Role of Gut Microbiome-Host Metabolic Interactions in Metabolic Diseases

Thesis submitted by

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ABSTRACT

The metabolic phenotype of any complex organism is dependent on a complex series of host and gut microbial gene (microbiome) interactions with diet. The microbiome itself can be affected by environmental factors such as stress, exposure to xenobiotics, lifestyle, and alterations in the mammalian-microbial-metabolic axis are associated with changes in disease factors.

To understand further the impact of the commensal microbiota on the host metabolism, germ-free (GF) animals, inoculated with individual bacterial strains or complex microbiota, and conventional mice were characterised using $^1$H NMR spectroscopy and UPLC-MS-based metabolic profiling approaches, and by recording the physiological and immunological parameters.

Higher systemic level of $(D)$-3-hydroxybutyrate and lower levels of circulating VLDL were observed in GF compared to conventional animals, indicating that the absence of gut microbiota stimulated lipolysis while it inhibited hepatic lipogenesis. Subsequently, the best inoculation procedure was obtained by inoculating single bacterial strains into individual animals followed by allowing the animals to exchange their microbes. Metabolic fingerprints showed that a 9 bacteria community is more able to regulate lipoprotein and circulating lipid levels compared to a 3 bacteria community. The origin of the inocula (mouse or human) impacts differently on the host metabolism since humanised mice were strongly disturbed (higher plasma triglyceride level) and displayed metabolic profiles similar to GF mice. Conversely, mouse-associated animals were physiologically, immunologically and metabolically similar to conventional animals. Finally, metabolic profiles and gut microbiota composition were statistically regressed and helped to identify specific bacteria, such as *Ruminococcus lactaris* and *Faecalibacterium prausnitzii*, and their putative role in the host homeostasis.

To conclude, these results confirmed the influence of gut microbes on the host physiology and metabolism. This PhD thesis provides new insights into the role of gut bacteria to understand the microbial-related mechanisms that participate in the host lipid metabolism, potentially leading to development of obesity.
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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<th>Description</th>
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<tr>
<td>1D</td>
<td>One dimension</td>
</tr>
<tr>
<td>2D</td>
<td>Two dimensions</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigens</td>
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<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cells</td>
</tr>
<tr>
<td>APCI</td>
<td>Atmospheric Pressure Chemical Ionisation</td>
</tr>
<tr>
<td>APS</td>
<td>Adenosine 5'-phosphosulfate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BA</td>
<td>Bile acid</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown Adipose Tissue</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BMRB</td>
<td>Biological Magnetic Resonance Data Bank</td>
</tr>
<tr>
<td>BW</td>
<td>Body Weight</td>
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<tr>
<td>BWG</td>
<td>Body Weight Gain</td>
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<tr>
<td>CA</td>
<td>Cholic acid</td>
</tr>
<tr>
<td>CDCA</td>
<td>Chenodeoxycholic acid</td>
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<tr>
<td>CI</td>
<td>Chemical Ionisation</td>
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<tr>
<td>COSY</td>
<td>Correlation Spectroscopy</td>
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<tr>
<td>CPMG</td>
<td>Carr-Purcell-Meiboom-Gill</td>
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<td>DCA</td>
<td>Deoxycholic Acid</td>
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<tr>
<td>DMA</td>
<td>Dimethylamine</td>
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<td>dNDP</td>
<td>Deoxynucleoside diphosphate</td>
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<td>Deoxynucleoside monophosphate</td>
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<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
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<tr>
<td>DA</td>
<td>Discriminant analysis</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EI</td>
<td>Electron Ionisation</td>
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<td>ESI</td>
<td>Electrospray Ionisation</td>
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<tr>
<td>FAB</td>
<td>Fast Atom Bombardment</td>
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<tr>
<td>FID</td>
<td>Free Induction Decay</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FT</td>
<td>Fourier Transformation</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>Fourier transform-ion cyclotron resonance</td>
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<tr>
<td>GC</td>
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<tr>
<td>GDCA</td>
<td>Glychodeoxycholic acid</td>
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<tr>
<td>GF</td>
<td>Germ-Free</td>
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<tr>
<td>GI</td>
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<tr>
<td>GM</td>
<td>Gut Microbiota</td>
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<tr>
<td><em>H. pylori</em></td>
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<td>HFD</td>
<td>High-Fat Diet</td>
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<tr>
<td>HPLC</td>
<td>High-Pressure Liquid Chromatography</td>
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<tr>
<td>HMBC</td>
<td>Heteronuclear Multiple Bond Correlation</td>
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<tr>
<td>HMDB</td>
<td>Human Metabolome Data Base</td>
</tr>
<tr>
<td>HR-MAS</td>
<td>High Resolution-Magic Angle Spinning</td>
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<tr>
<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
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<td>HSS</td>
<td>High Strength Silica</td>
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<tr>
<td>HTS</td>
<td>High-Throughput Sequencing</td>
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<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IEL</td>
<td>Intestinal epithelial lymphocyte</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>LCA</td>
<td>Litocholic acid</td>
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<tr>
<td>LC-MS</td>
<td>Liquid-Chromatography Mass Spectrometry</td>
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<tr>
<td>LDL</td>
<td>Low-Density Lipoprotein</td>
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<td>Lipoprotein Lipase</td>
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<td>β-MCA</td>
<td>Beta-Muricholic acid</td>
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<td>MALDI</td>
<td>Matrix-Assisted Laser Desorption Ionisation</td>
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<tr>
<td>MIS</td>
<td>Mucosal Immune System</td>
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<td>Mesenteric Lymph Node</td>
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<td>NMR</td>
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<tr>
<td>O-PLS-DA</td>
<td>Orthogonal projection to latent structures discriminant analysis</td>
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<td>OSC</td>
<td>Orthogonal Signal Correction</td>
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<td>PAG</td>
<td>Phenylacetylglycine</td>
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GENERAL INTRODUCTION

The phenotype of a complex organism called the “host” is the result of the expression of its genome ($2.5 \times 10^4$ genes) together with the expression of the symbionts’ genome, called the “microbiome”. This specific virtual organ is affected by environmental factors such as diet, stress, exposure to xenobiotics, lifestyle, etc. The host is a complex network of metabolic pathways and in some cases even the smallest deregulation or maladapted microbial ecology can trigger diseases such as atherosclerosis, cancer, diabetes, hypertension, inflammatory bowel diseases (IBD), obesity or osteoporosis.

The phenotype can be characterised by its metabolic status, e.g. healthy or diseased, and can be influenced by modifying the function and expression of the microbiome (by modifying the balance of the micro-organisms which compose the microbiome), or by adjusting the impact of environmental factors. Diet is a crucial way to modulate the phenotype of the host, since it plays a role in both the host and the symbionts’ metabolism. Technical advances in both sequencing techniques and metabolic profiling platforms offer new possibilities for characterising the role of the microbiome.

The mechanisms of action of foods, food supplements and chemicals on the metabolism are generally well known. Nevertheless, how the symbiont participates in and impacts on the host homeostasis is poorly understood. In particular, the specific functions of bacteria in the particular context of the introduction of individual bacteria or complex microbiota into a defined host, and their impact on the host metabolism, have to be determined.
OBJECTIVES

This thesis has four main objectives:

1) To investigate the impact of the commensal microbiota on the metabolism of the host at the metabolic level, with a specific focus on the brown adipose tissue.

2) To investigate modifications of germ-free mouse metabolism inoculated with specific individual bacterial strains or complex microbiota from murine or human donors.

3) To identify and characterise the functions of specific bacteria and their role in maintaining the host homeostasis, by establishing specific correlations between host metabolic signatures and defined bacteria residing in the host gut.

4) To decipher the role of these bacteria and the mechanisms leading to the development of obesity, in the specific context of health and disease.
THESIS STRUCTURE

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Background
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Germ-Free model
Bile acid metabolism

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Figure 1: Thesis general overview
Chapter I  Introduction

The gut microbiota (GM) constitute a complex and highly dynamic internal ecosystem that perpetually interacts with the host\(^1\,^2\,^6\). They contribute to numerous and diverse mammalian processes including regulation of gastrointestinal (GI) development, defence against pathogens, production of vitamins for the host, maturation of the immune system and fermentation of non-digestible dietary fibres such as dietary polysaccharides and carbohydrates which provide energy to the host\(^1\,^6\,^7\). From a metabolic viewpoint, the intense bacterial activity within the colon makes it one of the most metabolically active organs in the body\(^3\). These interactions between the intestinal microbiota and the host are important for our understanding of biological processes, such as the maintenance of the host homeostasis. This symbiosis is the result of a long co-evolutionary process leading to a stable and regulated interplay between the host and its gut microorganisms. As a result of this co-evolution, the host gut actually contains more DNA derived from its microbial counterparts than from its own genome\(^8\). In addition, it is considered as the largest reservoir of microorganisms in the body, consisting of bacteria, fungi, viruses, yeasts, archaea and protozoan organisms (collectively called the microbiome\(^9\)). Bacteria account for the largest proportion of these microorganisms, and account for \(~60\%\) of the faecal mass in the colon\(^2\).

The microbiome is known to impact beneficial actions as well as producing potentially dangerous chemicals i.e. carcinogenic compounds and toxic micrometabolites. Indeed, certain gut microorganisms are known to produce chemicals which can be toxic for the host, mainly in the form of phenols, indoles and amines. The GM can also inactivate certain drugs\(^10\). In addition, it has been reported that the microbiome may be involved in the development of several diseases and metabolic disorders such as gastritis, gastric ulcers, obesity or even certain cancers\(^11\,^20\). Despite the obvious link between GM and host metabolism, the mechanisms by which they impact host homeostasis remains unclear. It is therefore crucial to elucidate the role of the microbiome in health and disease in order to control their harmful effects and to promote their beneficial functions in the host. In this context, metabonomics is the best approach currently available, as it allows us to study both the nature of host and bacterial metabolites produced and their kinetic
changes, which represent real end-points of physiological regulatory processes\textsuperscript{21-24}. Metabonomics is based on the acquisition of a metabolic fingerprint of a biological matrix (i.e. biofluid or tissue), by analytical techniques, combined with multivariate data analysis. This can highlight metabolic perturbations and variations of a defined system and provides a global overview of the metabolic state of a system, including contributions from the GM\textsuperscript{24}.

Gut microbial-mammalian interactions have been extensively studied by metabonomic approaches in the last couple of years to enhance understanding of these complex symbiotic relationships\textsuperscript{25-29}. This has been achieved using a top-down systems-biological approach in order to characterise the impact of the presence or absence of the gut microbial species onto the metabolism of the host system at multiple levels, i.e. multiple organs. For instance, Claus \textit{et al} have demonstrated recently the temporal systemic effect of conventionalisation of germfree (GF) animals compared to conventional animals using a $^1$H NMR metabolic approach\textsuperscript{29}.

The following sections aim to illustrate the specificity of host-GM symbiosis and to present the current knowledge about the microbiome. In addition, the metabolism of bile acids (BAs) will subsequently be discussed as they are known to be significantly modified and deconjugated by the action of the GM\textsuperscript{25,30,31}. 
1. Current knowledge of the gut microbiota

The mammalian gut is colonised by a diverse collection of microorganisms which constitute the GM. These mainly anaerobic microbes are intimately involved in several host physiological processes and are qualified as crucial, when considering the interaction between host metabolism and microorganisms. Dubos et al developed in the 60’s the concept that the indigenous microbiota have two types of relationships with the animal or human host. The first called autochthonous microbiota – corresponds to a symbiosis between the bacteria and the host thanks to a long process of adaptation throughout the evolution, directly responsible for their maintenance at a very high and constant level. The second one is derived from the fact that some bacteria have a certain infectivity level and are dangerous for the living systems. They have the particular ability to establish themselves successfully into the host. However, their presence triggers a defensive response leading to their limitation to a very low level, or even to their irrevocable elimination.

1.1 Techniques to study gut microbes

Following this first hypothesis about the gut colonisation and bacteria establishment, the importance and diversity of the GM were underestimated until the development of the anaerobic glove box. This technique allowed scientists to carry out studies in an anaerobic environment (complete suppression of the oxygen coming from the atmosphere) that facilitated working with the anaerobes, which dominate the lower gut. Recent reports indicate that the gut microbial species are composed of about 1000 different species of mainly Bacteria and Archaea. Originally, the gut microbial species were analysed by cultivating them on selective media to determine the strains present in a sample. However, it appeared that most of them have restrictive growth conditions and are anaerobes, making them almost impossible to grow under classical culture conditions. One approach, called Denaturing Gradient Gel Electrophoresis (DGGE), consists of a DNA extraction step, followed by enzymatic digestion and electrophoretic separation on a polyacrylamide denaturing gel. The bands on the gel will separate based on the relative similarity of the base pair content. However, this technique is mostly used for the identification of proteins and cannot provide information about the identity of all bacterial species.
Therefore, molecular sequencing techniques are needed for this as it has been found that 70 to 80% of the gut microorganisms appear to be unculturable. Another technique, called FISH (Fluorescent In Situ Hybridization), is used to specifically investigate the microbial composition and detect bacteria in faeces, tissues or gut compartments by using ribosomal RNA (rRNA)-based fluorescent probes. Finally, the main technical advance in this field has been the development and widespread use of 16S rRNA sequencing. Indeed, the emergence of methods based on 16S rRNA gene sequences has facilitated the understanding of species composition because these sequences contain regions conserved across all bacterial species, interspersed with variable sequences specific to different bacterial types. This rRNA is abundant in faecal matter, and displays hypervariable regions which are able to be amplified prior to sequencing. Therefore, the 16S rRNA gene has been recognised as a universal and phylogenetically relevant marker and has allowed the development of molecular tools to facilitate the study of gut microorganisms. In addition, sequencing of community metagenomic DNA by high-throughput sequencing (HTS) allows the scientific community to create phylogenetic trees for analysing metabolic or functional information from different types of samples (faeces, lumen, etc). This technique is powerful enough to identify the global set of microbial species that are present in a sample. 16S rRNA sequencing techniques and the parameters used to avoid bias coming from the DNA extraction techniques (i.e. differential bacterial lysis) will be explained in detail in the section on Analytical strategies. This approach has notably been used to characterise Helicobacter pylori (H. pylori), a common gastric pathogen linked to gastric and duodenal ulcers, gastritis, and gastric cancer, and which is able to survive in the acidic gastric environment. Indeed, it has been shown recently that 13 bacterial phyla were associated in the human GM with a low abundance or even absence of H. Pylori, indicating that this species could severely impact the gut microbial composition in humans.

1.2 Co-evolution, tolerance and protection against pathogens

Nowadays, the GM are part of a highly developed and integrated system which can protect the host against potential pathogens. Indeed, the intestine can be thought as a complex and living system protecting the host against external assault.
It is considered by the scientific community as intelligent and able to send, receive and understand messages. Alfred Binet has defined intelligence as “the range of processes involved in adapting to the environment”. This defensive system is composed of 3 major constituents: the microbiota, the mucosal barrier and the local immune system. Each of these constituent parts has a well-defined and essential role in this process.

The commensal GM play a major role in protecting against the invasion of exogenous pathogenic bacteria simply by prior colonisation of the GI tract. Indeed, several competitions are taking place in this tract, such as bacterial competitions for colonisation sites and for nutrient resources. The most important benefit remains that the gut microbes make it more difficult for exogenous bacterial species to establish themselves in the GI tract and cause disease. This is called “colonisation resistance” defined as competition between the indigenous and exogenous bacteria, seeking to establish themselves in the tract, for consuming carbon and energy sources and adhering to the glycan layer. In addition, intestinal microorganisms are crucial for the integrity of the epithelial barrier and development of the gut immune system and its composition, and their ecological structure plays a central role for the establishment of immunological characteristics such as intestinal epithelial lymphocyte (IEL), and Ig-M and Ig-A producing cells. The mechanisms behind this are not completely understood at present, but are linked to bacterial colonisation of the gut. Three different molecular signalling steps have been reported as crucial in this process: the bacteria - bacteria dialog, the bacteria - mucin interaction, and the bacteria - host cross-talk. The mucosal barrier, also called the intestinal mucosa, is the second layer of protection for the body. It forms a barrier representing the main site of interaction with foreign substances (e.g. food) and exogenous microorganisms. Nevertheless, it is not well known what kinds of bacteria are responsible for the development and activation of the mucosal immune system (MIS). Indeed, several characteristics of the intestinal microbiota are not yet fully understood, i.e. the bases of the host specificity of intestinal bacterial species, differences in the microbial composition related to age, differences in the microbial distribution along various parts of the intestine and the nature of the unculturable bacteria. The MIS is arguably one of the most important sites of mammalian immune activation. It encompasses the entire GI tract and its role is to provide a “barrier” function to pathogenic microbes. Mucosal surfaces in the gut are subject to
a continuous barrage of antigenic substances from not only pathogenic microorganisms but from particulate food antigens (Ag) and a multitude of commensal microbiota. It is also imperative that the animal distinguishes between “harmful” and harmless” Ag in order to mount an effective immune response to the former and to avoid an inappropriate immune response to the host.

Multicellularity is based on the ability of a group of cells with the same genetic background to distinguish self from non-self. This is carried out by what is called the “primitive innate immune system”. It resulted from the development of an elaborate immune system which can adapt to novel Ag by recombining segments of immunoglobulins. The gene responsible for this adaptation seemed to be from bacterial origin and incorporated into the eukaryotic genome through a specific gene transfer. It is well reported that multicellular organisms have co-evolved with microorganisms. Most of these “mammalian-microbial interactions” are taking place at the MIS level, with a wide distribution along the GI tract.

A specificity of the MIS compared to the systemic immune system is its ability to monitor its surrounding by sampling the contents of the intestinal lumen. Mucosal induction sites such as the lamina propria, isolated lymphoid follicles and the Peyer’s Patches (PP) conduct the majority of Ag sampling. Ag are produced by Antigen Presenting Cells (APC) which, among other responses, induce Immunoglobulin (Ig) production by plasma cells in the Mesenteric Lymph Node (MLN) and other immune structures. Igs are proteins produced by plasma cells, which can recognise and bind to an antigen to neutralise it or label it for degradation. Mammals have five distinct Ig isotypes: IgA, IgD, IgG, IgE and IgM. Ig A is a secretory immunoglobulin which exists as a dimer and is the most abundantly produced in mucosal secretions. Its primary function is to be a non-inflammatory protection against microbial antigens at mucosal surfaces. Secretory IgM is a pentamer with low affinity and high avidity, for which the presence in mucosal secretions indicates a response to novel Ag in the GI tract. Ig-D has been described as an “ancestral surveillance system”, but with a not well understood function; it is implicated in monitoring invasion of respiratory pathogens. Finally, Ig-E is involved in the immune response to extracellular parasites, whereas IgG is the main Ig located in serum (about 75%) and is considered as having little importance compared to IgA in the MIS. Mucosal tolerance to commensal microbes can be seen as a basal trait in mammalian evolution and the host elaborated synergistic relationships with their gut microorganisms. Therefore, it led Lederberg to
introduce the term “superorganism” to describe the mammalian complement of symbiotic organisms and its host. Indeed, it is crucial to consider mammalian physiology and the consequences of external intervention under the term “metagenome”.

Finally, the local immune system is represented by the association with the gut and is considered as the primary immune organ of the body. This specific system is able to recognise and reject the pathogens, whilst distinguishing between “dangerous” and “friendly” bacteria. Further, this is the site of intervention of prebiotics collaborating with endogenous barriers to reinforce the system of defence.

1.3 Gastrointestinal tract and short chain fatty acid production

The interactions that occur between this complex microbial community and the human host have become a major focus of scientific research due to an increased incidence of diseases associated with deficient, modified or compromised intestinal microbiota. The GI tract is defined as “a specialised tube divided into various well-defined anatomical regions extending from the lips to the anus” and has become a highly interesting field of study. In terms of bacterial population, the stomach is the site of the lowest amount of bacteria because of its high acidity (pH around 1). This has the consequence of destroying the majority of bacteria passing through and acts as a defensive mechanism for the organism. At the opposite extreme, the intestine has an average pH around 8 (with the exception of the colon which displays a pH of 6.5) and is the extended site of permanent interaction between the host, nutrients and microbiota, characterised by the digestion of foods and absorption of nutrients. In addition, it has also necessarily evolved to have robust defences against invading pathogens. The gastric emptying phenomenon helps many bacteria to survive and join the duodenum where only a limited number of bacteria are present (pH around 7). Indeed, in order to proliferate, bacteria need energy-providing nutrients to grow and reproduce. For this reason, a small multiplication of bacterial species is observed in the small intestine. However, the colon harbours the highest abundance of microbes as it is a rich site in terms of access to nutrients derived from foods, cellular materials and metabolites derived from cellular activities. Two other important processes take place in the colon: the fermentation of undigested food due to a direct contact with the bacteria and the
production of metabolites that directly depend on available substrates. The main available substrates for microbial fermentation are carbohydrates, from which they can produce short chain fatty acids (SCFAs) composed mainly of acetate, butyrate and propionate\textsuperscript{58,60}. SCFAs are, along with carbon dioxide, methane and hydrogen, the main end products of microbial fermentation of undigested carbohydrates. These SCFAs are largely produced in the proximal region and serve as energy sources for the host. It has been reported that approximately 5\% of the SCFAs are excreted in faeces and that they provide 10 to 30\% of the basal metabolic requirements such as energy for hepatocytes, colonocytes and peripheral tissues\textsuperscript{59-61}. They are known to provide beneficial effects in the body and to be involved in many basal metabolic processes i.e. hepatic regulation of sugars, supply of cell energy, lipid hydrolysis, protein degradation leading to the production of amino acids, vitamin production, etc\textsuperscript{59}. Butyrate is a preferred energy source for colonic epithelial cells, whereas propionate is mainly processed by hepatocytes in the liver as a substrate for gluconeogenesis. Acetate, which can also be endogenously produced, reaches the general circulation and enters peripheral tissues to serve as an energy source\textsuperscript{59,60}.

Environmental and internal factors directly impact the composition of the gut microbial species and consequently the nature of the products, which are absorbed via the intestinal tract into the general circulation. The gut, the microbial species which inhabit it and the environmental conditions controlling the interplay between them are at the origin of a highly integrated and regulated ecosystem. Nevertheless, the equilibrium between these partners is vital, unstable and may be broken by any minor disruptions leading to a perturbation of the whole system.

2. Germ-free models and modulation of the gut microbiota composition

The role and functions of GM have been a longstanding enigma. Therefore, techniques were evolved from biological cultures to the development of bioreactors, as well as the development of animal models with controlled microbiota. Continuous cultures were developed in the 1950s and allowed researchers to understand the modifications of the environment caused by the presence and growth of
microorganisms. A culture system connecting 3 chemostat bioreactors has been developed to reproduce some of the physiological conditions of the GI tract in vitro, and determine the chemical transformations occurring during the digestion process. Nevertheless, this system does not mimic the natural absorption and the impact of mammalian metabolism, and cannot therefore be considered as entirely representative of the metabolism of a substance. Therefore, the GF mouse model was first developed in the 1950’s, and was validated as a model to assess the effects of the colonisation of microbial species on the mammalian host, as these animals have never been in contact with any microorganisms before colonisation.

2.1 Germfree animals as a “living test tube”

GF models are obtained by caesarean section into sterile plastic-film isolators, as the mammalian foetus is essentially free of culturable bacterial species at the time of birth. GF animals are considered as highly interesting as they provide a “living test tube” offering the possibility to be colonised by one bacterium (mono-association), several bacteria (bi, tri-associations or even more) or an open environment allowing us to study the interaction between the host and microbial species colonising the gut. These approaches are called association and colonisation studies, respectively, whereas contamination corresponds to the unwanted introduction of microbial species into GF animals. These models colonised with known and defined microbial species are called “gnotobiotic animals” which means “known life”. Interestingly, the colonisation process starts shortly after birth and several microbial species become established into the GI tract as soon as suckling begins. For instance, it has been originally shown in the 60s that lactobacilli and anaerobic streptococci – two of the most abundant bacterial groups culturable presently – reached their maximum population after 12 days of colonisation confirming a rapid colonisation and highlighting a crucial role of the first days of colonisation on the establishment and development of host characteristics.

2.2 Germfree animal characteristics

GF and conventional animals are known to be histologically, anatomically and physiologically distinct. Indeed, GF rodents display unusual gut morphology i.e. a larger caecum (the GF caecum can be tenfold larger than the conventional rodent
caecum), decreased weight of intestinal wall, decreased intestinal surface area, decreased size of liver, heart and adrenal glands and of blood volume as well as thinner intestinal villi and lamina propria when compared to their conventional counterparts. From a physiological and biochemical aspect, GF rodents present an altered mucosal enzyme pattern, lower pH of intestinal contents, decreased basal metabolic rate, decreased synthesis of vitamin K and B complexes, and no Peyer’s patches or BA transformation in the gut. Finally, GF rodents have immunological abnormalities i.e. lower peristalsis and depression of several immune responses such as lower production of immunoglobulin-A producing cells, responsible for the lymphocytes maturation and activation in the lamina propria.

These animals are also characterised by a decreased inflammatory response and blood clearance of microorganisms when compared to their conventional counterparts. It is evident that GF animals colonised with defined microorganisms or even a complex GM can never be considered as conventional animals due to the lack of influence of the gut microbiota on GF rodent metabolism (immunology, physiology, morphology,) during the period which precedes the acquisition of microbial species by the animals. Therefore, gnotobiotic models are considered as “artificial models”. Nevertheless, comparisons of GF and conventional mice have been studied extensively to obtain a deeper understanding of the symbiotic relationship between host and gut microbes and to reveal the important effects that they exert on the host metabolism. In addition, these models have also delineated important basic ecological principles operating in the GI tract and revealed the dramatic effect that the microbial community exerts on the morphological, biochemical, physiological and immunological development of its host. It is well known that gut microbes have both beneficial and detrimental effects on the host metabolism and homeostasis. Indeed, they offer bacterial interference and colonisation resistance to the host while stimulating the immune response to respond more quickly to a pathogen invasion. Furthermore, the presence of microbial species helps to maintain the intestinal mucosal integrity and intestinal tract peristalsis, and convert dietary carcinogens into noncarcinogen molecules. Interestingly, some carcinogen synthesis is microbially controlled, i.e. nitrosamine synthesis. In addition, the gut microbes contribute significantly to the synthesis of vitamin K and B12 complexes which are involved in posttranslational modification,
especially for blood coagulation and in the normal functioning of the brain and nervous system, respectively. At present, the complex of biological mechanisms, defined as symbiosis between the GM and the host, is beginning to be well understood. Indeed, several studies have manipulated the indigenous GM introduced by using GF models in order to establish and decipher the basic mechanisms that maintain this biological equilibrium and gain a deeper understanding of the complex relationship between the gut microbial species and its host. In this context, a particular focus will be performed on the brown adipose tissue (BAT) metabolism since it is central in energy metabolism. This tissue is crucial in the regulation of the energy expenditure, as it produces heat by oxidising fatty acids, and will be introduce later (chapter III). In addition, bile acids (Bas) are known to be strongly modified by the gut microorganisms and the study of their metabolism is therefore crucial in this project.

3. Bile acid metabolism and gut microbiota

3.1 Bile acids composition and function

BAs are saturated, hydroxylated C-24 cyclopentanephenanthrene sterols with a steroid backbone, and are amphipathic – they possess both hydrophilic and hydrophobic properties (Table 1). They are synthesised exclusively from cholesterol in hepatocytes of the liver. Like all amphipathic molecules, they can self-associate in water to form aggregates, called micelles, which can in turn emulsify lipids in the form of mixed micelles.

BAs are secreted by the liver into the gallbladder and then released into the intestinal lumen following ingestion of a meal. The conversion of cholesterol into BA is a multi enzyme-dependent mechanism which is crucial for the absorption of dietary lipids and vitamins as well as hepatic bile formation. This process includes oxidative cleavage of the cholesterol side chain, resulting in the conversion of an isooctane moiety (side chain with eight carbons) into an isopentanoic moiety (side chain with five carbons) and addition of hydroxyl groups to the nucleus. Mammals present a shortening of the C₈ side chain of cholesterol to a C₅ side chain directly involved in the process leading to BA pools composed mainly by C₂₄ BAs. The BA synthetic pathway produces two primary bile acids, by most vertebrate species. one
is usually cholic acid (CA, 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid) and the second one is muricholic acid, exclusively in the mouse (Figure 2)\textsuperscript{67}. Indeed, in rodents, various alternative hydroxylation reactions generate other primary BAs such as the α-, β- and γ-muricholic acids (Figure 2 and table 1). This second bile acid could be chenodeoxycholic acid (CDCA, 3α, 7α-dihydroxy-5β-cholan-24-oic acid) in rat, human and hamster, ursodeoxycholic acid in bear or hyodeoxycholic acid in pig\textsuperscript{67}. In the presence of 12α-hydroxylation activity, cholesterol is converted to cholic acid. In the absence of this activity, a conversion to muricholic acid (mouse) or another bile acid cited previously is observed, depending on the organism in which this conversion is taking place. Therefore, the level of 12α-hydroxylase in the liver is known to regulate the relative amounts of these two bile acids. In addition, it has been shown in the mouse model that CA mediates the feedback regulation of this conversion\textsuperscript{67}. Finally, BAs are, preceding their secretion, metabolised by the liver where they are conjugated as N-acyl amidates (peptide linkage) to glycine or taurine (glyco- and tauro-conjugated, respectively): a modification that triggers a decrease of pKa to 5. At pH=7, conjugated BAs are fully ionised and may be termed bile salts\textsuperscript{30,68}. 

\textsuperscript{43}
Figure 2: Summary of bile acid metabolism in the liver and intestinal lumen of rodents with a specific focus on the role of gut microorganisms\(^{27,30,67}\).

Key: CA: Cholic Acid; \(\alpha\)-MCA: alpha-muricholic acid; \(\beta\)-MCA: beta-muricholic acid; HCA: Hyocholic acid; TCA: taurocholic acid; GCA: glycocholic acid; DCA: deoxycholic acid; LCA: lithocholic acid; HDCA: Hyodeoxycholic acid; UDCA: ursodeoxycholic acid; TCDCA: taurochenodeoxycholic acid; GCDCA: glycochenodeoxycholic acid; T-\(\alpha\)-MCA: tauro-alpha-muricholic acid; T-\(\beta\)-MCA: tauro-beta-muricholic acid.

Following the ingestion of a meal, release of a specific cytokine called cholecystokinin from the duodenum stimulates the contraction of the gallbladder leading to the release of bile into the duodenum. Fat digestion and absorption occur in the duodenum, jejunum and proximal ileum. Bile salts are complexed with plasma proteins upon entering the bloodstream and then return to the liver \(^{68}\). They are
cleared upon reaching the liver and rapidly secreted into bile. This whole process is known as enterohepatic circulation and is considered as a key point of BA metabolism (Figure 2)\textsuperscript{25,69,70}. This results from the efficient reabsorption of these compounds in the terminal ileum – called the BA pool. This process constantly regulates the concentration of BAs in order to maintain an efficient digestion in the intestinal lumen. Each molecule is used several times during this process, as most of the BAs transported by hepatocytes are “old” BAs, which were previously synthesised and secreted into the bile. Interestingly, the level of efficiency for intestinal absorption of BAs is about 95%, as 5% is lost per day in the faeces. This loss is compensated for by a daily production to maintain the bile acid pool at a constant level\textsuperscript{30}.

The synthesis and function of BAs are well known and they are involved in many key biological reactions in the host. For instance, BAs were identified in 1999 as the natural ligands for the nuclear receptor farnesoid X receptor (FXR). This receptor mediates the suppression of bile acid synthesis as it binds BAs and is involved in the regulation of gene expression related to bile acid and lipid metabolism\textsuperscript{67}. Furthermore, they are also involved in the control of fat emulsification (in collaboration with enzymes), in energy homeostasis, and in lipid, glucose and energy metabolism\textsuperscript{31,71}. Indeed, it has been shown that BAs administration, and especially CA, to C57Bl6/J mice fed with a high-fat diet (HFD) significantly reduced weight gain, with a significant impact on epididymal White Adipose Tissue (WAT), mesenteric WAT and intrascapular BAT. These results showed that BAs increase energy expenditure in BAT and are involved in the prevention of obesity and resistance to insulin\textsuperscript{71}.

### 3.2 Specific focus on bile physiology

Bile is a key biofluid in the body and constantly interacts with gut bacteria. Furthermore, bile has a regulatory action on commensal and pathogenic microorganisms, which may regulate their abundance in the intestinal tract according to 4 different parameters: variations in pH, low oxygen levels, limitation of nutrient access and elevated osmolarity\textsuperscript{70}.

Bile is a digestive secretion acting as biological detergent and playing a central role in the emulsification and solubilisation of lipids. It is a yellow/green
aqueous solution composed of organic and inorganic compounds whose major constituents are BAs, cholesterol, phospholipids and biliverdin. Immunoglobulin A and mucus are secreted into bile to avoid bacterial growth and adhesion. It is well known that bile is synthesised in the liver, and then stored into the gallbladder at high concentrations. It is released into the duodenum upon the ingestion of a meal by the host. It is notable that BAs constitute approximately 50% of bile organic components. Interestingly, bile is also considered as an excretory fluid useful to eliminate substances that cannot be efficiently excreted in urine, due to their binding to proteins or their insolubility (i.e. cholesterol, carried in blood and attached to albumin proteins), therefore making this biofluid a crucial target for bile acid characterisation and the understanding of the interaction between gut microbes and host metabolism.

3.3 Modification of bile acids by the gut microbiota

Intestinal bacteria produce the secondary BAs and are implicated in the high diversity of the BA pool. Indeed, during the passage of BAs through the gut, conjugated BAs can be transformed by the indigenous microbial species (Figure 2). The three main alterations of BAs by the GM are:

- Deconjugation, which results in cleavage of the amino acid side chain liberating free BAs, called unconjugated BAs (Figure 2).
- 7-α-dehydroxylation, which occurs at the position 7 of the skeleton, and corresponds to the removal of an OH group converting therefore CA to DCA and CDCA to LCA, respectively (Figure 2).
- 7-α-dehydrogenation converts CDCA to 7-oxolithocholic acid which can then be epimerised by bacterial enzymes to form ursodeoxycholic acid (Figure 2).

Deoxycholic acid (DCA, 3α, 12α-dihydroxy-5β-cholan-24-oic acid) and lithocholic acid (LCA, 3α-dihydroxy-5β-cholan-24-oic acid) are considered in human as the main secondary BA and they are derived directly from cholic and chenodeoxycholic acids, respectively (Figure 2 and Table 1). Two mechanisms and anatomical sites of BAs synthesis are known to date: the so-called primary BAs formed from cholesterol in the liver, and the production of secondary BAs by the
modification of primary BAs by bacterial enzymes in the intestine (Figure 2)\textsuperscript{25,27}. Nevertheless, some of the secondary BAs generated by the gut microbes are potentially toxic and/or mutagenic. The hypothesis is that these can disturb the normal and indigenous microbiota of the host leading to diseases and intestinal problems such as diarrhoea, mucosal inflammation and activation of carcinogens in the intestinal tract.
Table 1: Structural information and listing of main BAs found in rodents and humans are presented. Names, abbreviations, type (primary, secondary and tertiary) and additional structural information about radicals are given.25,67

Other studies have demonstrated that secondary BAs, solely produced via transformation by intestinal microbial species, can accumulate to a high amount in
faeces and biofluids (i.e. blood and bile) and contribute to colon cancer, gallstones and GI diseases. The potential exists to alter the bile acid pool by targeting key enzymes in the 7α/β-dehydroxylation pathway through the development of pharmaceutical BAs biologically active in bacteria. In addition, glyco- and tauroconjugated BAs can be considered as substrates for microbial metabolism. Unlike glycine, taurine contains a sulfonic acid moiety which is reduced and dissimulated to hydrogen sulphide by the deconjugation. This molecule is highly toxic and related to an increase of colonocyte tumours.

Metabonomic approaches have been used previously to characterise the effects of drug interventions or microbiota inoculation on BA metabolism and has highlighted strong effects in rodent models. Indeed, Swann et al demonstrated in kidney, liver and heart that each tissue has a specific bile acid profile and that the absence of gut microorganisms tends to shift the balance to almost only taurine-conjugated species in the rat. Interestingly, antibiotic treatments seem to impact bile acid metabolism in a similar manner confirming therefore the specific role played by the GM as a regulator of the host metabolism. This work complemented a study by Martin et al who demonstrated that mice colonised with human baby microbiota (HBM) displayed higher levels of tauroconjugated BAs compared to normal mice, due to a lower deconjugation activity from the microorganisms colonising the gut.
Chapter II  Analytical strategies

1. NMR fundamental principles

Two main analytical platforms are currently used to generate data to be analysed by a metabolic profiling approach: nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography coupled with mass spectrometry (LC-MS). They both present the advantage of measuring a wide range of molecular compounds called metabolites (<1000 Daltons).

$^1$H NMR spectroscopy is a powerful tool for achieving the compositional study of complex biological samples, and presents the advantage of being non-destructive. This means that a sample can be analysed by NMR in the first instance and then be subject to further analysis, for instance mass spectrometry which will be detailed later. NMR is highly reproducible, provides a rapid analysis of a wide range of metabolites in virtually the same conditions as in vivo, and with a very low number of preparation steps. NMR spectroscopy delivers a snapshot of the metabolic composition of biofluids and tissues.

NMR spectroscopy takes advantage of the magnetic properties of certain nuclei and records the energy difference between the spin states. Applying an external magnetic field, all these nuclei adopt a spin orientation parallel or anti-parallel to this applied magnetic field. These 2 spin states are non-equivalent in terms of energy. The energy difference is proportional to the nuclear magnetic moment and the magnetic field strength (see Equation 1), and the populations of these levels are governed by the Boltzmann distribution.

\[ \Delta E = \left( \frac{\hbar \gamma}{2\pi} \right) B_0 \]

where $\hbar$ is the Planck’s constant, $\gamma$ is the gyromagnetic ratio and $B_0$ the magnetic field.

The signal between 2 spin states depends directly on the excess of the spin populations between these two energy levels (Figure 3), and its intensity is proportional to the total number of nuclei, and therefore the concentration, of the sample: thus, NMR is an inherent quantitative technique. It is possible to force some nuclei of the lower energy level to adopt a higher energy level by applying the exact
radiofrequency energy to the nuclei, $\nu_1$. When the radiofrequency is withdrawn, nuclei relax to recover their equilibrium energy state. This is characterised by the release of an oscillating radiofrequency signal which gives the NMR signal, called Free-Induction-Decay (FID). A Fourier Transformation (FT) is then applied to convert the time domain signal into the frequency domain and thus to obtain an NMR spectrum\textsuperscript{73,74}.

