly, it is unclear whether *Peptostreptococaceae* or *Streptococaceae* growths are directly responsible for the raise of colonic amino acid degradation. Several *in vitro* tests have shown that *Peptostreptococaceae* are able to degrade several amino acids such as ornithine, lysine and arginine (<http://www.metacyc.org/META/NEW-IMAGE?type=ORGANISM&object=TAX-186804>) but no evidence of tyrosine digestion by *Peptostreptococaceae* or *Streptococaceae* has been reported

yet. HF diet may independently activate the proliferation of these bacterial families, as well as gut bacterial amino acid degradation.

The potential role of *Peptostreptococaceae* and *Streptococaceae* families in the production of *p*-cresol also needs to be investigated. In particular, it is important to identify which bacterial species within *Peptostreptococaceae* and *Streptococaceae* are involved in amino acid degradation. Fermentors are commonly used to study the anaerobic fermentation by bacterial cultures. A complex mix of bacteria (for instance, derived from fecal cultures) or a selected group of bacteria can be introduced in the chamber with fermenting substrate such as proteins or fibres. More advanced cultures (such as artificial gut) have also been developed to reproduce the physical, anatomical and nutritional characteristics of a gastrointestinal tracts³⁰⁸. Although *in vitro* experimental conditions do not entirely reproduce the complex environment in the gut and the bacterial cross-feeding, these techniques would be useful to better understand the role of *Peptostreptococaceae* and *Streptococaceae* on gut bacterial tyrosine degradation, alone or in a mixed culture. Ultimately, *in vivo* experiments would be necessary to validate the biological mechanism linking *Peptostreptococaceae*, *Streptococaceae*, amino acid degradation end-products, and host physiology.

5. PERSPECTIVES ON HUMAN HEALTH

This PhD thesis further highlights the role of mitochondrial homeostasis in obesity. It also shows the potential interactions between gut microbiota and host mitochondrial oxidative pathways. The identification of early and stable biomarkers predicting the differential adaptation to HF diet in C57BL/6 mice provides promising perspectives for weight gain management and personalized nutrition. If these markers are confirmed in human studies, they could be used to anticipate the individual or population responses to an obesogenic environment (high calorie diets, low physical activity).

This PhD thesis also demonstrates that prebiotics are efficient in limiting mouse obesity development and therefore could be proposed as dietary complements for weight gain management in animals and humans. However, it is important to note that the prebiotic consumption correspond to 24 mg/d per mouse in our experiment. It is unsure whether this fibre proportion can be easily applied to other animal models and to humans in particular. The recommended dietary reference intake of total fibre is 25 g/d for young women and 38 g/d for young men. It has been previously

found that high fibre ingestion may confer side effects such as bloating, gas production and discomfort in the human bowel, which limits prebiotic usage³⁰⁹. Interestingly, in a recent study, obese women treated with 15 g/d of inulin type fructans for 3 months showed modest changes in lipid metabolism but no significant effect on weight gain¹¹⁴. Therefore, additional research focused on studying the optimal fibre portion and/or designing new prebiotics are also important to find the optimum prebiotic intervention in humans.

In addition, it has been suggested that prebiotics improve mouse physiology by disturbing the gut microbiota-host physiology interactions involving *Peptostreptococaceae* and *Streptococaceae* activity, *via* metabolites derived from bacterial tyrosine degradation (PAG, PCS) and IL-1 β related inflammatory pathways. Further *in vitro* and *in vivo* analyses are required to validate this hypothesis. Based on this mechanism, new therapies specifically targeting effectors of the inflammation and/or the modulation of bacterial species could be developed to alleviate obesity development. However, the human gut ecosystem and its function exhibits great differences compared with the mouse gut ecosystem⁸¹. Therefore it is uncertain whether the same combination of prebiotics will have a comparable effect on obesity in human.

Finally, several urinary metabolites (PAG, PCS, and PCG) and a fecal bacteria family (*Streptococaceae*) were found to predict at early stage the prebiotic efficiency in reducing mouse obesity. These markers could also present a great potential for personalized nutrition if they are transferable to human populations as they would assess at early stage the effect of prebiotic intervention on obesity. However, considering that human subjects present greater genetic variability and are subjected to a less-controlled environment than a single mouse strain, gut microbiota-host metabolic interactions might be complex and less predictable. In addition, it is also important to note that PAG, PCS and PCG are diet-dependent markers as they derived from gut protein and amino acid degradation. These metabolites may not be observed in experiments using low protein diets. Nevertheless, it is known that the western diet responsible for the obesity pandemic in humans is based on the intake of high fat, high sugary diets and red meat which contains a large protein content^{1,18}. Hence, the gut microbial reshaping by prebiotics is likely to affect gut microbial amino acids degradation in human population during obesity development. However, these markers may not be effective predictors of weight gain and metabolic disturbances in vegans. In addition, given the significant difference in gut microbial ecosystem between humans and rodents, the effects of prebiotics may lower other end-products of gut microbial amino acid degradation in human model.

To conclude, considerable efforts are needed to test the reliability of these results in humans. In particular, studying prebiotic effects on the human metabolome and gut bacterial functionalities on epidemiological scale will be valuable to underpin the individual responses to different prebiotic treatments, depending on genetic, environmental, and gut microbial factors. The new mechanisms of gut microbiota and host metabolic interactions, and the concept of prediction of the physiological outcomes under DIO proposed in this PhD thesis, offer novel perspectives in healthcare management and personalised nutrition.

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APPENDIX 1:

SUPPLEMENTARY DATA AND 2D NMR ASSIGNMENTS OF THE BODY WEIGHT VARIABILITY STUDY (CHAPTER III)

Table S1: Diet composition.

Products	Gm (%) (g/100kg)		
	Baseline (3437)	Low Fat (D12450B)	High Fat (D12492)
Proteins	18.5	19.5	26.2
Fibres (cellulose)	4.5	4.7	4.7
Fat	4.5	4.3	34.9
Carbohydrates	54.2	66.3	26.3
Minerals	41.9	4.3	4.3
Vitamins	40.13	0.95	0.95
Choline	200	0.19	0.19
Energy	3226.6 kcal/kg	3850 kcal/kg	5240 kcal/kg

Table S2: Comparison of cumulative food intake (grams) between HF diet and LF diet fed mice NR mice and SR mice.

cumulative food intake		
	7 days	60 days
LF group (mean $\pm$ stdev)	18.8 $\pm$ 1.4	131.5 $\pm$ 6.8
HF group (mean $\pm$ stdev)	17.5 $\pm$ 2.4	110.9 $\pm$ 7.5
Wilcoxon-Mann-Whitney test LF vs HF (<i>p</i> value)	0.0001	9.12 10 ⁻¹²
NR group (mean $\pm$ stdev)	17.4 $\pm$ 4.26	107.0 $\pm$ 4.9
SR group (mean $\pm$ stdev)	18.4 $\pm$ 1.06	116.1 $\pm$ 6.5
Wilcoxon-Mann-Whitney test NR vs SR (<i>p</i> value)	0.0013	0.001

Table S3: Comparison of cumulative energy intake (kcal) between HF diet and LF diet fed mice NR mice and SR mice.