**Figure 3:** Principles of NMR spectroscopy: schematisation of the Larmor precession (A) and distribution of protons according to their energy level in a magnetic field $B_0$ (B). Adapted from Hore et al.\textsuperscript{75}

A) When a magnetic field $B_0$ is applied to an atom with a nuclear spin, the spin axis can precess around the axis of the magnetic field $B_0$. This notion is known as the Larmor precession characterised by the Larmor frequency $\nu$ (Hz) and an angular speed $\omega_0$ (in radian).

B) All NMR active nuclei possess angular momentum which is quantised, i.e. it can take only certain values, from which a magnetic moment ($m$) is derived. $m$ can take values from $I$, $I-1$, ..., $-I$, and thus for $I=1/2$ the values of $m$ can be $+1/2$ and $-1/2$. In a magnetic field ($B_0$) this moment can align with or be in opposition to the field, giving rise to two energy levels.

Metabonomics mainly employs $^1$H NMR spectroscopy, which makes the application of NMR valuable for structural elucidation of a wide range of biomolecules. Indeed, NMR spectroscopy is useful in chemical structure identification because each proton is directly influenced by its chemical and therefore
electronic environment. The applied magnetic field induces an electric current around the nucleus that generates in turn a local magnetic field in the opposite direction of the applied magnetic field. This difference between the applied and the local magnetic field is created by this nuclear shielding\textsuperscript{73-75}. Therefore, the chemical shift is defined as \textit{nuclear shielding} divided by \textit{applied magnetic field}. Knowledge of chemical shifts then enables identification of the molecules present in a complex mixture.

The resonance of one single nucleus in an isolated environment will give a single peak, termed a singlet. However, neighbouring nuclei in a molecule interact to generate spin-spin interactions via their own magnetic moments ($J$, in Hertz). These interactions result in the splitting of the NMR signal (doublet, triplet, quadruplet or multiplet) as shown in figure 4 below.
Figure 4: Overview of the shielding in a $^1$H NMR spectrum.

A) Example of a standard 100 MHz $^1$H NMR spectrum of ethanol in D$_2$O (with TMS).

B) Examples of spin orientations for the CH$_2$ group (2) and CH$_3$ group (3) with an applied magnetic field $B_o$.

A standard 100 MHz $^1$H NMR spectrum of ethanol, which has a CH$_3$CH$_2$OH raw structure, has been acquired (Figure 4.A). The methyl group (CH$_3$) has 8 different combinations of spin orientations which lead to the formation of a quartet (see Figure 4.A and 4.B.3), whereas the methylene group (CH$_3$) has 4 different possibilities of spin orientations which lead to the formation of a triplet (see Figure 4.A and 4.B.2). The multiplicity is derived from the number of discrete magnetic moments (m.m.), whereas the amplitude value derived from the number of possibilities of that particular magnetic moment. The labile alcoholic proton (OH) is not seen because it exchanges with the deuterium protons of the solution, reducing its resonance. Residual water resonance is due to the 0.01% of H$_2$O still present in D$_2$O solution.
1.1. One Dimensional NMR spectroscopy (1D NMR)

Each 1D NMR experiment can be divided into three parts: the preparation time, the radiofrequency pulse (RF pulse) and detection time (Figure 5). During the phase of preparation, the spin system is brought to a defined state and reaches “equilibrium”. Afterwards, an excitatory RF pulse is applied, and then the FID is acquired during the relaxation time corresponding to the return of the spins to their equilibrium state. Usually, the experiment is repeated several times to sum up the FIDs as a series of scans, defined as identical experiments in order to improve the signal to noise ratio and to null artefacts due to imperfect coil geometry. A FT is finally applied to convert the time-domain to frequency-domain and obtain an NMR spectrum (Figure 5)\textsuperscript{73-75}.

![Figure 5](image.png)

**Figure 5:** The essential elements of the single-pulse NMR experiment: the preparation time, the RF pulse with a specific excitation angle and the data acquisition time of the FID.

Due to the high number of overlapping signals limiting the efficient assignments of a large number of metabolites, 2D NMR spectroscopy represents a useful approach to identify metabolites present in a complex biological matrix by dispersing NMR signals into a second dimension\textsuperscript{76}.
1.2. Solid state NMR experiment

Solid state NMR spectroscopy is particularly useful to analyse biopsies of tissues without extraction into a liquid phase, preserving therefore the overall structure of samples\textsuperscript{72,77}. The main difficulty in solid state NMR is the spatial orientation of biological structures relative to the external magnetic field. This results in anisotropic interactions which disturb the spectrum. Indeed, spectra are dominated by strong homonuclear dipolar interactions which broaden the signal. In order to resolve the resonances, it is necessary to remove these anisotropic interactions and this is achieved by applying High Resolution-Magic Angle Spinning (HR-MAS). In this approach, a sample is spun at the so-called magic angle $\Theta = 54.7^\circ$, at a high frequency typically of 5000-6000Hz, to remove spinning side bands out of the spectral shift window, to increase spectral resolution and information content, and to minimise the line broadening effects due to the heterogeneity of the sample, macroscopic field inhomogeneities and anisotropic line broadening\textsuperscript{78}. A review regarding this technique has been published by Schnell and Spiess\textsuperscript{79}.
2. UPLC-MS analysis

MS is generally coupled with a prior separation technique, such as gas chromatography (GC) and high-pressure liquid chromatography (HPLC), or more recently, ultra-high-pressure liquid chromatography (UPLC)\textsuperscript{80}. Biological samples are complex mixtures containing a wide range of metabolites and therefore producing complex mass spectra. Introducing a prior separation stage reduces the complexity of the mass spectra by performing a metabolite separation into a time dimension before mass separation\textsuperscript{81-83}. This can enable the separation of isomers during the chromatography step according to their physico-chemical properties (such as structure and polarity).

2.1. Liquid Chromatography

LC-MS analysis is mostly performed using reversed-phase (RP) gradient chromatography. This chromatography method employs a column with a non-polar stationary phase, for instance ethylene-bridged or alkyl-derivatised silica particles. The elution is usually achieved by a mixture of water and organic solvent (e.g. acetonitrile or methanol), either with an isocratic flow or with a gradient (high aqueous to high organic), which allows changing the polarity of the mobile phase during the analysis. RP separations are suitable for the analysis of medium and low polarity analytes, but are inappropriate for the analysis of highly polar molecules, which are not retained well or eluted in the void volume of the RP column.

UPLC employs smaller particles (<2\(\mu\)m) and higher pumping pressures than HPLC (up to 15000 psi), which results in higher chromatographic resolution, increased sensitivity and faster analysis\textsuperscript{80}. RP-UPLC separations are often carried out on BEH C18 or C8 columns. These columns provide stability over a wide pH range and produce sharp peaks. However, high polar compounds are not well retained on these columns and are therefore co-eluted at the beginning of the run, leading to ion suppression.

In order to improve the retention of polar compounds, a RP-chromatography with a High Strength Silica (HSS) T3 column can be used (Figure 6). This column provides a more efficient retention and separation of organic polar compounds than the other columns used in reversed-phase chromatography, while also offering a good retention for hydrophobic species. When more information is required on polar
compounds, in order to improve the metabolome coverage, one can use an HILIC (Hydrophilic Interaction Chromatography) column, which is sometimes called reverse-phased reverse chromatography. A study comparing BEH C18, BEH HILIC and HSS T3 columns for UPLC-MS-MS analysis of glutathione and its endogenous derivatives (polar compounds) from liver and plasma samples by New and Chan concluded that the HSS T3 column was the most suitable column considering chromatographic retentivity, peak shape, MS sensitivity, and resolution. This is the column chosen for this study (Figure 6).

![Figure 6: Composition of HSS T3 (C18) column.](image)

### 2.2. Principles of Mass Spectrometry

The use of MS in the metabolic profiling field is based on the measure of the mass-to-charge ratio of molecules that are charged. The molecules are ionised in order to generate charged molecules or fragments that are then measured. A mass-spectrometer exerts forces on charged particles called ions by using both magnetic and electric fields in a vacuum.

![Figure 7: Diagram of the different components of a mass-spectrometer.](image)

The interface is placed between the source and the analyser in order to perform the ionisation, focusing and transmission of the ions.
A basic mass-spectrometer is composed of three modules called the source, where the molecules are transformed into gas phases and ionised, the analyser, where the ions are separated based on their mass-to-charge ratios by applying electromagnetic fields, and the detector, where the ions are counted and their abundances are determined. The final step is the recording of the signal in order to analyse it (Figures 7 and 8).

**Figure 8:** Diagram of the molecule ionisation, separation and detection processes through a mass-spectrometer.

The injection of the samples can be either direct or by combination with chromatographic techniques such as gas-chromatography (GC), capillary electrophoresis (CE), high pressure liquid chromatography (HPLC) and ultra-high pressure liquid chromatography. Each technique is suited to different sample classes based on their physical and chemical properties. GC-MS is mainly used for the separation of volatile compounds analysis whereas HPLC and UPLC-MS are mainly designed for the separation of liquid samples, and CE-MS is a more specific chromatographic technique which allows separation of molecules based on their size-to-charge ratio inside a small capillary, driven by an electric field.

The source is a module where the ionisation of the molecule is taking place. It depends on the nature of the sample and the information which is required for the analysis. The ionisation has to be adapted to the nature of the molecules to optimise the analysis further (Figure 8). Indeed, EI (electron ionisation), CI (chemical
ionisation) and APCI (Atmospheric Pressure Chemical Ionisation) are adapted to small volatile and non thermo-sensitive molecules, whereas the FAB (Fast Atom Bombardment) is adapted to molecules with a molecular weight less than 6000 Da (Daltons). In addition, two soft ionisation modes are generally used in biology to preserve the biomolecule integrity and non-covalent complexes, as they tend to produce mass spectra with little fragment-ion content: the ESI (electrospray ionisation) and MALDI (Matrix-Assisted Laser Desorption Ionisation).

![Schematisation of the electrospray ionisation (ESI) principle.](image)

**Figure 9:** Schematisation of the electrospray ionisation (ESI) principle.

In the ESI source, the solution is sprayed across a high potential difference at a few kilovolts from a needle into an orifice in the interface. A strong electric field is applied to the tip of a narrow capillary where the liquid sample emerges. The ions are then desolvated by the combination of heat and gas flows as the solvent is stripped away. This type of ionisation has the ability to produce a number of multiple charged ions, which increases with the molecular weights. ESI produces ions free of solvent. A glow flow perpendicular to the ion projection is used to reduce the spray dispersion and leads the sample to the mass analyser. Thanks to a Z spray system, which corresponds to a Z shape block formed by the path of the ions, the neutral molecules and contaminants are removed, and only charged ions enter into the vacuum (Figure 9).
The charged ions are subsequently transferred to the analyser which leads to their separation according to their mass-to-charge ratios, and their abundances are then counted at the detector. The output is an isotopic profile which shows the isotopic distribution and isotope composition for each fragment constituting a molecule. The number of peaks and their intensities depends directly on the number of atoms and the source natural isotopic abundance. They are characteristic from the ionic elementary composition and are useful to determine the net formula of a molecule. Analysers can either be a quadrupole, ion trap, time-of-flight (TOF) or Fourier transform-ion cyclotron resonance (FT-ICR), or combination of them, for example Q-TOF or a triple quadrupole (QQQ). In this study, a LCT Premier TOF (Waters) operated in ESI+ and ESI- modes was used for most MS analysis, as well as a Q-TOF Premier (Waters) to perform fragmentation. Q-TOF analysers are able to provide structural information of unknown compounds with high mass accuracy. A TOF instrument has been used as it is able to accelerate the ions to the same potential, and then separate them based on their mass-to-charge ratios, due to an applied electric field (Figure 9). Once inside the analyser, lighter ions always reach the detector before heavier ions for the same level of ionisation. By measuring the time of flight, the TOF is able to calculate the ion mass. Following detection, the final result can be displayed as a chromatogram obtained from ion energy converted into an electrical signal by the detector (Figure 9).

Nevertheless, one of the main problems encountered with LC-MS is called ion suppression. Indeed, some molecules are better at carrying charges than others which mean that the ionisation process of complex substance mixtures is not in an equal amount for all the particles. Therefore, some proportions of ionised species will obtain more charge than expected given their concentration, while others will not appear on the chromatogram.
3. Pattern recognition techniques for data with multiple variables

3.1. General principles

Metabonomics is defined as “the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification”\textsuperscript{24} and enables together with genomic, transcriptomic and proteomic (defined as the “omics” technologies) approaches new scientific possibilities to characterise the molecular basis of a host (plant, animal, human) in a particular context such as diseases, pharmaceutical treatment, nutritional intervention, etc\textsuperscript{90,91}. Indeed, the host metabolism is a complex network of metabolic reactions under permanent regulation that is called homeostasis. Even the simplest effective disruption can lead to diseases such as atherosclerosis, diabetes, osteoporosis and hypertension. The application of a metabolomic approach in the particular field of nutrition aims at preventing such diseases by measuring the metabolic impact of nutritional interventions, rather than e.g. drug treatment\textsuperscript{24,90-93}.

This approach has opened new horizons and understanding in the domain of nutrition. It is based on the use of two major analytical platforms to study the metabolic composition of biological matrices: nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Metabolic profiling approaches in metabonomics are mainly based on these two spectroscopic platforms, as they offer structural and conformational information relevant to chemical classes and compounds\textsuperscript{24}. Unlike gene expression or proteomic data, which might only indicate the potential for physiological change, metabolites and their kinetic changes represent real end-points of physiological regulatory processes. In consequence, measurements of metabolites are a valid option to assess physiological changes which take place within a biological system, i.e. ingestion of foods. Modern nutrition aims to promote improvement of health and disease prevention. In this context, metabolic profiling is efficient for studying the metabolic phenotype, which directly reflects aspects of the consumer dietary habits\textsuperscript{90,91}. Nutritional metabonomics or nutrimetabonomics appears in the field of nutritional research as an effective approach for the classification of dietary responses in populations and personalised nutritional management\textsuperscript{90-92}.
3.2. Multivariate Data Analysis

Multivariate Data Analysis (MVDA) is defined as the observation and analysis of more than one statistical variable at a time and is commonly applied to extract information from the complex data matrices generated by “omics” techniques. This thus employs mostly linear projection based analysis such as Principal component analysis (PCA) and partial least squares analysis, also called projection to latent structures (PLS), which are commonly used in pattern recognition analysis and are widely employed to detect variations between samples and classes.

3.2.1. Principal Component Analysis

PCA is an unsupervised technique because no a priori knowledge or class information is used to fit the model. Mathematically, PCA is defined as an orthogonal linear transformation, which projects the initial data in Euclidean space into a compressed space described by fewer dimensions. The aim of PCA is to reduce the dimensionality of a dataset by describing its main sources of variation (latent variables). The first principal component (PC) captures the most important source of variation in the measurement matrix (X), the second PC is the most important source of variation after the contribution of the first PC has been removed from X, and so on for the following components (Figure 10). By construction, successive components are orthogonal to each other.
Figure 10: Principal Component Analysis (Reprinted from “Principal Component Analysis”).

A: The observations blue points (i.e. NMR data) can be seen as a cloud of points in a variable space.

B: Calculation of the first two components (noted as PC 1 and PC 2).

C (left): PC 1 and PC 2 define together a plane which includes all data. All observations are projected onto this two dimensional plane known as scores points.

C (right): Visualisation of two groups on the scores plot.

Graphically, the PCA can be interpreted via 2 projection plots, the “scores plot” and the “loadings plot”. The linear combination of variables used to calculate a component is called the loadings (P) while the coordinates of the points in the new system projected on these components give the scores (T).

\[ X = TP^T + E \] (PCA model)

where X are the metabolic profiles, P is the loadings, T is the scores and E is the residual matrix.

- The scores can be interpreted as the projection of the individual observations onto the PCs. Therefore, the scores can be used to represent the
relation between samples using the global set of data, and can reveal differences or similarities between the samples (time trajectories, group segregation and outliers).

- The loadings are the projection of the variables onto the PCs. They can be interpreted as the weights of the variables in each PC but also represent the share of variance of the variables explained by each PC. Loadings are used to identify variables that weight highly in the discrimination of interest groupings and permits the interpretation of the spectroscopic differences and similarities. They are the linear combination of observations used to calculate a component (P).

In the nutrimetabonomics field, the effects are often subtle with relatively low amplitude and their observation is difficult with unsupervised methods alone. Therefore, it is relatively common to use supervised methods which require a priori knowledge such as the class of the sample\(^99\).

### 3.2.2. Supervised methods

PLS is a supervised technique based on the multiple linear regression model, used to capture the linear relationship between the output variables, \(Y\), and the input variables, \(X\). The main advantage of this method is its possibility to deal with a number of input variables higher than the number of observations, which is the case in a NMR-based or MS-based metabolic profiling method, by finding latent factors accounting for the largest amount of variation associated with the predictive \(Y\)\(^97\). PLS can either be used in classical regression mode or in discriminant mode (PLS-DA). Discriminant analysis (DA) aims to find a linear combination of features which characterise or maximally separate two or more classes.

O-PLS is an extension to the PLS method with an integrated Orthogonal Signal Correction (OSC) filter. O-PLS removes variation from \(X\) that is orthogonal to \(Y\). Orthogonal signal correction is a useful tool to remove unwanted factors, identified or not, which are orthogonal to \(Y\): these factors based on structured variance are also called structured factors\(^97,98\). The orthogonal components of O-PLS correspond to the orthogonal factors and their interpretation can lead to their identification. Orthogonal factors could have biological origin (i.e. age, gender, etc) or can be produced by experimental bias (MS sample run). The O-PLS analysed data are then easier to interpret with fewer and more relevant predictive components\(^97,98\).

The validity of the model against overfitting is tested by computing cross-validation parameters. The standard 7-fold cross-validation method repeatedly
leaves out a seventh of the sample and predicts them back into the model to check the robustness of the model.

An O-PLS-DA coefficients plot highlighting the variables involved in the discrimination of the classes can be calculated according to the method described by Trygg et al.$^{97,98}$ The coefficients plots are back-projected as a pseudo-NMR spectrum to facilitate the interpretation. The line intensity and direction corresponds to the mean-centered model representing the covariance of the variables. Additionally, each data variable is plotted with a colour code which relates to the correlation with class discrimination as calculated from the correlation matrix to form a pseudospectrum. Cold colours such as blue show a weak correlation and hotter colours such as red show the most significant correlation (Figure 11).

![Figure 11: A typical example of an O-PLS-DA coefficient plot.](image)

The colour-code corresponds to the degree of correlation of the peaks with the discrimination between the two classes. Each peak is made up of several variables. For example, peaks 1 and 2, representing metabolite-specific chemical shift regions, are highly positively correlated with class 2 affiliation. Peak 3 is moderately positively correlated with elevated class 1 samples, whereas peak 4 shows no correlated variation due to class, although elevated in class 1 (Figure 11).
Three essential parameters are used to characterise the robustness of the models and to determine their validity:

- $R^2_X$: the coefficient of determination. The $R^2_X$ value shows how much of the variation in the dataset is explained by the model.
- $R^2_Y$: the predicted percentage of the response.
- $Q^2_Y$ corresponds to the cross-validation parameter. It allows the validity of the model to be tested against overfitting. The $Q^2_Y$ value represents the predictability of the models and relates to its statistical variability: it is the cross-validated predicted percentage of the response. The $Q^2_Y$ can be used to estimate the significance of a PLS and O-PLS regression using resampling by permutation of $Y$. The distribution of the $Q^2_Y$ obtained by permutation simulates the null hypothesis and allows it its comparison with the actual $Q^2_Y$ obtained from the original dataset and inference. As a consequence, an inference on the significance of the regression can be given.

### 3.3. Metabolite identification

To identify a candidate biomarker, the use of a defined process is required. First of all, a biomarker is generally a biological entity considered as an indicator of a certain biological state. It is a characteristic that is measured and evaluated as an indicator of normal biological processes, pathogenic processes, or for example a metabolic response after a dietary intervention.

The major problem is that the identified components in nutrition are almost never ingested as a unique identity distinct one from the others but always in complex mixtures. The use of 1D NMR experiments combined with in-house standards, data from the literature and existing databases such as the Human Metabolome Data Base (HMDB): [http://hmdb.ca/](http://hmdb.ca/), or the Biological Magnetic Resonance Data Bank (BMRB): [http://www.bmrb.wisc.edu](http://www.bmrb.wisc.edu) are frequently useful in identifying the metabolites. Nevertheless, metabolite identification is sometimes complex due to the signal multiplicity and the overlap of resonances in a 1D NMR spectrum, or to a highly complex set of signals for a new molecule. In this context, one solution is the use of multidimensional NMR experiments. 2D acquisition allows the observation of homo- and heteronuclear coupling, generally $^1H-^{13}C$ coupling. NMR bi-dimensional techniques with $^1H-^1H$ homonuclear coupling such as COSY and TOCSY are often used, and heteronuclear $^1H-^{13}C$ coupling techniques such as HSQC and HMBC are also helpful.
Alternatively, separation techniques such as solid phase extraction (SPE) and high performance liquid chromatography (HPLC) are used to resolve the compounds and assist identification of the biological sample composition, using a following analytical step e.g. NMR, MS, etc\textsuperscript{105-107}. Moreover, NMR experiments can potentially be combined with other analytical platforms like mass spectrometry usually coupled with different chromatographic systems such as liquid chromatography (LC) or gas chromatography (GC). Finally, the STOCSY approach developed by Cloarec et al (Statistical Total Correlation Spectroscopy)\textsuperscript{108} is a useful tool, which complements 1D and 2D NMR experiments, to identify the discriminant metabolites. This approach uses the collinearity, i.e. very high correlation, between the spectroscopic variables which are signals from the same molecule or signals of interdependent molecules, such as molecules present in a same metabolic pathway, but corresponds to variation to be present across a number of samples.
4. **Fundamental principles of 16S rRNA sequencing using high-throughput sequencing**

DNA sequencing based on 16S rRNA genes appears as a useful method to identify unambiguously the bacteria that colonised the host, especially, but not only, at the gut level\textsuperscript{8,32,42,45,109-112}. Metagenomics, defined as the genetic studies of microbial communities, will ultimately significantly contribute toward the understanding of human health and disease. This field consists of the identification of genomes from mixed microbial communities that reside in plants, environmental niches or animal hosts. It uses the genomes to identify gene regulation, as well as specifically identifying members of the microbial community\textsuperscript{113}. Therefore, the integration of both metagenomics and metabonomics often displays new insights in the understanding of host and commensal bacteria interaction\textsuperscript{22}.

DNA sequencing technologies based on pyrosequencing are an emerging tool providing possibilities for deeply sequencing data collections in order to obtain microbial identification. This technique is based on the amplification by Polymerase Chain Reaction (PCR) of specific genetic targets such as hypervariable regions located in the bacterial 16S rRNA genes. They are then sequenced by a technique called pyrosequencing described hereafter (Figure 12)\textsuperscript{113}. 


Figure 12: Principles of pyrosequencing chemistry and biochemical reactions involved in DNA sequencing. Adapted from Petrosino et al.\textsuperscript{113}

Key: DNA: deoxyribonucleic acid; dNTP: deoxynucleoside triphosphate; dNDP: deoxynucleoside diphosphate; dNMP: deoxynucleoside monophosphate; PPi: inorganic pyrophosphate; APS: Adenosine 5'-phosphosulfate; ATP: Adenosine triphosphate; ADP: adenosine diphosphate; AMP: adenosine monophosphate.

A) A DNA template is hybridised to a primer and incubated in the presence of DNA polymerase, ATP sulfurylase, luciferase and apyrase, adenosine 5' phosphosulfate (APS) and luciferin. One of the four deoxynucleotide triphosphates, called dNTPs, initiates the second step which consists of the incorporation by the DNA polymerase of the complementary dNTPs onto the template.

B) ATP sulfurylase, incubated at the beginning quantitatively converts PPi to ATP in the presence of adenosine 5' phosphosulfate. ATP is considered as the fuel for the conversion of luciferin to oxyluciferin that generates visible light in amounts directly proportional to the amount of ATP. The light released by this reaction is detected by camera and analysed by a specific program.

C) Unincorporated nucleotides and ATP are eliminated by the apyrase and the reaction can restart from the beginning with the next nucleotide.

D) Finally, the nucleotide sequence will be reconstructed based on the light sequence that has been detected. The intensity is proportional to the number of nucleotides that have been incorporated.
It is noteworthy that DNA pyrosequencing has the limitation that it is not able to sequence very long stretches of DNA, as sequences do not access 100-200 bases with actual high-throughput pyrosequencing techniques. Therefore, the applications for microbial identification have focused on hypervariable regions and particularly on 16S rRNA genes, within the small ribosomal-subunit RNA genes, as it gives a very specific fingerprint\textsuperscript{113}. These specific hypervariable regions are used to identify different types of bacteria. Indeed, the 16S rRNA gene is easier to characterise thanks to the combination of conserved primer-binding sites and 9 variable sequences (Figure 13).

\textbf{Figure 13:} Representation of hypervariable (V) and conserved (C) regions in the 16S rRNA gene. The lengths of hypervariable regions generally varied from 50 to 100 bases.

Key: The 16S rRNA gene is made of a succession of conserved regions (C1 to C9) and 9 hypervariable regions (V1 to V9) that are targeted for pyrosequencing.

- This double arrow indicates an example for primer selection in order to perform DNA amplification by PCR.
- This arrow indicates an example of primer selection for sequencing.

Nowadays, the microbiota is considered as a vast and complex ecosystem composed of approximately 800-1000 species per individual. Nevertheless, the main drawback is that an estimated 60% of the human intestinal bacteria are still unidentified and 80% of the identified bacteria are considered as unculturable\textsuperscript{113}. This represents a considerable proportion of bacteria residing in the host and therefore is a major concern for this type of analysis. Finally, recent findings indicate that the population of bacteria in the indigenous community is restricted to a subset of all bacteria, and that bacterial populations are not randomly distributed within the host cavities or on the host\textsuperscript{114,115}. For instance, it has been demonstrated that predominant species are different between the GI tract and the genitourinary tract\textsuperscript{114}. 
A promising approach which has been developed in this PhD thesis corresponds to the integration of metagenomics and metabonomics data in order to decipher the metabolic capabilities and activities of a microbial community. This aims at improving the knowledge about the interaction between the host metabolism and its gut microbiota, and to identify bacteria involved in specific metabolic functions. The principle of this approach will be detailed in the corresponding chapter.

In this overall context, a metabolic profiling approach is the unifying method of choice since it combines the use of two complementary platforms, NMR and MS, to acquire a metabolic fingerprint of a biological matrix by non-selective analytical techniques with applied multivariate statistics. It therefore provides a holistic approach towards understanding the global metabolism of the host. These approaches are complemented by information regarding the composition of the gut microbiota (16S rDNA sequencing). The following chapters aim to apply this combination of methods to study the different ways gut microbes can influence the physiology of the host.
Chapter III  Metabolic Investigation of the effect of GF state and gender on the physiology and metabolism of C3H mice

Objectives

1) To assess the effect of the gut microbiota on the energy metabolism of the host.

2) To investigate the putative interaction of gut microbes with the gender of GF and conventional C3H mice.

3) To characterise the Brown Adipose Tissue (BAT) $^1$H NMR metabolic profiles of lean GF and conventional phenotypes.

4) To investigate the correlation of BAT metabolic profiles with the physiological parameters (BW and TBFC) of both GF and conventional C3H mice.
1. Introduction

Currently, the main approach to better understand the relationship between the gut microbial species and the metabolism of the host is the use of GF models (Chapter II). Their extensive study has aimed to better characterise the symbiotic relationship that has been established between the host and its gut microbes. GF animals have been validated as a model to assess the effects of the colonisation of microbial species on the metabolic fingerprint of the host\textsuperscript{26,63}. In a recent study, the multi-compartmental metabolic profiles of conventional and GF mice have been studied using a $^1$H NMR-based metabonomic approach and demonstrated the significant contribution of this approach. Indeed, Claus et al demonstrated that the GM impact the host metabolism at both local and global levels and exert an interesting effect on the metabolism of the liver, which is central in the host\textsuperscript{26}. In addition, a recent work demonstrated also that C57Bl6/J male GF mice had a lower total body fat content (TBFC) than their conventional counterpart and that they were resistant to diet-induced obesity (DIO)\textsuperscript{5}. This mechanism was attributed to an elevated level of Fiaf which is a circulating lipoprotein lipase inhibitor, normally selectively suppressed in the gut epithelium by the GM\textsuperscript{5}. This resistance to DIO was involved in the protection against obesity induced by a Western-style, high-fat and sugar-rich diet, through the stimulation of fatty acid metabolism\textsuperscript{5,65}.

However, although several biofluids and tissues including urine, plasma, liver, kidney and ileum have been characterised, the metabolism of the adipose tissue and especially the BAT, which is central in energy metabolism, has not been characterised in the studies cited previously. This tissue is crucial in the regulation of the energy expenditure as it produces heat by oxidising fatty acids \textit{via} uncoupling the proton gradient in mitochondria from the production of ATP\textsuperscript{116}. Therefore, BAT contributes to thermogenesis by producing heat instead of energy\textsuperscript{116}. This tissue has been deeply investigated by $^1$H and $^{13}$C NMR in rats in the 90’s since Zancanaro et al showed resonances attributable to triglycerides and conducted a comparison with similar fractions in white adipose tissue\textsuperscript{117}. However, the hydrophilic fractions of BAT were not assessed by these authors and therefore an investigation of the polar metabolites remains lacking. As a matter of fact, BAT has long been considered as having no relevance in human physiology for several decades because it was not easily discernable and found in spots spread out in different sections of the body.
However, the recent discovery of Nedergaard et al. has now demonstrated its presence and activity in adult humans, with the main depots localised in the supraclavicular and neck regions\textsuperscript{118}. In addition to its role and difference with WAT previously described (Chapter II), investigating BAT is also particularly relevant since this tissue is considered as a new target to fight against obesity. Indeed, Cypess et al demonstrated in 2009 that humans have functionally active BAT and that its amount was inversely correlated with the body mass index (BMI) of these people, especially in older people\textsuperscript{119}. BAT plays a role in adult human metabolism, a finding which has not been further explored and which opens many possibilities for future pharmacological treatments\textsuperscript{119,120}. In addition, BAT and its uncoupling protein UCP1 have been recently linked to the development of obesity in C57Bl6/J mice after genetic ablation of UCP1. One other consequence of this ablation was the full elimination of the diet-induced thermogenesis effect in this strain of mice\textsuperscript{121}.

Interestingly, this tissue was associated also with triglyceride clearance from blood as BAT plays a regulator role for triglyceride-rich lipoproteins clearance and for the control of blood lipid abundance\textsuperscript{122}. Therefore, BAT could have the potential for reducing or eliminating the risks of metabolic syndrome development\textsuperscript{123}. In this context, a $^1\text{H}$ NMR metabolic profiling approach was applied in order to investigate the influence of GF state and gender on energy metabolism in urine, plasma, liver and BAT of C3H mice.
2. Material and Methods

2.1. Animal handling

All animal studies were carried out under the Swiss legislation on animal experimentation and appropriate national guidelines at the Nestle Research Centre (Lausanne, Switzerland). GF C3H females (Charles River, France) were mated in isolators. At birth, half of the breed (10 males and 10 females) were transferred to a conventional environment in order to start acclimatisation and colonisation of their gut by microbial species. All animals were fed with the standard chow diet A03 (produced by the company SAFE, for Scientific Animal Food and Engineering, and described in the appendix II). At 8 week old, spot urine was collected by massaging the urine bladder and the abdomen. Simultaneously, animals were weighed and in vivo EchoMRI (Magnetic Resonance Imaging, Echo Medical Systems, Houston, Texas) calibrated with canola oil was recorded to measure the total body fat content (TBFC). Measurement was repeated twice for each individual mouse and the average was taken as the final value of total body fat content (TBFC). Mice were then euthanized by decapitation to collect intrascapular BAT. Samples were snap frozen in liquid nitrogen and stored at -80°C until analysis.

2.2. $^1$H NMR acquisition of biofluids and BAT extracts

25 µL of urine samples were diluted into 35 µL of a deuterated phosphate buffer solution at pH 7.4 (0.2M NaH$_2$PO$_4$, Na$_2$HPO$_4$, 0.05% of sodium 3-trimethylsilyl-1-[2,2,3,3-$^2$H$_4$] propionate (TSP), 70% D$_2$O) before transferring to 1.7mm diameter capillary tubes for $^1$H NMR acquisition. Apolar metabolites were extracted from BAT using a binary mix of chloroform/methanol (1:1). This protocol has been established based on previous work from Folch and Bligh and Dyer on lipid extraction from biological matrices. The organic phase was washed with an equivalent volume of water to clean the lipophilic phase and extract the hydrophilic compounds. After 5 minutes of centrifugation at 13000 rpm, the supernatant, containing the hydrophilic phase, was freeze-dried and resuspended in 550µL of D$_2$O while the dried residue of the organic phase was resuspended in 600µL of CDCl$_3$ prior to analysis by NMR spectroscopy.

$^1$H NMR spectra of biofluids and BAT were acquired on a 600 MHz Bruker Avance spectrometer operating at 600.13 MHz and a constant temperature of 300 K
using a standard 1D pulse sequence (recycle delay (RD)-90°-t1-90°-tm-90°-acquire FID) with water suppression applied during RD of 2 s and mixing time (tm) of 100 ms and a 90° pulse set at 10.50 µs. Spectra were acquired using 256 scans for urine and BAT hydrophilic extracts and 128 scans for BAT lipophilic extracts into 32 k data points with a spectral width of 12 000 Hz. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line broadening before applying Fourier transformation.

2.3. High resolution magic angle spinning $^1$H NMR spectroscopy of liver

$^1$H NMR spectra of intact liver samples were acquired on a 600 MHz Bruker spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 600.13 MHz by high resolution magic angle spinning technique and using a Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence with water pre-saturation. FIDs were collected into 32 K data points using 128 scans with a spectral width of 12000 Hz and were multiplied by an exponential function corresponding to 1 Hz line broadening.

2.4. Data analysis

2.4.1. Data pre-processing

Spectra were processed using TOPSPIN 2.0 software package (Bruker Biospin, Rheinstetten, Deutschland). All spectra were manually phased, baseline corrected and calibrated to TSP ($\delta$ 0.00) for urine and BAT hydrophilic extracts, to chloroform ($\delta$ 7.26) for BAT lipophilic extracts and to glucose ($\delta$ 5.223) for plasma and liver samples. The region between $\delta$ 0.5-9 was imported into Matlab software (Version 7.5, The Mathworks Inc., Natwick, MA) for statistical analysis.

For urine samples, resonances corresponding to water and urea signals ($\delta$ 4.6-5.9) were removed before dataset analysis. For BAT extracts, methanol, water and chloroform resonances were also removed before analysis of the dataset ($\delta$ 2.3-2.4, $\delta$ 4.6-5.2 and $\delta$ 7-7.5 respectively). All $^1$H NMR spectra were aligned using an in-house algorithm\textsuperscript{128}. As described before,\textsuperscript{129} all data were analysed with 32k data points and normalised to the total peak area (urinary and hydrophilic extracts of BAT) or using the probabilistic quotient (plasma, liver and lipophilic extracts of BAT)\textsuperscript{130}.
2.4.2. Multivariate statistics

Models were constructed using PLS and O-PLS-DA using unit variance (UV) scaling in Simca-P +11.5 software (Umetrics, Umea, Sweden) and Matlab 7.0.1 software (The Mathworks, Inc.) using an in-house algorithm. The robustness of the models was characterized using the model parameters detailed previously (chapter II): $Q^2_Y$, $R^2_Y$ and $R^2_X$. The validity of the models against overfitting has been tested by computing cross-validation parameters, and all models have been validated using permutation tests (perm = 1000).

In order to help the interpretation, the cross-validated scores plots (Tcv) and O-PLS-DA coefficients plots were used as detailed before (chapter II) and according to the method described by Trygg et al.97,98.
3. Results

The measurement of the total body weight (BW) and total body fat content (TBFC), and the acquisition of $^1$H NMR metabolic profiles on urine, plasma, liver and BAT of C3H mice were performed at 8 weeks.

3.1. Physiological data

To determine the effects of GF state and gender on BW and TBFC of C3H mice, 8-week old GF and conventional mice were weighed before measurement of TBFC by MRS spectroscopy (EchoMRI$^\text{TM}$). These measurements have been performed at the Nestle Research Centre and I have analysed the corresponding data. Females were significantly lighter than males in both groups (Figure 14.A, p values < 0.05), and to the same extent, as they displayed 18% less BW in the conventional group and 22% less BW in the GF group. Furthermore, both male and female GF mice were significantly lighter compared to their conventional counterpart (Figure 14.A, p values < 0.05) and again to the same extent, as they displayed respectively 13% and 17% less BW than conventional animals.

The TBFC was then assessed in both groups (Figure 14.B). Conventional females had a lower amount of fat (11%) compared to conventional males, and GF males had a significantly lower TBFC compared to conventional animals as they displayed 21% less of fat (p value < 0.05). These results matched with previous findings showing that C57Bl6/J conventional animals contain 42% more total body fat when compared to their GF counterpart despite an inferior daily consumption of a chow diet of 29%. Interestingly, no sexual dimorphism in the TBFC was observed in the GF group, and no significant difference in body fat content was observed between conventional and GF females.

Finally, a larger dispersion of the TBFC values was observed in the conventional group when compared to the GF animals. Indeed, in conventional mice, the range of TBFC extended from 8% to 22% whereas the difference was less important in GF animals with values varying from 10% to 14% (Figure 14.B and supplemental table 2). This observation suggests that GF animals present a better TBFC homogeneity and that could be due to a fat mass development which is more homogenous in GF animals due to the absence of gut microorganisms whereas this fat formation is more heterogeneous and GM-dependent in conventional animals.
Figure 14: Sexual dimorphism on body weight (A) and Total Body Fat Content (TBFC, B) in female (yellow open box-plot) and male (green box-plot) conventional and germ-free mice. (n=10/group).
To increase understanding of this TBFC difference specific to male animals, an investigation was performed to determine whether it was due to the effect of one factor or to the interaction between the two factors, the gender and the microbial status of C3H mice. Therefore, an analysis of variance (ANOVA) was performed to test a potential interaction between both of these factors. ANOVA is a generation of statistical models useful to determine the factors that have a significant influence, in this case on BW and TBFC. An analysis of variance organises and directs the analysis, allowing easier interpretation of results.

**Table 2:** Effect of gender and microbial status on body weight and TBFC in C3H mice: summary of ANOVA

<table>
<thead>
<tr>
<th></th>
<th>Body weight</th>
<th>Pr(&gt;F)</th>
<th>TBFC</th>
<th>Pr(&gt;F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td>2.099e-10 ***</td>
<td></td>
<td>0.230805 (N.S)</td>
</tr>
<tr>
<td>Colonisation</td>
<td></td>
<td>2.550e-07 ***</td>
<td></td>
<td>0.004911 **</td>
</tr>
<tr>
<td>Interaction</td>
<td></td>
<td>0.9021</td>
<td></td>
<td>0.307050</td>
</tr>
</tbody>
</table>

Student’s t-test: * p<0.05; ** p<0.01; *** p<0.001, N.S. = Non Significant.

The results generated by these analyses of variance showed that (table 2):

- The gender and microbial status have both an impact on the BW of C3H mice as the ANOVA displayed a p < 0.05. However, these factors do not interact together as the p value obtained (p = 0.9) was superior at the level of significance generally considered for an ANOVA analysis (0.05).
- The colonisation effect is the only significant factor that has an effect on the TBFC (p value < 0.001). In addition, gender and microbial status do not interact significantly on the TBFC (p value = 0.3).

### 3.2. Characterisation of urinary metabolic profiles and statistical analysis

Urine collected from the 4 different groups have been characterised by $^1$H NMR spectroscopy and the assignments of the metabolites were achieved by the use of spectral databases and literature$^{26,131,132}$.

Spectra from urine samples contain prominent resonances from metabolites involved in numerous major metabolic pathways. Indeed, the spectra are dominated by a variety of resonances from organic acids such as acetate, citrate and hippurate,
and further metabolites including taurine, aliphatic amines such as methylamine (MMA), dimethylamine (DMA), trimethylamine (TMA) and trimethylamine-N-oxide (TMAO). Other metabolites such as creatine, creatinine, succinate and amino acids (i.e. alanine, arginine and lysine) were also assigned in mouse urine from conventional and GF animals.

In order to visualise the similarities and dissimilarities within the data set, a PCA model using 4 principal components was carried out. The scores plot of the first two components is presented below as they generated the biggest variations in the data set (Figure 15).

![PCA scores plot derived from $^1$H NMR urinary spectra from urine of conventional males ■, germ-free males □, conventional females ● and germ-free females ○).](image)

The largest source of variation was driven by gender as the biggest discrimination was observed on PC1, which was responsible for 22% of the total variation, between male animals in blue and female animals in red. Then, the second component PC2, responsible for 14% of the total variation, did not allow the visualisation of any discrimination between the 4 groups (Figure 15). 3 samples in the conventional group and 2 samples in the GF group were not collected. In
addition, two major outliers (one GF male and one GF female) were observed due to their higher dilution factor and therefore biased the model, even after a normalisation step. They were both removed before any further analysis.

In order to decipher the metabolic variations in the urinary metabolic profiles associated to gender and microbial status, an O-PLS-DA model using three predictive components and one orthogonal component was carried out on the total set of individuals. This model has been validated by permutation multiple testing (perm = 1000, p value < 0.001) and its robustness was satisfactory as described by the model parameters: $R^2_X = 0.26$, $R^2_Y = 0.56$ and $Q^2_Y = 0.44$. 
Figure 16: 3D and 2D O-PLS-DA scores plots derived from $^1$H NMR spectra from urine of conventional males ■, germ-free males □, conventional females ● and germ-free females ○.

The strongest variation was observed between male animals in blue and female animals in red according to the 1st component (Tcv1). Another segregation was observed between GF (open symbols) and conventional animals (closed symbols) according to the 2nd component (Tcv2). Finally, the third component isolated again GF (open symbols) from conventional animals (Tcv3).
A good discrimination was observed between the 4 groups according to the projection of the cross-validated scores on the three first components (Figure 16.A). The biggest variation was dominated by the gender effect as the largest discrimination was observed between male animals in blue and female animals in red according to the 1st component (Tcv1, Figures 16.B and C). Another segregation was observed between GF (open symbols) and conventional animals (closed symbols) according to the 2nd component (Tcv2, Figures 16.B and D). Finally, the third component isolated again GF animals from conventional animals (Tcv3, Figures 16.C and D).

Pairwise models defined as two by two comparisons were then generated to simplify data interpretation, and their associated loadings plots have been summarised in Table 3 (correlation coefficients are displayed). The variations in metabolites listed in columns 3 and 4 were attributed to gender dimorphism as conventional females and males were compared in the column 3, and GF females and males were compared in the column 4. Based on the same approach, variations in metabolites defined in columns 5 and 6 were attributed to microbial status as GF and conventional males were compared in column 5, and GF and conventional females were compared in column 6. Finally, discriminant metabolites that are common to more than two columns could be attributed to a putative metabolic interplay between the gender and microbial status of these mice.
<table>
<thead>
<tr>
<th>Metabolites</th>
<th>δ^1H (p.p.m.)</th>
<th>(+) F Conv vs M Conv (-)</th>
<th>(+) F GF vs M GF (-)</th>
<th>(+) M GF vs M Conv (-)</th>
<th>(+) F GF vs F Conv (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-HCA</td>
<td>7.34</td>
<td>- 0.90</td>
<td>- 0.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-HPPA sulfate</td>
<td>6.92</td>
<td>- 0.91</td>
<td>- 0.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAG</td>
<td>7.38</td>
<td>- 0.82</td>
<td>- 0.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-ketoisocaproate</td>
<td>2.19</td>
<td>- 0.72</td>
<td>- 0.77</td>
<td>+ 0.82</td>
<td>+ 0.79</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.01</td>
<td>- 0.92</td>
<td>- 0.80</td>
<td>+ 0.73</td>
<td></td>
</tr>
<tr>
<td>Isovaleric acid</td>
<td>2.03</td>
<td>- 0.85</td>
<td>+ 0.71</td>
<td>+ 0.86</td>
<td></td>
</tr>
<tr>
<td>Putrescine</td>
<td>1.80</td>
<td>- 0.85</td>
<td>- 0.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R^2X</td>
<td>0.40</td>
<td>0.35</td>
<td>0.48</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>R^2Y</td>
<td>0.94</td>
<td>0.93</td>
<td>0.96</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Q^2Y</td>
<td>0.79</td>
<td>0.47</td>
<td>0.67</td>
<td>0.73</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3**: Summary of variations of metabolite signals in urine samples.

*Correlation coefficients with the highest discriminant axis for the metabolites involved in the difference between the positive group (+) and the negative group (-) are shown.*

**Gut microbiota affected a urinary marker of mitochondrial activity**

Putrescine (δ 1.80 and 3.05) was observed in higher concentrations in the urinary profile of male mice compared to females in both groups. The sulfate-conjugate of m-hydroxyphenylpropionic acid (m-HPPA sulfate, δ 2.51, 2.90, 6.92 and 7.22), 3-hydroxycinnamate (δ 6.92, 7.02 and 7.33), which both result from the metabolism of polyphenols (i.e. chlorogenic compounds) into bioavailable compounds by the gut microorganisms, and phenylacetylglucose (δ 3.65 and 7.35) were observed in higher concentrations in the urinary profile of conventional mice compared to GF mice while the level of isovaleric acid (δ 0.92 and 2.03), a marker of mitochondrial activity, was higher in GF animals (Table 3 and Figure 17). For instance, this metabolite plays a role in a disease called isovaleric acidemia, which results in the isovaleric acid accumulation in urine and serum due to a deficiency of the specific mitochondrial enzyme responsible for the dehydrogenation of isovaleryl-CoA\textsuperscript{133}. 

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85
Gut microbial urinary co-metabolites are affected by an interplay between microbial status and gender

Analysis of O-PLS-DA loadings plots derived from $^1$H NMR metabolic profiles of urine showed that several differences might be attributed to an interplay between the microbial status (GF or conventional) and the gender (female or male) as shown in Table 3. Indeed, 2-ketoisocaproate (2-kic, δ 0.94, 2.18 and 2.64) and isoleucine (δ 0.95 and 1.26) were significantly more concentrated in urine of male mice compared to female animals (Table 3 and Figure 17). In addition, 2-kic was found in higher concentration in urine of GF animals compared to their conventional counterpart whereas isoleucine was found only in higher concentrations in urine of GF females compared to conventional females. These variations were not only specific to the gender or microbial status of C3H mice and revealed the interplay between microorganisms and mouse gender (Table 3 and Figure 17).
Figure 17: Concentration differences (A) between males (yellow) and females (green) mice and (B) between GF (red) and conventional (black) mice derived from integration of $^1$H NMR spectra from urine samples of C3H mice. Mean peak intensities +/- SEM, Student’s t-test: * p < 0.05; ** p < 0.01; *** p < 0.001.
Regressions against the body weight and the total body fat content of C3H mice

In order to investigate the relationship between mice TBFC and BW and their metabolic phenotypes, regression of the $^1$H NMR metabolic urinary profile was performed against BW and TBFC. $^1$H NMR urinary profiles were weakly correlated to the BW ($Q_2^Y/R_2^Y = 0.5$) and even less correlated to the TBFC ($Q_2^Y/R_2^Y = 0.18$), and will not be further discussed.

3.3. Characterisation of Brown Adipose Tissue metabolic profiles

3.3.1. Lipophilic extracts

3.3.1.1. $^1$H NMR spectroscopy

A typical example of a $^1$H NMR spectrum obtained from the lipophilic extract of BAT from an 8 week old male mouse is shown in figure 18. Assignments of the metabolites were achieved based on the literature$^{100,101,131,132}$. Spectra of the lipophilic fraction were clearly dominated by the resonances assigned to fatty acid acyl chains of different lengths (C2, C4, C6, C8, etc) and glycerol backbone belonging to unsaturated and polyunsaturated triglyceride acyl. There are also free, mono and polyunsaturated fatty acids, carbonyls, aldehydes and fatty acids (Figure 18).
Figure 18: $^1$H NMR spectrum (600MHz) of a lipophilic extract from a brown adipose tissue sample of a conventional mouse.

3.3.1.2. Statistical analysis

In order to visualise the similarities and dissimilarities among BAT lipophilic extracts, a PCA model using 4 principal components was carried out. Scores plot of the first two components is presented below as they generated the biggest variations in the data set (Figure 19).
Figure 19: PCA scores plot derived from $^1$H NMR spectra of BAT lipophilic extracts from conventional males ■, germ-free males □, conventional females ● and germ-free females ○.

The PCA scores plot displayed above highlighted a correct discrimination between GF animals (open symbols) and conventional males (closed blue symbols) according to the PC1 which generated 21% of the total variation. Again, the same discrimination was observed on the PC2 which generated 15% of the total variation of the model. Nevertheless, no discrimination was observed on the 2 first components between conventional females (closed red symbols) and the three other groups as conventional females were widely distributed (Figure 19).

Then, pairwise comparisons have been generated to reveal the variations of metabolites involved in the discrimination between groups, which have been summarised in the table 4 below.
3.3.2. Aqueous extracts

3.3.2.1. $^1$H NMR spectroscopy

Interestingly, this thesis gives the first attempt to characterise $^1$H NMR profiles of BAT hydrophilic extracts. Indeed, BAT lipophilic extracts have been characterised before and their similarity with white adipose tissue has been studied.$^{117}$ Nevertheless, hydrophilic extracts of BAT have not been characterised to date. Two examples of $^1$H NMR spectra obtained from the hydrophilic extract of BAT from 8 week old conventional and GF male mice are shown in figures 20 and 21, respectively. The spectra are dominated by the resonances of amino acids (alanine, glutamine, histidine, leucine, isoleucine, phenylalanine, tyrosine and valine), organic acids (($D$)-3-hydroxybutyrate, acetate, lactate), steroid derivatives, essential compounds of the membranes (choline and its phosphodervatives phosphocholine and glycerophosphocholine) and compounds related to energy metabolism (glycerol, creatine and creatinine)$^{131, 132}$. 

<table>
<thead>
<tr>
<th>Chemical functions / Resonances</th>
<th>F Conv (+) vs M Conv (-)</th>
<th>F GF (+) vs M GF (-)</th>
<th>F GF (+) vs F Conv (-)</th>
<th>M GF (+) vs M Conv (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$(CH_3)_n$ (0.995 to 1.01 ppm)</td>
<td>+ 0.54</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(CH_3)_n$ (1.01 to 1.02 ppm)</td>
<td>+ 0.58</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$(CH_2)n$ (1.2 to 1.4 ppm)</td>
<td></td>
<td>- 0.41</td>
<td>- 0.58</td>
<td></td>
</tr>
<tr>
<td>$CH_2-CH=CH$ (2.03 to 2.10 ppm)</td>
<td></td>
<td>+ 0.43</td>
<td>+ 0.68</td>
<td></td>
</tr>
<tr>
<td>$CH_2-CO$ (2.37 to 2.395 ppm)</td>
<td>+ 0.60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$CH=CH-CH_2-CH=CH$ (2.74 to 2.785 ppm)</td>
<td>(Q$^2$Y &lt; 0)</td>
<td>+ 0.71</td>
<td>3.27 to 3.35 ppm</td>
<td>- 0.56</td>
</tr>
<tr>
<td>3.69 to 3.76 ppm</td>
<td>- 0.60</td>
<td>- 0.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$R^2X$</td>
<td>0.64</td>
<td>0.56</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>$R^2Y$</td>
<td>0.51</td>
<td>0.74</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>$Q^2Y$</td>
<td>0.32</td>
<td>0.54</td>
<td>0.64</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Summary of variations of metabolite signals in brown adipose tissue lipophilic extracts, with the highest discriminant power for each model (correlation values are displayed, N.S.: Non Significant).
Figure 20: $^1$H NMR spectra of a BAT hydrophilic fraction of a conventional male mouse: 0.7 - 4.5 ppm (A) and 5 – 9 ppm (B). The region between 5 and 9 ppm has been vertically scaled up 20 times.
Figure 21: $^1$H NMR spectra of a BAT hydrophilic fraction of a germ-free male mouse: 0.7 - 4.5 ppm (A) and 5 – 9 ppm (B). The region between 5 and 9 ppm has been vertically scaled up 20 times.
Interestingly, it is noteworthy that no glucose was observed in the hydrophilic extracts of BAT. This is consistent with previous findings showing that glucose enters brown adipocytes after insulin or norepinephrine activation which allow the translocation of glucose transporters from the intracellular storage to plasma membrane\textsuperscript{134}. In addition, (\textit{D})-3-hydroxybutyrate and myo-inositol were observed in the $^1$H NMR spectrum of GF animal but not in the conventional counterpart. These results will be discussed later in the discussion chapter.

### 3.3.2.2. Statistical analysis

In order to visualise the intrinsic similarities and dissimilarities among the hydrophilic extracts of BAT, a PCA model using 4 principal components was carried out on all groups (Figure 22).

![Figure 22: PCA scores plot derived from $^1$H NMR spectra of BAT hydrophilic extracts from conventional males ■, germ-free males □, conventional females ● and germ-free females ○.](image)

Scores plot of components 2 and 3 were displayed above as the 1\textsuperscript{st} component did not show a good discrimination between all groups for unknown reasons. A good discrimination was observed between GF (open symbols) and conventional animals (closed blue symbols) on the second component PC2, which
explained 10% of total variation. Another segregation was observed on PC3 between 4 conventional males and the other samples. This may be due to a lower amount of metabolites extracted during the sample preparation, but not to a higher dilution factor as all samples have been prepared following exactly the same procedure. Nevertheless, these 4 samples have not been excluded as they were not clearly outliers on the first two components. It is noteworthy that conventional females (red closed symbols) were widely distributed as observed previously in BAT lipophilic extracts (Figure 22).

Then, pairwise comparisons were generated to reveal the variations of metabolites involved in the discrimination between groups (coefficients plots are displayed), and were summarised in the table 5.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>d^1H (p.p.m.)</th>
<th>(+) F Conv vs M Conv (-)</th>
<th>(+) F GF vs M GF (-)</th>
<th>(+) M GF vs M Conv (-)</th>
<th>(+) F GF vs F Conv (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(D)-3-hydroxybutyrate</td>
<td>1.21</td>
<td>+ 0.65</td>
<td>+ 0.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.31</td>
<td>+ 0.73</td>
<td>+ 0.70</td>
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Table 5: Summary of variations of metabolite signals in BAT hydrophilic extract samples. 
*Correlation coefficients with the highest discriminant axis for the metabolites involved in the difference between the positive group (+) and the negative group (-) are shown.*
Effect of sexual dimorphism and gut microorganisms on BAT metabotypes

Analysis of the loadings plots from hydrophilic extracts of BAT showed metabolite variations in response to the microbial status. GF animals presented higher levels of myo-inositol ($\delta 3.60$), ($D$)-3-hydroxybutyrate ($\delta 1.21$ and $2.31$) and glutamate ($\delta 2.35$) and lower relative levels of lactate ($\delta 1.33$) when compared to conventional animals (Table 5 and Figure 23). Gender-based variation was also observed since amino acids such as glutamate and lysine were more concentrated in conventional females compared to conventional males (Table 5). In contrast, no sexual dimorphism was observed in GF animals as there was no difference between male and female GF animals. Finally, higher levels of aspartate, choline, phosphocholine and glycerophosphocholine were observed in GF males compared to conventional males (Table 5 and Figure 23). Interestingly, these variations were not visible in female mice, which might be due to a specific interplay between the mouse microbial status and gender.

![Graph showing concentration differences between GF (red) and conventional (black) mice derived from integration of $^1$H NMR spectra from BAT samples of C3H mice. Mean peak intensities +/- SEM, Student’s t-test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.](image-url)
3.3.3. $^1$H NMR metabolic profiles of BAT predict BW and TBFC

To investigate the relationship between mice TBFC and BW and the rodent metabolic phenotypes, PLS regressions of the $^1$H NMR metabolic profiles of hydrophilic and lipophilic extracts of BAT were performed against BW or TBFC (Figures 24 and 25).

Figure 24: Cross-validated scores derived from the O-PLS regression of $^1$H NMR spectra obtained from lipophilic extracts of BAT against the total BW (A) and the TBFC (B) of conventional and germ-free C3H mice (conventional males ■, germ-free males □, conventional females ○ and germ-free females ●).
As observed in figures 24.A and 25.A, the Y-axis defined as the total BW clearly discriminated conventional males, which had the highest body weight, from GF males and conventional females, having an intermediate body weight, and from GF females having the lowest body weight. Regressions of lipophilic and hydrophilic extracts versus the total BW were validated by permutation tests (perm = 1000) and the following parameters: $R^2_X=0.56$; $Q^2_Y=0.42$; $R^2_Y=0.58$ and $R^2_X=0.47$; $Q^2_Y=0.39$; $R^2_Y=0.51$, for lipophilic and hydrophilic extracts, respectively. The projection of the cross-validated scores (Tcv) obtained from the O-PLS regression of the lipophilic profiles against the BW of these mice highlighted an excellent separation between the conventional (closed symbols) and GF animals (open symbols, Figure 24.A). The projection of the Tcv resulting from the O-PLS regression of the hydrophilic extracts against the total BW of these mice discriminated clearly the conventional males (blue closed symbols) from the 3 other groups (Figure 25.A). Based on the pairwise comparison, this discrimination was associated with higher levels of lactate and glycerol in conventional males and ($D$)-3-hydroxybutyrate in GF males (Figure 26.A).

A similar modelling approach was applied to TBFC. Both models presented in figures 24.B and 25.B were validated by permutation tests (perm = 1000) and by the following parameters: $R^2_X=0.15$; $Q^2_Y=0.40$; $R^2_Y=0.68$ and $R^2_X=0.23$; $Q^2_Y=0.32$; $R^2_Y=0.58$, for lipophilic and hydrophilic extracts, respectively. As observed in figures 24.B and 25.B, the Y-axis, defined as the TBFC, highlighted an overlapping between the conventional females, GF males and females. However, conventional male animals displayed a higher amount of TBFC compared to the 3 other groups as observed previously in figure 15.B. In addition, the projection of the Tcv obtained from the O-PLS regression of the lipophilic extract regressed against the TBFC of these mice highlighted a good separation between conventional males and their GF counterpart (Figure 24.B). In contrast, no discrimination was observed between female animals because of a high dispersion of conventional female Tcv. As observed with total BW, the projection of the Tcv obtained from the regression of hydrophilic extracts against the TBFC discriminated the conventional and GF mice (Figure 25.B).
Figure 25: O-PLS regression of $^1$H NMR spectra obtained from hydrophilic extracts of BAT against the total BW (A) and the TBFC (B) of conventional and germ-free C3H mice (conventional males □, germ-free males □, conventional females ●, and germ-free females ○).
Based on the pairwise comparison, this discrimination was associated with higher levels of lactate, glycerol and lysine in conventional males and higher levels of (D)-3-hydroxybutyrate in GF males (Figure 26).

**Figure 26:** O-PLS-DA loadings plot derived from the regression of $^1$H NMR spectra from BAT hydrophilic extracts of conventional and GF males against the BW (A) and the TBFC (B) of C3H mice.
To summarise, $^1$H NMR metabolic profiles of BAT successfully reflected the variation in BW and TBFC in this rodent model. Individuals with the highest TBFC and BW presented higher levels of glycerol and lactate and lower levels of (D)-3-hydroxybutyrate.

Altogether these results showed that components of the $^1$H NMR metabolic profiles of BAT (lipophilic and hydrophilic extracts) from conventional and GF animals are correlated to the total BW and TBFC of C3H mice. This finding clearly indicates that $^1$H NMR metabolic profiling of BAT predicts the total BW and TBFC of this strain of mice.

3.4. Hepatic and plasma concentration of (D)-3-hydroxybutyrate is affected in GF mice

In order to decipher whether the increase of the ketone body (D)-3-hydroxybutyrate was a local response to the GF state or a systemic pattern, the plasma and hepatic metabolic profiles were also investigated. These metabotypes revealed a significant increase of (D)-3-hydroxybutyrate in both compartments in GF animals compared to their conventional counterpart (Figures 27 and 28).

![Figure 27: Hepatic (D)-3-hydroxybutyrate concentration derived from $^1$H NMR relative quantification to the total spectral area in conventional (Conv) and germ-free (GF) animals. Mean peak intensities +/- SEM are plotted, p value = 0.001.](image)
In GF plasma profiles, this increase of ketone body was concomitant to a remarkable decrease of VLDL but not of HDL, as shown by Figure 28. Altogether, these results demonstrate the systemic increase of \((D)-3\)-hydroxybutyrate in GF animals.

**Figure 28:** Partial \(^1\)H NMR plasma metabolic profile derived from conventional (black) and GF (red) animals. HDL: High Density Lipoprotein; VLDL: Very Low Density Lipoprotein.
4. Discussion

The aim of this study was to characterise the effect of the GM on the energy metabolism of C3H mice by assessing their metabolic profiles in urine, plasma, liver and BAT in response to their GF status. In addition, I aimed to investigate the relationship existing between the BAT metabolic profiles and the BW and TBFC of these mice assessed at 8 weeks to determine whether they were reflected by BAT metabolic profiles.

4.1. Sexual dimorphism and gut microbiota impact on the urinary and BAT 

\(^1\)H NMR metabolic profiles

In the present study, the aim was to characterise the impact of sexual dimorphism on urinary and BAT metabolic profiles. Sexual dimorphism is known to play a crucial role in the understanding and prediction of gender-based responses in the host. It has been shown that the gender background severely impacts the host responses to pharmaceutical treatment or even disease development and their study has helped to characterise their relevance as models of toxicity and disease\(^{135,136}\). For instance, Stanley et al. showed in urine of rats that male animals are metabolically more active than female with prolonged pharmaceutical activity for several drugs\(^{135}\). Moreover, they indicated that sexual dimorphism in rat plasma was attributed to higher levels of plasma lipoproteins in males compared to their female counterparts\(^{135}\).

In the present study, putrescine - known to be a microbial co-metabolite derived from the GM - was observed in higher levels in urine of male animals when compared to females. Nevertheless, it is likely that the differential levels of putrescine observed in this study were due to an increased synthesis in males by the prostate gland through a testosterone-dependant mechanism as previously observed in rats\(^{137}\). In addition, a higher excretion level of \(m\)-HPPA sulfate was observed in urine of conventional mice which is concordant with previous publications as this metabolite has already been reported in lower concentrations in C3H GF mice\(^{26}\). \(M\)-HPPA sulfate is a bioavailable compound obtained from the catabolism of polyphenols and chlorogenic acids by the gut microbes. Here, the variations of \(m\)-HPPA sulfate reflect the crucial role of the GM in the production of metabolites from the digestion of dietary nutrients\(^{26}\).
4.2. Brown adipose tissue in the context of obesity and metabolic disorders

Based on the BAT metabolic profiles, choline, phosphocholine and glycerophosphocholine were observed in higher levels in GF males when compared to their conventional counterpart (Table 4). These metabolites are known to be involved into the formation and maintenance of cellular membranes through structural lipids. Choline is an essential constituent of cell membranes and phosphocholine and glycerophosphocholine are storage forms of choline in the cytosol where they also act as osmoprotectants. This difference may be explained by a variation of brown adipocytes size between GF and conventional male animals. Indeed, conventional animals are fatter than GF animals and displayed a higher lipid storage capacity. This is likely to be linked to the higher size and a lower number of cells in conventional BAT compared to GF adipocytes and explained the higher levels of choline, phosphocholine and glycerophosphocholine in hydrophilic extracts of GF animals when compared to their conventional counterpart.

In addition, a strong correlation was established between $^1$H NMR metabolic profiles of BAT and both BW and TBFC of C3H mice. This finding therefore highlighted a link between BAT metabolism and its involvement in weight management and metabolic disorders, and complemented recent results rehabilitating the role of BAT in adult humans. As explained before, BAT has long been considered as having no relevance in humans. Nevertheless, recent publications demonstrated that this tissue plays a central role in obesity and metabolic disorders. Moreover, it has been demonstrated that BAT controls triglyceride clearance in mice as approximately 50% of ingested triglycerides are taken up by this tissue, whilst the muscle takes up about 30%, the liver 15% and the WAT 5%. Moreover, BAT is involved in the disposal of glucose in obese mice exposed to the cold, since approximately 75% of the ingested glucose are taken up by this tissue, whereas the muscle takes up about 10%, the liver 8% and WAT, brain and heart equally take up the remaining 7%. Therefore, the ability of the BAT metabolic profiles to predict the BW and TBFC of C3H mice reinforces the emerging interaction of this tissue with obesity and metabolic disorders.
4.3. The gut microbiome affect the regulation of β-oxidation in the BAT

Hydrophilic extracts of BAT revealed higher levels of (D)-3-hydroxybutyrate and lower levels of lactate in GF animals compared to their conventional counterpart (Table 4). This information was complemented by a strong elevation of (D)-3-hydroxybutyrate in plasma and liver of GF animals compared to conventional animals (Figures 27 and 28). (D)-3-hydroxybutyrate is a ketone body produced in the mitochondria of adipocytes and hepatocytes initiated by the condensation of 2 molecules of acetyl-coA derived from the β-oxidation of lipids (Figure 29). This metabolite is well-known to play a central role in the host energy homeostasis as it acts on noradrenaline receptor to inhibit BAT thermogenesis and regulate appetite\textsuperscript{140-142}. In fact, its appetite suppressant effect has been reported twenty years ago\textsuperscript{142}. In 1998, Canas et al. demonstrated that norenadrenaline-induced thermogenesis was inhibited by (D)-3-hydroxybutyrate in lean rats, but not in obese rats\textsuperscript{141}. Finally, lactate derives, in peripheral tissues, from the conversion of pyruvate produced by glycolysis. The retro-conversion of lactate to pyruvate is not possible in brown adipocytes as the redox potential of this reaction is favourable only in the liver (Figure 29)\textsuperscript{143}. Lactate will thus travel to the liver where it will form glucose through the gluconeogenic pathway in the fasting state. Interestingly, adipose tissue is a major site of glucose conversion to lactate and adipose mass is known to be higher in obesity. Therefore, lactate overproduction could be associated to metabolic abnormalities that are related to obesity development\textsuperscript{144}.

It has been demonstrated that the regulation of the β-oxidation in the BAT is under the control of a norepinephrine-dependent mechanism. Indeed, lipolysis and fatty acids release are norepinephrine-induced in brown adipocytes and it has been demonstrated previously that the free FA are then transferred into the mitochondria where they are β-oxidised\textsuperscript{116}. Norepinephrine is a neurotransmitter which plays a central role in the BAT as it induces stimulation of the thermogenesis in brown adipocytes including β-oxidation of the fatty acids\textsuperscript{145,146}. Both TCA cycle and β-oxidation occurring in mitochondria generate FADH and NADH which are ultimately oxidised by the respiratory chain (Figure 29)\textsuperscript{116}. This results in a pumping out of protons from the mitochondria towards its double membrane generating a gradient of $H^+$ that drives the $H^+$ back into the mitochondria through UCP1. UCP1 is functionally the equivalent of an $H^+$ transporter but is uncoupled from the production of ATP. This
uncoupling protein is specific to brown fat where it plays an essential role in the tremendous heat-generating capacity of the tissue\textsuperscript{116}.

Several factors can influence the activation of lipid $\beta$-oxidation in mammals. As previously described, $\beta$-oxidation in the BAT is primarily induced by cold. Since all animals’ cages were housed in a temperature controlled husbandry, the variation in room temperature is unlikely to be the reason for this discrepancy. Therefore, the metabolic differences previously described are expected to be related to the lack of GM in GF animals. In conventional animals, GM produce short chain fatty acids (SCFA) (i.e. acetate, butyrate and propionate) derived from the fermentation of carbohydrates\textsuperscript{59-61}. Dietary carbohydrates such as resistant starches and dietary fibers, defined as the non digestible plant foods, are ideal substrates for the fermentation process in the gut leading to the production of SCFA\textsuperscript{61}. Whereas butyrate is a preferred energy source for colonic epithelial cells\textsuperscript{59,60}, propionate is mainly processed by hepatocytes in the liver as a substrate for gluconeogenesis and acetate, which is also endogenously produced, reaches the general circulation and enters peripheral tissues such as the brown adipocytes (Figure 29). In addition, acetate and propionate have been reported as inhibitors of lipolysis and stimulators of lipogenesis in both adipocytes (Figure 29) and hepatocytes\textsuperscript{147,148}. It has also been reported that acetate has similar hepatic effects. Moreover, its entrance into peripheral tissues has major metabolic consequences as it inhibits lipolysis \textit{in vivo} and \textit{in vitro}\textsuperscript{148}. Therefore, the main hypothesis is that the metabolic disruptions observed in the GF host are the consequence of the missing production of SCFA by the GM. This results in the stimulation of lipolysis activity (i.e. $\beta$-oxidation) in both the liver and the BAT (increased (D)-3-hydroxybutyrate production) and in the hepatic inhibition of lipogenesis (decreased circulating VLDL levels, Figure 28). It is noteworthy that the levels of plasma HDL were not affected, which indicates that the reverse cholesterol transport was not influenced by the absence of the GM in the GF host.
Figure 29: Impact of the absence of GM on the regulation of β-oxidation in BAT.

Key: FA: Fatty Acid; SCFA: Short Chain Fatty Acid; TCA: Tricarboxylic Acid; VLDL: Very Low Density Lipoprotein.
5. Conclusion

This study confirmed that metabonomics is a well-suited approach to characterise the metabolic profiles of C3H mice and to correlate these data with physiological parameters such as BW and TBFC. This project provided new insights for characterizing the energy metabolism of the host and highlighted the future role of BAT in the understanding of these mechanisms. It has been demonstrated that the GM abolished the sexual dimorphism observed for TBFC. In addition, $^1$H NMR metabolic profiles of BAT from conventional and GF animals were correlated to the total BW and TBFC of C3H mice and were therefore excellent predictors of BW and TBFC in the C3H mouse strain. The lack of GM resulted in a higher systemic level of (D)-3-hydroxybutyrate and lower levels of circulating VLDL. These results indicated that the absence of GM stimulated lipolysis while it inhibited hepatic lipogenesis (Figure 29).

It has been demonstrated in this study that the GM and the host metabolism interact differently according to the mouse gender, with a direct and measurable impact on the urinary, plasma, hepatic and BAT metabolotypes of these animals. The importance of studying the metabolism of BAT has been recently enhanced and appears as a key possibility in terms of developing pharmaceutical treatments. In the context of improving the knowledge about host-microbiome interaction and its link with energy metabolism, BAT could play a significant role for future research and the understanding of mechanisms and development of treatments against obesity.

The next chapters will aim at improving the understanding of the interaction between the host and the GM in the particular context of GF animals colonised by different types of gut microbial species. The metabolic signatures of colonisation with known microbiota (inoculation with several defined bacteria forming a simplified flora) or complex microbiota (inoculation with mouse or human GM) have been investigated and are reported in the following chapters (Chapters IV, V and VI).
The 3 following projects aim at characterising and completing what is already known about the interaction between the gut microbiota and the host metabolism and at assessing the role of specific intestinal microbial species in the development of obesity. The project BAMBEE, an acronym used for Bile Acids Modulation for Boosting Energy Expenditure, is based on an approach involving the modulation of intestinal microbiota in mouse models (Figure 30).

The first study included the test of a completely defined mixture of bacteria selected on specific criteria (chapter III: material and methods) called the Nestle Simplified Flora (NSF). The best inoculation procedure was implemented based on the results obtained with several pilot studies. The optimum procedure was further used to perform a flora modulation experiment for testing the impact of individual bacterial strains on host weight management and metabolism in mice colonised with the NSF.

Then, two different studies were designed to investigate the impact of the origin of the inocula (mouse, lean human and obese human) on the global host metabolism. Indeed, in a first project, the impact of C57Bl6/J mouse inoculation with lean or obese human intestinal microbiota has been investigated on the physiological and metabolic status of the host. Then, in a follow-up study, the impact of the timepoint of colonisation (early-life versus post-weaning) and the origin of the colonised microbial species (mouse versus human) on the physiology, immunology and metabolism of C3H mice was investigated.

**Figure 30:** Overview of the BAMBEE project.
Chapter IV  Impact of individual bacterial strains on host weight management and metabolism in mice colonised with a completely defined microbiota

1. Introduction

The quest for the perfect mouse model to study the relationships between mammals and their bacterial inhabitants, at the laboratory scale, has been a technical adventure for decades. The search for an animal model capable of mimicking the conventional state, i.e. keeping the essential microbial functionality, but at the same time remaining a simple system to work with has been the subject of several research groups around the globe. These models are crucial to determine how animal-bacteria symbioses and bacteria-bacteria interactions operate. Notwithstanding the differences in the gut microbial makeup in animals, substantial knowledge has been accumulated to date indicating a close association between the intestinal bacteria and a variety of functions: gut bacteria have been reported to shape the systemic immune system$^{149}$ and mucosal immune response$^{150}$, modulate several morphological and physiological aspects of the intestine$^{151}$, induce metabolic activity of mucosal epithelial cells$^{152}$, ferment unused energy substrates$^{153,154}$, prevent the growth of harmful, pathogenic bacteria$^{155,156}$, produce vitamins and hormones$^{6,7}$, and metabolise diet carcinogens and pro-carcinogens$^{157}$.

Human flora-associated animals have been used as a means to mimic more closely the interactions in the human gut. Several models have been developed, and it is now possible to find a wide variety of systems reflecting either the adult or infant microbiota in mice, rats or pigs with different levels of complexity$^{27,158-162}$. Unfortunately, most studies lack a detailed description of the microbial composition (especially in the more complex models), and often fail to describe how they compare to the original faecal inoculum, or how accurately they represent the human microbiome$^{29}$. Two recent reports have addressed this caveat$^{163,164}$.

With the advent of the genomic era, the understanding of ecological systems like that of the gut has been expanded through a modern holistic approach that
incorporates a variety of “omics” techniques (metagenomic, transcriptomic, proteomic and metabolomic). These approaches can provide valuable information about the contribution of the microbial symbionts to the host metabolic phenotypes. In this context, metabonomics will enable measurement of metabolites in order to assess physiological changes which take place within a specific biological system, i.e. GF mice inoculated with a mixture of bacteria. These approaches have been already used in several animal studies to characterise host-microbial interactions contributing to a better understanding of this complex ecological niche. Ultimately, a model consisting of a few bacterial strains whose genome has been sequenced provides a simple yet suitable basis for this holistic approach. Knowledge of bacterial genomes is of particular interest since they could help to develop new powerful therapeutic approaches to fight against infectious diseases. When selecting the bacterial strains, however, one has to be wary of the interactions between them that can potentially impair the establishment of a stable microbiota, affect the metabolism of the host and jeopardize the accuracy of the model.

The purpose of the present study was (1) to evaluate different methods for obtaining a stable mouse model of simplified human microbiota using culturable bacteria, readily available from culture repositories, and of known genome, that reflects as closely as possible the dominant commensal bacterial makeup found in adult human faeces; and (2) to determine the impact of different procedures of inoculation, leading to various profiles of bacterial establishment in the gut, on the urinary and plasma metabolite profiles of the host.
2. Material and methods

2.1. Animals husbanding and bacteria selection

All animal studies were carried out under the Swiss legislation on animal experimentation and appropriate national guidelines at the Nestle Research Centre (Lausanne, Switzerland) using protocols approved by the Federal Veterinary Office. GF C3H mice were purchased from Charles River laboratories (l'Abresle, France). 6- to 8-week-old mice were housed in flexible film GF isolators and fed autoclaved standard rodent chow diet with free access to sterilised water. After one week of acclimatisation, mice were randomised by weight and by litter and then transferred to gnotobiotic isolators where the intervention studies took place.

All bacterial strains were of human intestinal origin and were selected to mimic proportion of bacterial phyla found in healthy human faeces: Bacteroides thetaiotaomicron DSM 2079, Bacteroides vulgatus DSM 1447, Bifidobacterium longum NCC 2705, Clostridium scindens DSM 5676, Collinsella aerofaciens DSM 3979, Escherichia coli HS, Eubacterium ventriosum DSM 3988, Faecalibacterium prausnitzii DSM 17677, Lactobacillus rhamnosus NCC 4007 and Ruminococcus hansenii DSM 20583 (Table 6). They have been chosen because they are all commensal to humans, are prominent within the gut intestinal tract and mimic human adult distal gut microbiota, are non pathogens, their sequences are fully available and they mimic the common distribution of the dominant groups found in human faeces.
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**Table 6**: Bacterial strains used for mouse inoculation with their respective growth media and primers used for real-time PCR enumeration.