Cumulative energy Intake		
	7 days	60 days
LF group (mean $\pm$ stdev)	72.80 $\pm$ 8.1	510 $\pm$ 51.83
HF group (mean $\pm$ stdev)	73.5 $\pm$ 12.5	535.7 $\pm$ 39.6
Wilcoxon-Mann-Whitney test LF vs HF (<i>p</i> value)	0.0001	1.84 10 ⁻⁹
NR group (mean $\pm$ stdev)	83.2 $\pm$ 6.9	541.49 $\pm$ 27.6
SR group (mean $\pm$ stdev)	100.1 $\pm$ 19.8	599.1 $\pm$ 42.7
Wilcoxon-Mann-Whitney test NR vs SR (<i>p</i> value)	0.00630	0.00038

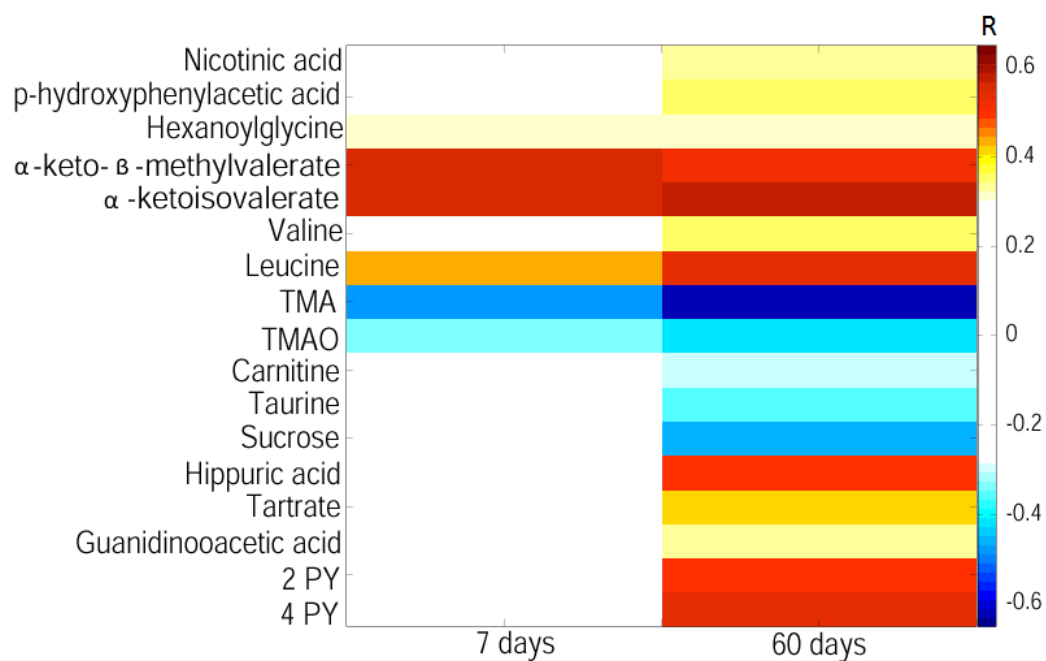


Figure S1: Correlation heatmap highlighting the significant correlations between energy intake and urinary metabolic profiles of LF and HF-fed mice at 7 days and 60 days after diet switch. Correlation values for each metabolite are displayed by color code. (Gradient of red color for metabolites positively correlated with energy intake and gradient of blue colors for metabolites negatively correlated).

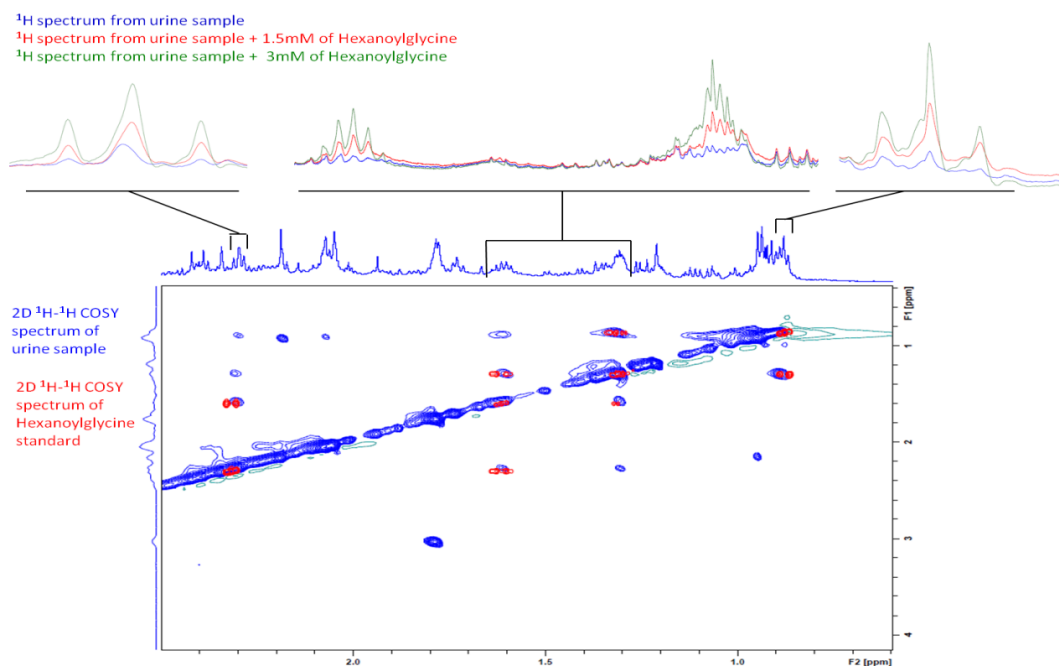


Figure S2: Identification of hexanoylglycine in urine metabolic profile by 2D ¹H-¹H COSY NMR experiment and experiments of spiking;

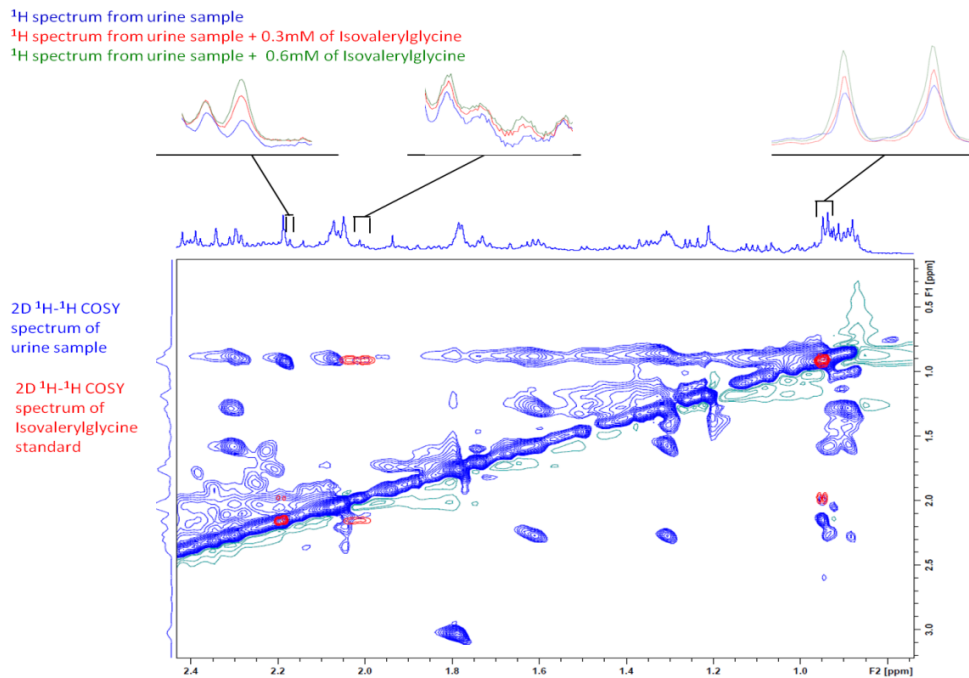


Figure S3: Identification of Isovalerylglycine in urine metabolic profile by 2D ¹H-¹H COSY NMR experiment and experiments of spiking.