\(^a\) primers targeting strain-specific genomic sequences located outside of 16S rRNA gene regions; \(^b\) ref 62; \(^c\) ref 63; \(^d\) enriched Brain Heart Infusion medium; \(^e\) Yeast extract Casitone Fatty Acids medium; \(^f\) MRS medium; \(^g\) LB, Luria Broth.
2.2. Study design

This study was designed in order to obtain the best suitable inoculation procedure for the animals (Figure 31). Indeed, a frozen bacterial cocktail was thawed and given intragastrically to 5 mice, placed in isolator A, at four different times during the 1st week, at days 0, 2, 5 and 7. In isolator B, a mixture containing the 10 bacterial strains was given to 5 mice twice during the 1st week and then a mixture of the strains that did not persist after one week was given intragastrically on week 2 (bacteria were quantified in faeces at the end of week 1 to determine those which did not persist). Then, a mixture of the bacterial strains that did not persist according to the preliminary study i.e. NCC 2705, DSM 5676, DSM 3979, DSM 3988, DSM 17677, NCC 4007 and DSM 20583 was given twice during the 1st week to 5 mice in isolators C, followed by gavage on week 2 with strains DSM 2079, DSM 1447 and HS and those that did not persist according to faecal levels measured at the end of week 1. Finally, in isolator D (5 male mice) and E (5 female mice), each animal received a single strain of the mixture twice during the 1st week, and then all animals in each isolator were housed together in the same isolator, on week 2, in order to stabilise the gut flora ecosystem though natural behaviour such as coprophagy (Figure 31). For all the isolators, fresh faecal samples were collected from individual animals every 3 days and weight; food and water intakes were measured throughout the study. On week 8, spot collections of urine were made at two different time-points during the day and animals were euthanized without fasting under isoflurane anaesthesia to collect blood and bile. Biofluids were collected from individual animals and quickly stored at -80°C until further use for metabonomic purpose.
Figure 31: Procedure of the inoculation experiment.

Five groups of six-week-old C3H GF male (groups A-D) and female (group E) mice were distributed into 5 GF isolators where they were housed in individual cages. Mixed bacterial suspensions were inoculated by intragastric gavage at several time-points between days 0 and 14 into mice from groups A to C. Mice from groups D and E were inoculated at days 0 and 2 with pure single bacterial suspensions (1 strain per mouse) and were housed together at day 14 to allow inter-individual microbiota transfer.

2.3. Preparation of bacterial suspensions and quantification of bacteria

Cultures of individual bacterial strains were synchronized in order to obtain early stationary phase cultures on the day of inoculation. 50 ml cultures were synchronised in order to obtain the appropriate biomass of each strain to prepare the mixtures. Cultures were centrifuged at 10 000 × g during 10 min at 4 °C, and pellets were resuspended in 5 ml of sterile Ringer solution (Oxoid, Basel, Switzerland). After reading absorbance and assessing bacterial morphology under the optical microscope, 500 µl of each suspension were collected to prepare the cocktails (final volume 5 ml). A sample of these suspensions was used to quantify the actual bacterial load. The cocktails were finally aliquoted in individual tubes, one for each animal. However, some modifications were introduced depending on the study protocol: in some cases, the cocktail was frozen at -80 °C and thawed and aliquoted into individual tubes (one for each animal) the day of the experiment; alternatively, the cocktail was prepared containing some of the 10 strains; or simply, a single-strain suspension was prepared for each animal. Doses of 300 µl of either bacterial
inoculum, corresponding to a total of approximately 10^9-10^{10} cells, were administered to each animal using sterile stainless-steel stomach tubes.

DNA was extracted from 1 ml of bacterial culture pellets and approximately 50 mg of faeces and caecal content samples using the EZ1 DNA Tissue kit in combination with the BioRobot EZ1 workstation (Qiagen, Switzerland). Briefly, samples were suspended in 400 µL buffer G2 containing 50 mg mL^{-1} of lysozyme (Sigma, Switzerland) and 0.3 g of 106 microns glass beads (Sigma, Switzerland). Homogenization was performed in a Bead Beater (Biospec, USA) for 1 min at maximal speed. Homogenates were incubated for 15 min at 37°C and then centrifuged for 5 min at 10,000 × g. Supernatants were transferred to a new tube and further purified. Real-time PCR analyses were performed with an ABI 7700 Sequence Detection System (Applied Biosystems). Reaction mixtures were performed in a final volume of 25 µL containing 12.5 µL of SynsiMixPlus SYBR 2X mastermix (Bioline, UK), 300 nM of forward and reverse primers and 1 µl of extract. PCR cycling conditions consisted of 94°C for 5 min and 40 cycles of 94°C for 20 s, 55°C for 20 s and 72°C for 50 s. A melting curve analysis was done after amplification. Standard curves were made by plotting C_t values as a function of serial dilutions of base 10 log-transformed amounts of purified genomic DNA from each strain. The number of equivalent genome copies in each sample was determined by comparing the C_t values to the standard curve.

2.4. NMR acquisition parameters

25 µL of urine samples and 25 µL of plasma samples were diluted into 25 µL of a deuterated phosphate buffer solution at pH 7.4 (0.2M NaH_2PO_4, Na_2HPO_4, 0.05% TSP, 70% D_2O) before transferring to capillary tubes for ^1H NMR acquisition. For urine samples, ^1H NMR spectra were acquired on a 600MHz Bruker Avance spectrometer operating at 600.13 MHz and at a constant temperature of 300K using a standard 1D pulse sequence (recycle delay (RD)-90°-t1-90°-t_m-90°-acquire free induction delay (FID)) with water suppression applied during RD of 2s and mixing time (t_m) of 100 ms and a 90° pulse set at 10 µs. For plasma samples, ^1H NMR spectra were acquired on a 600MHz Bruker Avance spectrometer operating at 600.13 MHz and a constant temperature of 300K using two different pulse sequences with a common pulse set at 10.50µs:
- First, a standard pulse sequence was used with water suppression identical to the one detailed above for the urine samples in order to acquire a global spectrum of detectable metabolites. Spectra were obtained with a relaxation delay of 2.0 s and a mixing time tm of 100ms.

- Then, a Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence with water suppression was used to remove the resonance caused by metabolites with a high molecular weight (mainly lipids and lipoproteins resonances). Spectra were measured using a spin-echo loop time \(2\pi t\) of 19.2 ms, a \(d_{20}\) of 0.0004, a number of loops \(L_4\) of 100, and a relaxation delay of 2s. All spectra were acquired using 256 scans into 32k data points with a spectral width of 12 000 Hz. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line broadening before applying Fourier transformation.

2.5. Data analysis

Spectra were processed with TOPSPIN 2.0 software package (Bruker Biospin, Rheinstetten, Deutschland). All spectra were manually phased, baseline corrected and calibrated to TSP \(\delta 0.00\) for urine and the \(\text{CH}_3\) group of lactate \(\delta 1.33\) for plasma. The region between \(\delta 0.5-9\) was imported into Matlab software (Version 7.5, The Mathworks Inc., Natwick, MA).

Resonances corresponding to water and urea signals \(\delta 4.6-5.9\) for urine samples and to water \(\delta 4.6-5\) for plasma samples were removed before dataset analysis. As described before, the spectral data were digitised into 32k data points and normalised to the total peak area for urine and CPMG spectra of plasma samples, and on the background noise for the standard 1D acquisition of plasma samples. Models were constructed using O-PLS-DA using unit variance (UV) scaling on Simca-P +11.5 software (Umetrics, Umea, Sweden) and Matlab 7.0.1 software (The Mathworks, Inc.). The robustness of the models was characterised using the model parameters: \(Q^2_Y\), cross-validated predicted percentage of the response; \(R^2_Y\), predicted percentage of the response; \(R^2_X\), variation of \(X\) explained by the model, and all models were validated using permutation tests \(\text{perm} = 1000\).

In order to help the interpretation, the cross-validated scores plots \(T_{cv}\) were used to visualise the relationship between samples in order to reveal clustering. In addition, O-PLS-DA coefficients plot highlighting the variables involved in the
discrimination of the classes was calculated according to the method described by Trygg et al.\textsuperscript{97} and explained in the previous chapter.
3. Results

3.1. Optimisation of the inoculation procedure to obtain a simplified gnotobiotic mouse model

Based on a 1st experiment, ten intestinal bacterial strains of human origin were inoculated by oral gavage to 14 GF mice housed in sterile isolators. Only 3 bacterial strains (Escherichia coli HS, Bacteroides vulgatus DSM1447, and Bacteroides thetaiotaomicron DSM2079) successfully colonized the mice until the end of the study (Figure 32).

**Figure 32:** Colonization pattern in mice from the 1st inoculation experiment.

Individual bacterial strains were quantified by real-time PCR in faeces collected between days 5 and 42 post-inoculation and values are expressed as \( \log_{10} \) (cells) g\(^{-1}\) of faeces. Dots represent bacterial counts in individual mice.

Considering the low success of bacterial colonisation observed in the first experiment, a second experiment was designed to test alternative methods of inoculation that would lead to improved colonization efficiency (see Material and methods, 2.2).

Animals from group A received the mixed bacterial suspensions prepared from frozen stocks during the first two weeks. Although initially all strains were widely detected in faeces, only 3 strains persisted in all 5 mice at the end of the study (Figure 33). Notably, the detected strains were the same as those that successfully colonized mice in the first experiment (Figure 32, i.e. Escherichia coli HS, Bacteroides vulgates DSM1447, and Bacteroides thetaiotaomicron DSM2079).
Figure 33: Colonization pattern in mice from groups A.

Individual bacterial strains were quantified by real-time PCR in feces collected between days 3 and 70 post-inoculation and values are expressed as $\log_{10}(\text{cells})\ g^{-1}\$ of feces. Dots represent bacterial counts in individual mice. Mean values are shown as lines between dots. The detection limit is approximately $10^6\ \text{cells}\ g^{-1}$.

Similar results were observed among the animals in group B, which were inoculated twice during the first week with mixed fresh culture suspensions, following the same protocol as for the first experiment. Animals in group C were inoculated twice during the first week with a bacterial mix prepared from fresh cultures without the 3 strains showing high persistence in the first experiment. This protocol was tested in order to favor colonization by less performing bacterial strains. The 3 strains that colonized mice in the first experiment were only given to the mice 2 weeks after the initial gavage. Although a considerable length of time was allowed before inoculating these 3 “strong” strains, it was impossible to recover any of the initial 7 strains after day 23. The resulting profile both in caecal contents and feces was similar to that obtained in groups A and B.

Animals in groups D and E (D, males; E, females) were inoculated twice during the first week with single-strain bacterial suspensions and on day 14 they were mixed with other mice from the same group to allow cross-transmission of
bacterial strains. Both groups exhibited similar colonization patterns. Results from group D mice are summarized in Figure 34. 

**Figure 34:** Colonization pattern in mice from groups D.

Individual bacterial strains were quantified by real-time PCR in feces collected between days 3 and 70 post-inoculation and values are expressed as $\log_{10}(\text{cells}) \, \text{g}^{-1}$ of feces. Dots represent bacterial counts in individual mice. Mean values are shown as lines between dots. The detection limit is approximately $10^6$ cells g$^{-1}$.

Before allowing all the animals to be housed together (day 14), some strains were not detected in feces of the corresponding animals. However, almost all of the strains were systematically detected in all of the animals a few days after the animals were housed together, except for the strain *Faecalibacterium prausnitzii* DSM17677. In group E, the strain *Collinsella aerofaciens* DSM3979 was also not detected. At the end of the experiment (day 70), fecal bacteria counts ranged from $10^8$ to $10^{11}$ cells g$^{-1}$.

On day 56, three GF mice were introduced in the cages containing mice from groups D and E. One week after being in contact with their gnotobiotic counterparts, newly added mice adopted the full set of bacteria, which corresponded respectively to 8 bacteria (group E) and 9 bacteria (group D, Figure 35). These results indicated that bacterial strains colonized the new animals more easily after they got
established in the gut of the donors. Fecal bacterial counts of these receiver mice were comparable to those from the donors.

![Graph](image)

**Figure 35:** Transfer of the simplified microbiota from colonized mice to GF mice. Individual bacterial strains were quantified by real-time PCR in feces collected at days 2, 7 and 14 after transfer of the GF mice and values are expressed as $\log_{10} (\text{cells} \cdot g^{-1})$. Numbers in parenthesis indicate day post-inoculation of group D mice. Dots represent values from individual mice. Mean values are shown as lines between dots. The detection limit is approximately $10^6$ cells g$^{-1}$.

**3.2. Physiological data**

Inoculated C3H mice were weighed at regular intervals from day -1 to day 70 and corresponding body weights are displayed in Figure 36. Interestingly, male mice exhibited systematic significant higher body weight compared to female mice from day -1 to day 70 (p values < 0.001). It is notable that, before inoculation of human faecal preparations to GF mice, significant differences were already observed between males and females. No significant differences of body weight were observed between male mice harbouring 3 bacteria and those harbouring 9 bacteria (Figure 36).
**Figure 36:** Bar plots representing the body weight of male mice harbouring 3 bacteria (groups A, B and C: light blue, dark blue and green, respectively), male mice harbouring 9 bacteria (group D, black) and female mice harbouring 8 bacteria (group E, red) from day -7 to day 42.

Mean peak intensities +/- SEM, Student’s t-test: * p<0.05; ** p<0.01; *** p<0.001. Since multiple comparisons were performed, corresponding p-values were corrected using Bonferroni’s corrections.
3.3. Urinary metabolic profiles

3.3.1. $^1$H NMR spectroscopy

Examples of $^1$H NMR urine spectra obtained from 12 week old inoculated mice are shown in Figures 37 and 38: A. male mouse with 9 bacteria; B. male mouse with 3 bacteria; C. female mouse with 8 bacteria established in its gut. Urine spectra contain a variety of resonances from organic acids such as acetate, butyrate, citrate, hippurate and keto-isovalerate, and further metabolites including taurine, aliphatic amines such as methylamine (MMA), dimethylamine (DMA, trimethylamine (TMA) and trimethylamine-$N$-oxide (TMAO). Other metabolites such as creatine, creatinine, succinate, phenylacetylglycine and amino acids (alanine, arginine and lysine) were also assigned in urine of C3H mice (Figures 37 and 38, respectively).
**Figure 37**: Partial $^1$H NMR urine spectra (600 MHz) of A) a male mouse harbouring 9 bacteria, B) a male mouse harbouring 3 bacteria and C) a female mouse harbouring 8 bacteria. Aliphatic region (0.5-4.5 ppm) is displayed.
Figure 38: Partial $^1$H NMR urine spectra (600 MHz) of A) a male mouse harbouring 9 bacteria, B) a male mouse harbouring 3 bacteria and C) a female mouse harbouring 8 bacteria. Aromatic region (5-9 ppm) has been vertically expanded 4 times.
3.3.2. Statistical analysis

First, a PCA model using 6 components was generated to visualise the variation in the dataset and identify potential outliers as well as similarities and dissimilarities within the dataset. The projection of the 2 first components was plotted below as they generated the maximum of inter-individual variability (Figure 39). Interestingly, a good discrimination was observed between males and females on the PC1 (29.3% of the global variation) whereas the second principal component (PC2) did not allow to discriminate the groups. PCs 3, 4, 5 and 6 are not shown as they did not further enable visualisation of any discrimination between the five groups. Finally, the groups with 3 bacteria established in the gut were overlapped even though different inoculation procedures were used (groups A, B and C). This may indicate that their urinary metabolic profiles were similar, but this needs to be confirmed by a deeper analysis using a supervised statistical approach (O-PLS-DA model).

Figure 39: PCA scores plot derived from $^1$H NMR spectra from urine of colonised mice with 3 bacteria (groups A, B and C), 9 bacteria (group D) and 8 bacteria established in the gut (group E).
In order to determine the metabolic signatures associated with each specific profile of bacteria in the gut, a supervised statistical approach was used. Firstly, pairwise models were generated in order to compare each pair of groups within the 5 groups. Interestingly, no discrimination was observed between groups A, B and C (table 7) and confirmed therefore the hypothesis generated according to the PCA scores plot that inoculation methods used for groups A,B and C had little effect on the urinary metabolite profile. The mice with 3 bacteria established in their gut presented similar urinary metabolic profiles independently to the inoculation procedure used to colonise them.

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Number of components</th>
<th>$Q^2$</th>
<th>$R^2$</th>
<th>$R^2_X$</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine ABC vs D</td>
<td>1 PC, 1 OC</td>
<td>0.86</td>
<td>0.93</td>
<td>0.37</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Urine D vs E</td>
<td>1 PC, 1 OC</td>
<td>0.89</td>
<td>0.93</td>
<td>0.45</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

**Table 7:** Summary of O-PLS-DA $Q^2$, $R^2_Y$ and $R^2_X$ values corresponding to pairwise model comparisons between male mice with 3 bacteria (groups A,B and C), male mice with 9 bacteria (group D) and female mice with 8 bacteria established in the gut (group E) in urine.

*Keys: PC: Predictive Component; OC: Orthogonal Component.*

Groups A, B and C were then considered as one group for supervised statistical analysis. In order to characterise the impact of the microbial diversity on urinary metabolic profiles, an O-PLS-DA model was carried out using 2 predictive components and 1 orthogonal component (Figure 40). The robustness of this model was confirmed by the following parameters: $Q^2 = 0.77$, $R^2_Y = 0.85$, $R^2_X = 0.43$ and validated using permutation tests (p value < 0.001, perm = 1000).
Figure 40: O-PLS-DA scores plot derived from $^1$H NMR spectra from urine of colonised mice with 3 bacteria (groups A, B and C), 9 bacteria (group D) and 8 bacteria established in the gut (group E).

The visualisation of the cross-validated scores plot on the first component (Tcv 1) indicated variation due to gender dimorphism as it mainly discriminated female mice harbouring 8 bacteria from male animals harbouring 9 bacteria (Figure 40). Then, the second component (Tcv 2) displayed a variation corresponding to the gut microbiota biodiversity as it highlighted a good discrimination between males with 3 bacteria (groups A, B and C) from both males with 9 bacteria and female mice with 8 bacteria established in the gut (Figure 40: groups D and E, respectively).

Impact of gender differences on metabolism of C3H mice

First, an O-PLS-DA comparison was generated between $^1$H NMR metabolic profiles of urine from females harbouring 8 bacteria and males harbouring 9 bacteria. This model was validated using permutation tests ($p$ value < 0.001, perm = 1000) and its robustness was confirmed by the following parameters: $Q^2_Y = 0.89; R^2_Y = 0.93; R^2_X = 0.45$. 

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Analysis of the loadings plot highlighted higher levels of butyrate (δ 0.90 and 2.18), trimethylamine (δ 2.885) and several amino acids such as lysine (δ 1.50) and methionine (δ 2.14) as well as hydroxy-isovalerate (δ 1.21) and N- and O-acetylglycoproteins (δ 2.04 and δ 2.06 respectively) in male mice colonised with 9 bacteria (Figure 41, at the bottom) compared to female mice (Figure 41, at the top). Higher levels of 2-oxo-isocaproate (δ 0.94 and 2.61), trimethylamine-N-oxide (TMAO, δ 3.27) and two unassigned metabolites (δ 3.599 and δ 3.952, respectively) were observed in female mice when compared to male mice (Figure 41). Combination of data from literature, databases such as HMDB and BMRB, in-house standards, 2D NMR experiments (TOCSY, COSY and HSQC) on selected samples and STOCY were used to attempt to identify these two metabolites, without success.

**Impact of gut microbiota biodiversity on metabolism of C3H mice**

An O-PLS-DA comparison was generated between $^1$H NMR metabolic profiles of urine from male mice harbouring 3 bacteria and male mice harbouring 9 bacteria (Figure 42). This model was validated by permutation test (perm =1000, p value < 0.001) and its robustness was confirmed by the following parameters: $Q^2_Y=0.86$; $R^2_Y=0.93$ and $R^2_X=0.37$. 

**Figure 41**: O-PLS-DA loadings plot derived from $^1$H NMR spectra from urine of male mice with 9 bacteria (group D, at the bottom) and female mice with 8 bacteria established in the gut (group E, at the top).
**Figure 42**: O-PLS-DA loadings plot derived from $^1$H NMR spectra from urine of male mice with 3 bacteria (groups A, B and C, at the bottom) and male mice with 9 bacteria established in the gut (group D, at the top).

A: 0.5 - 4.2 ppm.
B: zoom on the aromatic region (6-9 ppm) and scaled up 10 times.

Analysis of the O-PLS-DA loadings plot presented in Figure 42 reflected various metabolic signatures according to the number of bacteria which did colonise the mouse gut. Indeed, a higher level of dimethylamine ($\delta$ 2.726), creatinine ($\delta$ 3.04) and an unassigned metabolite ($\delta$ 6.96) was observed in male mice with 3 bacteria (Figure 42: groups A, B and C, at the bottom) compared to male mice with 9 bacteria established in the gut (Figure 42: group D, at the top). Conversely, a higher level of
formate ($\delta$ 8.46) and two amino acids, glutamate ($\delta$ 1.494) and lysine ($\delta$ 1.604), was observed in the group D when compared to groups A, B and C (Figure 42).

3.4. Plasma metabolic profiles

3.4.1. $^1$H NMR spectroscopy

Examples of $^1$H NMR plasma spectra obtained from a 12 week old mice with 9 bacteria established in the gut are shown in Figure 43 (standard 1D pulse sequence acquisition) and (CPMG acquisition). Plasma standard 1D acquisition displays mainly lipid resonances corresponding to saturated and unsaturated compounds as well as lipoproteins such as Very-Low-Density-Lipoproteins (VLDL) like and Low-Density-Lipoproteins (LDL) like, and resonances from sugars such as $\alpha$- and $\beta$-glucose (Figure 43.A).

Other metabolites in lower concentrations such as acetate, lactate, pyruvate, and amino-acids (alanine, glutamine, leucine, isoleucine, and valine) as well as choline and energy compounds such as creatine were also assigned in mouse plasma in the CPMG spectra (Figure 43.B).
Figure 43: $^1$H NMR plasma spectra (600 MHz) of a male mouse with 9 bacteria established in the gut: A) Standard 1D acquisition and B) CPMG acquisition.
3.4.2. Statistical analysis

To determine the metabolic signatures in plasma associated to the gut microbiota biodiversity, pairwise models were generated between the 5 groups. Similarly to urinary results, no discrimination was observed between groups A, B and C, characterised by 3 bacteria established in the gut. Therefore, they were considered as only one group for further statistical analyses (Table 8).

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Number of components</th>
<th>$Q^2_Y$</th>
<th>$R^2_Y$</th>
<th>$R^2_X$</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma: Standard 1D ABC vs D</td>
<td>1 PC 1 OC</td>
<td>0.56</td>
<td>0.74</td>
<td>0.47</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Plasma: Standard 1D D vs E</td>
<td>1 PC 1 OC</td>
<td>0.70</td>
<td>0.84</td>
<td>0.58</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Plasma: Standard 1D A vs B</td>
<td>-</td>
<td>&lt; 0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A vs C</td>
<td>-</td>
<td>&lt; 0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B vs C</td>
<td>-</td>
<td>&lt; 0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Plasma: Standard 1D ABC vs D</td>
<td>1 PC 1 OC</td>
<td>0.57</td>
<td>0.73</td>
<td>0.16</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Plasma: CPMG D vs E</td>
<td>1 PC 1 OC</td>
<td>0.79</td>
<td>0.96</td>
<td>0.22</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table 8: Summary of O-PLS-DA $Q^2_Y$, $R^2_Y$ and $R^2_X$ values corresponding to plasma pairwise model comparisons between male mice with 3 bacteria (groups A,B and C), male mice with 9 bacteria (group D) and female mice with 8 bacteria established in the gut (group E).


Then, two O-PLS-DA models were built using 2 predictive components and 1 orthogonal component and the robustness of these models was confirmed by the following parameters, $Q^2_Y = 0.56$, $R^2_Y = 0.74$, $R^2_X = 0.47$ (standard 1D acquisition) and $Q^2_Y = 0.57$, $R^2_Y = 0.73$, $R^2_X = 0.16$ (CPMG acquisition), and validated using permutation tests (p values < 0.001, perm = 1000). Based on standard 1D
acquisition (figure 44.A), two main sources of variation were observed: gender differences and gut microbiota biodiversity. Indeed, the visualisation of the cross-validated scores plot on the first component (Tcv 1) displayed a variation due to the common effect of gender differences and 1 missing bacterium as it discriminated females harbouring 8 bacteria from male animals harbouring 9 bacteria. Then, the second component (Tcv 2) displayed a variation corresponding to the gut microbiota biodiversity as it highlighted a good discrimination between males harbouring 3 bacteria (groups A, B and C) from males harbouring 9 bacteria (group D, Figure 44.A). Based on CPMG acquisition (Figure 44.B), I have been able to identify readily the sexual dimorphism as the strongest variation in the dataset as the first component (Tcv 1) discriminated male from female animals. Nevertheless, I did not observe a strong discrimination in male animals between those colonised with 3 bacteria and those colonised with 9 bacteria (Figure 44.B).
Figure 44: O-PLS-DA scores plot derived from $^1$H NMR spectra from plasma (A: standard 1D acquisition; B: CPMG acquisition) of colonised mice with 3 bacteria (groups A, B and C), 9 bacteria (group D) and 8 bacteria established in the gut (group E).
Impact of gender-based dimorphism on plasma metabotypes of C3H mice

First, an O-PLS-DA comparison was generated between $^1$H NMR metabolic profiles of plasma (standard 1D acquisition) from female mice harbouring 8 bacteria and male mice harbouring 9 bacteria (Figure 45). This model was validated by permutation test ($p$ value $< 0.001$, perm = 1000) and its robustness was confirmed by the following parameters: $Q^2_Y = 0.70$; $R^2_Y = 0.84$; $R^2_X = 0.58$.

Figure 45: (A) O-PLS-DA loadings plot derived from $^1$H NMR standard 1D spectra from plasma of male mice with 9 bacteria (group D, at the bottom) and female mice with 8 bacteria established in the gut (group E, at the top) and (B) zoom on the unassigned metabolite $^1$H NMR resonance ($\delta$ 4.132) involved in the discrimination between the two groups (males in black, females in red).
Analysis of the O-PLS-DA loadings plot derived from $^1$H NMR standard 1D acquisition of plasma showed higher levels of cholesterol ($\delta$ 0.66), lipids (mainly from Very-Low-Density-Lipoproteins, $\delta$ 0.87 and 1.29, and unsaturated lipids, $\delta$ 5.27 and 5.29), choline ($\delta$ 3.207) and one unassigned metabolite ($\delta$ 4.132) in male mice (Figure 45.A, at the bottom) when compared to their female counterpart (Figure 45.A, at the top). As observed in Figure 45.B above, the unassigned metabolite ($\delta$ 4.132) corresponds to a broad singlet that is much broader than typical small molecule metabolites. One hypothesis may be the presence of a larger molecule, such as a protein, with a proton that has been chemically exchanged, and would typically be a $-\text{NH}$ proton. Complementary experiments performed on a representative sample from a male mouse highlighted the probability of an $-\text{NH}$ proton in exchange with the proton of the HOD since downfield shifts were observed when the acquisition temperature was decreased (Appendix I). Finally, O-PLS-DA loadings plot derived from CPMG spectra highlighted higher levels of butyrate ($\delta$ 0.93) and lower levels of lactate ($\delta$ 1.32 and 4.097), alanine ($\delta$ 1.48) and taurine ($\delta$ 3.27 and 3.42) in the plasma of male mice when compared to their female counterpart and the variations have been summarised in table 9.

**Impact of the gut microbiota biodiversity on plasma metabotypes of C3H mice**

An O-PLS-DA comparison was generated between $^1$H NMR metabolic profiles of plasma (standard 1D acquisition) from male mice harbouring 3 bacteria and male mice harbouring 9 bacteria (Figure 46). This model was validated by permutation test ($p$ value $< 0.001$, perm = 1000) and its robustness was confirmed by the following parameters: $Q^2_Y = 0.56$; $R^2_Y = 0.74$; $R^2_X = 0.47$. 
Analysis of the O-PLS-DA loadings plot derived from $^1$H NMR spectra of plasma (standard 1D acquisition) showed higher levels of lipids (mainly Very-Low-Density-Lipoproteins, VLDL, $\delta$ 0.83, 0.87 and 1.29, and unsaturated lipids, $\delta$ 5.27 and 5.29) and choline ($\delta$ 3.207) in male mice colonised with 3 bacteria (Figure 46, at the bottom) compared to male mice colonised with 9 bacteria (Figure 46, at the top). O-PLS-DA loadings plot derived from CPMG spectra highlighted higher levels of creatine ($\delta$ 3.039) and proline ($\delta$ 3.351 and 4.136) in mice with 9 bacteria compared to mice with 3 bacteria established in the gut. In addition, variations observed in urine and plasma (standard 1D and CPMG acquisitions) are all summarised in the table 9 below and correlation values for each metabolite are displayed.
<table>
<thead>
<tr>
<th>Metabolites</th>
<th>$\delta^1$H (p.p.m.)</th>
<th>(-) 3 strains vs 9 strains (+)</th>
<th>(-) 8 strains vs 9 strains (+)</th>
<th>Biological matrices</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (C$_{18}$, HDL)</td>
<td>0.671 (m)</td>
<td>+ 0.77</td>
<td>P (std)</td>
<td></td>
</tr>
<tr>
<td>Lipids (mainly VLDL)</td>
<td>0.89 (t)</td>
<td>- 0.76</td>
<td>+ 0.88</td>
<td>P (std)</td>
</tr>
<tr>
<td></td>
<td>1.28 (t)</td>
<td>- 0.75</td>
<td>P (std)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.58 (m)</td>
<td>- 0.70</td>
<td>P (std)</td>
<td></td>
</tr>
<tr>
<td>Lipids (CH$_2$-C=C)</td>
<td>2.02 (m)</td>
<td>- 0.75</td>
<td>+ 0.82</td>
<td>P (std)</td>
</tr>
<tr>
<td>Lipids (CH$_2$-CO)</td>
<td>2.24 (m)</td>
<td>- 0.72</td>
<td>P (std)</td>
<td></td>
</tr>
<tr>
<td>Lipids (C=C-CH$_2$-C=C)</td>
<td>2.755 (m)</td>
<td>- 0.73</td>
<td>+ 0.87</td>
<td>P (std)</td>
</tr>
<tr>
<td>Choline</td>
<td>3.207 (s)</td>
<td>- 0.78</td>
<td>+ 0.92</td>
<td>P (std)</td>
</tr>
<tr>
<td>Glyceryl of Lipids (CH$_2$-CO-R)</td>
<td>4.082 (m)</td>
<td>- 0.67</td>
<td>+ 0.79</td>
<td>P (std)</td>
</tr>
<tr>
<td>Choline (Lipids) (O-CH$_2$)</td>
<td>4.29 (m)</td>
<td>- 0.69</td>
<td>P (std)</td>
<td></td>
</tr>
<tr>
<td>Unsaturated lipids (=CH-CH$_2$-CH$_2$)</td>
<td>5.313 (m)</td>
<td>- 0.75</td>
<td>+ 0.87</td>
<td>P (std)</td>
</tr>
<tr>
<td>(=CH-CH$_2$-CH$_2$)</td>
<td>5.33 (m)</td>
<td>- 0.74</td>
<td>P (std)</td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>3.27 (t)</td>
<td>- 0.80</td>
<td>P (cpmg)</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>1.48 (d)</td>
<td>- 0.65</td>
<td>P (cpmg)</td>
<td></td>
</tr>
<tr>
<td>Creatine</td>
<td>3.039 (s)</td>
<td>+ 0.63</td>
<td>P (cpmg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.93 (s)</td>
<td>+ 0.69</td>
<td>P (cpmg)</td>
<td></td>
</tr>
<tr>
<td>Unassigned</td>
<td>4.132 (s)</td>
<td>+ 0.76</td>
<td>P (cpmg)</td>
<td></td>
</tr>
<tr>
<td>n-Butyrate</td>
<td>0.88 (t)</td>
<td>+ 0.72</td>
<td>+ 0.83</td>
<td>U</td>
</tr>
<tr>
<td>2-oxo-isocaproate</td>
<td>0.94 (d)</td>
<td>- 0.75</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>Hydroxy-isovalerate</td>
<td>1.21 (s)</td>
<td>+ 0.76</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>N-acetyl glycoproteins</td>
<td>2.04 (s)</td>
<td>+ 0.85</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>O-acetyl glycoproteins</td>
<td>2.06 (s)</td>
<td>+ 0.89</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>2.89 (s)</td>
<td>+ 0.89</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>Trimethylamine-N-oxide</td>
<td>3.27 (s)</td>
<td>- 0.85</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>1.92 (m)</td>
<td>+ 0.83</td>
<td>+ 0.85</td>
<td>U</td>
</tr>
<tr>
<td>Glutamine</td>
<td>2.144 (d)</td>
<td>+ 0.76</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>Dimethylamine</td>
<td>2.72 (s)</td>
<td>- 0.77</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>Creatinine</td>
<td>3.046 (s)</td>
<td>- 0.77</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>Allantoin</td>
<td>5.39 (s)</td>
<td>- 0.59</td>
<td>- 0.76</td>
<td>U</td>
</tr>
</tbody>
</table>
Table 9: Summary of variations of urinary (U) and plasma (P) metabolite signals obtained by pairwise comparisons between animals harbouring 3 (pooled data from groups A, B and C), 9 (group D) and 8 (group E) bacterial strains. Correlation values are displayed and include highest discriminant power for each model. Results were derived from $^1$H NMR spectra in urine and plasma (standard 1D [std] and CPMG acquisitions).

Key: s: singlet; d: doublet; t: triplet; m: multiplet. $\delta$ $^1$H (p.p.m.): value of chemical shift.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$\delta$ (p.p.m.)</th>
<th>d (p.p.m.)</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formate</td>
<td>8.46</td>
<td>+ 0.71</td>
<td>U</td>
</tr>
<tr>
<td>Unassigned</td>
<td>5.124</td>
<td>+ 0.84</td>
<td>U</td>
</tr>
</tbody>
</table>
4. Discussion

The complex bacterial communities that inhabit the gut are known to have a strong relationship with their host and are classified as mutualistic symbionts\textsuperscript{168}. Their beneficial impact on our physiology spans from protection against pathogenic attack\textsuperscript{169,170} to development of the immune system\textsuperscript{171,172}, and energy and vitamin supply\textsuperscript{5,7,173}, while apparently benefiting themselves by controlling the composition of their microbial environment, rich in nutrients and maintained at a stable temperature. It is not only because of these attributes but also because of their remarkable biodiversity that gut microbiologists have been trying to develop methods to study this symbiotic relationship with man.

4.1. GF animals have competition for colonisation by microorganisms

In this context, a bacterial cocktail was designed based on the criteria detailed previously (see Material and methods section). Because standard methods that have been used for inoculation did not result in a complete colonisation, a \textit{sui generis} approach was used, \textit{i.e.} unique in its characteristics, expecting to achieve high and diverse colonisation levels in our rodent colonies. Each animal was initially gavaged with one different bacterial strain to allow for a successful colonisation in this former GF environment. After a few weeks, all 10 animals were allowed to socialise. Taking advantage of the coprophagic habits of the rodents, the main expectation was to promote a diversified bacterial colonisation in all of the animals. This approach was applied to both male and female animals, contained in separate isolators to avoid territorial issues at the time of housing the animals together. This approach proved to be much more successful than successive inoculations of the whole bacterial mixture (either freshly prepared or from thawed frozen stocks), or a step-wise inoculation of subgroups from the mix. With the exception of one strain in the male animals, and two strains in the females, all other members of the cocktail reached over $10^9$ cells g\textsuperscript{-1} of faeces or caecal contents at the end of this study. In addition, GF animals introduced to these isolators on day 56 of the study, quickly acquired a similar bacterial community to that of the donor animals, suggesting that the transferred microbial community had achieved a significant level of stability and adaptation to the host gut environment.
One can speculate that the intestinal tract of GF animals provide no competition for colonisation by incoming microorganisms, making it relatively easy to deliberately colonise these animals with a few defined microbial species. This belief was challenged in this study, and prompted us to develop this specific inoculation procedure. Indeed, bacteria-bacteria interactions, even in the simplest bacterial consortia, must be considered as part of the equation and may be the reason why it has been often difficult to create and maintain a stable human flora-associated model. In contrast to these results, two recent reports described the development of simplified microbiota models in mice and rats that were obtained by direct inoculation of mixed-strain bacteria suspensions containing fifteen and seven strains, respectively. The discrepancy compared to these studies might be due to differences in the interaction of the bacterial strains composing each simplified microbiota that might lead to varying degrees of selective pressure on less performing strains. It is important to note, however, that other studies were in agreement with our results suggesting that competitive interaction between members of a simplified microbiota might prevent establishment of less performing strains.

4.2. Metabolic profiles as a key approach to monitor the metabolism of mice colonised with individual bacterial strains

In the present study, a stable model was obtained by testing different inoculation procedures and then investigated their specific impact on metabolic profiles of the host. Marked metabolic differences were observed in urine and plasma between mice harbouring 3 and 9 bacteria. Interestingly, the discrimination between the 3 groups of mice was stronger in urine (Figure 40) than in plasma, independently of the acquisition (standard 1D or CPMG acquisition, Figure 44). This confirmed that urine is essential in a metabolic profiling approach as it is the biofluid where hydrophilic metabolites are mainly eliminated through all regulatory processes taking place in the host body. However, the metabolic profiling of faecal samples from these mice could have provided new information about the regulation of the host metabolism. Finally, CPMG acquisitions, based on a specific sequence removing anisotropic interactions which are caused by strong homonuclear dipolar interactions resulting in a broader signal, are used to display very small metabolites frequently not observed in standard 1D acquisition. Therefore, the strongest discrimination observed in the O-PLS-DA model derived from standard 1D compared
to CPMG acquisitions are explained by the importance of lipid and cholesterol resonances which drive the discrimination between males and females, and between males harbouring 3 or 9 bacteria. This result is correlated to the higher body weight observed in male mice (independently of the number of bacteria established in their gut) compared to their female counterpart.

First, a disruption of the lipid metabolism was observed in this strain of mice as male mice with 3 bacteria in the gut displayed significantly higher levels of lipids and lipoproteins, especially Very-Low-Density-Lipoproteins (VLDL) and Low-Density-Lipoproteins (LDL) compared to those colonised with 9 bacteria. These results suggested that increasing microbial diversity is associated with a more efficient regulation of lipoprotein levels and circulating lipids which may have implications for weight management. GF animals, for instance, have lower levels of VLDL compared to conventional animals (Chapter III). Interestingly, variations in plasma lipoproteins levels matched those of SCFA, butyrate, and formate in urine. SCFA are formed by microbial fermentation of dietary polysaccharides, principally into acetate, propionate and butyrate as well as other organic acids such as formate. Interestingly, it has been previously demonstrated that they have beneficial effects on host metabolism. Indeed, some SCFA may help to reduce developments of gastrointestinal disorders, cancer and cardiovascular disease. Butyrate is known for having a trophic function on intestinal epithelial cells, and is considered the preferred source of energy for colonocytes. In addition, based on animal and cell line studies, it has been shown that this metabolite prevented colon cancer and adenoma development. In addition, butyrate added to GF colonocytes rescued their deficit in mitochondrial respiration and prevented them from undergoing autophagy.