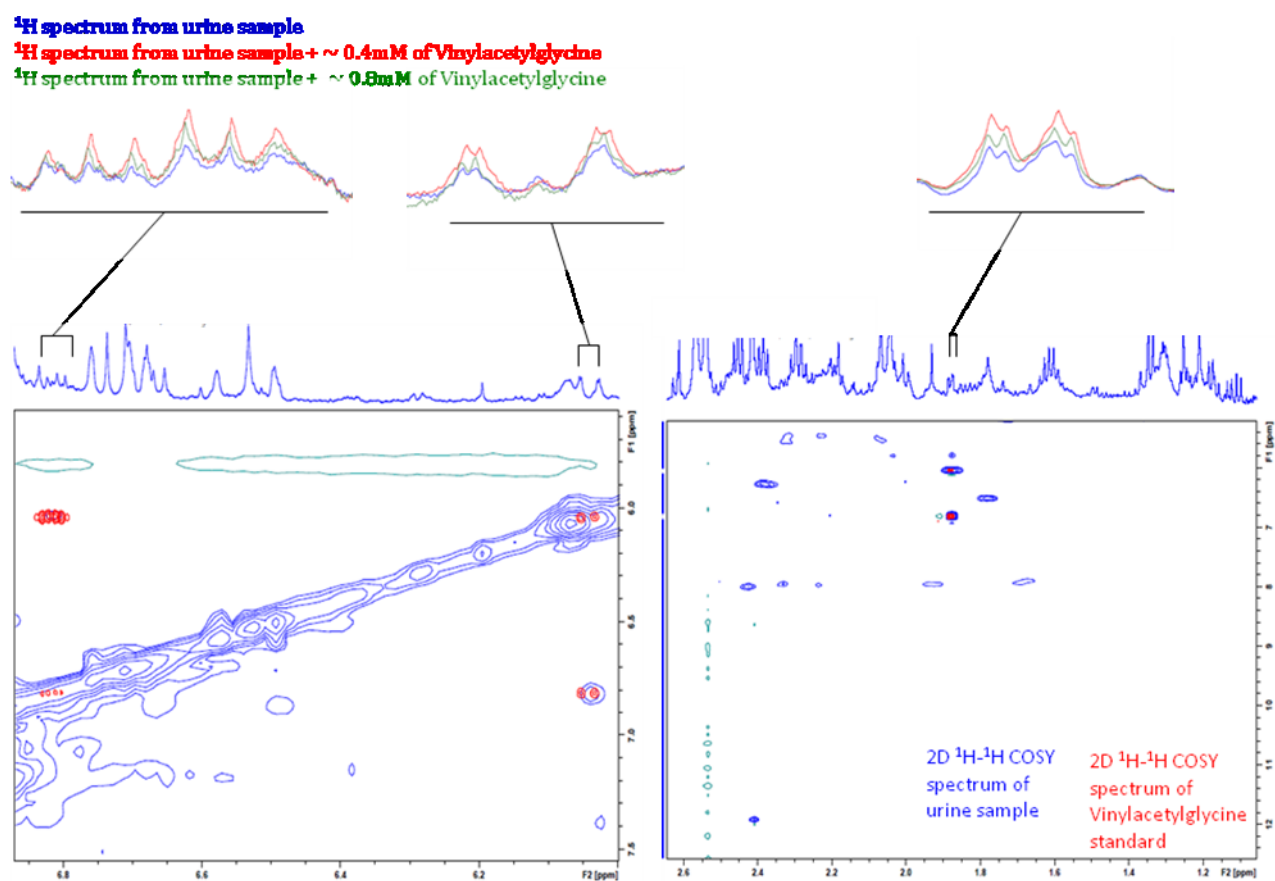


Figure S4: Identification of Vinylacetylglycine in urine metabolic profile by 2D ^1H - ^1H COSY NMR experiment and experiments of spiking.

Table S4: value of Q^2Y and R^2Y and validation of models.

group	Timepoint	component	Q^2Y	R^2Y	Permutation test (1000 tests): p value
OPLS-DA LF vs HF	day 0	1	negative	x	x
	7 days	1+1	0.83	0.89	$1.0 \cdot 10^{-3}$
	60 days	1+3	0.93	0.98	$5.0 \cdot 10^{-4}$
OPLS-DA NR vs SR	day 0	1	0.045	0.3821	x
	7 days	1+2	0.25	0.51	0.046
	60 days	1+2	0.40	0.8572	0.01

Table S5: Mean ratios $\pm$ standard error of integrals at day 7 (i.e day 7/day 0) or day 60 (i.e day 60/day 0) for each group and for each metabolite used in the data mapping (figure 4).

metabolites	^1H ppm	7 DAY LF			7 DAY HF			7 DAY NR			7 DAY SR			60 DAY LF			60 DAY HF			60 DAY NR			60 DAY SR		
Isovalerylglycine	0.92	0.97	$\pm$	0.01	1.27	$\pm$	0.02	1.38	$\pm$	0.01	1.13	$\pm$	0.02	0.80	$\pm$	0.02	1.20	$\pm$	0.014	1.32	$\pm$	0.02	1.06	$\pm$	0.01
α -keto- β -methylvalerate	1.99	1.16	$\pm$	0.01	1.54	$\pm$	0.02	1.48	$\pm$	0.02	1.52	$\pm$	0.02	1.01	$\pm$	0.01	1.37	$\pm$	0.014	1.27	$\pm$	0.02	1.35	$\pm$	0.02
α -ketosiovalerate	1.12	1.46	$\pm$	0.01	2.05	$\pm$	0.02	1.94	$\pm$	0.03	2.01	$\pm$	0.02	1.33	$\pm$	0.01	2.03	$\pm$	0.022	1.88	$\pm$	0.02	1.97	$\pm$	0.02
Isoleucine	1.029	1.37	$\pm$	0.02	1.62	$\pm$	0.02	1.56	$\pm$	0.02	1.61	$\pm$	0.03	1.20	$\pm$	0.02	1.46	$\pm$	0.017	1.45	$\pm$	0.02	1.44	$\pm$	0.02
Valine	1.05	1.04	$\pm$	0.02	1.18	$\pm$	0.01	1.21	$\pm$	0.01	1.13	$\pm$	0.01	0.82	$\pm$	0.01	1.06	$\pm$	0.012	1.04	$\pm$	0.01	1.04	$\pm$	0.01
Leucine	0.97	1.40	$\pm$	0.01	1.86	$\pm$	0.01	1.91	$\pm$	0.01	1.77	$\pm$	0.01	1.09	$\pm$	0.01	1.64	$\pm$	0.013	1.62	$\pm$	0.01	1.53	$\pm$	0.01
Citrate	2.57	1.09	$\pm$	0.05	1.12	$\pm$	0.05	1.06	$\pm$	0.03	0.93	$\pm$	0.04	1.14	$\pm$	0.04	1.40	$\pm$	0.093	0.85	$\pm$	0.02	1.42	$\pm$	0.10
Succinate	2.410	0.212	$\pm$	0.004	0.272	$\pm$	0.007	0.260	$\pm$	0.004	0.292	$\pm$	0.008	0.186	$\pm$	0.003	0.238	$\pm$	0.008	0.182	$\pm$	0.003	0.301	$\pm$	0.010
α -ketoglutarate	2.45	0.62	$\pm$	0.01	0.72	$\pm$	0.01	0.72	$\pm$	0.01	0.70	$\pm$	0.01	0.59	$\pm$	0.01	0.68	$\pm$	0.012	0.65	$\pm$	0.01	0.70	$\pm$	0.01
Cis.Aconitate	3.12	0.86	$\pm$	0.03	0.83	$\pm$	0.02	0.89	$\pm$	0.03	0.75	$\pm$	0.02	0.98	$\pm$	0.04	0.84	$\pm$	0.027	0.74	$\pm$	0.03	0.86	$\pm$	0.02
Fumarate	6.53	0.102	$\pm$	0.005	0.128	$\pm$	0.003	0.145	$\pm$	0.003	0.134	$\pm$	0.004	0.074	$\pm$	0.002	0.118	$\pm$	0.004	0.097	$\pm$	0.002	0.127	$\pm$	0.006
Carnitine	0.88	0.98	$\pm$	0.01	1.17	$\pm$	0.01	1.20	$\pm$	0.01	1.19	$\pm$	0.01	1.01	$\pm$	0.01	1.30	$\pm$	0.015	1.40	$\pm$	0.02	1.27	$\pm$	0.02
Acyl-carnitine	3.23	1.09	$\pm$	0.01	1.20	$\pm$	0.01	1.19	$\pm$	0.00	1.19	$\pm$	0.01	1.17	$\pm$	0.01	1.24	$\pm$	0.022	1.20	$\pm$	0.01	1.30	$\pm$	0.03
Hexanoylglycine	3.2	1.54	$\pm$	0.03	2.13	$\pm$	0.04	2.19	$\pm$	0.03	1.78	$\pm$	0.04	1.18	$\pm$	0.03	2.03	$\pm$	0.035	2.11	$\pm$	0.04	1.60	$\pm$	0.02
vinylacetylglycine	1.88	0.85	$\pm$	0.01	1.08	$\pm$	0.01	1.17	$\pm$	0.01	0.98	$\pm$	0.01	0.77	$\pm$	0.01	1.06	$\pm$	0.013	1.16	$\pm$	0.02	0.96	$\pm$	0.01
N methyl nicotinamide	9.27	1.20	$\pm$	0.05	1.20	$\pm$	0.05	1.12	$\pm$	0.04	1.39	$\pm$	0.08	1.14	$\pm$	0.04	1.22	$\pm$	0.054	1.29	$\pm$	0.06	1.28	$\pm$	0.08
Nictinurate	9.25	1.02	$\pm$	0.03	0.92	$\pm$	0.02	0.90	$\pm$	0.02	0.83	$\pm$	0.02	1.37	$\pm$	0.04	2.04	$\pm$	0.053	1.81	$\pm$	0.06	2.04	$\pm$	0.06
2 PY	6.66	0.39	$\pm$	0.01	0.48	$\pm$	0.01	0.48	$\pm$	0.01	0.46	$\pm$	0.01	0.38	$\pm$	0.01	0.78	$\pm$	0.022	0.70	$\pm$	0.01	0.88	$\pm$	0.03
4 PY	6.70	0.091	$\pm$	0.002	0.128	$\pm$	0.003	0.134	$\pm$	0.003	0.119	$\pm$	0.002	0.082	$\pm$	0.002	0.172	$\pm$	0.004	0.151	$\pm$	0.004	0.178	$\pm$	0.005

Table S6: List of *p value* obtained from pair-wise comparison between LF, HF, NR and SR mice of integral ratios at day 7 or day 60 using non parametric Wilcoxon-Mann-Whitney test.

metabolites	¹ H ppm	HF vs LF		NR vs SR		LF vs NR		LF vs SR		HF vs NR		HF vs SR	
		7 d a y s	60 d a y s	7 d a y s	60 d a y s	7 d a y s	60 d a y s	7 d a y s	60 d a y s	7 d a y s	60 d a y s	7 d a y s	60 d a y s
Isovalerylglycine	0.92	6.40E-06	5.55E-08	0.03059	0.01855	2.97E-05	6.66E-05	0.11837	0.00108	0.18269	0.28954	0.06272	0.03831
α-Keto-β-methylvalerate	1.99	1.15E-07	3.66E-08	0.93139	0.6251	0.00574	0.01013	0.00001	0.00001	0.66706	0.27049	0.69952	0.59391
α-Ketosiovalerate	1.12	1.30E-10	4.66E-12	0.79609	0.88588	0.0011	0.00027	3.78E-06	8.36E-08	0.48207	0.27049	0.64941	0.1869
Isoleucine	1.029	0.00146	0.00048	0.84075	0.75209	0.07934	0.03482	0.11048	0.01614	0.57815	0.63021	0.53124	0.90953
Valine	1.05	0.01486	2.35E-05	0.50801	0.79609	0.05918	0.0171	0.29308	0.00556	0.84199	0.48681	0.50898	0.70703
Leucine	0.97	3.75E-13	2.14E-15	0.34074	0.31192	4.51E-08	4.51E-08	1.04E-06	3.88E-08	0.74498	0.83892	0.23998	0.04724
Citrate	2.57	0.57098	0.2912	0.31192	0.09564	0.78718	0.23283	0.20972	0.90746	0.79309	0.52791	0.23319	0.11364
Succinate	2.41	0.01665	0.13615	0.93139	0.00478	0.03889	0.72398	0.04049	0.00495	0.82561	0.14274	0.75098	0.07316
α-Ketoglutarate	2.45	0.05000	0.05100	0.88588	0.50801	0.34777	0.4164	0.17498	0.05199	0.95816	0.61509	0.80357	0.64319
cis.Aconitate	3.12	0.94751	0.26136	0.54577	0.37116	0.72398	0.12441	0.72717	0.6063	0.69786	0.29939	0.68686	0.78642
Fumarate	6.53	0.00195	0.0002	0.37116	0.70883	0.0031	0.05918	0.01781	0.02601	0.2196	0.20298	0.81687	0.68122
Carnitine	0.88	0.00029	2.43E-07	0.02602	0.01855	0.00424	0.00018	0.24903	0.01192	0.68239	0.88939	0.04567	0.00622
Acyl-carnitine	3.23	0.00057	1.23E-06	0.70883	0.19153	0.00494	0.00063	0.01781	0.00052	0.65187	0.21067	0.88407	0.60607
Hexanoylglycine	3.2	0.00343	0.02112	0.02139	0.001883	0.0171	0.21657	0.02849	0.11837	0.66706	0.52791	0.68686	0.9234
Vinylacetyl glycine	1.88	1.05E-07	4.89E-11	0.01295	0.0484	2.03E-06	3.02E-06	0.00868	7.15E-05	0.07974	0.33028	0.08471	0.08831
N-methyl-nicotinamide	9.27	0.67339	0.36448	0.9771	0.88588	0.91733	0.78718	0.62978	0.62978	0.99163	0.92329	0.95212	0.69408
Nictinurate	9.25	0.55317	0.0022	0.84075	0.43662	0.60322	0.21657	0.41444	0.03117	0.87494	0.30946	0.63712	0.90953
2PY	6.66	0.03406	2.17E-10	0.58484	0.47159	0.06538	1.21E-05	0.29308	1.82E-05	0.71345	0.6765	0.62492	0.56994
4PY	6.7	7.77E-05	3.57E-11	0.19153	0.50801	0.0011	0.00063	0.02158	3.78E-06	0.36133	0.43481	0.45555	0.99303

APPENDIX 2:

SUPPLEMENTARY DATA OF THE PREBIOTIC STUDY

(CHAPTER IV, V, VI)

Table S1: Diet composition.