Interestingly, the bacterium *Faecalibacterium prausnitzii* DSM 17677 (also called *Fusobacterium prausnitzii*), which failed to establish in the inoculated mice, has been reported as a butyrate producer. Indeed, it has been shown that the ability for producing butyrate is widely distributed among Gram-positive anaerobic bacteria that inhabit the host gut, including *F. prausnitzii*. It is particularly relevant to notice that urine samples from mice harbouring 3 and 9 bacteria displayed butyrate resonances despite the absence of *F. prausnitzii* indicating that other bacteria within the simplest cocktail (*Escherichia coli* HS, *Bacteroides vulgatus* DSM 1447, and *Bacteroides thetaiotaomicron* DSM 2079) are able to produce butyrate. Animals harbouring 9 strains displayed higher levels of butyrate when compared to mice
colonised with 3 strains, suggesting that colonisation with those 9 strains results in a more efficient butyrate production\textsuperscript{59,178,181}. This phenomenon could be attributed to an improvement or gain of function due to the bacteria association and bacteria-bacteria interactions. Interestingly, it has been reported that many butyrate producers from the gut environment can also produce hydrogen and formate, with a direct dependence on the environmental conditions.

Unlike butyrate, the metabolic function of formate has not been well documented and its potential beneficial role is not known to date. Nevertheless, Macfarlane \textit{et al.} (2006) reported that formate was one of the principal products of dietary polysaccharide fermentation by the species \textit{Bifidobacterium breve} under carbohydrate-limited conditions\textsuperscript{59}. This result tends to indicate that the difference of formate production observed in this study could be due to the lower amount of carbohydrates digested by the gut microbial species in mice with 3 bacteria when compared to those with 9 bacteria established in the gut. Nevertheless, this result would have to be complemented in the future by the comparison with formate production in conventional C3H animals. In addition, it has been suggested by Holmes \textit{et al.} that formate could play a role in blood pressure regulation\textsuperscript{182}. Indeed, endogenous formate is the product of one-carbon metabolism\textsuperscript{183} whereas this metabolite could also be produced by fermentation of dietary fibre by the gut microbiota\textsuperscript{184}. These authors demonstrated that formate was inversely associated with both systolic and diastolic blood pressure, suggesting a role in the capacity of regulating blood pressure in humans\textsuperscript{182}. Finally, it is noteworthy that urinary and plasma metabolic profiles of this strain of mice highlighted major perturbations in energy metabolism and amino acids pool regulation. Indeed, variations in the levels of creatine and creatinine was observed – possibly indicating a perturbation of energy metabolism in mice with the simplest microbiota, as well as higher levels of amino acids such as proline, lysine and glutamine in mice colonised with 9 bacteria compared to those containing only 3 bacteria in their gut.

Although the observed differences in metabolite profiles were stronger between males and females than between mice with different microbial diversity, this study showed that high intestinal microbial biodiversity is associated with different metabolic regulation and control than animals harbouring a simpler gut microbial makeup. Higher levels of lipids and lipoproteins were observed in mice harbouring 3 bacteria compared to animals harbouring a more complex microbiota, suggesting
that a simpler gut microbial makeup displayed metabolic signatures that could be associated to the development of obesity, diabetes and metabolic disorders.\textsuperscript{185}

Finally, comparable weight gain was recorded in all of the animals regardless of their microbiota, but animals with low bacterial diversity had larger caeca. Large caecum sizes have been systematically reported for GF or mono-colonised rodents since the first production of a GF mouse in the late 50s.\textsuperscript{186,187} This phenomenon has been associated with the absence of bacterial components stimulating the caecal muscle tone,\textsuperscript{64} and accumulation of secretion of water and electrolytes,\textsuperscript{188} among other causes. The reduction in caecum size associated with increasing microbiota diversity is indicative of a clear difference in functional properties between the two microbiota.
5. Conclusion

The complex bacterial communities inhabiting our guts are considered as our mutualistic symbionts. They impact beneficially on the host physiology at several levels while apparently benefiting themselves by controlling the composition of their microbial environment, rich in nutrients and maintained at a stable temperature. In the present project, a gnotobiotic mouse of human simplified microbiota has been created, maintained and metabolically characterised.

Previous adaptation of bacterial strains to the host’s gut environment (monocolonisation) may predict the successful establishment of a more complex microbiota in the host and will be suitable for further works. Indeed, it could be used to study the improvement of the host-gut microbiota symbiosis, i.e. testing a pre/probiotic given by gavage to mice that have been previously colonised, according to the inoculation procedure described in this project, to determine the potential beneficial impact of this product on the host metabolism (weight gain or loss, fat composition, etc).

This study will also provide new information regarding the development of gnotobiotic models, and studies of microbial biodiversity and associated metabolic regulations and signatures could certainly have broad implications on personalised nutrition and biomedical research. The results obtained here are of further value when considered together with those reported in the two following chapters, which aimed at identifying the impact of complex microbiota, from mouse and human origins, on the metabolism and physiology of the host.
Chapter V Investigation of the impact of mouse colonisation with lean and obese human intestinal microbiota on the physiological parameters and metabolic profiles of C57Bl6/J mice

1. Introduction

Animal studies have demonstrated a well established role for characterising the influence of intestinal microbiota in health and disease\textsuperscript{158,189,190}. Nevertheless, the functions of the human intestinal microbiota remain complicated to characterise. Indeed, in human studies, several factors such as genetic, environmental and dietary conditions, and the number of human volunteers used to make the stock solution for the inoculum are difficult to harmonise making the intestinal microbiota a highly interesting field to study\textsuperscript{190}. In addition, inoculated animals can be exposed to pathogens or toxic substances that cannot be used with human volunteers for evident ethical reasons. Studies in this field have demonstrated that a human intestinal microbiota, once established in the animal gut, could be maintained for a long period and strongly modified the conversion of bile acids and immunological capacity of the host\textsuperscript{189,190}.

It has been previously demonstrated that a human baby flora could establish itself in the host intestinal gut and therefore impact the gut microbiota-host metabolism cross-talk. Indeed, Martin et al showed strong alterations of bile acid metabolism in ileum as well as disruptions of plasma lipid and hepatic triglyceride metabolisms suggesting that the microbiome modulate the absorption, storage and energy harvest from the diet\textsuperscript{27}. Nevertheless, the impact of different origin of human microbiota on the host metabolism has not been investigated to date. Therefore, the aim of this study is to specifically characterise the metabolic phenotypes of GF mice inoculated with two types of human microbiota (5 donors), derived from lean and obese human participants. In parallel, physiological parameters of the host were
recorded, and microbiota composition was characterised by 16S rRNA sequencing. Therefore, by combining this microbial information with host metabolic profiles, this study may provide new insights for understanding the complex relationship existing between the host and its microbial community, and propose mechanistic explanations for microbiota-mediated effects.

2. Material and methods

2.1. Study design

![Study design of the human flora comparison study.](image)

Figure 47: Study design of the human flora comparison study.

15 C57Bl6/J conventional mice and 30 C57Bl6/J GF mice were purchased from Charles Rivers’s laboratories (France). After one week of adaptation, 30 GF mice have been separated into two groups of 15 GF mice (week 0) and inoculated with human flora from lean people (5 different donors) or obese people (5 different donors) at week 1. Urine was collected throughout the study at weeks 0, 2, 4 and 6 whereas plasma and liver were collected at week 6 after sacrifice (Figure 47).

2.2. $^1$H NMR acquisition

2.2.1. Biofluids

Aliquots of 20 µL of urine samples and 30 µL of plasma samples were diluted into 30 µL of a deuterated phosphate buffer solution at pH 7.4 (0.2M NaH$_2$PO$_4$, Na$_2$HPO$_4$, 0.05% of sodium 3-(tri-methylsilyl)-propionate-2,3,-d$_4$ (TSP), 70% D$_2$O) before transferring 50 µL of mixed solution to 1.7mm diameter capillary tubes for $^1$H NMR acquisition.

All biofluid $^1$H NMR spectra were acquired on a 600 MHz Bruker Avance spectrometer operating at 600.13 MHz and at a constant temperature of 300 K using a standard 1D pulse sequence (urine and plasma samples) with water suppression applied during RD of 2 s, mixing time ($t_m$) of 100 ms and a 90° pulse set at 10.50 µs,
as well as using a CPMG acquisition pulse sequence (plasma samples only) as described before (chapter IV). Spectra were acquired using 256 scans for urine and plasma samples into 32 k data points with a spectral width of 12 000 Hz. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line broadening before applying Fourier transformation.

2.2.2. Liver samples

50 mg of each tissue sample was weighed and extracted using a protocol established based on previous work from Folch and Bligh and Dyer\textsuperscript{124-127}, and previously used for extracting BAT (Chapter III).

All \textsuperscript{1}H NMR spectra were acquired using the same approach as previously described (Chapters III and IV). Spectra were acquired using 256 scans for liver hydrophilic extracts and 128 scans for liver lipophilic extracts into 32 k data points with a spectral width of 12 000 Hz. The FIDs were multiplied by an exponential function corresponding to 0.3 Hz line broadening before applying Fourier transformation.

2.3. Data analysis

Spectra were processed using TOPSPIN 2.0 software package (Bruker Biospin, Rheinstetten, Deutschland). All spectra were manually phased, baseline corrected and calibrated to TSP (δ 0.00) for urine and liver hydrophilic extracts and to chloroform (δ 7.26) for liver lipophilic extracts. The region between δ 0.5-9 was imported into Matlab software (Version 7.5, The Mathworks Inc., Natwick, MA) for statistical analysis. Then, urine, plasma and liver data were analysed using the same approach as previously described (Chapters III and IV).

2.4. UPLC-MS analysis

2.4.1. Sample preparation and data analysis

For plasma samples, metabolites were extracted using a standard protocol method described previously\textsuperscript{191}. Cold methanol (3:1) was added to 50 µL of plasma and the solution was placed in freezer for 20 minutes. Then, samples were centrifuged for 10 minutes at 16,000g. 150 µL of supernatant were transferred to microtubes and dried down using Speedvac for 30 minutes at 45°C. After
resuspension in 50 µL of H₂O, samples were vortexed, sonicated for 10 minutes to aid the resuspension, centrifuged for one minute at 16,000g and transferred into 96-well plates for UPLC-MS analyses.

For bile samples, 50 µL of bile samples were diluted in H₂O (3:1), vortexed, centrifuged for 10 minutes at 16,000g and 150µL of mixed solution were transferred into 96-well plates for UPLC-MS analyses. Due to the high concentration of the bile samples and to avoid saturation problems, bile samples were then diluted by a factor of 50 in H₂O. After UPLC-MS analysis, raw data from plasma and bile samples were processed separately using XCMS by performing peak picking, peak matching within the samples, non-linear retention time correction and filling of missing data¹⁹².

### 2.4.2. Acquisition parameters

Analyses were performed on an Acquity UPLC System (Waters) coupled with an LCT Premier Time-of–Flight mass spectrometer (TOF-MS, Waters), operated in the positive (ESI+) and negative (ESI-) electrospray ionisation modes. Wellplates were kept at 4°C during the analyses.

The chromatography was carried out at 40°C on a Waters Acquity UPLC HSS T3 column (1.8 um, 2.1*100 mm) with the following solvent system: A = 0.1% formic acid in water, B = 0.1% formic acid in acetonitrile. The gradient was performed at a flow rate of 0.5 ml/min using a 26 minute gradient for plasma analysis with an injection volume of 5 µL (Table 10).

<table>
<thead>
<tr>
<th>t (minutes)</th>
<th>% A</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>99.9</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>99.9</td>
<td>0.1</td>
</tr>
<tr>
<td>12</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>13</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>17</td>
<td>0.1</td>
<td>99.9</td>
</tr>
<tr>
<td>21</td>
<td>0.1</td>
<td>99.9</td>
</tr>
<tr>
<td>22</td>
<td>5.0</td>
<td>95.0</td>
</tr>
<tr>
<td>23</td>
<td>99.9</td>
<td>0.1</td>
</tr>
<tr>
<td>26</td>
<td>99.9</td>
<td>0.1</td>
</tr>
</tbody>
</table>

**Table 10:** Gradient composition for the analysis of the plasma and bile samples.
ESI conditions were: source temperature 120°C, desolvation temperature 350°C, cone gas flow 20L/hr, desolvation gas flow 900L/hr, capillary voltage 3200 V for ESI+ and 2400V for ESI-, cone voltage: 35 V. The instrument was set to acquire over the m/z range 50-1000 in V mode with scan time of 0.2 s and an interscan delay of 0.01 s. Data were collected in centroid mode. Leucine/Enkephalin (200pg/µL, in acetonitrile: water 50:50, 0.1% formic acid) was used as a lock mass with an analyte-to-reference scan ratio of 4:1. The instrument was calibrated before analyses using 0.5mM sodium formate solution. The order of injection of samples was randomised and one injection of 10 µL of water (blank) was performed every 5 samples. Analyses were run in one batch in positive and negative mode. Four blanks were injected at the beginning and at the end of the run. Also, in order to monitor the performance of the UPLC-MS system, the Quality Control (QC) samples approach was used according to the approach previously reported. The QC sample was prepared by combining equal aliquots from all samples that were analysed. This QC sample was injected at the beginning of the analytical run (at least ten injections) in order to condition the chromatographic column, and periodically throughout the run every ten injections in order to assess the analytical variability.

2.5. Microbiota profiling

2.5.1. DGGE analysis

The method used was derived from the one published by Muyzer et al. DNA was isolated from faeces or colon of the three groups (conventional, lean inoculated and obese inoculated mice) at D7, D14, D21 and D42 using the FastDNA® SPIN Kit and the FastPrep® Instrument (Qbiogene, Inc., CA). The experiments described below have been performed at the Nestle Research Centre and I have analysed the corresponding data.

The V1/V2 and V4 variable regions of the 16S rDNA gene of bacteria were amplified by PCR using a combination of commercially synthesised primers (Sigma Genosys, UK), P2 (5’-ATTACCGCGGCTGCTGG-3’) and P3 (5’-CGCCCGCGGCGGCGCGGCCGGCGGGCAGGGGCACGGGGGCGCCTACGGGAG GCAGCAG-3’) that contains the GC-clamp. PCR amplification was performed with a MJ Research PTC-200 Peltier Thermal Cycler (GRI, Essex, UK) using a PCR mixture (50 µL) of 10 µL of 5° MgCl₂-free reaction buffer (Promega, Southampton, UK), 1 µL of deoxyribonucleoside triphosphates (dNTPs, 10 mM; Promega), 3 µL of
MgCl₂ (25 mM, Promega), 2.5 µL of each primer (10 µM), 0.2 µL of Taq DNA polymerase (5 U/µL; Promega) and 5 ng of template DNA. Touchdown PCR was performed with an initial denaturation step of 94°C for 5 min. Following the hot start which minimizes nonspecific annealing primers to nontarget DNA, 2 cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 1 min were run. Then, the annealing temperature was subsequently decreased by 1 °C every second cycle until a touchdown temperature of 55°C, at which point 5 additional cycles were carried out (27 cycles in total). A final elongation step at 72°C for 7 min was also performed. All amplification products were analysed by gel electrophoresis on 1.5% (w/v) agarose gel containing ethidium bromide (0.4 mg/mL).

DGGE was performed with BDH DGGE V20-HCDC Unit (Merck Eurolab Ltd., Dorset, UK). Amplicons were separated on 8% (w/v) polyacrylamide gels (acrylamide-bisacrylamide 37.5:1, BioRad, UK). A linear denaturing gradient of 30% to 70% (100% denaturant was defined as 7M urea and 40% (v/v) deionised formamide) was used. Electrophoresis was performed at a constant voltage of 200V at 60°C for 16 hours.

2.5.2. 16S rDNA pyrosequencing

DNA was extracted from colon and faecal samples using the GNOME kit (BIO 101, La Jolla, CA, USA) as previously described. The experiments described below have been performed at the Nestle Research Centre and I have analysed the corresponding data. The influence of the priming sequences on the microbiota profile was studied and two sets of primers were selected, pV12-B and pV4 and designed as previously proposed. For each set of primers, two 50 µL PCRs were prepared, containing 1X Expand Long Template buffer 1, 50 µM of each dNTP (Roche Applied Science, Basel, Switzerland). To each reaction a minimum of 2 ng of DNA template was added. PCR amplifications were performed in GeneAmp PCR System 9700 (Applied Biosystems Inc, Foster City, CA, USA). The PCR parameters were 94°C for 5 min, 25 cycles of 94°C for 30 sec, annealing temperature for 30 sec and 72°C for 30 sec, followed by 72°C for 7 min.

After pooling the two PCRs, 10 µL of PCR product were visualised on agarose gel (1.2% in TBE buffer) stained with SYBR Safe (Invitrogen, Eugene, Oregon, USA). Then, PCR products were sent to Beckman Coulter Genomics (Grenoble, France) where equal amounts of each were pooled and sequenced by
the GS FLX System (Roche). Low quality reads were identified using criteria adapted from Huse et al.\textsuperscript{198}.

1) Undefined sequence key; these reads could not be unambiguously assigned to any of the PCR samples.
2) Lack of recognisable 5' primer sequence.
3) More than one error in 5' primer sequence.
4) Average quality score below 25.
5) Presence of ambiguous characters (N').
6) Sequence reads not matching the expected length were excluded. First, primer sequences were removed using the vectorstrip software (-besthits 1, -mismatch 30, -besthits parameters). All reads with less than 100 bp were excluded. 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 excluded.
7) Reads without a BLAST (1) hit to the ARB\textsuperscript{29} 16S DNA database (Evalue cut-off:10^{-10}) were discarded, since these likely do not represent regions of 16S DNA genes. All high-quality reads were classified into Bergey's taxonomy using the RDP-Classifier (80% confidence cut-off). Sequences that could not be assigned to any known 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 grouped into Operational Taxonomic Groups (OTUs) based on their best BLAST hit to full-length reference 16S DNAs. First, reads were compared to all type strain 16S DNAs from the RDP database using BLAST. Second, all reads with a perfect match (sequence-identity of 100%) to the same reference 16S DNA were grouped into one OTU. In an analogous manner, all remaining reads were iteratively grouped into OTUs using sequence-identity cut-offs of 98%, 95%, 90%, 80% and 70%. Each generated OTU was labelled by the type-strain of the respective best-matching reference 16S DNA and the sequence-identity cut-off employed.
3. Results

3.1. Physiological parameters

Conventional and inoculated C57Bl6/J mice were weighed at regular intervals from day -7 to day 42 (Figure 48). Surprisingly, conventional animals exhibited higher body weight compared to GF mice at day -5 only. Before inoculation of human faecal preparations to GF mice, no significant difference was observed between conventional and GF mice at days -7, -3, -1 and 0. Significant differences were observed between conventional and inoculated mice at days 3, 6, 28, 35 and 42 (Figure 48). Interestingly, conventional mice were significantly lighter than lean inoculated (7.1%) and obese inoculated (5.4%) mice at the end of the experiment (p values = 0.0102 and 0.029, respectively). However, no significant BW difference was observed between lean and obese inoculated mice throughout this study (p values > 0.05). It is noteworthy that the food intake of these animals was recorded and no differences were observed between all groups.
Figure 48: Bar plots representing the body weight of conventional (black, Conv), lean inoculated (blue, LHF) and obese inoculated (red, OHF) mice from day -7 to day 42.

Mean peak intensities +/- SEM, Student’s t-test: * p<0.05; ** p<0.01; *** p<0.001.
In addition, cholesterol, free fatty acid and plasma triglyceride levels of these mice were measured at day 42. Conventional mice displayed significantly lower levels of triglycerides compared to lean inoculated (14.6%) and obese inoculated (15.9%) mice (p values = 0.001 and 0.006, respectively). No significant differences in cholesterol and free fatty acids levels were observed between the three groups (Figure 49).

![Figure 49: Bar plots representing cholesterol, triglyceride and free fatty acid levels of conventional (black, Conv), lean inoculated (blue, LHF) and obese inoculated (red, OHF) mice. Mean peak intensities +/- SEM, Student’s t-test: * p<0.05; ** p<0.01; *** p<0.001.](image)

3.2. $^1$H NMR-based metabolic profiling approach

3.2.1. Urinary metabolic profiling

3.2.1.1. $^1$H NMR acquisition

Examples of $^1$H NMR urine spectra obtained from conventional mice, ex-GF mice inoculated with lean human microbiota and ex-GF mice inoculated with obese human microbiota (10 week old) are shown in Figures 50 and 51. Urine spectra contain a variety of resonances from organic acids such as acetate, butyrate, citrate, hippurate and keto-isovalerate, and further metabolites including taurine, aliphatic amines such as methylamine (MMA), dimethylamine (DMA, trimethylamine (TMA) and trimethylamine-N-oxide (TMAO). Other metabolites such as creatine, creatinine, succinate, phenylacetylglutamine and amino acids (alanine, arginine and lysine) were also assigned in urine of C57Bl6/J mice using databases$^{100, 101}$ 2D experiments and literature$^{131, 132}$. 

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Figure 50: Partial $^1$H NMR spectra (600 MHz) of a urine sample of a conventional mouse.
A: 0.5-4.5 ppm.
B: 5-8.5 ppm.
Figure 51: $^1$H NMR spectrum (600 MHz) of a urine sample of a mouse inoculated with lean human microbiota.
A: 0.5-4.5 ppm.
B: 5-8.5 ppm.

3.2.1.2. Statistical analysis

First, a PCA model was generated on the total set of individuals in order to visualise the similarities and dissimilarities within the dataset. Excellent discrimination was observed between GF animals (pre-inoculation mice, red triangles) and conventional (black squares) / inoculated mice (blue and red circles)
according to the 2nd principal component which generated 11.5% of the total variation (Figure 52). It is noteworthy that the 1st principal component did not allow any discrimination as samples from all groups were spread along this component. Finally, 3 conventional mice and 1 inoculated mouse were considered as outliers due to the low sample volume necessitating a higher dilution factor and were removed from further statistical analyses (Figure 52, bottom right of the PCA plot).

![PCA scores plot](image)

**Figure 52**: PCA scores plot derived from $^1$H NMR urinary spectra of GF mice (pre-inoculation, ▲), conventional mice (■), lean inoculated mice (●), and obese inoculated mice (●).

PC 1 = principal component 1; PC 2 = principal component 2.

Then, the specific comparison between lean inoculated mice and obese inoculated mice was investigated to determine how the origin of the human inocula impacts the host metabolism. Therefore, pairwise comparisons were generated at time-points 2, 4 and 6 using O-PLS-DA analysis. The parameters have been summarised in the table 11 below.
<table>
<thead>
<tr>
<th>Time-Point</th>
<th>Group comparisons</th>
<th>PC</th>
<th>OC</th>
<th>Q²Y</th>
<th>R²Y</th>
<th>R²X</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GF vs others</td>
<td>1</td>
<td>1</td>
<td>0.92</td>
<td>0.96</td>
<td>0.39</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>2</td>
<td>Lean In. Vs Obese In.</td>
<td>1</td>
<td>1</td>
<td>&lt; 0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Conventional vs Inoculated</td>
<td>1</td>
<td>1</td>
<td>0.76</td>
<td>0.95</td>
<td>0.16</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>3</td>
<td>Lean In. Vs Obese In.</td>
<td>1</td>
<td>1</td>
<td>&lt; 0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Conventional vs Inoculated</td>
<td>1</td>
<td>1</td>
<td>0.68</td>
<td>0.92</td>
<td>0.22</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>4</td>
<td>Lean In. Vs Obese In.</td>
<td>1</td>
<td>1</td>
<td>0.43</td>
<td>0.83</td>
<td>0.21</td>
<td>0.009</td>
</tr>
<tr>
<td>4</td>
<td>Conventional vs Lean In.</td>
<td>1</td>
<td>1</td>
<td>0.51</td>
<td>0.90</td>
<td>0.29</td>
<td>0.001</td>
</tr>
<tr>
<td>4</td>
<td>Conventional vs Obese In.</td>
<td>1</td>
<td>1</td>
<td>0.57</td>
<td>0.87</td>
<td>0.29</td>
<td>0.002</td>
</tr>
</tbody>
</table>

**Table 11:** Summary of Q²Y, R²Y and R²X parameters from the O-PLS-DA models generated from urine samples of conventional, lean and obese inoculated mice. PC: Predictive component; OC: Orthogonal component.

To complement these pairwise comparisons generated at each time-point, and based on the metabolic similarities observed between both human-associated groups at time-points 2 and 3, an O-PLS-DA model with 2 predictive components (Y1 = class, Y2 = time) and 1 orthogonal component was carried out to determine the impact of the time and inoculation on the urinary metabolic profiles. Interestingly, a good discrimination was observed according to the predicted scores plot on the 1<sup>st</sup> component (Tcv 1) and 2<sup>nd</sup> component (Tcv 2) between conventional and both inoculated groups at time-points 0 (conventional vs GF), 2, 4 and 6 weeks after inoculation (Figure 53).

Among the conventional group, a strong separation was observed between the three first time points (0, 2 and 4 weeks) and the last urine collection (6 weeks) according to Tcv 1. Interestingly, this segregation according to time was similar in both inoculated groups according to Tcv1. As already observed on PCA scores plot (Figure 52), GF animals (blue and red circles) were strongly discriminated from their inoculated and conventional counterparts according again to Tcv1 (Figure 53).
Figure 53: O-PLS-DA scores plot on the two first components (Tcv 1 and Tcv 2) derived from $^1$H NMR urinary spectra from conventional (black), lean inoculated (blue) and obese inoculated mice (red) showing progression of metabolite profiles with time.

Key: TP1: circles; TP2: triangles; TP3: diamond; TP4: square.

**Metabolites correlated to gut microbiota activity strongly discriminated conventional from both inoculated groups**

Before inoculation, GF mice displayed higher levels of an unassigned metabolite ($\delta$ 3.599) and lower levels of taurine ($\delta$ 3.27(t) and 3.43(t)), trimethylamine ($\delta$ 2.88), hippurate ($\delta$ 3.97(s), 7.56(t), 7.64(t) and 7.84(d)), phenylacetylglucine ($\delta$ 7.36(t), 7.42(m)) and indoxyl-sulfate ($\delta$ 7.19(t), 7.26 (t), 7.49(d), 7.69(d)) compared to their conventional counterpart (Figure 54).
At 2 weeks after inoculation, creatine, phenylacetylglucose and hippurate were again observed in higher concentration in conventional animals compared to both inoculated groups, whereas an unassigned metabolite (δ 1.21, singlet) was observed in higher levels in ex-GF mice (Figure 55.A and .B). At 4 weeks after inoculation, creatine, isoleucine, indoxyl-sulfate, hippurate and phenylacetylglucose were
observed in lower concentrations in human-associated animals compared to their conventional counterparts (Figure 55.C and D).

Figure 55: O-PLS-DA loadings plots derived from $^1$H NMR spectra of urine from conventional (at the bottom) and inoculated (at the top) mice, at 2 weeks (A and B) and 4 weeks after inoculation (C and D).
At 2 and 4 weeks after inoculation, conventional animals were characterised by higher levels of phenylacetylglycine ($\delta$ 7.36 (t)) compared to human-associated mice, either inoculated with lean or human microbiota (Figure 56.B and .D). Interestingly, lean inoculated mice displayed higher levels of trimethylamine, citrate and succinate compared to their conventional counterpart (Figure 56.A) whereas obese inoculated mice displayed higher levels of creatinine, citrate, dimethylamine and succinate compared to their conventional counterparts (Figure 56.C).
Figure 56: O-PLS-DA loadings plots derived from $^1$H NMR spectra of urine (last time-point) from conventional (at the bottom, all), lean inoculated mice (at the top, A and B) and obese inoculated mice (at the top, C and D) at 6 weeks after inoculation.
Finally, 6 weeks after inoculation, human-associated mice with lean microbiota were discriminated from those inoculated with obese human microbiota. Higher levels of oxo-glutarate (δ 2.45(t) and 3.01 (t)) and citrate (δ 2.56 (d) and 2.68(d)) and lower levels of dimethylamine (δ 2.72 (s)) and allantoin (δ 5.40 (s)) were observed in lean inoculated compared to obese inoculated mice (Figure 57). Interestingly, no variation of metabolites in the aromatic region (6-9 ppm) was observed, suggesting a similar gut microbial activity in both inoculated groups, and corresponding data were therefore not shown.

![Figure 57: O-PLS-DA loadings plot derived from $^1$H NMR spectra of urine (6 weeks after inoculation) from lean inoculated (at the bottom) and obese inoculated mice (at the top) at 6 weeks after inoculation.](image)

### 3.2.2. Plasma

#### 3.2.2.1. $^1$H NMR acquisition

Examples of $^1$H NMR spectra obtained from plasma samples of mice have been detailed in the previous section (chapter IV / Results).

#### 3.2.2.2. Statistical analysis

In order to visualise the metabolic variations associated to the origin of the inocula, pairwise comparisons were generated in mouse plasma between conventional, lean inoculated and obese inoculated mice using an O-PLS-DA algorithm. Interestingly, conventional animals were significantly discriminated from
both lean human and obese human associated-animals. Nevertheless, lean and obese inoculated mice were not successfully discriminated by this approach. Therefore, a single O-PLS-DA model integrating all three datasets was generated using one predictive and one orthogonal component and showed a good discrimination between conventional and both inoculated groups (Figure 58.A). This model was validated by permutation tests (p value = 0.001, perm = 1000) and based on the following parameters: $Q^2_Y = 0.39$; $R^2_Y = 0.92$ and $R^2_X = 0.13$.

The model revealed that conventional animals displayed higher levels of (D)-3-hydroxybutyrate ($\delta 1.20$ (d)), acetate ($\delta 1.91$ (s)), isoleucine ($\delta 0.93$ (t)) and valine ($\delta 0.96$ (d)) and lower levels of alanine ($\delta 1.48$ (d)), lactate ($\delta 1.33$ (d)) and an unassigned metabolite ($\delta 1.07$ (s)) when compared to human-associated animals (Figure 58.B). It is noteworthy that no variation of triglycerides level was observed between these groups that could have matched what has been previously measured. One hypothesis may be the use of a standard 1D acquisition that is not perfectly adapted for the characterisation of large molecules, and not diffusion experiments that are generally well suited for this kind of approach.
Metabolites | $\delta^1$H (p.p.m.) | (+) Conventional vs Inoculated mice (-)
--- | --- | ---
(D)-3-hydroxybutyrate | 1.20 | + 0.62
Acetate | 1.91 | + 0.67
Alanine | 1.48 | - 0.6
Isoleucine | 0.93 | + 0.57
Lactate | 1.33 | - 0.45
Valine | 0.96 | + 0.58
Unassigned | 1.07 | - 0.69

**Figure 58**: O-PLS-DA model derived from $^1$H NMR spectra of plasma from conventional (A, □) and inoculated mice (B, lean inoculated (●) and obese inoculated (○) mice).
A: Plot corresponding to the observed versus cross-validated (T1 / Tcv1) scores.
B: Summary of metabolite variations.
3.2.3. Liver

3.2.3.1. \(^1\)H NMR acquisition

3.2.3.1.1. Aqueous extracts

Examples of \(^1\)H NMR spectra obtained from aqueous extracts of liver samples of a conventional mouse and an inoculated mouse with lean microbiota are presented in Figures 59 and 60, respectively. Spectra are mainly dominated by resonances from glucose (\(\alpha\)- and \(\beta\)-glucose) and organic acids such as acetate, lactate and \((D)-3\)-hydroxybutyrate, and further metabolites including taurine, glutamine and oxidised glutathione. Other metabolites such as amino acids (alanine, isoleucine, leucine, phenylalanine, and valine), creatine and creatinine could be observed in the liver of C57Bl6/J mice. Assignments were achieved based on the literature and the use of databases\(^{26,100,101,131,132}\).
**Figure 59:** Partial $^1$H NMR spectra (600 MHz) of a liver aqueous extract of a conventional mouse. The aromatic region (B) has been vertically expanded *2.

A: 0.5-4.5 ppm.

B: 5-9 ppm. This region has been vertically expanded *2.
Figure 60: Partial $^1$H NMR spectra (600 MHz) of a hepatic aqueous extract of an inoculated mouse.

A: 0.5-4.5 ppm.
B: 5-9 ppm. This region has been vertically expanded *2.
3.2.3.1.2. Lipophilic extracts

A typical example of a $^1$H NMR spectrum obtained from the lipophilic extract of liver from a conventional mouse is shown in Figure 61. Assignments were achieved based on the literature and the use of databases$^{100,101,199,200}$.

$^1$H NMR spectra of the lipophilic fraction were mainly dominated by resonances assigned to fatty acyl chains of different lengths, but mainly from long-chain and very-long-chain, and by glycerol backbone belonging to unsaturated and polyunsaturated triglyceride acyls. Resonances from mono and polyunsaturated fatty acyls, carbonyls, aldehydes and fatty acyls that are not triglycerides were also observed in lipophilic extracts of C57Bl6/J mouse livers (Figure 61).
Figure 61: $^1$H NMR spectra (600 MHz) of hepatic lipophilic extracts of a conventional mouse (A) and inoculated mouse (B).
3.2.3.2. Statistical analysis of both extracts

In order to visualise the metabolic variations associated with the origin of the inocula, pairwise comparisons have been generated in both extracts (aqueous and lipophilic extracts) between conventional, lean inoculated and obese inoculated mice using an O-PLS-DA approach.

Conventional mice were discriminated from both lean and obese inoculated mice, independently of the extracts that were analysed (Figure 62). Nevertheless, lean and obese inoculated mice were not discriminated by an O-PLS-DA approach, neither in lipophilic nor in aqueous extracts ($Q^2_Y < 0$). Therefore, similarly to plasma, two O-PLS-DA models were generated using one predictive and one orthogonal component, for both extracts, to compare conventional and inoculated mice. These models were validated by permutation tests ($p$ values = 0.009 and 0.01 for lipophilic and aqueous extracts, respectively; perm = 1000) and based on the following parameters: $Q^2_Y = 0.43$; $R^2_Y = 0.76$ and $R^2_X = 0.54$ for lipophilic extracts, and $Q^2_Y = 0.36$; $R^2_Y = 0.96$ and $R^2_X = 0.52$ for aqueous extracts. It is noteworthy that the model performed in the aqueous extracts presented a non-negligible overfit ($Q^2_Y / R^2_Y = 0.38$) which is observed as well in Figure 62.A, and should therefore be carefully interpreted.
Interestingly, conventional mice were discriminated from both inoculated groups, in lipophilic and aqueous extracts, according to the projection of the observed scores (T1) and the cross-validated scores on the 1st component (Tcv1). As explained before, no discrimination was observed between both inoculated groups independently of the analysed extracts (Figure 62.A and .B).

Figure 62: OPLS-DA scores plot derived from $^1$H NMR spectra of aqueous extracts (A) and lipophilic extracts (B) of liver samples of conventional (■), lean inoculated (■) and obese inoculated (■) mice.
By specifically investigating the discrimination between conventional and human-associated animals in the hepatic hydrophilic extracts, phosphocholine ($\delta$ 3.22 (s) and 4.23 (m)) and trimethylamine-N-oxide ($\delta$ 3.27(s)) were observed in lower levels, whereas bile acids ($\delta$ 0.7(s) and 1.05(s)) were observed in higher levels in inoculated animals compared to their conventional counterparts (Figure 63).

**Figure 63:** O-PLS-DA loadings plot generated from $^1$H NMR spectra of hepatic aqueous extracts of conventional (at the bottom) and inoculated mice (at the top).
The same approach has been applied to lipophilic extracts of liver and inoculated mice displayed fattier livers than conventional animals as highlighted above by higher levels of CH$_2$ and CH$_3$ groups, triglycerides, glycerol backbone and cholesterol ester (Figure 64) in human-associated compared to normal mice.

![O-PLS-DA loadings plot](image-url)

**Figure 64:** O-PLS-DA loadings plot generated from $^1$H NMR spectra of liver lipophilic extracts of conventional (at the bottom) and inoculated mice (at the top).

### 3.3. UPLC-MS-based metabolic profiling approach

#### 3.3.1. Plasma samples

##### 3.3.1.1. Global profiling

As illustrated in Figure 65, a typical TIC chromatogram of plasma obtained by an UPLC-MS analysis is mainly dominated by phospholipids and lipids which are eluted between 14 and 18 minutes according to the use of a 26 minutes gradient described in table 10. Bile acids have a retention time between 8 minutes for the first eluted and 13 minutes for the latest eluted (Figure 65).
Figure 65: Representative TIC chromatograms of plasma obtained by UPLC-MS analysis from a conventional mouse (black, at the bottom), lean inoculated mouse (blue, in the middle) and obese inoculated mouse (red, at the top).

The global bile acid profile obtained by this approach does not highlight any obvious differences between the 3 groups. As a consequence, it is necessary to use statistical analysis tools such as a non-supervised method (i.e. PCA) and a supervised method (i.e. PLS-DA) in order to visualise the variations of concentrations between the three groups by a global profiling approach. Nevertheless, variations between the 3 groups were hypothesised according to bile acid targeted profiling that will be detailed in the following section.
3.3.1.2. Targeted bile acid profiling

Figure 66: Zoom on the bile acids elution window on a typical chromatogram obtained by a UPLC-MS analysis from a conventional mouse (black, at the bottom), lean inoculated mouse (blue, in the middle) and an obese inoculated mouse (red, at the top).

7 different bile acids were identified in plasma based on their m/z, their retention time and the comparison with standards. 3 unconjugated bile acids (cholic acid, CA; α-muricholic acid, α-MCA; deoxycholic acid, DCA) have been observed together with 4 tauroconjugated bile acids (taurocholic acid, TCA; α-tauromuricholic acid, α-TMCA; taurodeoxycholic acid, TDCA; taurohyodeoxycholic acid, THDCA). As expected, no glycoconjugated bile acids have been observed in the plasma of these mice since this model is known for producing marginal levels of glycine conjugates of bile acids (Table 12).
<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>m/z</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholic acid</td>
<td>CA</td>
<td>407.3</td>
<td>11.37</td>
</tr>
<tr>
<td>α-Muricholic acid</td>
<td>α-MCA</td>
<td>407.3</td>
<td>10.15</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>DCA</td>
<td>391.3</td>
<td>13.40</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>TCA</td>
<td>514.3</td>
<td>9.78</td>
</tr>
<tr>
<td>α-Tauromuricholic acid</td>
<td>α-TMCA</td>
<td>514.3</td>
<td>8.66</td>
</tr>
<tr>
<td>Taurodeoxycholic acid</td>
<td>TDCA</td>
<td>498.3</td>
<td>11.32</td>
</tr>
<tr>
<td>Taurohyodeoxycholic acid</td>
<td>THDCA</td>
<td>498.3</td>
<td>9.64</td>
</tr>
</tbody>
</table>

Table 12: Summary of the 7 identified bile acids in plasma samples based on their mass over charge ratio (m/z) and retention time.