D12450B, D12492 and D09072901-06

Rodent Diets With 10 and 60 kcal% Fat and With Added Fiber Mixes

Product #			Low fat with				High fat with				Mix A		Mix B			Mix C			Mix D	
		D12450B		D09072901		D12492		D09072902		D09072903		D09072904		D09072905		D09072906				
	%	gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal	
Protein		19	20	18	20	26	20	25	20	23	20	23	20	23	20	23	20	25	20	
Carbohydrate		67	70	64	70	26	20	25	20	31	20	29	20	30	20	30	20	25	20	
Fat		4	10	4	10	35	60	33	60	30	60	31	60	31	60	31	60	33	60	
Total			100		100		100		100		100		100		100		100		100	
kcal/gm		3.8		3.7		5.2		4.9		4.5		4.6		4.6		4.9				
Ingredient		gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal	gm	kcal	
Casein, 80 Mesh		200	800	200	800	200	800	200	800	200	800	200	800	200	800	200	800	200	800	
L-Cystine		3	12	3	12	3	12	3	12	3	12	3	12	3	12	3	12	3	12	
Corn Starch		315	1260	315	1260	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Maltodextrin 10		35	140	35	140	125	500	125	500	60.5	242	106.25	425	96	384	125	500			
Dextrose		0	0	0	0	0	0	0	0	0	0	0	0	0	0	35.1	140			
Lactose		0	0	0	0	0	0	0	0	0	0	0	0	0	0	32.3	129			
Galactose		0	0	0	0	0	0	0	0	0	0	0	0	0	0	1.34	5			
Sucrose		350	1400	350	1400	68.8	275	68.8	275	0.6	2	0	0	58.8	235	0	0			
Cellulose, BW200		50	0	100	0	50	0	100	0	100	0	100	0	100	0	100	0			
Martin Fiber Mix A		0	0	0	0	0	0	0	0	211	532	0	0	0	0	0	0			
Martin Fiber Mix B		0	0	0	0	0	0	0	0	0	0	140	350	0	0	0	0			
Martin Fiber Mix C		0	0	0	0	0	0	0	0	0	0	0	0	100	156	0	0			
Soybean Oil		25	225	25	225	25	225	25	225	25	225	25	225	25	225	25	225	25	225	
Lard		20	180	20	180	245	2205	245	2205	245	2205	245	2205	245	2205	245	2205	245	2205	
Mineral Mix S10026		10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0	
Dicalcium Phosphate		13	0	13	0	13	0	13	0	13	0	13	0	13	0	13	0	13	0	
Calcium Carbonate		5.5	0	5.5	0	5.5	0	5.5	0	5.5	0	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	16.5	0	16.5	0	16.5	0	16.5	0	16.5	0	
Vitamin Mix V10001		10	40	10	40	10	40	10	40	10	40	10	40	10	40	10	40	10	40	
Choline Bitartrate		2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	2	0	
FD&C Yellow Dye #5	0.05	0		0	0	0	0	0.025	0	0.025	0	0	0	0	0	0	0	0.0025	0	
FD&C Red Dye #40	0	0		0.05	0	0	0	0.025	0	0	0	0.025	0	0.005	0	0	0	0	0	
FD&C Blue Dye #1	0	0		0	0	0.05	0	0	0	0.025	0	0.025	0	0	0	0	0	0.0025	0	
Total		1055.05	4057	1105.05	4057	773.85	4057	823.85	4057	902.15	4058	876.3	4057	884.805	4057	823.745	4057			

Formulated by Research Diets, Inc.

MartinF01.for.xls

Low fat with cellulose: LF diet

High fat with cellulose: HF diet

Mix A: Galactosyl-oligosaccharides prebiotic blend (GOS)

Mix B: Galactosyl-oligosaccharides and cow milk oligosaccharides prebiotic blend (GOS_CMO)

Mix C: Fructosyl-oligosaccharides and inulin prebiotic blend (FOS_IN)

Mix D: galactose-lactose-dextrose for HF_S diet.

Table S2: Percentage of calories (kcal) brought by fat, carbohydrates, proteins and vitamins in each diet.

groupe	diet	total Kcal	Protein% (ckal)	Fat %(kcal)			Carbohydrate % (kcal)							Vitamins
				soybean oil	lard	total fat	corn starch	Maltodextrine	Sucrose	other sugars	other fibre	cellulose	total carboyhdrate	
A	LF	4057	20	5	4	9	31	3	34	0	0	0	70	1
B	HF	4057	20	5	54	59	0	12	7	0	0	0	19	1
C	HF_S	4057	20	5	54	59	0	12	0	7	0	0	19	1
D	HF_GOS	4058	20	5	54	59	0	6	0	0	13	0	19	1
E	HF_GOS_CMO	4057	20	5	54	59	0	10	0	0	8	0	18	1
F	HF_FOS_IN	4057	20	5	54	59	0	9	6	0	4	0	19	1

Table S3 results of non parametric Wilcoxon-Mann-Whitney test for weight gain (A.) and cumulative food efficiency (B.) (Chapter IV).

A. Body weight gain										
<i>p values</i>	D7	D14	D21	D28	D35	D42	D49	D56	D63	D70
LF vs HF	5.70E-11	1.6.E-9	1.80E-08	3.32E-06	1.00E-13	3.10E-15	2.00E-16	1.29E-08	3.32E-06	4.80E-02
LF vs HF_S	5.30E-06	2.93E-05	5.11E-05	4.47E-06	3.20E-07	8.40E-09	2.90E-11	1.26E-08	4.47E-06	9.00E-03
LF vs HF+GOS	0.4	0.82	0.42	0.00087	0.5	0.43	0.000723	2.30E-03	8.70E-04	0.0051
LF vs HF+FOS_IN	0.156	0.2	0.99	0.00088	0.039	0.0414	0.024	6.90E-04	1.18E-03	0.00329
LF vs HF+GOS_CMO	0.36	0.47	0.62	0.0012	0.016	0.035	0.00426	4.10E-03	8.70E-03	0.00146
HF vs HF+GOS	4.40E-10	1.40E-08	3.90E-07	4.49E-06	3.00E-11	5.10E-12	6.10E-14	1.29E-08	4.99E-06	3.08E-03
HF vs HF+FOS_IN	8.60E-06	1.50E-07	1.50E-06	5.02E-06	3.00E-11	8.48E-11	6.60E-13	9.02E-08	5.02E-06	6.94E-03
HF_S vs HF+GOS_CMO	0.016	0.03924	3.96E-02	1.57E-05	5.80E-04	1.30E-05	3.80E-06	2.50E-06	6.67E-05	4.90E-02
HF vs HF_S	0.069	0.1529	3.86E-01	3.00E-03	8.20E-03	1.00E-02	2.00E-03	6.60E-03	3.00E-03	8.90E-01

B. cumulative food efficiency										
<i>p values</i>	D7	D14	D21	D28	D35	D42	D49	D56	D63	D70
LF vs HF	1.21E-07	1.32E-08	1.19E-07	6.97E-09	6.02E-10	6.02E-10	2.07E-10	1.52E-11	3.22E-12	1.00E-12
LF vs HF_S	0.000369	4.59E-06	2.65E-08	4.34E-10	2.36E-10	2.36E-10	1.17E-09	1.94E-11	5.82E-12	9.15E-11
LF vs HF+GOS	0.139236	0.8791	0.008161	0.003984	0.001497	0.001497	0.000162	1.69E-05	6.09E-06	4.54E-06
LF vs HF+FOS_IN	0.199014	0.894663	0.022852	0.005097	0.00704	0.00704	0.000105	9.66E-05	3.58E-06	4.26E-06
LF vs HF+GOS_CMO	0.013683	0.059146	0.004432	0.00095	0.000569	0.000569	0.000586	0.000409	3.01E-05	7.51E-06
HF vs HF+GOS	2.45E-07	7.86E-09	7.62E-06	2.13E-06	3.16E-07	3.16E-07	9.02E-08	1.09E-08	1.75E-08	1.10E-08
HF vs HF+FOS_IN	5.33E-06	7.73E-08	8.57E-06	6.52E-07	1.25E-07	1.25E-07	2.40E-07	1.30E-08	2.42E-08	1.51E-08
HF_S vs HF+GOS_CMO	0.070722	0.004976	4.80E-05	4.90E-06	3.53E-05	3.53E-05	5.36E-06	4.91E-06	3.09E-06	3.18E-05
HF vs HF_S	0.160352	0.024572	0.290605	0.145861	0.031026	0.059855	0.022596	0.018676	0.010385	0.822345

Table S4: Goodness of fit (R^2Y) and prediction (Q^2Y) for pair-wise OPLS-DAs at baseline (D0)

The pair-wise OPLS DAs models at D0 show no significant differences in urinary, and fecal metabolic profiles between the different diet groups. No plasma sample at baseline was run and no ^1H NMR plasma metabolic profiles were performed before diet intervention.