3.3.1.3. Statistical analysis

In order to investigate the quality of my dataset, a PCA model was calculated using 6 principal components. As expected, the quality control samples (QCs) were clustered on the scores plot indicating a good repeatability of the experiments. In addition, PC1, which generated 14.9% of the total variation, discriminated readily the conventional mice from the two inoculated groups. However, no discrimination was observed by PCA between lean inoculated and obese inoculated mice. It is noteworthy that 3 samples within the obese inoculated group plotted just outside the 95% confident ellipse, and were not considered as outliers for further analysis (Figure 67).
A PLS-DA model has been generated in order to establish whether the two inoculated groups could be differentiated from each other. The robustness of the model was supported by the following parameters, $Q^2_Y=0.46$; $R^2_Y=0.48$ and $R^2_X=0.19$, and validated by permutation tests ($p$ value $< 0.001$, perm = 1000). Interestingly, conventional mice were readily discriminated from the two inoculated groups according to the first latent component (Figure 68.A, PLS 1), the features directly involved in the discrimination were then identified (Figure 68.B). Nevertheless, as the second latent component PLS2 displayed a $Q^2_Y < 0$ and was not interpreted, it was still not possible to discriminate the inoculated groups from each other (Figure 68.A).
Figure 68: PLS-DA scores plot (A) and loadings plot (B) derived from the analysis of plasma samples analysed by UPLC-MS (negative-ion mode). Two discriminant features were identified as two bile acids, deoxycholic acid and α-muricholic acid (B).
As observed in Figure 68.B, 14 features were outlined as the main discriminant variables. It is noteworthy that they do not correspond necessarily to 14 different metabolites since two or more features could represent the same metabolite due to fragmentation taking place in the source of the analyser. Interestingly, two bile acids, DCA and $\alpha$-MCA, were identified in higher concentration in conventional mice compared to human-associated mice and were involved in the discrimination between these groups (Figure 68.B).

![Deoxycholic acid](image)

**Figure 69:** Raw formula of deoxycholic acid and its potential formate and sodium adducts observed as fragments after a UPLC-MS experiment. 

*Based on the same principle, the formate adduct of $\alpha$-MCA was observed with a m/z ratio of 407.3+46 = 453.3.*

Based on these global profiling results, the first hypothesis based on the inability of lean and obese humanised animals to recover the level of bile acids observed in conventional mice was confirmed. Therefore, univariate statistics have been used to target bile acid composition in plasma in order to determine more specifically the impact of both origins of inocula onto the bile acid metabolism.
Figure 70: Bile acids profiling in plasma samples obtained from UPLC-MS analysis (negative ion mode).

Mean peak intensities +/- SEM, n=15. Student’s t-test: * p<0.05; ** p<0.01; *** p<0.001. Keys: α-MCA = α-muricholic acid; α-TMCA = α-taumuricholic acid.

Interestingly, conventional animals displayed significantly different plasma bile acid profiles from lean and obese inoculated mice. As expected, DCA and α-MCA were more highly concentrated in conventional animals compared to the two other groups (Figure 70, p value < 0.001). In addition, a higher level of one tauroconjugated bile acid, TDCA, was observed in conventional animals compared to ex-GF mice (Figure 70, p value < 0.05) whereas T-α-MCA varied in the opposite way as conventional animals displayed a significant lower level of T-α-MCA compared to both lean inoculated mice and obese inoculated mice (Figure 70, p value < 0.05). Finally, TCA was the only bile acid that did not vary between conventional and lean inoculated mice as it was observed in higher concentrations only in the obese inoculated group (Figure 70, p value < 0.05).
3.3.2. Bile samples

3.3.2.1. Global profiling

The same bile acid profiling approach was applied to bile samples collected after sacrifice at 6 weeks. Typical chromatograms obtained by an UPLC-MS analysis of bile from a conventional mouse (Figure 71, black, at the bottom), a GF mouse inoculated with lean human microbiota (blue, in the middle) and a GF mouse inoculated with obese human microbiota (Figure 71 red, at the top) are presented below. As illustrated by the Figure 71 below, a typical chromatogram of bile obtained by an UPLC-MS analysis is mainly dominated by bile acids which are eluted between 8 and 13 minutes according to the use of a 26 minutes gradient described in table 10.

Figure 71: Representative chromatograms of bile obtained by UPLC-MS analysis from a conventional mouse (black, at the bottom), lean inoculated mouse (blue, in the middle) and obese inoculated mouse (red, at the top).

Interestingly, the global profiles obtained by this approach highlighted a high level of one bile acid specifically eluted at 8.67 minutes and this variation can be readily observed on the chromatograms displayed above (Figure 71). No other variations were easily observed and it was therefore necessary to use statistical
analysis tools such as non supervised method (i.e. PCA) and supervised method (i.e. PLS-DA) to visualise the statistical differences between the three groups.

**3.3.2.2. Targeted bile acid profiling**

12 bile acids have been identified in bile based on their m/z, retention time and by comparison with standards (Table 13). 4 unconjugated bile acids (cholic acid, CA; α-muricholic acid, α-MCA; β-muricholic acid, β-MCA; γ-muricholic acid, γ-MCA), one glycoconjugated bile acids (glycocholic acid, GCA) and 7 tauroconjugated bile acids (taurocholic acid, TCA; α-taumuricholic acid, α-TMCA; γ-taumuricholic acid, γ-TMCA; THDCA / TUDCA, taurohyodeoxycholic acid / tauroursodeoxycholic ; taurodeoxycholic acid, TDCA; taurohydroxycholic acid, THCA; taurochenodeoxycholic acid, TCDCA) have been observed in bile samples. However, it was not possible to discriminate THDCA and TUDCA using the gradient previously described due to their similar retention time (9.78 and 9.77 minutes, respectively).
<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>m/z</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
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<td>cholic acid</td>
<td>CA</td>
<td>453.3</td>
<td>11.36</td>
</tr>
<tr>
<td>$\alpha$-muricholic acid</td>
<td>$\alpha$-MCA</td>
<td>453.3</td>
<td>10.13</td>
</tr>
<tr>
<td>$\beta$-muricholic acid</td>
<td>$\beta$-MCA</td>
<td>453.3</td>
<td>10.29</td>
</tr>
<tr>
<td>$\gamma$-muricholic acid</td>
<td>$\gamma$-MCA</td>
<td>453.3</td>
<td>10.47</td>
</tr>
<tr>
<td>glycocholic acid</td>
<td>GCA</td>
<td>464.3</td>
<td>10.26</td>
</tr>
<tr>
<td>taurocholic acid</td>
<td>TCA</td>
<td>514.3</td>
<td>9.80</td>
</tr>
<tr>
<td>$\beta$-tauromuricholic acid</td>
<td>$\beta$-TMCA</td>
<td>514.3</td>
<td>8.66</td>
</tr>
<tr>
<td>$\gamma$-tauromuricholic acid</td>
<td>$\gamma$-TMCA</td>
<td>514.3</td>
<td>8.50</td>
</tr>
<tr>
<td>taurohyodeoxycholic acid / tauroursodeoxycholic acid</td>
<td>THDCA / TUDCA</td>
<td>498.3</td>
<td>9.77</td>
</tr>
<tr>
<td>taurodeoxycholic acid</td>
<td>TDCA</td>
<td>498.3</td>
<td>11.34</td>
</tr>
<tr>
<td>taurohydroxycholic acid</td>
<td>THCA</td>
<td>514.3</td>
<td>9.10</td>
</tr>
<tr>
<td>taurochenodeoxycholic acid</td>
<td>TCDCA</td>
<td>498.3</td>
<td>11.02</td>
</tr>
</tbody>
</table>

**Table 13:** Summary of the 12 identified bile acids in bile samples based on their mass over charge ratio (m/z) and retention time.

In order to confirm the observations made from the raw chromatograms, the intensity of each bile acid was averaged (+/- SEM) and multiple comparisons using Student t-test were generated between the three groups (Figure 72).
Figure 72: Bile acids profiling in bile samples (A and B) obtained from UPLC-MS analysis (negative ion mode) and (A) dominated by the two primary bile acids with (B) a specific zoom on strong variations of minor tauroconjugated bile acids.

Mean peak intensities +/- SEM, n=15, Student’s t-test: * p<0.05; ** p<0.01; *** p<0.001. Keys: a-MCA = α-muricholic acid; TaMCA = α-tauromuricholic acid; TwMCA = γ-tauromuricholic acid; b-MCA = β-muricholic acid.

It is noteworthy that the bile acid profile in bile is strongly dominated by two tauroconjugated forms, TCA and T-β-MCA, followed by the unconjugated bile acids including cholic acid and several muricholic acids, whereas the only glycoconjugated form detected in bile was in very low concentration (GCA). Firstly, significant variations of tauroconjugated bile acids were observed since conventional mice displayed higher levels of T-γMCA and TDCA (p values < 0.01) and lower levels of T-β-MCA, TCDCA and THCA (p values < 0.001) compared to both inoculated groups. Interestingly, obese inoculated mice showed a higher level of THCA (p value < 0.001) and a lower level of TCDCA (p value < 0.001) compared to lean inoculated mice. Finally, CA was the only unconjugated bile acid that varied systematically between groups as it was observed in significantly higher concentration in conventional compared to inoculated mice (p value < 0.001, Figure 72).

Finally, the importance of each bile acid family within the three different groups is displayed in Figure 73. Bile acid profiles were dominated by
tauroconjugated forms (96% in conventional animals and up to 99% in lean and obese inoculated mice). It is noteworthy that the proportion of glycoconjugated bile acid (GCA) is similar in all groups (less than 1%) whereas the main difference subsisted in a higher proportion of unconjugated bile acids in conventional animals compared to the inoculated groups (about 1% in both inoculated groups and up to 3% in the conventional group).

**Figure 73:** Bar-plot representative of the percentage of each bile acid family (unconjugated, glycoconjugated and tauroconjugated) for each group of mice (conventional, lean inoculated and obese inoculated mice).

In bile, it has been previously reported that the control of the ratio of CA over MCA is crucial in the mouse as it regulates the overall hydrophobicity of the BA pool, involved in the regulation of cholesterol absorption and biosynthesis in the liver. Two main pathways are involved in this regulation and called the classic and the alternative biosynthetic pathways: the classic one leads to the production of CA whereas the alternative one leads to the production of MCA in the mouse, which is more hydrophilic. Therefore, the overall hydrophobicity of the bile acid pool, which regulates the cholesterol absorption and synthesis in the liver, is determined by the ratio of these two primary bile acids. Nevertheless, they are then conjugated to glycine, the minor form in the mouse, and taurine, the major form, prior to their secretion in bile. Therefore, the ratio of TCA over TMCA was evaluated to determine the specific bile acid composition in each group (Figure 74).
Figure 74: Bar-plot representative of the TCA over TMCA ratio in conventional (black), lean inoculated (blue) and obese inoculated mice (red). Mean of measured intensities +/- SEM is presented. *** p value<0.001.

It is worth mentioning that conventional animals displayed a TCA/TMCA ratio of 2:1 that was significantly different compared to this ratio in the two inoculated groups that was approximately 1:1 (Figure 74, p value < 0.001). Finally, no difference of TCA/TMCA ratio was obtained between lean and obese inoculated mice (Figure 74, p value = 0.3).

3.3.2.3. Statistical analysis

A multivariate data analysis approach was used to characterise the biliary metabolic profiles of these mice by an UPLC-MS analysis. A first unsupervised approach (PCA) using 4 principal components allowed the separation of conventional animals from lean and obese inoculated mice. However, both inoculated mice overlapped according to the two first components which generated the maximum of variation in the dataset (34.3% and 20.8% for PC1 and PC2, respectively). It is noteworthy that four samples (two from each inoculated group) plotted with conventional animals for unknown reasons (Figure 75).
Figure 75: PCA scores plot derived from UPLC-MS analysis of bile samples at week 6 post inoculation.

An O-PLS-DA model was generated using 2 components and its robustness was indicated by the following parameters, $Q^2_Y = 0.71$, $R^2_Y = 0.91$ and $R^2_X = 0.74$ (Figure 76) and the model has been validated by permutation tests ($p$ value < 0.001, perm = 1000). Interestingly, an excellent discrimination was observed between conventional, lean and obese human inoculated mice according to the projection on PLS1 and PLS2 (Figure 76.A). This separation was notably associated with higher levels of cholic acid (CA) and β-muricholic acid (β-MCA) in conventional animals compared to both inoculated groups (Figure 76.B).
Figure 76: PLS-DA model derived from UPLC-MS analysis of bile samples.

A: Scores plot corresponding to the projection of the two first latent components PLS 1 and PLS 2. Colour legend is indicated in the Figure 76.A.

B: Corresponding loadings plot showing that cholic acid (CA) and β-muricholic acid (β-MCA) were identified in the features responsible for the discrimination between conventional and inoculated mice.
3.4. Gut microbiota composition

The sequencing of the gut bacterial composition is considered as the most efficient way to precisely characterise the bacterial species colonising the gut of the host. In the present study, a 16S rDNA sequencing approach coupled with High-Throughput-Sequencing was used as it allows a quick, robust and efficient sequencing of the gut bacterial community. In the present study, the faecal bacteria families in human donors and mouse receivers were sequenced.

3.4.1. Gut microbiota composition in human donors

In this project, 5 lean and 5 obese human donors were used to inoculate GF mice following the protocol detailed previously (Chapter V, Material and Methods section). It is noteworthy that one donor was consistently used to inoculate three different GF mice. The characteristics of human donors and description of identity of inoculated mice are given in the table below.

<table>
<thead>
<tr>
<th>Group</th>
<th>Donor codes</th>
<th>Age</th>
<th>Body weight (kg)</th>
<th>Size (m)</th>
<th>BMI</th>
<th>Receiver mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>2</td>
<td>39 y.o.</td>
<td>58.7</td>
<td>1.7</td>
<td>20.3</td>
<td>L1.1, L1.2, L1.3</td>
</tr>
<tr>
<td>Lean</td>
<td>4</td>
<td>46 y.o.</td>
<td>58</td>
<td>1.57</td>
<td>23.5</td>
<td>L1.4, L1.5, L1.6</td>
</tr>
<tr>
<td>Lean</td>
<td>13</td>
<td>55 y.o.</td>
<td>62</td>
<td>1.64</td>
<td>23.1</td>
<td>L1.7, L1.8, L1.9</td>
</tr>
<tr>
<td>Lean</td>
<td>5</td>
<td>37 y.o.</td>
<td>56.9</td>
<td>1.61</td>
<td>22.0</td>
<td>L2.1, L2.2, L2.3</td>
</tr>
<tr>
<td>Lean</td>
<td>1</td>
<td>43 y.o.</td>
<td>66</td>
<td>1.67</td>
<td>23.7</td>
<td>L2.4, L2.5, L2.6</td>
</tr>
<tr>
<td>Obese</td>
<td>7</td>
<td>44 y.o.</td>
<td>76.9</td>
<td>1.54</td>
<td>32.4</td>
<td>OB1.1, OB1.2, OB1.3</td>
</tr>
<tr>
<td>Obese</td>
<td>9</td>
<td>47 y.o.</td>
<td>109</td>
<td>1.68</td>
<td>38.6</td>
<td>OB1.4, OB1.5, OB1.6</td>
</tr>
<tr>
<td>Obese</td>
<td>6</td>
<td>32 y.o.</td>
<td>88.7</td>
<td>1.67</td>
<td>31.8</td>
<td>OB2.1, OB2.2, OB2.3</td>
</tr>
<tr>
<td>Obese</td>
<td>11</td>
<td>53 y.o.</td>
<td>69</td>
<td>1.51</td>
<td>30.3</td>
<td>OB2.4, OB2.5, OB2.6</td>
</tr>
<tr>
<td>Obese</td>
<td>12</td>
<td>37 y.o.</td>
<td>75</td>
<td>1.57</td>
<td>30.4</td>
<td>OB2.7, OB2.8, OB2.9</td>
</tr>
</tbody>
</table>

Table 14: Characteristics (age, body weight, size and BMI) of human donors used to inoculate GF mice.
All faecal samples from lean and obese donors (n=10) were sequenced by technicians from the Nestle Research Center (Switzerland) and the results presented in Figure 77 are the results of my own analysis. In the lean donor group, similar bacterial family profiles were observed within 4 donors, which are donors 1, 2, 4 and 13, with a relatively good distribution within the bacterial profile of the main bacterial families: *Bacteroidia Prevotellacea* and *Bacteroidaceae*, *Clostridia Lachnospiraceae* and *Clostridiaceae*, and *Unclassified* bacteria. Surprisingly, donor 5 was significantly different from the 4 other donors since it was mainly dominated by *Bacteroidia Prevotellacea* (Figure 77). Similar results were observed in obese human donors as donors 7, 9, 11 and 12 displayed profiles dominated by *Bacteroidia Bacteroidaceae*. Surprisingly, donor 6 displayed a higher proportion of *Unclassified* bacteria and *Bacteroidia Prevotellacea*, and a lower proportion of *Bacteroidia Bacteroidaceae* compared to the 4 other groups (Figure 77).
3.4.2. Gut microbiota composition in mouse receivers

The gut microbiota composition was determined in conventional, lean inoculated and obese inoculated mice at days 7, 14, 21 and 42 and bacterial families were identified based on the protocol previously described (Chapter V, Material and methods) and presented in Figure 78 below.
Figure 78: The family-level composition of the gut microbiota obtained by 16S rDNA sequencing in faecal samples of conventional, lean inoculated and obese inoculated groups (average composition). 4 different time-points are displayed: day 7 (D7), day 14 (D14), day 21 (D21) and day 42 (D42). Data at D14 were not collected for the conventional group.

First of all, the 16S rDNA composition of faecal samples showed that this composition is relatively stable in conventional mice over the timeline of this experiment whereas the proportions of each family evolved significantly in both lean and obese inoculated mice (Figure 78). Indeed, in conventional mice, the proportion of unclassified family decreased slightly from 59.74% to 51.82% to the profit of both Bacteroidia prevotellaceae and rikenellaceae families. The proportions of unclassified bacteria represented only 1.14% and 1.63% in lean and obese
inoculated mice, respectively. This is due to the fact that human databases are more developed than murine databases making the comparison of both microbiota complicated. In addition, lean inoculated mice were marked by a massive decrease of *Bacteroidia bacteroidaceae* between day 7 (68.38%) and day 42 (42.61%). This was accompanied by a strong increase of *Bacteroidia prevotellaceae* and a slight rise of *Clostridia lachnospiraceae* over time to reach 22.34% and 8.80% of the total bacterial account, respectively. It is noteworthy that the proportion of the other bacterial families remained stable between day 7 and day 42. In obese inoculated mice, 75% of the total account of bacteria was dominated by *Bacteria bacteroidaceae* at 7 days after inoculation, whereas this proportion slightly decreased over time to reach 57.43% at the end of the experiment (day 42). This was correlated with an increase of the family *Clostridia lachnospiraceae* (from 8.79% at day 7 to 13.75% at day 42, Figure 78). *Bacteroidia bacteroidaceae* remained the most abundant family of bacteria in both human-associated animals, independently of the origin of the inocula (Figure 78).

### 3.4.3. Multivariate statistical analysis

In order to visualise the variations associated with the origin of the microbiota inoculated in the mouse gut, an O-PLS-DA model using one orthogonal and one principal component was carried out in caecum and faecal samples. Both models were validated by permutation tests (p values < 0.001 for both models, perm = 1000), and the robustness of these models was confirmed by the following parameters: $Q^2_Y = 0.47$; $R^2_Y = 0.62$ and $R^2_X = 0.30$ for cecum samples, and $Q^2_Y = 0.49$; $R^2_Y = 0.51$ and $R^2_X = 0.30$ for faecal samples.

Firstly, the three groups of mice were discriminated in both matrices, as PLS1 displayed a good separation between conventional and both human-associated mice (Figures 79.A and B). In addition, lean inoculated were discriminated from obese inoculated mice in cecum and stool samples according to the second latent component (PLS2, Figures 79.A and B). Therefore, this result indicated that the discrimination and quality of the sequencing seemed to be robust between cecum and stool samples. However, the discriminant families varied slightly between both matrices. In cecum, it was associated at the family level with higher levels of *Bacteroidia odoribacter* and *alstipes*, *Bacilli lactobacillus*, *Unclassified* and *Remaining* bacteria in conventional mice, of Gammaproteobacteria
*Escherichia/shingella* and *Clostridia ruminococcus* in lean inoculated mice and of *Clostridia dorea* and *megamonas* as well as *Bacteroidia prevotella* in obese inoculated mice. At the opposite, in faecal samples, it was associated with higher levels of *Bacteroidia odoribacter* and *alistipes*, *Bacilli lactobacillus* and *Unclassified* bacteria in conventional mice, of *Clostridia ruminococcus* in lean inoculated mice and of *Bacteroidia prevotella* and *Remaining* bacteria in obese inoculated mice (Figures 80.A and B). Finally, it is noteworthy that the discrimination observed between the three groups of mice is stronger in gut microbiota composition than in the metabolic profiles of the conventional and inoculated hosts.
**Figure 79**: PLS-DA scores plots of 16S rDNA data from data in cecum samples (A) and in stool samples (B).
Figure 80: PLS-DA loadings plots derived from 16S rDNA data of cecum samples (A) and stool samples (B).
3.5. Correlation of gut microbiota composition with metabolic profiles

In order to evaluate the correlation between the metabolic profiles of conventional and inoculated mice and their gut microbiota composition (16S rDNA data grouped into Operational Taxonomic Units (OTUs)), an O-PLS-based integration method was used\textsuperscript{29, 97}. Regression models between both urinary and hepatic metabolic profiles from each group of mice and OTUs were computed.

3.5.1. Correlations between hepatic metabolic profiles and 16S rDNA data

3.5.1.1. Lipophilic extracts

\textsuperscript{1}H NMR metabolic profiles of liver hydrophilic extracts from conventional (n=16), lean inoculated (n=14) and obese inoculated mice (n=12) were regressed separately against 16S rDNA data grouped into OTUs.

Regression models of hepatic lipophilic profiles from conventional models (\textsuperscript{1}H NMR spectra, n=16) against OTUs data (n=232) were computed. Interestingly, \textit{Alistipes finegoldii} correlated positively with hepatic lipophilic profiles of conventional mice and was positively associated with glycerol (CH group and backbone of glycerol) and cholesterol (Figure 81).
In addition, *Coproccocus comes* 90 correlated positively with hepatic metabolic profiles of conventional mice and was negatively associated with triglycerides, glycogen, lipids, cholesterol, methanol and bile acids (Figure 82.A). Surprisingly, it strongly correlated with methanol residues. Therefore, the corresponding resonance (δ 3.32 to 3.38) was removed from the dataset and a new correlation was performed: the same negative associations were observed with triglycerides, glycogen, lipids, cholesterol and bile acids (Figure 82.B).
Figure 82: Loadings plot derived from the O-PLS regression between hepatic $^1$H NMR lipophilic extracts of conventional animals ($n=16$) and 16S rDNA data grouped into OTUs of *Coprococcus comes* 90.
Regression models of hepatic lipophilic profiles from lean human-associated models \( (^1H \text{NMR spectra, } n=13) \) against OTUs data \( (n=193) \) were computed. A total of 8 correlations were validated based on their statistical parameters \( (Q^2_Y, R^2_Y \text{ and } R^2_X) \) and permutation tests \( (\text{perm } = 1000) \).

*Ruminococcus callidus* was identified as correlating positively with hepatic lipophilic profiles of lean human-associated models and this correlation was negatively associated with fatty acids, cholesterol, terminal CH$_3$ groups and triglycerides (Figure 83).

![Figure 83: Loadings plot derived from the O-PLS regression between hepatic $^1H$ NMR lipophilic extracts of lean associated-animals (n=14) and 16S rDNA data grouped into OTUs of *Ruminococcus callidus* 90.](image)
Regression models of hepatic lipophilic profiles from obese human-associated models (¹H NMR spectra, n=12) against OTUs data (n=181) were computed. It is worth mentioning that 10 significant correlations were obtained and validated based on their statistical parameters ($Q^2_Y$, $R^2_Y$ and $R^2_X$) and permutation tests (perm = 1000). Among these correlations, Faecalibacterium prausnitzii was observed as correlating positively with hepatic lipophilic profiles. Interestingly, this correlation was associated positively with triglycerides, glycerol, cholesterol, and CH$_2$ groups from lipids (Figure 84).

**Figure 84:** Loadings plot derived from the O-PLS regression between hepatic $^1$H NMR lipophilic extracts of obese human-associated animals (n=12) and 16S rDNA data grouped into OTUs of Faecalibacterium prausnitzii 90.
In addition, two other bacteria, *Parasporobacterium faucivorans* 90 and *Bacteroides massiliensis* 98, displayed significant correlations that have been associated with higher levels of CH$_2$ groups in fatty acyl chains (δ 1.60, Figure 85).

**Figure 85:** Loadings plot derived from the O-PLS regression between hepatic $^1$H NMR lipophilic extracts of obese human-associated animals (n=12) and corresponding 16S rDNA data grouped into OTUs of *Parasporobacterium faucivorans* 90 and *Bacteroides massiliensis* 98.
3.5.1.2. Hydrophilic extracts

$^1$H NMR metabolic profiles of hepatic hydrophilic extracts from conventional (n=16), lean inoculated (n=12) and obese inoculated mice (n=12) were regressed separately against 16S rDNA data grouped into OTUs.

In conventional animals, *Clostridium aldenense* 80 and *Barnesiella viscericola* 80 correlated positively with metabolic profiles from conventional animals and were negatively associated with lactate, glutamine and phosphocholine (*Clostridium aldenense* 80) and with lactate and myo-inositol (*Barnesiella viscericola* 80, Figure 86).

**Figure 86:** Loadings plot derived from the O-PLS regression between hepatic $^1$H NMR hydrophilic extracts of conventional animals (n=16) and corresponding 16S rDNA data of *Clostridium aldenense* 80 and *Barnesiella viscericola* 80.
In addition, regression models of hepatic hydrophilic profiles from lean human-associated models (\(^1\)H NMR spectra, n=12) against OTUs data (n=193) were computed. *Blautia wexlerae* correlated positively with glucose and negatively with hypotaurine and lysine. Lysine was observed as correlating negatively together with lactate with *Clostridium clostridioforme* 90. In addition, branched-chain amino acids (isoleucine, leucine and valine), acetate, alanine, creatine and glutamate were positively associated with *Phascolarctobacterium faecium* 98 (Figure 87).
Figure 87: Loadings plot derived from the O-PLS regression between hepatic $^1$H NMR hydrophilic extracts of lean animals (n=12) and corresponding 16S rDNA data of Blautia wexlerae 98, Clostridium clostridioforme 90 and Phascolarctobacterium faecium 98.
Finally, regression models of hepatic hydrophilic profiles from obese human-associated models ($^1$H NMR spectra, n=12) against OTUs data (n=193) were computed. Interestingly, *Roseburia inulinivorans* 90 and *Ruminococcus lactaris* 95 correlated positively with glucose. In addition, the latter was observed as correlating positively with lactate and negatively with (D)-3-hydroxybutyrate (Figure 88).

**Figure 88**: Loadings plot derived from the O-PLS regression between hepatic $^1$H NMR hydrophilic extracts of obese-associated animals (n=12) and corresponding 16S rDNA data of *Roseburia inulinivorans* 90 and *Ruminococcus lactaris* 95.
4. Discussion

The aim of this project was to determine the impact of two types of human microbiota, one from lean human subjects and one from obese human subjects, on the metabolism of the host. Therefore, inoculated mice have been physiologically and metabolically characterised by recording their body weight, body weight gain and total body fat content throughout the study and by generating multiple $^1$H NMR metabolic profiles of biofluids and liver. Finally, I targeted specifically their bile acid metabolism in bile and plasma using an UPLC-MS approach and their microbial content in faeces was determined using a 16S rDNA sequencing approach.

4.1. Human inoculated mice differ strongly from normal mice

$^1$H NMR metabolic profiles of urine, plasma and liver (both aqueous and lipophilic extracts) from C57Bl6/J mice highlighted strong similarities between mice inoculated with lean human microbiota and obese human microbiota. Indeed, no discriminations were observed between both human-associated groups in urine, plasma and liver samples. Conversely, significant differences were observed between inoculated and conventional mice in both biofluids and liver, independently of the hydrophilic and lipophilic extracts. In urine, several metabolites related to the gut microbiota activity, (i.e. hippurate, phenylacetylglycine and trimethylamine) were observed in higher concentrations in conventional animals compared to the two inoculated groups. These results suggested that conventional mice display a complete, developed and functional gut ecosystem more able to convert dietary compounds to metabolites such as hippurate and phenylacetylglycine. Indeed, hippurate is formed by the detoxification of benzoate by glycine conjugation in the liver and kidney, which is then secreted in the renal tubular cells and excreted in urine. Originally, benzoate is formed through the conversion of dietary aromatic compounds by the gut microbial species$^{203}$. Phenylacetylglycine is considered as an end-product of gut bacterial amino acid metabolism as it results from the degradation of phenylalanine by the bacteria which reside in the gut$^{204}$. In plasma, a strong perturbation of the $\beta$-oxidation and branch-chain amino acid metabolism was noted since variations of lactate, acetate and $\beta$-hydroxybutyrate were observed between conventional and inoculated mice. This result was expected since the introduction of human bacteria to GF animals, which have never been in contact with any
microorganisms before, is known to strongly disrupt the metabolism of the host\textsuperscript{26,29}. This is characterised by perturbations of the whole metabolism including energy metabolism.

Finally, the variation observed in liver extract profiles showed that inoculated mice displayed fattier liver compared to the control group as indicated by higher levels of triglycerides, both saturated and unsaturated, and various lipid family in lipophilic extracts of conventional compared to human-associated animals\textsuperscript{166}. In addition, the hydrophilic extracts revealed higher levels of bile acids in human-associated mice compared to conventional mice. This phenotype is associated with GF animals and these results suggested that inoculated animals did not recover a normal bile acid metabolism, and therefore the inability of the human gut microbes to mimic functions of conventional microbiota. Therefore, an UPLC-MS approach has been applied to deeply investigate these perturbations and identify unambiguously the distribution of unconjugated, glyco- and tauroconjugated bile acids among these mice.

4.2. Bile acid metabolism indicates a “GF like” excretion pattern in inoculated mice

Based on an untargeted approach, the three groups of animals were discriminated according to their metabolic profiles generated by UPLC-MS in plasma and bile, since this was not possible using \textsuperscript{1}H NMR-based metabolic profiling approach. The difference with the results generated by a \textsuperscript{1}H NMR-based approach could be explained by the higher sensitivity of an UPLCMS analysis compared to a NMR acquisition (limit of detection: $10^{-9}$ to $10^{-15}$ g/l vs. $10^{-6}$ g/l, respectively) and the fact that the C18 axial methyl groups of several bile acids overlap in the 1D \textsuperscript{1}H NMR spectra. An UPLC-MS analysis is therefore more able to capture very subtle changes in the host, while the identification of the discriminant metabolites remained a very difficult task to perform since it could require MS\textsuperscript{n} experiments to identify fragmentations\textsuperscript{23,24,205,206}. Interestingly, two bile acids (DCA and $\alpha$-MCA) were highly influential in the discrimination between conventional and both inoculated groups, and were unambiguously identified in higher levels in normal mice compared to their human-associated counterparts. These results are surprising as DCA has been previously reported as being produced in high concentrations in humanised mice\textsuperscript{207}. Narushima et al demonstrated that DCA was observed in caecal contents of mice.
associated with deconjugating and 7-α-dehydroxylating bacteria, and identified them as member of the family *Bacteroides* or similar to *Clostridium hylemonae*. It has been demonstrated as well that DCA plays a role in colorectal tumor enlargement and in the promotion of proliferation of colonic carcinoma.

A UPLC-MS-based targeted approach was used to characterise specifically the bile acid metabolism of conventional and inoculated animals. In this context, a focus was performed on TCA/TMCA ratio. Indeed, this ratio is involved in the regulation of the overall hydrophobicity of the BA pool, and then regulates cholesterol absorption and biosynthesis in the liver. In the present study, conventional mice displayed a ratio of approximately 2:1 which is similar to that previously reported in conventional mice and rats. Both inoculated groups displayed a ratio of 1:1 which is similar to what has been reported in germ-free mice and rats. These results clearly indicate that human-associated models do not have the same regulation of BA metabolism than conventional mice for producing bile acids, even 6 weeks after inoculation. This might be explained by the inability of human gut microbes to mimic the functions of conventional microbiota. Therefore, inoculated mice may lack specific bacteria, or specific functions provided by a whole bacterial community, that are involved in bile acid synthesis control and regulation. For instance, it has been previously demonstrated that CYP8B1, the enzyme responsible for the regulation of TCA/TMCA ratio, is downregulated in GF animals. A recent study demonstrated that the mRNA expression level of *cyp8b1* (assessed by quantitative reverse transcription-PCR) was significantly lower expressed in GF animals and recovered a level observed in conventional animals after 20 days of colonisation. However, in the present study, it was not possible to perform such measurements and no information is available about CYP8B1 regulation and expression level of *cyp8b1*. Taken altogether, these results could support the hypothesis that human inocula are not adapted to a GF mouse host and this postulate would have to be confirmed by the follow-up study detailed in chapter VI.

### 4.3. *Faecalibacterium prausnitzii* as a key bacterium correlating with hepatic metabolic profiles

In this project, correlation of faecal 16S rDNA data grouped into OTUs with urinary and hepatic metabolic profiles of C57Bl6/J mice were performed. To implement these associations, an O-PLS-based integration method was used.
Significant correlations between specific bacteria and lipophilic and hydrophilic hepatic extracts of conventional or human-associated animals were obtained. The bacterium *Faecalibacterium prausnitzii* was observed as correlating positively with triglycerides, cholesterol, and CH\(_2\) groups from lipids. This result is of particular interest since this bacterium has been linked to colitis, Crohn’s disease and obesity in the literature. Indeed, Sokol et al. observed that this bacterium was significantly underrepresented in the microbiota of infectious colitis and active irritable bowel syndrome patients compared to healthy subjects. This may suggest a potential role for *Faecalibacterium prausnitzii* and the phylum *Firmicutes* in the maintenance of the gut homeostasis\(^{211}\). Firstly, this butyrate producer, known for having trophic functions and protective effect against cancer, could be crucial to gut homeostasis and considered as a protective factor for the gut mucosa. In addition, this bacterium was detected in higher levels in obese Indian children compared to non-obese participants\(^{212}\). This confirmed previous findings showing that *Firmicutes* have been consistently found in higher levels in obese humans compared to healthy and lean individuals. One hypothesis is based on the capacity of *Faecalibacterium prausnitzii* to increase energy recovery from undigested and unabsorbed carbohydrates, which would not have been involved otherwise in the dietary energy intake\(^{212}\). Interestingly, evidences have been collected about the role playing by *Faecalibacterium prausnitzii* in chronic diarrhoea, malnutrition and Crohn’s disease as lower counts of this bacterium were systematically reported in diseased patients compared to healthy subjects\(^{212-216}\). *Faecalibacterium prausnitzii* seems to be highly relevant to nutrition research since inulin, known and widely used as a prebiotic, has been reported to strongly stimulate the proportion of this bacterium in twelve human volunteers recruited in this study\(^{217}\). Finally, this bacterium has been shown to impact on human physiology. Indeed, it was recently associated with the modulation of eight urinary metabolites with very different structures (i.e. positive correlations with glycolate, 2-hydroxyisobutyrate, dimethylamine, 3,5-hydroxybenzoate, taurine and 3-aminoisobutyrate and negative correlations with glycine and lactate), indicating its potential to affect numerous host pathways and demonstrating its high functionality and activity in the microbiome\(^{218}\). Taken altogether, these data highlight that this bacterium is of major interest in the scientific community. *Faecalibacterium prausnitzii* may therefore play a crucial role in the protection against intestinal inflammation: one hypothesis is the secretion of specific metabolites, which are then
able to block NF-kB activation and IL-8 secretion, two factors involved in the process of intestinal inflammation and gut homeostasis\textsuperscript{213,219}. Another aspect that remains to be characterised is the capacity of \textit{Faecalibacterium prausnitzii} to be a predictive factor for IBD and to determine this bacterium’s putative function in the manipulation of the gut microbial content to fight against obesity.

\textbf{4.4. What is the correct number of donors to use?}

In the present study, results generated by a \textsuperscript{1}H NMR-based metabolic profiling approach revealed the inability to discriminate human-associated mice with lean or obese microbiota in most of biofluids and tissue. One hypothesis may be that about 30\% of the obese human population is considered as healthy and that therefore obese people cannot be considered as only one single metabolic group. Another hypothesis may be the influence of the initial experimental design. Indeed, 5 different human donors have been used in each group to inoculate 3 different GF mice. The aim of this experimental design was to generate a subset of samples that should be as representative as possible of the bacterial profiles found among the human population. Nevertheless, the metabolic profiles of the host obtained in humanised mice highlighted that this approach has generated high variability within each inoculated group due to inter-individual differences in bacterial profiles of the donors. Therefore, the approach consisting of using 5 different donors to inoculate GF animals may explain that only very subtle variations in urine of C57Bl6/J were observed between lean and obese humanised models (discrimination of both inoculated groups occurred only at the last time-point), and that they were not discriminated in plasma and liver\textsuperscript{220}. Interestingly, plasma composition is tightly controlled by homeostatic regulation to maintain the integrity of the organism, in comparison to urine, which contains end products of metabolism and whom the excretion is the reflection of dietary inputs and metabolic requirements\textsuperscript{220}. It is noteworthy that some variability was observed among the lean and obese human donors (one donor in each group displayed a different profile) but it had a low impact on the gut microbiota composition of the receiver population.
5. Conclusion

This study aimed to characterise the impact of mouse inoculation with human microbiota and at deciphering the functions of the human intestinal microbiota. I have demonstrated that the human microbiota, independently of its origin (from lean or obese people), perturbed strongly the physiology of the host by generating systemic perturbations of its metabolism, which deviated strongly from the metabolic baseline characterising conventional mice. Metabolic signatures that were generated in biofluids and tissue from humanised animals could be compared to phenotypes generally associated to the development of metabolic disease such as obesity, and the deregulation of the host homeostasis. In addition, regressions performed between hepatic metabolic phenotypes of the host and its gut microbiota composition (OTUs obtained by 16S rDNA sequencing) helped to identify bacteria that could be specifically used in the future to implement and complement dietary products (cereals, yoghurts, ice creams). Based on this study, a focus was performed on the characterisation of the impact of time-point of colonisation and the origin of microbiota on body composition and metabolism of the host, and therefore investigated specifically the effect of inoculation with mouse or human microbiota on both the physiological (body weight, body weight gain, fat content and plasma triglyceride levels) and immunological parameters of the host, and also on the metabolic profiles of C3H mice.
Chapter VI  Impact of time-point of colonisation and microbiota origin on the physiology, immunology and metabolism of C3H mice

1. Introduction

Recent studies have shown that the gut microbiota affect energy harvesting and fat storage suggesting a role in the development of obesity\textsuperscript{5,14-16,18,65}. Most compelling evidence comes from microbiota transplantation experiments performed in GF mice showing greater fat deposition in mice colonised with obese mice microbiota\textsuperscript{5,16}. Based on these results, a previous study was designed and revealed very subtle changes between GF mice inoculated with lean or obese human microbiota (chapter V). These results suggested particularly that the number of donor(s) used to colonise the GF host seemed to be crucial. In this follow-up study, the overall aim was to further explore the role of intestinal microbiota in the development of obesity and metabolic disorders and consisted of characterising the impact of mouse and human microbiota on the metabolism of the host. Only one human and one mouse donor have been be used in order to reduce inter-donor variations in this experiment. In particular, the following hypotheses derived from results obtained within the previous study were tested:

a) Post-weaning colonisation of GF mice with human microbiota lead to different body weight (BW), total body fat content (TBFC) and plasma triglyceride level compared to mice colonised with mouse microbiota, due to different functional properties of the two inocula.

b) Different complex microbiota transferred in the GF host (i) generate different metabolic fingerprints in biofluids collected from the C3H mice, that are dissimilar to those of GF and conventional mice and (ii) impact significantly the bile acid metabolism.
2. Material and Methods

2.1. Study design

Figure 89: Study design of the mouse vs human flora comparison study.

15 C3H conventional mice and 45 C3H GF mice were purchased from Charles Rivers laboratories (France). After one week of adaptation, 45 GF mice were separated into three groups of 15 GF mice housed in three different isolators (week 0). One group was kept at its GF state, one group was inoculated by gavage with human microbiota from a non-obese human subject (1 donor), and one group was inoculated by gavage with mouse microbiota from a non-obese mouse (1 donor). Urine was collected throughout the study at days -7 and 0 (pre-inoculation) to set up the metabolic baseline of these mice, and then 1, 3, 7, 14, 21, 28, 35 and 42 days after inoculation. Plasma and bile were collected after sacrifice at day 42 (Figure 89).

2.2. $^1$H NMR and UPLC-MS analyses

The procedure that was used to prepare and acquire urine, plasma and bile samples by both $^1$H NMR and UPLC-MS has been previously described (see chapters IV and V).

2.3. Immunological parameters

2.3.1. Lymphoid organ cell suspension preparation

The immunological experiments described below have been performed at the Nestle Research Centre and I have analysed the corresponding data. Spleens were collected and placed in ice-cold complete IMDM medium containing 10% foetal calf serum (FCS, Amimed), 50 μM β-mercaptoethanol, 100 U/mL penicillin and 100
µg/mL streptomycin (all from Sigma). Spleen cell suspensions were erythrocyte depleted by lysis with standard red cell lysis buffer. All Peyer’s patches (PP) were collected along the small intestine of every single mouse, then cell suspension was prepared by incubating samples for 90 minutes at 37°C with 2 U/mL collagenase (Liberase Blendzyme 3, Roche) in IMDM medium and 5 mM CaCl₂, then washed, resuspended in complete IMDM medium and passed through a cell strainer. Enumeration of viable spleen and PP cells was determined by Trypan Blue exclusion.

2.3.2. Flow cytometry analysis

Cells were washed and resuspended in PBS containing 1% FCS and 0.01% NaN₃ (staining buffer). Cell surface staining was performed on ice with appropriate antibody dilutions and fluorochrome combinations in staining buffer, after initial blocking of FCγ-receptors with anti-CD16/CD32 (BD Pharmingen). mAbs included anti-CD11b-FITC, PNA-FITC, anti-CD44-FITC, anti-CD3ε-PE, anti-FAS-PE, anti-CD11a-PE, anti-CXCR5-PerCP, anti-B220-APC and anti-CD4-APC (BD Pharmingen). Data were collected on a FACSCalibur flow cytometer and analysed using CellQuest software (Becton Dickinson) gated on viable lymphocytes. Total B cells were defined as B220⁺CD3ε⁻, total myeloid cells as CD11b⁺B220⁻CD3ε⁻, splenic B1 cells as B220⁺CD11b⁺, germinal center B cells (GC) as B220⁺PNAhiFAS⁺, and follicular helper CD4⁺ T cells (TFH) as CD4⁺CD44hiCD11a hiCXCR5⁺ cells.

2.3.3. B and T cell stimulation assays

Whole PP suspensions were cultured in triplicates in complete IMDM for 48 hours in 96 well flat bottom plate (200 µL, 10e5 cells/well). Specific B cell stimulations were performed with addition of 5 µg/mL of anti-CD40 antibody (clone FGK-45, kindly provided by A. Rolink, University of Basel, Switzerland). Specific T cell stimulations were performed in anti-CD3e coated 96 well flat bottom plate at 5 µg/mL 1hr at 37°C (clone 2C11, BD Pharmingen) with addition of 1 µg/mL of anti-CD28 antibody (clone 37.51, BD Pharmingen). After 48 hours of stimulation, supernatants were collected and frozen. Cells were pulsed with fresh complete medium with 1 µCi 3H-thymidine for 18-20 hours prior to harvesting. Mean value of triplicate were expressed in count per minutes (cpm) as a reflection of cell proliferation.
2.3.4. Analysis of secreted antibody and cytokine levels

Total secreted antibody levels from B cell stimulation assays were measured by ELISA. Microtiter plates were coated with anti-mouse IgA, IgG or IgM (Sigma). Free binding sites were blocked with PBS containing 20% FCS, 0.05% Tween 20 and 0.05% NaN₃. Serial dilution of supernatants were then added and bound antibodies detected following incubation with biotin-conjugated anti-mouse IgA, IgM or IgG (Southern Biotechnologies). The plates were further incubated with horseradish peroxidise-labelled streptavidin (KPL). Finally, the plates were developed with TMP peroxidase substrate (KPL) and read at 450 nm. Monoclonal purified antibodies from Sigma were used as standard. Total secreted cytokine levels from T cell stimulation assays were measured by standard ELISA for IL-17 and IL-22 according to manufacturer’s instructions whereas IL-1b, IL-2, IL-4, IL-5, IL-6, KC (mouse IL-8), IL-10, IL-12, IFNγ and TNFα were measured with multiplex ELISA using ultrasensitive multiplex Th1 / Th2 9-plex cytokine profiling kit (Meso Scale Discovery) according to manufacturer’s instructions.
3. Results

3.1. Physiological parameters

GF, conventional and inoculated C3H male mice were weighed every three days from day -7 to day 42 (Figure 90). As expected, conventional animals displayed significant higher body weight than GF and future inoculated animals at the beginning of the experiment. Interestingly, these differences remained between conventional and mouse-associated animals throughout the study, whereas no significant differences were observed between conventional and humanised mice from day 31 to day 42, and between conventional and GF mice at the last time-point (day 42). Mouse-associated animals were significantly lighter (4.5%) than humanised mice at day 42 only (Figure 90, p value = 0.0425). It is noteworthy that the food intake of these animals was recorded throughout the study and both humanised and GF mice displayed a higher food intake than mouse-associated animals (p value = 0.0083 and 0.0021, respectively). No differences were observed between the other groups.
Figure 90: Bar plots representing the average body weight of GF (red, 3A), conventional (black, 3C), humanised (blue, 3H) and mouse-inoculated (green, 3M) animals from day -7 to day 42.

Mean peak intensities +/- SEM, Student’s t-test: * p<0.05; ** p<0.01; *** p<0.001.

Since multiple comparisons were performed, the corresponding p-values were corrected using Bonferroni’s corrections.
In complement to the measurement of body weight, the cholesterol, free fatty acid and plasma triglycerides levels of these mice were measured at day 42 (Figure 91). Conventional mice displayed significantly higher levels of cholesterol (6.6%) and lower levels of free fatty acids (22%) compared to humanised mice (p values = 0.0175 and 0.009, respectively).

It is noteworthy that, at day 42, human inoculated mice displayed significantly higher levels of TGs compared to GF (30%, p-value < 0.001), conventional (25.8%, p-value < 0.01), and mouse-associated animals (23.4%, p-value < 0.01); no significant differences were observed between conventional, mouse and human inoculated mice (Figure 91).

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**Figure 91:** Bar plots representing the cholesterol, free fatty acid (FFA) and plasma triglyceride (TGs) levels measured at day 42 in C3H mice. (GF: red / 3A; conventional: black / 3C; humanised mice: blue / 3H; mouse-associated mice: green / 3M).

Mean peak intensities +/- SEM, Student’s t-test: * p<0.05; ** p<0.01; *** p<0.001.
3.2. Immunological parameters

Several immunological parameters of the host have been measured such as immune cell populations in Peyer’s patch, immunoglobulins in Peyer’s patch and cytokines which will be further presented.

Various Peyer’s patch cells are displayed in Figure 92, such as B-cells, CD4+ cells, T-cells and TFH. Statistical differences were observed for each class of cells between the levels measured in both conventional / mouse-associated animals and both GF / humanised mice. Mouse-associated and conventional animals had a significantly lower percentage of B-cells compared to both humanised and GF animals (p values < 0.001). Regarding CD4+ cells, T-cells and the percentage of T<sub>FH</sub> cells in CD4+, mouse-associated and conventional mice exhibited a significantly higher percentage compared to GF and humanised mice (p values < 0.001). In all the Peyer’s patch cell types, there was no significant difference between conventional and mouse-associated animals. Similarly, no significant differences were observed between GF and humanised mice (p values > 0.05).

Figure 92: Bar plot showing the Peyer’s patch cell profiles of C3H mice. Mean peak intensities +/- SEM. Student’s t-test: * p<0.05; ** p<0.01; *** p<0.001. Since multiple comparisons were performed, the corresponding p-values were corrected using Bonferroni’s corrections. Keys: GF mice: 3A (red); conventional mice: 3C (black); humanised mice: 3H (blue); mouse-associated animals: 3M (green).
The levels of three types of immunoglobulin (IgA, IgG and IgM) were then measured in the Peyer’s patches after sacrifice (Figure 93). It is noteworthy that significantly higher levels of IgA were observed in conventional and mice inoculated with mouse microbiota compared to both GF and humanised mice (p values < 0.001), whereas no significant differences of IgA levels were observed neither between conventional and mouse-associated animals, nor between GF and humanised mice. Finally, IgG and IgM levels were similar in the 4 groups (Figure 93).

![Figure 93: Bar plots showing the immunoglobulin profiles of C3H mice.](image)

Mean peak intensities +/- SEM. Student’s t-test: * p<0.05; ** p<0.01; *** p<0.001.

Keys: GF mice: 3A (red); conventional mice: 3C (black); humanised mice: 3H (blue); mouse-associated animals: 3M (green).

Several cytokines were measured in this experiment: the interleukin (IL) family, with IL-1β, IL-4, IL-5, IL-6, IL-12p40 and IL-22 subclasses, interferon-γ (IFN-γ) and TNF-α (Figure 94). GF mice displayed significantly lower levels of cytokines compared to mouse-associated animals, except for IL-5 cytokines which were not significantly different. Similarly, humanised mice displayed consistently lower levels of cytokines compared to mouse-associated animals. Conventional animals had significantly higher levels of IL-22 and IFN-γ compared to the three other groups. Similarly, GF mice exhibited lower levels of TNF-α compared to the three other groups (Figure 94).
Figure 94: Bar plot showing the cytokine profiles of C3H mice.

Mean peak intensities +/- SEM. Student's t-test: * p<0.05; ** p<0.01; *** p<0.001.

Since multiple comparisons were performed, the corresponding p-values were corrected using Bonferroni's corrections.

Keys: GF mice: 3A (red); conventional mice: 3C (black); humanised mice: 3H (blue); mouse-associated animals: 3M (green).
3.3. ¹H NMR-based metabolic profiling approach

3.3.1. Urine

3.3.1.1. ¹H NMR acquisition

A typical urinary spectrum has been already presented and detailed in the previous chapters (Chapters IV and V). A focus was performed at day 1 (1st day post-inoculation) to determine immediate specific responses of the GF host to the foreign inocula. Interestingly, human and mouse-associated animals produced high levels of formate at day 1 compared to both GF and conventional animals (Figure 95).

![Figure 95: (A) Zoom on the formate resonance (δ 8.46 ppm) of a ¹H NMR urinary spectrum derived and (B) integration of the corresponding formate resonance from GF (red), mouse inoculated (green), human inoculated (blue) and conventional (black) animals. Mean peak intensities +/- SEM. Student’s t-test: * p<0.05; ** p<0.01; *** p<0.001.](image)

To complement this observation, a follow-up of formate production was performed in all groups from day -14 to day 42. Interestingly, GF and conventional mice excreted consistently very low levels of formate throughout the study. Conversely, a peak of formate excretion was observed in inoculated animals at d1, independently of the origin of the inocula, instantaneously followed by a comeback to
the basal level at d3. Then, human and mouse-associated mice produced low levels of formate from d3 to d42 (Figure 96).

Figure 96: Average formate excretion pattern from d -14 to d 42 derived from the integration of the peak resonance of formate in urinary $^1$H NMR spectra from GF (red), humanised (blue), mouse-associated (green) and conventional (black) mice ($\delta$ 8.46).

3.3.1.2. Statistical analysis

3.3.1.2.1. Impact of the origin of the inocula

In order to determine the similarities and dissimilarities within these 4 groups of mice, a PCA was generated on the total set of individuals using 3 principal components and is presented in Figure 97. Interestingly, a good discrimination was observed between GF mice (red) and humanised (blue), mouse-associated (green) and conventional (black) animals according to the principal components 1 (PC1) and 2 (PC2). No discrimination was observed on PC3.
Figure 97: 3D PCA scores plot derived from all $^1$H NMR urinary spectra of GF (🔺), human inoculated (●), mouse inoculated (○) and conventional mice (□).

As no evident discrimination was observed between inoculated and conventional mice using a PCA approach, a supervised method was then used to characterise the metabolic variations associated with the origin of the inocula (mouse or human). Therefore, an O-PLS-DA model was generated using 3 predictive components and 1 orthogonal component and validated based on permutation tests (perm = 1000, p-value < 0.001) and the following parameters: $Q^2_Y = 0.45$; $R^2_Y = 0.56$ and $R^2_X = 0.40$. Interestingly, good discrimination was observed between the 4 groups according to the projection of the cross-validated scores on the three first components (Figure 98). The strongest source of variation was observed between GF animals, human inoculated and mouse inoculated/conventional mice, since these last two groups overlapped together on the first component (Tcv 1, Figures 98.A and B). Another segregation was observed between human inoculated mice and the three other groups according to the second component (Tcv 2, Figures 98.A and B). Finally, the third component isolated mice inoculated with mouse microbiota from the other groups (Tcv 3, Figures 98.A and B).
Figure 98: 3D (A) and 2D (B) O-PLS-DA scores plots derived from all $^1$H NMR urinary spectra of GF (▲), human inoculated (●), mouse inoculated (●) and conventional mice (■).
3.3.1.2.2. **Specific focus on day 1**

In order to determine the impact of the inoculation procedure and the early response of the host to this perturbation (one day after inoculation), an O-PLS-DA model using two predictive components and one orthogonal component was generated (Figure 99). The robustness of this model was supported by permutation tests (perm = 1000, p value < 0.001) and based on the following parameters: $Q^2_Y = 0.44$, $R^2_Y = 0.74$ and $R^2_X = 0.47$. In addition, the validity was confirmed by permutation tests (perm = 1000, p-value = 0.004). Conventional mice were strongly discriminated from the other groups on the 1st component (Tcv 1). Another segregation was observed between GF mice and the other groups according to the 2nd component (Tcv 2). Finally, no obvious separation was observed between humanised and mouse-associated animals on Tcv 1 and Tcv 2 (Figure 99).

![Figure 99: O-PLS-DA scores plot derived from $^1$H NMR spectra of GF (▲), mouse-associated (●), humanised (○) and conventional (■) animals at day 1 (1st day post-inoculation).](image)

Then, pairwise comparisons were generated between all groups. The robustness of these models was characterised by the parameters summarised in table 15 below, and all models were validated based on permutation tests (perm = 1000). However, the comparison between mouse-inoculated and conventional
animals presented a high overfit, based on a $Q^2_Y/R^2_Y$ ratio of 0.43 and a p-value of 0.048, and should be carefully interpreted.

<table>
<thead>
<tr>
<th>Group comparisons</th>
<th>PC</th>
<th>OC</th>
<th>$Q^2_Y$</th>
<th>$R^2_Y$</th>
<th>$R^2_X$</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GF vs MF</td>
<td>1</td>
<td>1</td>
<td>0.66</td>
<td>0.95</td>
<td>0.44</td>
<td>0.006</td>
</tr>
<tr>
<td>GF vs HF</td>
<td>1</td>
<td>1</td>
<td>0.55</td>
<td>0.94</td>
<td>0.38</td>
<td>0.016</td>
</tr>
<tr>
<td>GF vs Conventional</td>
<td>1</td>
<td>1</td>
<td>0.68</td>
<td>0.96</td>
<td>0.34</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HF vs MF</td>
<td>1</td>
<td>1</td>
<td>0.38</td>
<td>0.89</td>
<td>0.38</td>
<td>0.048</td>
</tr>
<tr>
<td>HF vs Conventional</td>
<td>1</td>
<td>1</td>
<td>0.65</td>
<td>0.92</td>
<td>0.37</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MF vs Conventional</td>
<td>1</td>
<td>1</td>
<td>0.33</td>
<td>0.94</td>
<td>0.56</td>
<td>0.045</td>
</tr>
</tbody>
</table>

**Table 15:** Summary of $Q^2_Y$, $R^2_Y$ and $R^2_X$ parameters for O-PLS-DA models at day 1 (first time-point post inoculation) and p-values derived from permutation tests (perm = 1000).

Analysis of loadings plots at day 1 showed metabolite variations in response to the microbial status of each group of mice. Indeed, GF animals displayed higher levels of keto-isocaproate, creatine, CHs groups from amino acids and one unassigned metabolite (3.599 ppm, singlet) compared to their conventional counterparts. Hippurate, methylamine, phenylacetylglycine, trimethylamine and taurine were observed in higher concentrations in conventional compared to GF animals (Figure 100).
Figure 100: O-PLS-DA scores plot derived from $^1$H NMR urinary spectra from GF (at the bottom) and conventional (at the top) mice at day 1.

A) 0.7-4.2 ppm.

B) 6.5-8 ppm. Aromatic region (B) has been expanded vertically *10.
At day 1, conventional mice displayed higher levels of hippurate and phenylacetylglycine and lower levels of formate and one unassigned metabolite (3.599 ppm) compared to mice inoculated with mouse microbiota (Figure 101).

**Figure 101:** O-PLS-DA scores plot derived from \(^1\text{H} \) NMR urinary spectra from mouse inoculated (at the bottom) and conventional (at the top) mice at day 1.

A) 0.7-4.2 ppm.

B) 6.5-8.6: aromatic regions (B) has been expanded *10.
Human inoculated mice were discriminated from mice inoculated with mouse microbiota and presented higher levels of N-acetylglycoprotein fragments and hippurate and lower levels of creatine, at day 1 (Figure 102).

**Figure 102**: O-PLS-DA scores plot derived from $^1$H NMR urinary spectra from mouse inoculated (at the bottom) and human inoculated (at the top) mice at day 1.

A) 0.7-4.2 ppm.

B) 6.5-8.5 ppm: aromatic region (B) has been expanded *10.
Analyses of loadings plots generated from all pairwise comparisons are summarised in the table 16 below.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>δ ¹H and multiplicity</th>
<th>(-) GF vs Conv (+)</th>
<th>(-) GF vs MF (+)</th>
<th>(-) GF vs HF (+)</th>
<th>(-) HF vs MF (+)</th>
<th>(-) HF vs Conv (+)</th>
<th>(-) MF vs Conv (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-hydroxyisovalerate</td>
<td>+ 0.82</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amino acid CHs</td>
<td>3.5 - 4 (m)</td>
<td>- 0.85</td>
<td>- 0.78</td>
<td>- 0.83</td>
<td>- 0.74</td>
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<td></td>
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<tr>
<td>Creatine</td>
<td>3.93 (s)</td>
<td>+ 0.71</td>
<td></td>
<td>+ 0.76</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Creatinine</td>
<td>4.06 (s)</td>
<td>- 0.87</td>
<td></td>
<td></td>
<td></td>
<td>- 0.69</td>
<td></td>
</tr>
<tr>
<td>Formate</td>
<td>8.46 (s)</td>
<td>+ 0.82</td>
<td>+0.49</td>
<td>- 0.53</td>
<td>- 0.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hippurate</td>
<td>7.56 (t)</td>
<td>+ 0.76</td>
<td>+ 0.72</td>
<td>- 0.84</td>
<td>+0.76</td>
<td>+0.76</td>
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</tr>
<tr>
<td></td>
<td>7.64 (t)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>7.84 (d)</td>
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<td></td>
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<tr>
<td>Indoxyl-sulfate</td>
<td>7.59</td>
<td>+ 0.81</td>
<td></td>
<td>+ 0.78</td>
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<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>1.93 (m)</td>
<td></td>
<td></td>
<td>- 0.69</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Keto-isocaproate</td>
<td>0.94 (d)</td>
<td>- 0.70</td>
<td>+ 0.74</td>
<td>- 0.70</td>
<td>- 0.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.62 (d)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Keto-isovalerate</td>
<td>1.141</td>
<td>+ 0.81</td>
<td></td>
<td></td>
<td>- 0.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-acetylglycoprot.</td>
<td>2.04 (s)</td>
<td>- 0.84</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>2.145 (s)</td>
<td></td>
<td></td>
<td>- 0.66</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylacetylglycerine</td>
<td>7.36 (t)</td>
<td>+ 0.75</td>
<td>+ 0.72</td>
<td>+ 0.82</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taurine</td>
<td>3.27 (t)</td>
<td>+ 0.82</td>
<td>+ 0.83</td>
<td>+ 0.80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.43 (l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>2.874 (s)</td>
<td>+ 0.78</td>
<td>+ 0.53</td>
<td></td>
<td>+ 0.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unassigned 1</td>
<td>3.599 (s)</td>
<td>- 0.81</td>
<td>- 0.64</td>
<td>- 0.76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unassigned 2</td>
<td>3.359 (s)</td>
<td>- 0.74</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unassigned 3</td>
<td>3.771 (s)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- 0.78</td>
</tr>
</tbody>
</table>

**Table 16:** Summary of metabolite variations derived from ¹H NMR urinary spectra of GF, mouse inoculated mice (MF), human inoculated mice (HF) and conventional mice (Conv) at day 1.

*(Correlation coefficients with the highest discriminant axis for the metabolites involved in the difference between the positive group (+) and the negative group (-) are displayed).*
Metabolites linked to gut microbial activity are highly involved in the discrimination between normal and both GF and inoculated animals

Systemic higher levels of hippurate were observed in conventional mice compared to the three other groups. In addition, hippurate was highly excreted in urine of humanised mice when compared to GF and mice inoculated with mouse microbiota. Hippurate is known to be the end-product of benzoate metabolism and its formation has been previously discussed in the previous chapter (Chapter V). Indoxyl-sulfate, phenylacetylglucine, taurine and trimethylamine were observed in higher concentrations in the urinary profiles of conventional mice compared to GF and humanised animals. Nevertheless, these metabolites did not vary between conventional mice and mice inoculated with mouse microbiota. Finally, one unassigned metabolite, with a singlet at 3.599 ppm, was systematically observed in higher concentrations in urine of GF mice when compared to conventional and both inoculated groups.

It is noteworthy that formate was observed in higher concentrations in urine profiles of humanised and mice inoculated with mouse microbiota when compared to GF or conventional animals. Nevertheless, the low correlation values obtained for the pairwise comparisons between GF and HF animals, and between HF and conventional animals (0.49 and 0.52, respectively) could be due to the high heterogeneity of formate excretion profiles in humanised mice previously observed in $^1$H NMR spectra.

### 3.3.1.2.3. Specific focus at week 4

Following the same approach, the impact of the inocula origin (mouse or human) on the metabolic profiles of the GF host was characterised at several weeks after the inoculation day. Therefore, an O-PLS-DA model using two predictive and one orthogonal component was generated to compare all groups at week 4. The robustness of the model was supported by the following parameters, $Q^2_Y = 0.75$, $R^2_Y = 0.88$ and $R^2_X = 0.50$. In addition, this model has been validated by permutation tests (p-value < 0.001, perm = 1000). Interestingly, GF mice were strongly discriminated from the other groups according to the 1st component (Tcv 1, Figure 103). Humanised mice were strongly discriminated from the other groups according to the 2nd component (Tcv 2, Figure 103). The third component was not significant and was therefore not plotted since it did not allow visualisation of any
discrimination between all groups. According to Tcv 1 and Tcv 2, it is noteworthy that mouse-associated were more similar to conventional animals compared to humanised animals (Figure 103).

![Figure 103: O-PLS-DA cross-validated scores plot (Tcv 1 vs. Tcv 2) derived from $^1$H NMR urine spectra of GF (▲), mouse-associated (●), humanised (●) and conventional (■) animals.]

Pairwise comparisons were generated on all groups to facilitate data interpretation. First of all, GF mice were compared to conventional (Figure 104), mouse inoculated (Figure 105) and human inoculated mice (Figure 106), respectively. As expected, GF mice were strongly discriminated from these three groups as detailed in table 17 (p value < 0.001).
<table>
<thead>
<tr>
<th>Group comparisons</th>
<th>PC</th>
<th>OC</th>
<th>Q²Y</th>
<th>R²Y</th>
<th>R²X</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>GF vs MF</td>
<td>1</td>
<td>1</td>
<td>0.91</td>
<td>0.99</td>
<td>0.40</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>GF vs HF</td>
<td>1</td>
<td>1</td>
<td>0.90</td>
<td>0.98</td>
<td>0.32</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>GF vs Conventional</td>
<td>1</td>
<td>1</td>
<td>0.92</td>
<td>0.98</td>
<td>0.45</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>HF vs MF</td>
<td>1</td>
<td>1</td>
<td>0.63</td>
<td>0.97</td>
<td>0.30</td>
<td>0.001</td>
</tr>
<tr>
<td>HF vs Conventional</td>
<td>1</td>
<td>1</td>
<td>0.85</td>
<td>0.97</td>
<td>0.39</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>MF vs Conventional</td>
<td>1</td>
<td>1</td>
<td>0.78</td>
<td>0.94</td>
<td>0.41</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table 17: Summary of Q²Y, R²Y and R²X parameters for O-PLS-DA models and p-values derived from permutation tests (perm = 1000) at week 4.

**Metabolites related to the gut microbiota activity are involved in the discrimination between GF mice and the other groups**

Loadings plots derived from these models highlighted that mainly metabolites related to the gut microbial activity were involved in the discrimination between GF mice and the three other groups. Indeed, the sulfo-conjugate of m-HPPA, indoxyl-sulfate and phenylacetylglycine were systematically observed in lower concentrations in GF mice compared to the three other groups. In addition, hippurate was observed in lower concentrations in GF mice compared to both conventional and humanised animals. Surprisingly, no variations of hippurate were observed between GF and mouse-associated animals suggesting that the gut microbes present in the mouse inocula are not able to produce hippurate.

In addition, GF mice displayed systemic higher levels of keto-isocaproate, creatinine, and CH groups of amino acids compared to the three other groups (Figures 104, 105 and 106). Conversely, other microbial co-metabolites accounted for the major differences between these groups since both methylamine and trimethylamine were observed in higher concentrations in conventional, humanised and mouse-associated animals compared to their GF counterparts. It has been reported that dietary choline is absorbed from the lumen of the small intestine via transporter proteins in the enterocytes\(^{221,222}\) and esterified to phosphocholine and
glycerosphocholine. Most of the renal choline is oxidised to betaine which acts as methyl-donor, whereas some choline is accumulated in the kidney, and gets excreted unchanged in the urine\textsuperscript{223}. Within the methylamine pathway, dietary choline is converted into TMA by the gut microbiota and is then further oxidised to TMAO in the liver. Some of these methylamines are eventually excreted into urine\textsuperscript{223}.

**Figure 104:** O-PLS-DA scores plot derived from $^1$H NMR urinary spectra from GF (at the bottom) and conventional (at the top) mice at week 4.

A.1) 0.7-4.5 ppm - A.2) 6.5-8 ppm: this region has been vertically expanded $\times 10$. 

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Figure 105: O-PLS-DA scores plot derived from $^1$H NMR urinary spectra from GF (at the bottom) and humanised (at the top) mice at week 4.

B.1) 0.7-4 ppm.

B.2) 6.5-8 ppm: this region has been vertically expanded *10.
Figure 106: O-PLS-DA scores plot derived from $^1$H NMR urinary spectra from GF (at the bottom) and mouse-associated (at the top) animals at week 4.

C.1) 0.7-4.5 ppm.
C.2) 6.5-8 ppm: this region has been vertically expanded *10.

Complementary pairwise comparisons allowed discrimination of conventional from both mice inoculated with mouse microbiota and humanised animals, respectively (Figures 107 and 108). Both models were statistically robust and validated by permutation tests (perm = 1000, table 17).
Hippurate was a robust discriminant metabolite as it was observed in higher levels in conventional animals compared to the two other groups (Figures 107 and 108). Higher concentrations of phenylacetylglucose and indoxyl-sulfate were observed in conventional compared to humanised animals. Conversely, no variations of these two metabolites were observed between conventional and mouse-associated animals (Figure 106). In addition, the sulfo-conjugate of m-HPPA was observed in higher concentrations in mice inoculated with mouse microbiota compared to normal mice. This result clearly indicates that the mouse inocula and the gut microbes related to them are more able to degrade polyphenols (i.e. chlorogenic compounds) into bioavailable compounds than conventional mice. In addition, glycine and taurine were observed in higher levels and lysine, methylamine and methionine in lower levels in conventional mice compared to humanised mice.
Figure 107: O-PLS-DA scores plot derived from $^1$H NMR urinary spectra from conventional (at the bottom) and humanised (at the top) mice at week 4.

A.1) 0.7-4.5 ppm.

A.2) 6.5-8 ppm: this region has been vertically expanded *10.
Finally, following the same approach, humanised mice were discriminated from mouse-associated animals ($Q^2_Y=0.63$, $R^2_Y=0.97$, $R^2_X=0.30$ and p value = 0.001) and this was correlated with higher levels of citrate, dimethylamine, glycine, hippurate, keto-isocaproate, lysine, methionine, N-acetylglucosamine, and 

Figure 108: O-PLS-DA scores plot derived from $^1$H NMR urinary spectra from conventional (at the bottom) and mouse-associated (at the top) animals at week 4. 

B.1) 0.7-4.5 ppm. 

B.2) 6.5-8 ppm: this region has been vertically expanded *10.
trimethylamine-\(N\)-oxide and lower levels of phenylacetylglucine, indoxyl-sulfate and taurine in humanised mice compared to mouse-associated animals (Figure 109).

\textbf{Figure 109:} O-PLS-DA scores plot derived from \(^1\)H NMR urinary spectra from mouse-associated (at the bottom) and humanised (at the top) animals at week 4.  
A.1) 0.7-4.5 ppm.  
A.2) 6.5-8 ppm: this region has been vertically expanded *10.
3.4. UPLC-MS approach to target bile acid metabolism

3.4.1. Plasma samples

As previously observed (Chapter V), a typical chromatogram of plasma obtained by an UPLC-MS analysis is mainly dominated by phospholipids and lipids which are eluted between 14 and 18 minutes according to a 26 minutes gradient (Figure 110 and table 10). Bile acids are eluted between 8 minutes and 13 minutes.

Figure 110: Representative chromatograms of plasma obtained by UPLC-MS analysis from a conventional mouse (black, at the top), a mouse inoculated with mouse microbiota (green, second from the top) a humanised mouse (blue, second from the bottom), and a GF mouse (red, at the bottom).
Eight different bile acids were identified based on their mass over charge ratios (m/z) and their retention time, and by comparison with standards that were analysed using the same 26 minute gradient (Table 18).

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>m/z</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholic acid</td>
<td>CA</td>
<td>407.3</td>
<td>11.94</td>
</tr>
<tr>
<td>β-Muricholic acid</td>
<td>β-MCA</td>
<td>407.3</td>
<td>11.06</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>TCA</td>
<td>514.3</td>
<td>9.74</td>
</tr>
<tr>
<td>β-Tauromuricholic acid</td>
<td>β-TMCA</td>
<td>514.3</td>
<td>8.71</td>
</tr>
<tr>
<td>ω-Tauromuricholic acid</td>
<td>ω-TMCA</td>
<td>514.3</td>
<td>8.65</td>
</tr>
<tr>
<td>Taurohyodeoxycholic acid</td>
<td>THDCA</td>
<td>498.3</td>
<td>9.64</td>
</tr>
<tr>
<td>Taurodeoxycholic acid</td>
<td>TDCA</td>
<td>498.3</td>
<td>10.80</td>
</tr>
<tr>
<td>Glycodeoxycholic acid</td>
<td>GDCA</td>
<td>448.3</td>
<td>12.39</td>
</tr>
</tbody>
</table>

**Table 18:** Summary of the 8 identified bile acids in plasma samples from all groups, based on their mass over charge ratio (m/z) and retention time.
For all groups, the intensity of each bile acid was measured and compared by univariate statistics. The results are summarised in Figure 111 below.

Figure 111: Bile acids profiling in plasma samples obtained from UPLC-MS analysis (negative ion mode). Mean peak intensities +/- SEM, Student's t-test: * p<0.05; ** p<0.01; *** p<0.001. Since multiple comparisons were performed, the corresponding p-values were corrected using Bonferroni’s corrections.

First of all, no significant variations of CA, TCA and GDCA were observed between the 4 groups of mice. Mainly tauroconjugated bile acids (T-β-MCA, T-ω-MCA, THDCA/TUDCA, and TDCA) and β-MCA were observed in different concentrations among these animals (Figure 111). These bile acids varied according to the same trend as they were observed in higher concentrations in both GF and humanised mice when compared to either conventional animals or mice inoculated with mouse microbiota. Interestingly, no significant differences were observed between GF and humanised animals, or between conventional and mouse inoculated mice (Figure 111).
3.4.2. Bile samples

As explained previously (chapter V), a typical bile chromatogram is mainly dominated by the two primary bile acids, which are TCA and TMCA in the mouse. TCA has a mass over charge ratio of 514.3 and a retention time of 8.54 minutes (Figure 112), and TMCA has a mass over charge ratio of 514.3 and a retention time of 9.54 minutes (Figure 112).

In bile, the approach that was applied aimed at specifically targeting the two main bile acids previously cited (Chapter V) and at characterising the ratio of TCA over TMCA (Figure 113). Indeed, as explained previously, this ratio determines the hydrophobicity of the bile acid pool and impacts the cholesterol synthesis and absorption in the liver. In the present project, significantly different ratios were observed between conventional and both GF and humanised mice (p values = 5.10^{-14} and 2.10^{-12}, respectively). Similarly, significant different ratios were observed in mouse-associated animals compared to both GF and humanised mice (p values = 4.10^{-9} and 7.10^{-8}, respectively). Nevertheless, no significant differences were observed between conventional mice and mice inoculated with mouse microbiota, or between GF and humanised mice (p values > 0.05, Figure 113).
Figure 113: Bar-plot representative of the TCA over TMCA ratio in GF (red), conventional (black), humanised (blue) and mouse-associated mice (green).

Mean peak intensities +/- SEM, Student’s t-test: * p<0.05; ** p<0.01; *** p<0.001.
4. Discussion

The global set of results generated in this study strongly suggest that mouse-associated animals are immunologically, physiologically and metabolically closer to normal mice than humanised animals. As expected, GF mice were metabolically discriminated from the three other groups and results observed in this follow-up study matched data that have been already published on mouse and rat models\textsuperscript{25,26,29,58}.

4.1. Humanised mice are immunologically and physiologically deregulated

The physiological and immunological data collected on these mice clearly suggested that mouse-associated mice have a more natural metabolism closer to conventional mice than humanised mice. First of all, significant higher BW were observed between conventional and GF animals throughout this study. In addition, humanised mice displayed a significantly higher body weight than mouse-associated animals, at 6 weeks after inoculation. Surprisingly, no difference was observed at previous time-points, which illustrates the necessity for the host to adapt to human and mouse inocula, and for the gut microbes to establish and develop themselves in the gut before having an impact on the physiology of the host. This is concordant with previous findings in C3H mice showing that conventional and conventionalised mice displayed significantly higher BW compared to their GF counterparts\textsuperscript{29}. However, similar body weights and significant differences of body fat were observed in C57Bl6/J mice between conventional and conventionalised mice\textsuperscript{5}. Furthermore, plasma triglycerides were measured in higher levels in humanised mice compared to the three other groups and could be linked to the higher body weight observed in this humanised model. Conventional animals displayed higher levels of cholesterol and lower levels of free fatty acids compared to humanised mice, whereas no variation of both parameters was observed between normal, GF and mouse-associated animals. These results highlight the inability of the human microbiota to properly regulate the cholesterol, fatty acid and triglyceride synthesis and absorption in C3H mice. This is of particular interest since triglycerides are not free in the plasma but are encapsulated in VLDL. The lipoprotein lipase (LPL) cleaves triglycerides in VLDL to form fatty acids and glycerol\textsuperscript{224}. As for cholesterol, it is a major component of blood lipoprotein, which can be synthesised by the liver or intestine or can come from the
diet. Cholesterol is transported in chylomicrons to the intestine, and in VLDL in the liver\textsuperscript{224}. Interestingly, this is related to the strong perturbation of bile acid metabolism observed in these animals, since this metabolism derives directly from cholesterol synthesis. It is worth mentioning that bile acids play a crucial role as a signalling molecule in the liver to regulate their release in faeces. Taken altogether, these results suggest an inability of humanised mice to properly regulate and control the metabolism of the host. These mice showed physiological parameters that could be associated with the development of metabolic disorders. Nevertheless, these results would have to be complemented by investigating the hepatic triglyceride levels in these mice by either NMR, as applied in chapter V, or MS-based approaches in order to provide new information on the triglyceride metabolism of these mice\textsuperscript{77,225,226}. The investigation of triglyceride quality by targeted approach such as GC-MS and UPLC-MS based lipidomic analyses\textsuperscript{227} could provide new insights on the host energy and lipid metabolism of GF, inoculated and conventional mice.