	OPLS-DAs	time points	components	R^2Y	Q^2Y
Urine	LF, HF	D0	1+1	0.5	-0.19
	LF, HF_S	D0	1+0	0.75	0.14
	HF, HF+GOS	D0	1+1	0.79	-0.32
	HF+ HF+FOS_IN	D0	1+0	-0.92	0.4
	HF_S + HF+ GOS_CMO	D0	1+1	-0.1	0.67
Feces	LF, HF	D0	1+2	0.98	0.08
	LF, HF_S	D0	1+0	0.9	0.14
	HF, HF+GOS	D0	1+1	0.97	-0.006
	HF+ HF+FOS_IN	D0	1+1	0.96	-0.28
	HF_S + HF+ GOS_CMO	D0	1+1	0.89	-0.15
plasma	LF, HF	D0	x	x	x
	LF, HF_S	D0	x	x	x
	HF, HF+GOS	D0	x	x	x
	HF+ HF+FOS_IN	D0	x	x	x
	HF_S + HF+ GOS_CMO	D0	x	x	x

Table S5: List of metabolites analysed with AbsoluteIDQ p180 Kit BiocratesLife Sciences AG and their corresponding abbreviations (Chapter IV)

Acylcarnitines (40)			
C0	Carnitine	C10:1	Decenoylcarnitine
C2	Acetylcarnitine	C10:2	Decadienylcarnitine
C3	Propionylcarnitine	C12	Dodecanoylcarnitine
C3:1	Propenoylcarnitine	C12:1	Dodecenoylcarnitine
C3-OH	Hydroxypropionylcarnitine	C12-DC	Dodecanedioylcarnitine
C4	Butyrylcarnitine	C14	Tetradecanoylcarnitine
C4:1	Butenylcarnitine	C14:1	Tetradecenoylcarnitine
C4-OH (C3-DC)	Hydroxybutyrylcarnitine	C14:1-OH	Hydroxytetradecenoylcarnitine
C5	Valerylcarnitine	C14:2	Tetradecadienylcarnitine
C5:1	Tiglylcarnitine	C14:2-OH	Hydroxytetradecadienylcarnitine
C5:1-DC	Glutaconylcarnitine	C16	Hexadecanoylcarnitine
C5-DC (C6-OH)	Glutaryl carnitine* (Hydroxyhexanoylcarnitine)	C16:1	Hexadecenoylcarnitine
C5-M-DC	Methylglutaryl carnitine	C16:1-OH	Hydroxyhexadecenoylcarnitine
C5-OH (C3-DC-M)	Hydroxyvalerylcarnitine (Methylmalonylcarnitine)	C16:2	Hexadecadienylcarnitine
C6 (C4:1-DC)	Hexanoylcarnitine (Fumaryl carnitine)	C16:2-OH	Hydroxyhexadecadienylcarnitine
C6:1	Hexenoylcarnitine	C16-OH	Hydroxyhexadecanoylcarnitine
C7-DC	Pimelylcarnitine	C18	Octadecanoylcarnitine
C8	Octanoylcarnitine	C18:1	Octadecenoylcarnitine
C9	Nonacylcarnitine	C18:1-OH	Hydroxyoctadecenoylcarnitine
C10	Decanoylcarnitine	C18:2	Octadecadienylcarnitine

Amino Acids (21)			
Ala	Alanine	Lys	Lysine
Arg	Arginine	Met	Methionine
Asn	Asparagine	Orn	Ornithine
Asp	Aspartate	Phe	Phenylalanine
Cit	Citrulline	Pro	Proline
Gln	Glutamine	Ser	Serine
Glu	Glutamate	Thr	Threonine
Gly	Glycine	Trp	Tryptophan
His	Histidine	Tyr	Tyrosine
Ile	Isoleucine	Val	Valine
Leu	Leucine		

Biogenic Amines (19)			
Ac-Orn	Acetylornithine	Nitro-Tyr	Nitrotyrosine
ADMA	Asymmetric dimethylarginine	OH-Pro	Hydroxyproline
SDMA	Symmetric dimethylarginine	PEA	Phenylethylamine
total DMA	Total dimethylarginine	Putrescine	Putrescine
alpha-AAA	alpha-Aminoadipic acid	Sarcosine	Sarcosine
Carnosine	Carnosine	Serotonin	Serotonin
Creatinine	Creatinine	Spermidine	Spermidine
Histamine	Histamine	Spermine	Spermine
Kynurenine	Kynurenine	Taurine	Taurine
Met-SO	Methioninesulfoxide		

Hexoses (1)	
H1	Hexose

Sphingolipids (15)			
SM (OH) C14:1	SM C18:0	SM (OH) C22:1	SM (OH) C24:1
SM C16:0	SM C18:1	SM (OH) C22:2	SM C26:0
SM C16:1	SM C20:2	SM C24:0	SM C26:1
SM (OH) C16:1	SM C22:3	SM C24:1	

Glycerophospholipids (90)			
lysoPC a C14:0	PC aa C34:1	PC aa C42:0	PC ae C38:2
lysoPC a C16:0	PC aa C34:2	PC aa C42:1	PC ae C38:3
lysoPC a C16:1	PC aa C34:3	PC aa C42:2	PC ae C38:4
lysoPC a C17:0	PC aa C34:4	PC aa C42:4	PC ae C38:5
lysoPC a C18:0	PC aa C36:0	PC aa C42:5	PC ae C38:6
lysoPC a C18:1	PC aa C36:1	PC aa C42:6	PC ae C40:1
lysoPC a C18:2	PC aa C36:2	PC ae C30:0	PC ae C40:2
lysoPC a C20:3	PC aa C36:3	PC ae C30:1	PC ae C40:3
lysoPC a C20:4	PC aa C36:4	PC ae C30:2	PC aa C40:4
lysoPC a C24:0	PC aa C36:5	PC ae C32:1	PC ae C40:5
lysoPC a C26:0	PC aa C36:6	PC ae C32:2	PC ae C40:6
lysoPC a C26:1	PC aa C38:0	PC ae C34:0	PC ae C42:0
lysoPC a C28:0	PC aa C38:1	PC ae C34:1	PC ae C42:1
lysoPC a C28:1	PC aa C38:3	PC ae C34:2	PC ae C42:2
PC aa C24:0	PC aa C38:4	PC ae C34:3	PC ae C42:3
PC aa C26:0	PC aa C38:5	PC ae C36:0	PC ae C42:4
PC aa C28:1	PC aa C38:6	PC ae C36:1	PC ae C42:5
PC aa C30:0	PC aa C40:1	PC ae C36:2	PC ae C44:3
PC aa C30:2	PC aa C40:2	PC ae C36:3	PC ae C44:4
PC aa C32:0	PC aa C40:3	PC ae C36:4	PC ae C44:5
PC aa C32:1	PC aa C40:4	PC ae C36:5	PC ae C44:6
PC aa C32:2	PC aa C40:5	PC ae C38:0	
PC aa C32:3	PC aa C40:6	PC ae C38:1	

Table S6: Goodness of fit (R^2Y) and prediction (Q^2Y) and p values for OPLS regression models predicting the cumulative food efficiency at 70 days after diet switch (CFE_70D) from urinary metabolic profiles at each time point (Chapter VI).

OPLS models with HF and HF+GOS fed mice (urine profiles)				
timepoints	cpnts	Q^2Y	R^2Y	p value
D7	3	0.64	0.9	$1. \cdot 10^{-3}$
D14	4	0.61	0.91	$1. \cdot 10^{-3}$
D21	3	0.44	0.86	$1. \cdot 10^{-3}$
D28	3	0.62	0.89	$1. \cdot 10^{-3}$
D35	3	0.65	0.94	$1. \cdot 10^{-3}$
D42	2	0.6	0.76	$1. \cdot 10^{-3}$
D49	4	0.7	0.93	$1. \cdot 10^{-3}$
D56	3	0.67	0.91	$1. \cdot 10^{-3}$
D63	3	0.66	0.93	$1. \cdot 10^{-3}$
D70	3	0.69	0.95	$1. \cdot 10^{-3}$
OPLS models with HF_S and HF+FOS_IN fed mice (urine profiles)				
timepoints	cpnts	Q^2Y	R^2Y	p value
D7	3	0.64	0.9	$1. \cdot 10^{-3}$
D14	4	0.58	0.87	$1. \cdot 10^{-3}$
D21	3	0.35	0.86	$1. \cdot 10^{-3}$
D28	2	0.65	0.8	$1. \cdot 10^{-3}$
D35	3	0.63	0.88	$1. \cdot 10^{-3}$
D42	4	0.6	0.95	$1. \cdot 10^{-3}$
D49	4	0.6	0.91	$1. \cdot 10^{-3}$
D56	3	0.67	0.89	$1. \cdot 10^{-3}$
D63	3	0.69	0.92	$1. \cdot 10^{-3}$
D70	3	0.72	0.92	$1. \cdot 10^{-3}$
OPLS models with HF and HF+GOS_CMO fed mice (urine profiles)				
timepoints	cpnts	Q^2Y	R^2Y	p value
D7	2	0.38	0.74	$1. \cdot 10^{-3}$
D14	4	0.39	0.73	$1. \cdot 10^{-3}$

D21	2	0.39	0.69	$1. \cdot 10^{-3}$
D28	4	0.37	0.95	0.01
D35	1	0.22	0.39	0.03
D42	2	0.42	0.71	$1. \cdot 10^{-3}$
D49	3	0.6007	0.91	$1. \cdot 10^{-3}$
D56	3	0.68	0.87	$1. \cdot 10^{-3}$
D63	2	0.48	0.8	$1. \cdot 10^{-3}$
D70	2	0.71	0.93	$1. \cdot 10^{-3}$

Table S7: Goodness of fit (R^2Y) and prediction (Q^2Y) and *p values* for OPLS regression models predicting the cumulative food efficiency at 70 days after diet switch (CFE_70D) from fecal metabolic profiles at each time point. (Chapter VI).

OPLS models with HF and HF+GOS fed mice (feces profiles)				
timepoints	cpnts	Q^2Y	R^2Y	<i>p value</i>
D7	3	0.44	0.99	0.01
D14	2	0.37	0.95	0.01
D21	3	0.19	0.99	0.048
D28	4	0.3	0.99	0.14
D35	2	0.34	0.94	0.03
D42	2	0.28	0.95	0.08
D49	2	0.14	0.96	0.39
D56	3	0.31	0.99	0.05
D63	4	0.38	0.99	0.06
D70	4	0.26	0.99	0.05
OPLS models with HF_S and HF+GOS_CMO fed mice (feces profiles)				
timepoints	cpnts	Q^2Y	R^2Y	<i>p value</i>
D7	7	-0.1	0.99	0.86
D14	1	0.25	0.97	0.14
D21	1	0.01	0.95	0.46
D28	7	-0.12	0.99	0.83
D35	3	0.16	0.99	0.24
D42	1	0.06	0.96	0.53

D49	1	0.15	0.94	0.32
D56	4	0.3	0.99	0.02
D63	3	0.34	0.99	0.08
D70	4	0.38	1	0.01
OPLS models with HF and HF+FOS_ IN fed mice (feces profiles)				
timepoints	cpnts	Q ² Y	R ² Y	<i>p value</i>
D7	3	0.31	0.99	0.06
D14	1	0.43	0.95	0.01
D21	4	0.31	0.99	0.13
D28	4	0.39	0.99	0.02
D35	2	0.47	0.97	0.01
D42	3	0.33	0.99	0.04
D49	4	0.47	0.99	0.03
D56	4	0.53	0.99	0.01
D63	4	0.28	0.99	0.15
D70	2	0.12	0.97	0.24

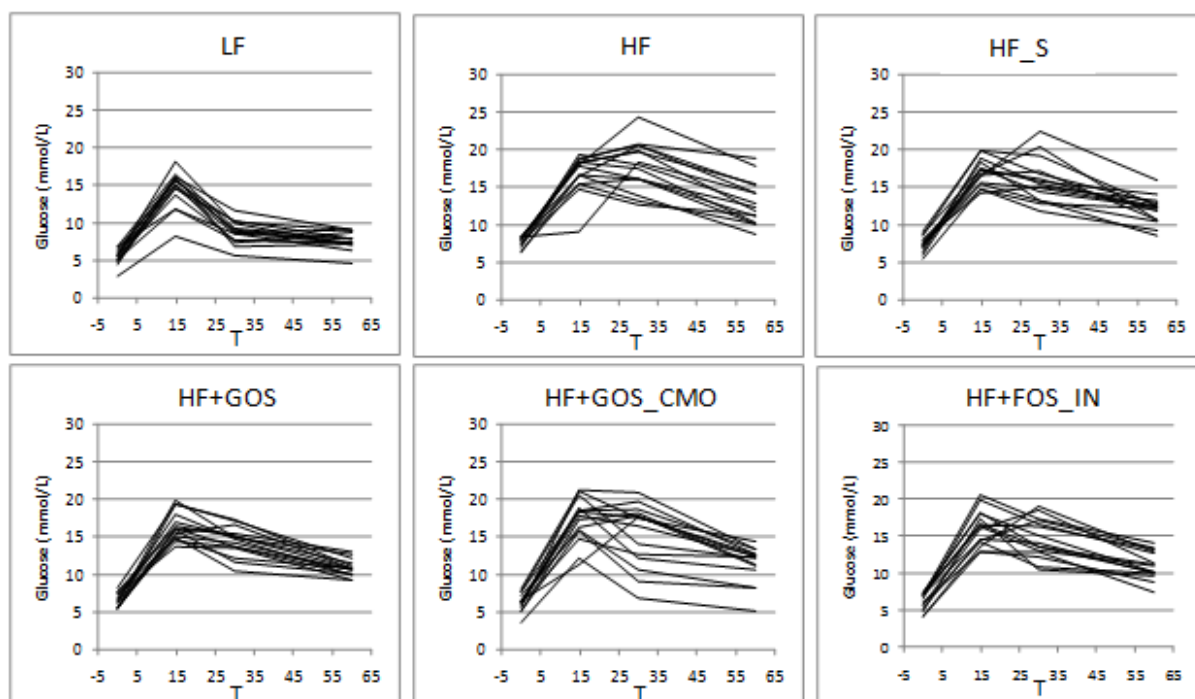


Figure S1 : Curves of glucose OGTT. LF: Low fat group, HF : High fat group; HF_S : HF + sugars, HF+GOS : HF + galactosyl-oligosaccharides, HF + GOS_CMO : HF + galactosyl-oligosaccharides and cow milk oligosaccharides, HF + FOS_IN : HF + Fructosyl-oligosaccharides and inulin. T: time in minutes

Table S8 : Statistics of OGTT glucose for each diet group

		N	Mean	SD	SEM	Median	Rob. SD	SE Median	Min	Max
Baseline insulin (ng/mL)	LF	15	0.35	0.22	0.06	0.34	0.20	0.07	0.00	0.80
	HF	15	0.95	0.93	0.24	0.88	0.57	0.18	0.00	3.75
	HF_S	15	0.69	0.48	0.12	0.56	0.41	0.13	0.00	1.83
	HF+GOS	15	0.42	0.21	0.05	0.37	0.18	0.06	0.00	0.83
	HF+ GOS_CMOS	15	0.30	0.23	0.06	0.32	0.30	0.10	0.00	0.64
	HF+ FOS_IN	15	0.44	0.29	0.08	0.41	0.29	0.09	0.00	1.00

		N	Mean	SD	SEM	Median	Rob. SD	SE Median	Min	Max
Max insulin (ng/mL)	LF	15	0.52	0.18	0.05	0.47	0.13	0.04	0.30	0.90
	HF	15	1.70	0.80	0.21	1.34	0.42	0.14	0.77	3.75
	HF_S	15	1.13	0.34	0.09	1.05	0.31	0.10	0.68	1.83
	HF+GOS	15	0.66	0.16	0.04	0.66	0.17	0.05	0.43	0.96
	HF+ GOS_CMOS	15	0.63	0.26	0.07	0.62	0.25	0.08	0.10	1.18
	HF+ FOS_IN	15	0.72	0.36	0.09	0.74	0.41	0.13	0.14	1.38
T30 insulin (ng/mL)	LF	15	0.39	0.27	0.07	0.38	0.24	0.08	0.00	0.90
	HF	15	1.56	0.81	0.21	1.32	0.70	0.23	0.00	3.12
	HF_S	15	1.01	0.40	0.10	1.03	0.37	0.12	0.02	1.67
	HF+GOS	15	0.56	0.25	0.06	0.59	0.22	0.07	0.00	0.96
	HF+ GOS_CMOS	15	0.41	0.30	0.08	0.42	0.39	0.13	0.00	0.87
	HF+ FOS_IN	15	0.69	0.36	0.09	0.74	0.43	0.14	0.14	1.38
T60 insulin (ng/mL)	LF	15	0.18	0.18	0.05	0.21	0.25	0.08	0.00	0.51
	HF	15	0.99	0.67	0.17	1.05	0.57	0.19	0.00	2.22
	HF_S	15	0.72	0.49	0.13	0.60	0.28	0.09	0.00	1.72
	HF+GOS	15	0.38	0.26	0.07	0.40	0.25	0.08	0.00	0.79
	HF+ GOS_CMOS	15	0.47	0.34	0.09	0.45	0.33	0.11	0.00	1.18
	HF+ FOS_IN	15	0.57	0.38	0.10	0.48	0.38	0.12	0.00	1.21

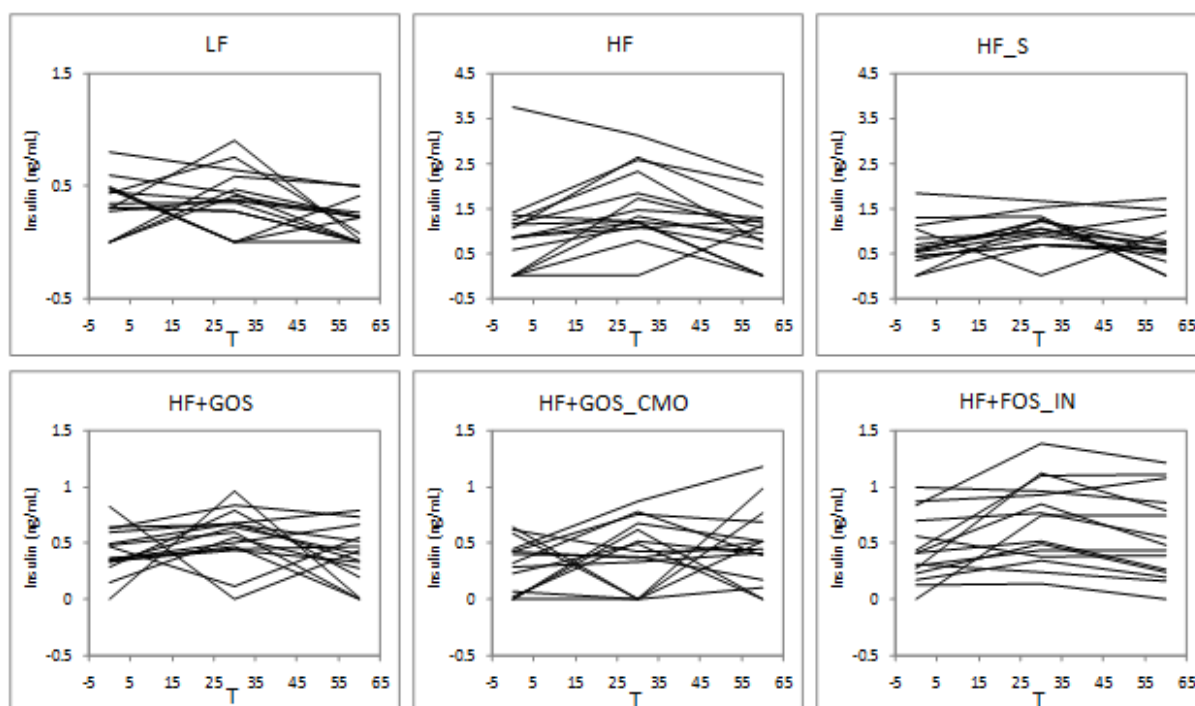


Figure S2 : Curves of insulin OGTT. LF : Low fat group, HF : High fat group; HF_S : HF + sugars, HF+GOS : HF + galactosyl-oligosaccharides, HF + GOS_CMO : HF + galactosyl-oligosaccharides and cow milk oligosachharides, HF + FOS_IN : HF + Fructosyl-oligosachharides and inulin. T : Time in minutes

Table S9 : Statistics of OGTT insulin for each diet group

		N	Mean	SD	SEM	Median	Rob. SD	SE Median	Min	Max
Baseline insulin (ng/mL)	LF	15	0.35	0.22	0.06	0.34	0.20	0.07	0.00	0.80
	HF	15	0.95	0.93	0.24	0.88	0.57	0.18	0.00	3.75
	HF_S	15	0.69	0.48	0.12	0.56	0.41	0.13	0.00	1.83
	HF+GOS	15	0.42	0.21	0.05	0.37	0.18	0.06	0.00	0.83
	HF+ GOS_CMOS	15	0.30	0.23	0.06	0.32	0.30	0.10	0.00	0.64
	HF+ FOS_IN	15	0.44	0.29	0.08	0.41	0.29	0.09	0.00	1.00

		N	Mean	SD	SEM	Median	Rob. SD	SE Median	Min	Max
Max insulin (ng/mL)	LF	15	0.52	0.18	0.05	0.47	0.13	0.04	0.30	0.90
	HF	15	1.70	0.80	0.21	1.34	0.42	0.14	0.77	3.75
	HF_S	15	1.13	0.34	0.09	1.05	0.31	0.10	0.68	1.83
	HF+GOS	15	0.66	0.16	0.04	0.66	0.17	0.05	0.43	0.96
	HF+ GOS_CMOS	15	0.63	0.26	0.07	0.62	0.25	0.08	0.10	1.18
	HF+ FOS_IN	15	0.72	0.36	0.09	0.74	0.41	0.13	0.14	1.38
T30 insulin (ng/mL)	LF	15	0.39	0.27	0.07	0.38	0.24	0.08	0.00	0.90
	HF	15	1.56	0.81	0.21	1.32	0.70	0.23	0.00	3.12
	HF_S	15	1.01	0.40	0.10	1.03	0.37	0.12	0.02	1.67
	HF+GOS	15	0.56	0.25	0.06	0.59	0.22	0.07	0.00	0.96
	HF+ GOS_CMOS	15	0.41	0.30	0.08	0.42	0.39	0.13	0.00	0.87
	HF+ FOS_IN	15	0.69	0.36	0.09	0.74	0.43	0.14	0.14	1.38
T60 insulin (ng/mL)	LF	15	0.18	0.18	0.05	0.21	0.25	0.08	0.00	0.51
	HF	15	0.99	0.67	0.17	1.05	0.57	0.19	0.00	2.22
	HF_S	15	0.72	0.49	0.13	0.60	0.28	0.09	0.00	1.72
	HF+GOS	15	0.38	0.26	0.07	0.40	0.25	0.08	0.00	0.79
	HF+ GOS_CMOS	15	0.47	0.34	0.09	0.45	0.33	0.11	0.00	1.18
	HF+ FOS_IN	15	0.57	0.38	0.10	0.48	0.38	0.12	0.00	1.21