Adaptive co-evolution of mammals and bacteria led to the establishment of symbiotic relationships that contributed to the development of the immune system in the host\textsuperscript{228,229}. The gut microbiota is known for playing a role in the education of the immune system, which allows efficient responses against pathogens\textsuperscript{230}. In the present study, the immunological parameters of C3H mice have been characterised and highlighted strong differences of immune cell population in Peyer’s patches cells, and of immunoglobulin and cytokine profiles between the four groups of mice. Indeed, conventional and mouse-associated animals displayed similar profiles of immune cell populations (B-cells, CD4+, T-cells and TFH), which strongly differed from those observed in both GF and humanised mice. B-cells are known as antibody producers whereas CD4+ are T helper (Th) cells and TFH are considered as a subset of Th cells. Similarly, IgA were observed in higher levels in conventional and mouse-associated animals compared to both GF and humanised mice, whereas no variations of IgG and IgM were observed among the groups. GF mice are known to display a decreased number of IgA, considered as lymphocytes producers in the lamina propria, compared to conventional animals\textsuperscript{58}. Interestingly, the similar level of IgA in GF and humanised mice (statistically lower than the one observed in both conventional and mouse-associated animals) constitutes a new finding. This result is of particular interest since IgA is the most abundant Ig isotype in mucosal secretions, and since plasma cells located in the gut lamina propria produce more IgA than any
other form of immunoglobulins\textsuperscript{231}. In addition, a previously reported response of the immune system to the microbial gut colonisation is the production of this specific IgA by the gut-associated lymphoid tissues (GALT)\textsuperscript{230,232}. IgA production could therefore be considered as a marker of the gut colonisation by microorganisms, and of their activity. Therefore, the immunological profiles obtained in this study suggested a different functionality of the gut microbiota depending on their origin – mouse or human – and an inability for the humanised mice to stimulate properly their immune system to reach levels observed in conventional and mouse-associated mice. This result was complemented by observed higher levels of CD4\(^+\) cells in both conventional and mouse-associated animals compared to both GF and humanised mice. CD4\(^+\) are defined as specific T cells that promote class-switching specifically to IgA, and potentially to specific adhesion molecules as well as factors that are derived from local environments and involved in the selective recruitment of precursors of mucosal IgA plasma cells\textsuperscript{231}. Finally, these IgA are also known for providing protection against mucosal pathogens and for acting in the maintenance of a diversified gut microbial community\textsuperscript{230}.

It is noteworthy that the interleukin profiles of these mice displayed significant variations according to the microbial status and the origin of the microbiota. IL-4, an interleukin secreted by macrophages to promote the differentiation of T helper cells, presented a similar profile to IgA and CD4\(^+\) cells since they were observed in higher levels in both conventional and mouse-associated animals compared to both GF and humanised mice. Very few works have been performed on the relation between gut microbiota and both cytokine and interleukin profiles. Nevertheless, these results are concordant with a previous publication showing that the indigenous microbiota contribute to the induction and maintenance of CD4\(^+\) T cells\textsuperscript{233}. Same variations of IL-22 were observed in these mice. Interestingly, in healthy mice, the T helper cell 17 (T\(_h\) 17) subset of T cells (CD4\(^+\)) are reported as responsible for the production of interleukin 17 (IL-17) and IL-22, which are enriched in the intestine. Indeed, many beneficial effects of the gut microbiota are modulated by microbial interactions with epithelial and immune cells in the intestine\textsuperscript{231}. Since humanised mice displayed similar levels of IL-4 and IL-22 compared to GF mice, this result showed the inability of humanised animals to mount a sufficient immune response to attack pathogens. Indeed, pro-inflammatory T cells in the intestine are evaluated by measuring the levels of CD4\(^+\) T cells.
Taken altogether, the physiological and immunological characterisation of C3H mice suggested that human microbes could not be suitable to the mouse host since they strongly deregulated the physiology of the host (higher levels of plasma triglycerides) and weakly promote the immune system development of the ex-GF mice. In comparison, mouse microbiota seemed to provide a set of functions sufficient to mimic, at a certain level, the functions of bacterial microbes residing in the gut of normal mice.

4.2. “GF like” bile acids excretion pattern in humanised mice strongly differ from mouse-associated mice

Bile acid targeted profiling in plasma and bile of C3H mice highlighted a strong perturbation of their metabolism due to the inoculation with either human or mouse inocula. Firstly, plasma tauroconjugated bile acid metabolism was stimulated in both GF and humanised mice as similar higher levels were observed in these two groups compared to both conventional and mouse-associated animals. These results confirmed plasma bile acid profiles detailed in the previous chapter (Chapter V) and results previously reported\(^\text{25,165,191,207,208}\). Tauroconjugated bile acids are the main conjugated family detected in mouse plasma\(^\text{234}\) and have been reported in higher levels in ex-GF mice colonised with human baby flora (HBF) compared to conventional mice\(^\text{165}\). In another mouse model, Want \textit{et al.} demonstrated that the profile of tauroconjugated bile acids was mainly perturbed after exposition to galactosamine\(^\text{191}\). In addition, the metabolism of this bile acid family was up regulated in colonised and antibiotics treated rats compared to their conventional counterparts\(^\text{25}\). Taken altogether, these results indicated that the modification of the gut microbial composition, and therefore its activity, by either colonisation or the use of antibiotics impacted in priority tauroconjugated bile acids in the murine host.

In addition, in the present study, bile acid profiles in mouse bile were characterised by similar TCA / TMCA ratios in both conventional and mouse-associated animals (2:1), which were statistically different from those observed in both GF and humanised mice (1:1). As previously explained, it has been reported that this ratio in GF mice and rats is of 1:1\(^\text{29,210}\). Therefore, the observed “conventional like” bile acid excretion pattern in mouse-associated animals is a new finding and matched ratio observed by Claus \textit{et al} in conventionalised mice, defined as GF mice transferred to an open environment to allow the environmental bacteria
to colonise their gut\textsuperscript{29}. Indeed, these animals were already reported as displaying a 2:1 TCA / TMCA ratio only 5 days after reconvencionalisation. In contrast, humanised C3H mice displayed in the present study a “GF like” bile acid excretion pattern, which is similar to what has been obtained previously in humanised C57Bl6/J mice (chapter V). As mentioned previously, this ratio is regulated by the enzyme CYP8B1, which is known to be downregulated in GF animals. Unfortunately, such measurement was not possible in the present study.

These results confirmed that the bile acid metabolism in the murine host is directly affected by the origin and activity of the gut microbiota. In addition, this study suggested that mouse microbiota seem to display functions that are similar to those of naturally colonised mice, and are able to mimic conventional BAs deconjugation in the intestinal lumen of the host. In contrast, human inocula do not seem to provide functions that could mimic the bacteria functions of conventional mice, in particular the conjugation activity, which is usually provided by the intestinal bacterial community\textsuperscript{30,31,235}.

4.3. Urinary metabolic phenotypes highlight similarities between normal and mouse-associated mice

GF mice are known to respond strongly metabolically to the inoculation of foreign inocula into their gastrointestinal tract by gavage\textsuperscript{165,166}. This process is more brutal than the process of open environment conventionalisation, which consists of moving a GF animal out of its isolator to allow the environmental bacteria to colonise it\textsuperscript{29}. In the present study, human and mouse-associated animals responded to the introduction of inocula by producing subsequently significantly high levels of formate one day post inoculation. Surprisingly, this level of formate returned to the basal level (defined in this case as the production of formate in GF and conventional mice) very quickly as no differences were observed between all groups 3 days after inoculation. The metabolism of formate is not well known to date and has been already discussed previously (chapter IV, Discussion). This could be due to the presence of specific formate-producer bacteria in the gut at day 1 precisely. This hypothesis would have to be further validated by obtaining the gut microbiota composition (16S rDNA pyrosequencing) of these mice and investigating the presence of a specific family of bacteria that could be reported as a formate-producer.
The urinary metabolic profiles obtained from all groups highlighted more similarities between mouse-associated and normal mice than between humanised and conventional mice. It is noteworthy that metabolites related to the gut microbiota activity such as hippurate, phenylacetylglycine and indoxyl-sulfate were involved in the discrimination between conventional and the three other groups. Hippurate and phenylacetylglycine metabolism have been described in the previous chapters (Chapters IV and V). Interestingly, indoxyl-sulfate originates from bacterial protein fermentation in the large intestine. Indeed, colonic microbiota are known for degrading tryptophan to indole, which is then transformed in 3-hydroxy-indole by hydroxylation. The majority of this compound is further sulfonated to form indoxyl-sulfate. These results suggested that conventional microbiota remained the most complete and that the mouse microbes established in the GF model is more able to mimic conventional functions compared to human-associated gut microbes.

4.4. One donor as the best approach to perform inoculation procedures with microbiota of human and animal origins

Taken together, the collection of physiological, immunological and metabolic parameters of human-associated and mouse-associated animals in this follow up study indicate that the use of a single donor seems to be the best approach to perform inoculation of complex microbiota in GF host. Indeed, this procedure avoids creating inter-individual differences. Therefore, the approach based on 5 different donors used to compare lean and obese-human associated animals (Chapter V) does not seem to be the most suitable procedure to compare the impact of two types of microbiota. Nevertheless, another procedure that has been previously used and demonstrated to be efficient consists of mixing altogether the faecal samples collected from different donors. This approach allows the creation of a mix that will be representative of the bacterial community of the donor (mouse, human).
5. Conclusion

This study presented an attempt to physiologically, immunologically and metabolically characterise the impact of complex microbiota (mouse and human origins) on the metabolism of ex-GF mice. It is worth mentioning that physiological variations observed in the mice (i.e. variations of body weight and plasma triglyceride levels) were also complemented by an immature immune system in humanised mice compared to both conventional and mouse-associated animals. In addition, these differences were directly reflected in the urinary metabolic profiles of C3H mice.

This collection of information is, to my knowledge, the first attempt of combining the study of immunological, physiological and metabolic parameters of the host. This approach is definitely suitable for this kind of study and will provide new insights in the characterisation of the symbiotic relationships between the host and its gut microbiota. Finally, this will contribute, in the future, in the understanding of the role of gut bacteria in metabolic disorders, such as obesity and diabetes, and the deregulation of the homeostasis.
Chapter VII  General Discussion

1. Summary of the results

Extensive characterisation of the interaction between the host and gut microbiota was conducted in the different projects detailed in this PhD thesis. I confirmed that NMR- and MS-based metabolic profiling approaches are suited to study this interaction. Indeed, these two high throughput complementary techniques were used, in the present thesis, for both a global (by NMR) and targeted (by MS) characterisation of the metabolic profiles in different biological matrices. Both NMR investigation of both biofluids (urine, plasma) and tissues (brown adipose tissue and liver), and MS analysis of plasma and bile, to specifically target their constituent bile acids, provided new insights into the metabolism of C57Bl6/J and C3H mice harbouring various functional intestinal gut microbes (i.e. conventional and GF states, different microbial composition and biodiversity, mouse and human origins).

By combining these two platforms, I demonstrated that the origin and biodiversity of the microorganisms residing in the host gut directly impact its metabolic phenotype. For instance, GF mice were strongly discriminated from conventional mice (chapters III and VI) and several discriminant urinary metabolites were identified in higher concentrations in the latter, such as \( m \)-HPPA sulphate and keto-isocaproate (chapters III and VI), and hippurate, indoxyl-sulfate and phenylacetylglycine (chapters V and VI). It is noteworthy that most of these discriminant metabolites are known to be gut microbial co-metabolites and confirmed previous findings\(^{26,29,203,204,236,238}\). In addition, an important outcome was the identification of similar variations of gut microbial co-metabolites independently of the genetic background of the mouse strain, i.e. C57Bl6/J and C3H mice. It is worth mentioning that few data are available about the metabolic characterisation of ex-GF animals inoculated with mouse or human gut microbes, making this project unique in this field. Hence, I demonstrated different gut microbial activities between GF, inoculated and normal mice since systematic variations of hippurate, phenylacetylgyglycine, indoxyl-sulfate and \( m \)-HPPA sulfate were observed in conventional animals when compared to their inoculated and GF counterparts. Surprisingly, mice inoculated with mouse microbiota produced higher levels of \( m \)-HPPA sulfate compared to normal mice. This result is probably due to different gut
microbiota composition and/or functionality between both groups, since m-HPPA sulfate is derived from the metabolism by the gut microbes of polyphenols into bioavailable compounds. This observation suggests that the transfer of mouse microbes from a unique donor to the different receivers could have favoured the development of specific bugs responsible for the production of m-HPPA sulfate.

In addition, an UPLC-MS-based metabolomic approach was applied in plasma and bile samples from C3H and C57Bl6/J mice. This analysis provided new information on biofluid composition and relative importance of unconjugated, glycoconjugated and tauroconjugated bile acids. Results collected throughout this thesis highlight a strong perturbation of the bile acid metabolism in GF and humanised animals that complemented previous results reported in both GF and conventional rats and mice. Moreover, this approach highlighted a disruption of the tauro-conjugated family metabolism, which is directly related to the gut microbial biodiversity according to previous findings in rodent models. Humanised C57Bl6/J and C3H mice displayed similar profiles of bile acid in plasma and bile (chapter V and VI) and, biliary TCA/TMCA ratios in both strains were very similar to those observed in GF mice, independently of the genetic background.

Finally, several studies and reviews have illustrated the symbiotic interaction between the host and its gut microbiota. In this context, the originality of this work was the integration of these metabolic phenotypes with OTUs generated by 16S rDNA pyrosequencing of the gut bacterial content, in conventional and ex-GF mice inoculated with lean or human microbiota. The beneficial input of this approach will be detailed below (section “impact and contribution to human physiology”). Results generated by NMR- and MS-based metabolic profiling approaches and 16S rDNA pyrosequencing have been interpreted in parallel with the physiological and immunological parameters of the mouse host and highlighted an impact of the microbial community on the immunology and physiology of the host measured at a relative long term (6 weeks after the beginning of the experiment).

2. Contribution of the gut microbiome to the host homeostasis

As expected, the introduction of gut microbes in a GF host led to a strong disruption of its metabolism, since this host has not been in contact before with any microorganisms. Interestingly, the establishment of these microbes seem to be dependent on several factors such as the inoculation procedure, the
identity and composition of the inoculated bacteria, the bacteria-bacteria interaction as well as the origin of the inocula\textsuperscript{158,160,163,174}. Indeed, it has been shown in this PhD thesis that different inoculation procedures impact the profile of bacteria established in the GF gut. In order to inoculate a cocktail of 10 individual bacteria, the best inoculation procedure was obtained by the inoculation of one bacterium per GF mice, and, then, by mixing the mice altogether (chapter IV). These results clearly suggested that the GF gut, firstly colonised by an individual bacterium, is stimulated and more able to be further colonised by other microorganisms. Therefore, these results showed that the statement considering that the GF host does not present competition for colonisation, making it relatively easy to deliberately colonise these animals with a few defined microbial species, is not correct. One hypothesis is that the inoculation of a cocktail of 10 bacteria creates a competition between the microorganisms for establishing themselves in the gut, and therefore strong or most suitable bacteria will dominate the weak ones in this particular environment. Other studies were in agreement with these results suggesting that competitive interaction between members of a simplified microbiota might prevent establishment of less performing strains\textsuperscript{175,176}. Interestingly, simplified microbiota, such as a community composed of 3 or 9 bacteria, generated different urinary and plasma metabolic profiles. Mice harbouring 3 bacteria displayed higher plasma levels of lipids and lipoproteins compared to those harbouring 9 bacteria (chapter IV) suggesting a better regulation of lipoprotein and circulating lipid levels with a more complete microbial community which may have implications for weight management and homeostasis regulation.

In the subsequent projects (Chapters V and VI), it is worth mentioning that complex microbiota from different origins established easily in the GF host. Indeed, lean and obese human microbiota inoculated to GF animals established themselves strongly and rapidly in the gut and impacted consequently the metabolism of the mice. Of note is the fact that 16S rDNA sequencing data obtained from human donors did not show the same profile between lean and obese human donors, illustrating the diversity of the microbiota in the human population that has been already reported\textsuperscript{4,12,15,43,164}. Indeed, each person harbour a distinct collection of bacterial species,\textsuperscript{240} and this could explain some of the heterogeneities observed within each group of donor. Based on the results obtained in the last project (chapter VI), mouse and human microbiota colonised efficiently the GF host since the

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metabolic profiles generated one day after inoculation were already different between the 4 groups. This assumption would have to be confirmed by the characterisation of the gut microbiota composition in C3H mice by 16S rDNA sequencing.

It is noteworthy that inocula of different origins induced various metabolic adaptations in the host on the short term and long term. Urinary metabolic profiles in C3H mice showed that mouse and human inoculated animals produced high levels of formate in response to the introduction of the microbiota, since it was observed at one day post-inoculation, only. No physiological perturbations were observed in the first days following the inoculation. Then, at 6 weeks after the beginning of the experiment, physiological changes were measured between conventional and inoculated C3H and C57Bl6/J mice in response to the adaptation of the host to these gut microbes and development of this symbiotic relationship. This was illustrated in C3H mice by systemic discriminations between mouse-associated and humanised mice throughout the study, whereas 6 weeks were necessary to discriminate urinary metabolic profiles between lean and obese human inoculated C57Bl6/J mice. These results suggested that the response of the host to the introduction of foreign microbes is strongly dependent on the diversity of the inocula (number of donors), its origin (mouse, human, lean, obese) as well as the timeline of the measurements. This is particularly illustrated by the impact of the microbiota on the physiological data since significant differences of body weight, total body fat content, and triglyceride levels were observed among ex-GF animals inoculated with different types of microbiota and conventional animals. The strongest effects of the inoculation on the physiological status of the host were systematically observed through long term measurement of the population (6 weeks after inoculation).

Taken altogether, results collected in the BAMBEE project indicated clearly that the gut microbial composition and biodiversity has a direct impact on the metabolism of the host and therefore play a crucial role in the homeostasis of C3H and C57Bl6/J mice. Interestingly, it has been demonstrated that gut microbes from a mouse donor, with the same genetic background as the receivers, provided functions similar to those of conventional animals since the immune system, bile acid metabolism and physiological parameters of these two mouse groups were similar. In contrast, humanised mice strongly differed immunologically, metabolically and physiologically from conventional and mice inoculated with mouse microbiota.
(Chapter VI). This is probably due to the differences in the enzymatic apparatus and intrinsic metabolism existing between humans and mice.

3. Impact and contribution to human physiology

The work presented in this PhD thesis aimed at improving our knowledge in the development of metabolic disorders and obesity in relationship with the gut microbiota. Therefore, in order to better understand the specific interaction between the host and its gut microbiota, a promising strategic approach was developed in this project, and was based on performing statistical correlations between mouse urinary and hepatic metabolic profiles and OTUs obtained from faecal 16S rDNA sequencing in both conventional and humanised animals. This approach aimed to identify variation in the concentrations of metabolites that were positively or negatively associated with one or several bacteria, and provided very interesting results in the present project. Indeed, I have been able to identify specific correlations between Faecalibacterium prausnitzii and lipids and triglyceride resonances in the lipophilic hepatic metabolic profiles of mice inoculated with obese human microbiota. Interestingly, this bacterium has reported already been reported to play a crucial role in Crohn’s disease and obesity in an Indian cohort. Similarly, Ruminococcus callidus was observed as correlating negatively with CH₃ groups of cholesterol and CH₂ groups from of fatty acyl chains in mice inoculated with lean human microbiota. Finally, Blautia wexlerae and Ruminococcus lactaris correlated positively with glucose resonances in the hydrophilic hepatic metabolic profiles of lean and human inoculated mice, respectively. This approach is very promising since the bacteria identified by this statistical approach could therefore be considered as potential probiotic candidates, which could further be tested in follow-up studies in mice and humans. Indeed, probiotics are defined by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as “live microorganisms, which when administrated in adequate amounts confer a health benefit on the host” and have been used for centuries in dairy-based and fermented products. Conversely, the same association defined prebiotics as “non-digestible foods that beneficially affect the host by selectively stimulating the growth or/and activity of one or more selective bacteria in the host” and could be also suitable for this type of application. It is of potential interest to modulate the composition of the gut microbiota by the ingestion of a dietary product (i.e. ice creams, yogurts, cereal bars, etc...) enriched with a
specific probiotic. To make such developments requires the confirmation that these findings will be transposable to the human organism. Pre and probiotics are already widely used in human nutrition\textsuperscript{166,217,241-249}. However, the effect of potential bacteria, identified in mouse studies, remains non evident on human physiology.

Finally, humanised mice have been initially developed and used in several domains to mimic human physiological conditions. Nevertheless, it is worth mentioning that the metabolic activities of the intestinal microbiota from an experimental model are different from the human intestinal microbiota. Therefore, it is always complicated to extrapolate results from animal studies to human physiology\textsuperscript{190}. First models were developed in the 80’s where human gut microbes were inoculated to GF rats and mice\textsuperscript{250-252}. Such approaches provided new insights into the understanding of certain diseases, host physiology, enzymatic activities, putrefactive products and SCFA metabolism\textsuperscript{158,253,254}. In addition, these humanised models led to major advances in the field of immunity, human haematopoiesis, cancer biology and regenerative medicine\textsuperscript{255}.

4. Future works and perspectives

The different approaches developed in this PhD thesis have globally generated promising new results on the interactions between the host and its microbiota. This thesis reinforces the link previously described between the immunological status, metabolism and gut microbiota of the host. Indeed, the immune-metabolic axis is at present relatively poorly studied and understood in both mice and humans. Constructive integration of metabolic, immune and gut bacterial data would help the understanding of this global network and would represent a major field of study for human physiology in the context of nutritional intervention. Since the host is a whole system functioning as a highly integrative network, in which all the components are related to each other and interacting together, understanding these mechanisms will help therefore to regulate its physiological processes and to maintain its homeostatic status.

Nowadays, based on studies previously published and results generated in this PhD thesis, animals colonised with different microbiota are at present well characterised. My opinion is that the scientific community needs to understand how a simple microbe influences and impacts the physiology, immunology and metabolism of the host. Therefore, approaches called mono-, bi- or tri-associations, defined as
the introduction of one, two or three bacteria in a GF host, respectively, can provide new insights about how bacteria, in a very simple consortium, interact with each other and the host\textsuperscript{256}. Indeed, the bacteria-bacteria dialog as well as their competition to colonise and establish in a new environment is a crucial factor for characterising this symbiotic relationship. This type of microbial community could provide further insights into the beneficial functions provided by bi-associated or tri-associated animals compared to a model harbouring only one bacterium. Therefore, these approaches would help to better characterise the impact of the microbial biodiversity on the physiology of the host harbouring a very simple consortium. However, it is worth mentioning that these associations are not representative of the gut microbiota composition, biodiversity and abundance generally observed in human and animal physiology \textsuperscript{(10\textsuperscript{14} bacteria)}.

It is noteworthy that the effect of diet on the host metabolism was not studied in this PhD thesis. Several studies demonstrated that the host diet plays a dramatic role in shaping the structure of microbial communities\textsuperscript{4,18,240}. Indeed, high-fat diet (HFD) and Western diet are known to rapidly reconfigure the gut microbial composition\textsuperscript{164}. Similarly, HFD have been reported for decades to induce global metabolic changes that could lead to muscle lipid accumulation involved in the induction of insulin resistance, obesity, type II diabetes and metabolic syndrome\textsuperscript{257-260}. Indeed, several publications demonstrated that a diet switch toward HFD leads to the modification of several metabolic pathways in order to optimise the processing of fat and to maintain energy homeostasis\textsuperscript{259,261}. In this context, it could be of potential interest to characterise the impact of a high-fat diet on a host harbouring a simple bacterial consortium (i.e. mono, bi or tri-assocations), a cocktail of individual bacterial strains (i.e. cocktail of bacteria used for the Nestle Simplified Flora or others) or a complex microbiota (i.e. lean or obese human/mouse origins). In addition, the strain specificity of these results remains to be determined since the study in C57Bl6/J and C3H mice were not conducted in parallel. This approach could provide new insights into the role of selected bacteria and genetic background in the development of obese phenotypes or in the resistance to diet-induced obesity (DIO).

Interestingly, GF animals are not the only model that could be used for this type of approach. Recently, the administration of a three-week course of a broad-spectrum cocktail of antibiotics has been used in a mouse model to remove the residing gut microbes\textsuperscript{262}. This approach presents several advantages compared to
the GF host: i) this model is easier to grow and to keep alive as it is not necessary to maintain these animals in isolators to avoid any contamination. ii) the model is cheaper as it required less animal facilities. For instance, it appeared that the C57Bl6/J mouse strain, which is expensive to grow, is very unstable under the GF state (high level of mortality in the first weeks of life, low level of survival to the inoculation of gut microbes, etc) iii) this model presents the advantage, compared to GF animals, that it has been in contact before with microorganisms from the environment. Therefore, there is no bias such as the morphological, physiological, biochemical and immunological characteristics which are known to be strongly different in GF mice compared to their conventional counterparts (Chapters III and VI)\textsuperscript{26,58}. 
REFERENCES


203. Mulder TP, Rietveld AG, van Amelsvoort JM. Consumption of both black tea and green tea results in an increase in the excretion of hippuric acid into urine. Am J Clin Nutr. 2005 Jan;81(1 Suppl):256S-60S.


APPENDIX I: SUPPLEMENTAL DATA

The figure below corresponds to $^1$H NMR acquisitions of a representative plasma sample from an ex-GF male mouse harbouring 9 bacteria in its gut. Several acquisitions were performed at various temperatures (from 275K to 305K). It is worth mentioning that the broad signal observed on the left side in Figure A shifted slowly to the right side when the acquisition temperature has been increased (Figures B, C, D and E, respectively). This observation confirmed that the unassigned metabolite corresponds to an –NH, -SH or –OH proton in slow exchange with the HOD protons. Indeed, by applying different acquisition temperature, the putative NH peak moved downfield and this phenomenon depended directly on the kinetics of the exchange process.
Appendix 1: Zoom on the specific region from $^1$H NMR spectra (4.1 to 4.14 ppm) acquired at 5 different temperatures (A: 305K, B: 300K, C: 290K, D: 280K, E: 275K).
APPENDIX II: FOOD COMPOSITION (CHOW DIET)

A03 DIET DATA SHEET

• DEFINITION
Diet for laboratory Rats and Mice

• PRODUCT OBJECTIVE
Rodent diet destined for growing and breeding animals (pregnant and nursing)

Distribution period:
• from birth, provide transition diet to A04 if necessary, during weaning.
• adult rodents during gestation and lactation.

Daily amount consumed: rats 15 to 22 g, mice from 6 to 10 g.
Method of distribution: ad libitum or rationed according to experimental protocols.

• PRODUCT PRESENTATION
10 mm diameter granulate

• PACKAGING

<table>
<thead>
<tr>
<th>Diet status</th>
<th>Packaging</th>
<th>Packing</th>
<th>Analytical sheet</th>
<th>Level of irradiation</th>
<th>Animal</th>
</tr>
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<tr>
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<td>10 kg</td>
<td>Paper bag</td>
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<td>No</td>
<td>45 kilograys</td>
<td>EOPS/IOPS/SPF Immunodepressed</td>
</tr>
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</table>

• MAINTENANCE CONDITIONS
Diet variant according to the status of the animal unit.
- CENTESIMAL COMPOSITION

- NUTRITIONAL COMPOSITION

Caloric intake (kcal/kg) 3200

Values are given as an indication only. They are average values.

### AMINO ACID VALUES
Calculated / kg

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<thead>
<tr>
<th>Quantity</th>
<th>Amino Acid</th>
</tr>
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<td>Arginine</td>
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<tr>
<td>3000 mg</td>
<td>Cystine</td>
</tr>
<tr>
<td>14600 mg</td>
<td>Lysine</td>
</tr>
<tr>
<td>4800 mg</td>
<td>Methionine</td>
</tr>
<tr>
<td>2600 mg</td>
<td>Tryptophan</td>
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<tr>
<td>12700 mg</td>
<td>Glycine</td>
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### FATTY ACID VALUES
Calculated / kg

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</tr>
<tr>
<td>900 mg</td>
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<tr>
<td>4500 mg</td>
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<td>10600 mg</td>
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<td>15300 mg</td>
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<td>Traces</td>
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## MINERAL AND VITAMIN CONTENT

Minerals calculated / kg

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<th>CMV val.</th>
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<td>mg</td>
<td>5 000</td>
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<td>Ca</td>
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<td>7 500</td>
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<tr>
<td>Na</td>
<td>mg</td>
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</tr>
<tr>
<td>K</td>
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<td>7 500</td>
<td>7 500</td>
</tr>
<tr>
<td>Mg</td>
<td>mg</td>
<td>1 990</td>
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</tr>
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</tr>
<tr>
<td>Fe</td>
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<td>Zn</td>
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<tr>
<td>Co</td>
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Vitamins calculated / kg

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<tr>
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<td>mg</td>
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<tr>
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<tr>
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<td>30</td>
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## MEAN TEST SHEET:

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<td>9</td>
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<tr>
<td>Variation from theoretical weight</td>
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<td>0,06</td>
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<tr>
<td>Resistance to crushing (kgf/cm²)</td>
<td>14,6</td>
<td>2</td>
<td>9 to 20</td>
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<td>Resistance to abrasing (%)</td>
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<td>(&gt; 97)</td>
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<td>(&lt; 3)</td>
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<td>Number of pellets burnt (kg)</td>
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<td>0</td>
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<tr>
<td>Incorporation of macro-mineral mix (Na)</td>
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<td>(9 to 14)</td>
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<td>0.8</td>
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<td>of which starch (%)</td>
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<td>of which total sugars (%)</td>
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<td></td>
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<td>21</td>
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<td>Arsenic - As (µg/kg)</td>
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<td>Selenium - Se (µg/kg)</td>
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<td>&lt; 5</td>
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<td>b HCH</td>
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SYNTHETIC PYRETHRINOIDS (µg/kg)

none
APPENDIX III: PUBLICATION AND PUBLISHED ABSTRACTS

Published articles

1st paper: Gut microbiota modulate the metabolism of Brown Adipose Tissue in mice. Mestdagh R.¹, Dumas M.E.¹, Rezzi S., Kochhar S., Holmes E.¹, Claus S.P.¹ Nicholson J. K.¹ (2011) Journal of Proteome Research

¹Division of Biomolecular Medicine, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, SW7 2AZ, London, UK.

²BioAnalytical Science Department, Nestlé Research Centre, Vers-Chez-Les-Blancs, Lausanne, Switzerland.

Abstract

A two by two experimental study has been designed to determine the effect of gut microbiota on energy metabolism in mouse models. The metabolic phenotype of germ-free (GF, n=20) and conventional (n=20) mice was characterized using a NMR spectroscopy-based metabolic profiling approach, with a focus on sexual dimorphism (20 males, 20 females) and energy metabolism in urine, plasma, liver and brown adipose tissue (BAT). Physiological data of age-matched GF and conventional mice showed that male animals had a higher weight than females in both groups. In addition, conventional males had a significantly higher total body fat content (TBFC) compared to conventional females while this sexual dimorphism disappeared in GF animals (i.e. male GF mice had a TBFC similar to those of conventional and GF females). Profiling of BAT hydrophilic extracts revealed that sexual dimorphism in normal mice was absent in GF animals, which also displayed lower BAT lactate levels and higher levels of (D)-3-hydroxybutyrate in liver, plasma and BAT, together with lower circulating levels of VLDL. These data indicate that the gut microbiota modulate the lipid metabolism in BAT as the absence of gut microbiota stimulated both hepatic and BAT lipolysis while inhibiting lipogenesis. We also demonstrated that ¹H NMR metabolic profiles of BAT were excellent predictors of BW and TBFC indicating the potential of BAT to fight against obesity.
Abstract

Rodent models harboring a simple yet functional human intestinal microbiota provide a valuable tool to study the relationships between mammals and their bacterial inhabitants. In this study, we aimed to develop a simplified gnotobiotic mouse model containing 10 easy-to-grow bacteria, readily available from culture repositories, and of known genome sequence, that overall reflect the dominant commensal bacterial makeup found in adult human feces. We observed that merely inoculating a mix of fresh bacterial cultures into ex-germ free mice did not guarantee a successful intestinal colonization of the entire bacterial set, as mice inoculated simultaneously with all strains only harbored 3 after 21 days. Several inoculation procedures were, therefore, tested and levels of individual strains were quantified using molecular tools. Best results were obtained by inoculating single bacterial strains into individual animals, followed by an interval of two weeks before allowing the animals to socialize to exchange their commensal microbes. Through this procedure, animals were colonized with almost the complete bacterial set (9/10). Differences in the intestinal composition were also reflected in the urine and plasma metabolic profiles, where changes in lipids, SCFA, and amino acids were observed. We conclude that adaptation of bacterial strains to the host’s gut environment
(mono-colonization) may predict a successful establishment of a more complex microbiota in rodents.
Published poster abstract

Metabolic investigation of the gender differences in energy metabolism of C3H mice in response to their germ-free status. Mestdagh R.¹, Claus S.P.¹, Dumas M.E.¹, Holmes E.¹, Nicholson J.K.¹. (2010) Society for General Microbiology, Spring Conference, Harrogate.

¹Division of Biomolecular Medicine, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, SW7 2AZ, London, UK.

Abstract

A two by two experimental study has been designed to determine the effect of gut microbiota on energy metabolism in mouse models. The metabolic phenotype of germ-free (GF, n=20) and conventional (n=20) mice was characterized using a NMR spectroscopy-based metabolic profiling approach, with a focus on sexual dimorphism (20 males, 20 females) and energy metabolism in urine, plasma, liver and brown adipose tissue (BAT). Physiological data of age-matched GF and conventional mice showed that male animals had a higher weight than females in both groups. In addition, conventional males had a significantly higher total body fat content (TBFC) compared to conventional females while this sexual dimorphism disappeared in GF animals (i.e. male GF mice had a TBFC similar to those of conventional and GF females). Profiling of BAT hydrophilic extracts revealed that sexual dimorphism in normal mice was absent in GF animals, which also displayed lower BAT lactate levels and higher levels of (D)-3-hydroxybutyrate in liver, plasma and BAT, together with lower circulating levels of VLDL. These data indicate that the gut microbiota modulate the lipid metabolism in BAT as the absence of gut microbiota stimulated both hepatic and BAT lipolysis while inhibiting lipogenesis. We also demonstrated that ¹H NMR metabolic profiles of BAT were excellent predictors of BW and TBFC indicating the potential of BAT to fight against obesity.
Metabolic monitoring of the colonisation of germ-free mice with human and mouse microbiota. Mestdagh R.¹, Rezzonico E.², Dumas M.E.¹, Claus S.P.¹, Sirohi S.², Holmes E.¹, Nicholson J.K.¹ (2011) Reseau Francais de Metabolomique et Fluxomique (RFMF).

¹Division of Biomolecular Medicine, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, SW7 2AZ, London, UK.
²BioAnalytical Science Department, Nestlé Research Centre, Vers-Chez-Les-Blancs, Lausanne, Switzerland.

Abstract
The gut microbiota play an essential role in the metabolism of the host as intestinal bacterial species are engaged in various metabolic aspects [1], and deregulation of bacterial metabolism may trigger metabolic disorders and diseases (i.e. obesity, diabetes, irritable bowel syndrome) [2]. Microbiota transplantation experiments performed in germ-free (GF) mice showed that individual intestinal microbiota differentially affect energy harvesting and fat storage [3,4], suggesting a potential role for the gut microbiota in the development of obesity.

The present study aims to compare the impact of conventionalization of GF mice with mouse and human intestinal microbiota on metabolic phenotypes and gut microbiota composition [5,6]. The metabolic profiles of GF, inoculated and conventional mice were characterized using a NMR-based metabolic profiling approach in various biofluids and tissues complemented with a targeted analysis of bile acids by UPLC-MS [7,8]. Conventionalization with different complex inocula led to distinct metabolic responses associated with unique microbiota profiles. In addition, urinary metabolic profiles displayed a strong discrimination between inoculated mice and control groups. Interestingly, mouse and human microbiota seemed to impact differently the host metabolism even at an early stage. Finally, the bile acid metabolism of the host was strongly affected and differed from conventional mice.

These results indicate that the origin (mouse or human) of the inocula impacts severely the host metabolism with a specific effect on the metabolism of bile acids. This study opens new insights regarding the understanding of the host metabolism-
gut microbiota interaction and the adaptation to colonisation with mouse or human microbiota.

Bibliography:


Oral communications


Metabolic monitoring of the colonisation of germ-free mice with human and mouse microbiota

Abstract

The gut microbiota play an essential role in the metabolism of the host as intestinal bacterial species are engaged in various metabolic aspects [1], and deregulation of bacterial metabolism may trigger metabolic disorders and diseases (i.e. obesity, diabetes, irritable bowel syndrome) [2]. Microbiota transplantation experiments performed in germ-free (GF) mice showed that individual intestinal microbiota differentially affect energy harvesting and fat storage [3,4], suggesting a potential role for the gut microbiota in the development of obesity.

The present study aims to compare the impact of conventionalization of GF mice with mouse and human intestinal microbiota on metabolic phenotypes and gut microbiota composition [5,6]. The metabolic profiles of GF, inoculated and conventional mice were characterized using a NMR-based metabolic profiling approach in various biofluids and tissues complemented with a targeted analysis of bile acids by UPLC-MS [7,8]. Conventionalization with different complex inocula led to distinct metabolic responses associated with unique microbiota profiles. In addition, urinary metabolic profiles displayed a strong discrimination between inoculated mice and control groups. Interestingly, mouse and human microbiota seemed to impact differently the host metabolism even at an early stage. Finally, the bile acid metabolism of the host was strongly affected and differed from conventional mice.

These results indicate that the origin (mouse or human) of the inocula impacts severely the host metabolism with a specific effect on the metabolism of bile acids. This study opens new insights regarding the understanding of the host metabolism-gut microbiota interaction and the adaptation to colonisation with mouse or human microbiota.
Bibliography:


