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# $^{1}$H-NMR SPECTROSCOPY TO INVESTIGATE THE EFFECTS OF FOOD ON ANIMALS AND HUMANS THROUGH METABOLOMICS

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**Abstract**

Metabolomics have proven highly effective for unravelling the complex metabolomic interactions between food and health. $^{1}$H-NMR has provided abundant information when the animal metabolism response to feed characteristics was evaluated or when the connection between health status of people and food composition was investigated.

As a first step of the PhD work, standard operating procedures (SOPs) were setup to investigate the metabolome of serum, feces, urine and meat by means of $^{1}$H-NMR. The SOPs were outlined so to be of general applicability for metabolomics investigation of any animal.

The SOPs gave the opportunity to face new projects which highlighted effects of food on the metabolism of animals and humans. In order to gain confidence about metabolomic investigations on animal studies, a simple trial focused on arginine supplementation for broiler chickens was setup at the first stage. We found that arginine supplementation improved food efficiency in fast-growing broiler chickens. Second, a complex trial relating to probiotics administration for horses was performed. Such trial was considered as an intermediate step to provide references for the final goal. As a result, some potential biomarkers suggested that a likely mechanism was linked to the change of energy source in muscle from carbohydrates to short-chain fatty acids.

Finally, the effects of probiotics on human health was investigated through two experiments aiming to provide evidences of probiotic supplementation on the treatments of gastrointestinal diseases. It was the first trial aiming to assess safety and efficacy of a mixture of probiotics for the treatment of infantile colic in exclusively breastfed infants. We found propylene glycol was considered as the potential candidate molecules of individuals supplemented with probiotics. In terms of the treatments of symptomatic uncomplicated diverticular disease (SUDD), short chain fatty acids production seemed to play a mainstay role in the complex metabolic pathway characterizing SUDD patients.

**Keywords:** Metabolomics, $^{1}$H-NMR, Biofluids, Food Metabolome, Food Supplementation, Animals Metabolism, Human Health

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# *Chapter 1. Introduction*

## ***Metabolomics***

Metabolomics is a powerful systems biology approach that aims to measure simultaneously all the low molecular weight metabolites (<900 Da) present in a biofluid or tissue. This approach to global untargeted and non-selected characterization of the metabolic phenotype allows the study of multidimensional biochemical responses of complex biological systems to genetic or environmental stimuli [1]. Metabolic profiling captures information from both intrinsic (genetics, protein expression) and environmental inputs (diet, gut microbiota), providing holistic information on the global system. This strategy has proven highly effective for unravelling the complex metabolic interactions between the food characteristics and health. Hence, metabolomics is a tool of particular interest to food researchers, given the vast impact of the gut microbiome on the bioavailability of food, medication and energy [2]. Metabolomics, along with other 'omic' approaches such as genomics, proteomics and transcriptomics, is increasingly showing potential in clinical settings as both a screening tool and a mean for mechanistic elucidation of disease pathways [3–5]. Metabolomics could be applied in an untargeted fashion, by using no selection or extraction of metabolites up-front to investigate all possible variables. Such systematic characterization is advantageous in metabolomics studies in which no prior information about metabolites is known or in which it still remains to be established whether several metabolites are linked to factors such as toxicity or disease [6].

## ***Platforms for Metabolomic Investigations***

Nowadays, nuclear magnetic resonance (NMR), liquid chromatography coupled to mass spectrometry (LC-MS) and gas chromatography coupled with mass spectrometry (GC-MS) are the three most used analytical approaches for metabolomic investigations. Each analytical platform has its own advantages and shortages, as summarized in Table 1.

Table 1. Summary of important advantages and limitations of NMR and MS in metabolomics applications.

| | **NMR** | **Mass Spectrometry** |
|---|---|---|
| Reproducibility | High | Low |
| Sensitivity | Low | High |
| Sample measurement | All metabolites at a detectable concentration level can be observed in one measurement. | Different ionization methods are required to maximize the number of detected metabolites. |
| Sample preparation | Minimal sample preparation. | More demanding. |
| Sample recovery | Nondestructive | Destructive but less amount of sample is need. |
| Quantitative analysis | Easy and directly | Complex |
| Number of detectable | Identify less than 200 metabolites | Detect thousands of different |

| | | |
|---|---|---|
| metabolites | | metabolites and identify several hundred |
| Targeted analysis | Both targeted and untargeted analyses, but commonly used for untargeted analyses. | Superior for targeted analyses |

In particular, high-resolution proton nuclear magnetic resonance ($^1$H-NMR) spectroscopy, when applied to metabolomic studies, has provided significant information when connections between health status of people and dietary habits were looked for [7,8]. It requires little or no sample preparation, it is cost effective, unbiased, rapid, robust and reproducible, quantitative, nonselective and nondestructive. A limitation of this analytical platform could be traced in the sensitivity, lower than the one of other techniques such as MS, which grants the generation of useful information from smaller samples [9].

An NMR profile contains qualitative and quantitative information on hundreds of different small molecules present in a sample at 1mM concentration [10]. NMR-based metabolomics, when untargeted, makes no assumption on the identity of the metabolites that are relevant for the selected study because information on the significant metabolic pattern features is directly obtained through statistical analysis of the NMR profiles [11].

***Standard Operating Procedure (SOP) for NMR-based Metabolomic Investigations***

Some physico-chemical properties of the samples are known to affect the NMR-based molecular profile, such as pH of sample, ionic strength, sample storage, concentration of analyte and reference, solubility and chemical interaction. Among the above properties, the pH of the sample plays a significant role in quantitative analysis. In fact, change in sample pH may cause a drift in the chemical shift of the desired signal, which may lead to overlapping of signals in the spectrum. For example, Miyataka *et al.* has evaluated the effect of pH on $^1$H-NMR spectroscopy of mouse urine, but only focusing on citrate and trimethylamine [12]. Xiao *et al.* has set up an optimized buffer system for NMR-based urinary metabolomics with effective pH control, chemical shift consistency and dilution minimization, which has been based on nine metabolites, namely acetate, hippurate, citrate, creatinine, DMA, TMA, glycine, histidine and urea [13]. From a review of the papers published on the topic, it seems that a limited number of researchers has been keen to investigate such potential source of error in metabolomics investigations, so that there is still the need for works that pay deeper attention to such issues.

Nowadays, the applications of NMR-based metabolomics are widespread, with publications appearing in many disciplines. However, the studies conducted still rarely follow specific and consolidated SOPs, so that results are hardly comparable, and the experiments are poorly

reproducible. Such phenomenon is crystal-clear, for example, when papers studying tea infusion metabolome are considered.

Most of papers published about NMR-based tea infusion metabolome follow a quite similar scheme. Fujiwara *et al.* analyzed 176 kinds of tea samples by extracting tea infusion at 75 ℃ for 3 minutes through an NMR-based metabolomic approach, but the final pH of the samples were not stated [14]. In order to evaluate the metabolomic profiling variances of green tea growing in three different areas, Lee *et al.* extracted tea samples at 60 ℃ for 30 minutes, and adjusted the final sample pH was equal to 7.0 before $^{1}$H-NMR analysis [15]. The same researcher also published two papers in 2011 referring to tea metabolome, while the final sample pH was adjusted to 6.40 [16] and 5.53 [17] respectively. Moreover, Lee *et al.* in 2015 published another $^{1}$H-NMR-based metabolomic paper to investigating the geography effect on green tea metabolites with the lack of final sample pH information [18].

We can see that even though many remarkable papers have been published, due to lack of SOP many key parameters are misleading or described insufficiently. Therefore, it is difficult for other researchers to follow their strategies, and even to set up comparative metabolomic investigations. For the other biomaterials, such limitations would also be faced through NMR-based metabolomic investigations.

In order to facilitate the development of metabolomics studies, it is not only necessary for us to publish data and conclusions for our current research, but also to provide a reference database for the further investigations. That's why, it is crucial for metabolomics publications to contribute to enrich metabolomics repositories, which is the best way to share and reuse data. According to the Metabolomics Standards Initiative (MSI) [19], these metabolomics databases should be open and ensure data traceability, reproducibility and interoperability. Among the high-throughput platforms employed for metabolomics, proton nuclear magnetic resonance ($^{1}$H-NMR) spectroscopy has been described as perfectly tailored for this purpose [20], because magnetic field, solvent and pulse sequence employed are the only variables modulating an NMR spectrum, thus granting a high reproducibility [21]. In order to maximize the advantages of $^{1}$H-NMR, it is crucial to set up NMR-based SOP, which is considered as the fundamental step for the further researches.

***The effect of food on animal metabolism***

Untargeted metabolomics observations can give simultaneously information about many aspects of animal metabolism. This surprising versatility allows to address through a single approach many important questions in animal science. Therefore, metabolomics is playing an increasingly important role on animals related researches. Particularly, the advantages of

metabolomics to noninvasively monitor subtle phenotypic changes, congenital phenotypic tendency and dietary effects makes it a powerful tool for evaluating the connection between food related metabolome and animal metabolism. Till now, there has been a abundant of publications focused on animal metabolomics that have obtained comprehensive results exploring how metabolomics and molecular-based phenotyping can help veterinarians, farmers, animal industry and researchers.

Jung *et al.* successfully distinguished beef originating from four countries by means of $^{1}$H-NMR-based metabolomics and several metabolites including various amino acids and succinate were found to be possibly regarded as biomarkers for discriminating the geographical origin of beef [22]. Kodani *et al.* obtained NMR-detected profiles of beef in Japanese black cattle with long-term aging duration, some linear correlations were found between concentration of molecules and aging duration [23]. Ritota *et al.* obtained metabolomic profiles of *longissimus dorsi* and *semitendinosus* muscles of four different cattle breeds by applying HRMAS-NMR, and excellently discriminated the muscle type between Buffalo and Chianina [24]. Zanardi *et al.* performed a $^{1}$H-NMR based metabolomic profiling on non-irradiated and irradiated beef, three metabolites, namely glycerol, lactic acid esters and tyramine or a *p*-substituted phenolic compound, were found as important biomarkers for classification of the irradiated and non-irradiated beef samples through multivariate data analysis [25].

Metabolomic investigations are not only suitable for characterizing animal products themselves, but they can also provide quantitative information for molecules in serum, urine and feces, in order to deeply understand the mechanisms of the connection of food characterization and animals' metabolism.

Hong *et al.* set up a $^{1}$H-NMR-based metabolomic assessment to evaluate probiotic effects in a colitis mouse model and found that the short chain fatty acids in feces of mice fed with probiotics increased comparing to the control group. Hence, the researchers concluded that the probiotics have protective effects against dextran sulfate sodium (DSS) induced colitis via modulation of the gut microbiota [26]. Martin *et al.* found that human baby microbiota (HBM) mice model differed from conventional and conventionalized microbiota mice models linked to lower short chain fatty acids, 5-aminovalerate, lactate and oligosaccharides, while higher choline and bile acids by $^{1}$H-NMR based fecal metabolomic investigations. In addition, probiotics administration of HBM mice was related to a particular amino acid pattern that can be connected to *L. paracasei* proteolytic activities, which enhanced a specific metabolism of carbohydrates, proteins and SCFAs [27].

***The effect of food on human health***

Biofluids, such as urine, feces and serum, are well used to evaluate the effects of food on humans by means of $^{1}$H-NMR. Among these biofluids, urine appears to be particularly useful. It is readily available, abundant, simple collectable, easily stored and noninvasive techniques. What's more, because of its chemical complexity, urine is particularly full of potential biomarkers coherent to various diseases. This makes it an important biofluid for monitoring or detecting disease processes. Foschi *et al.* characterized the urine metabolome of women with *Chlamydia trachomatis* (CT) uro-genital infection, comparing it with a group of CT-negative subjects and successfully identified several metabolites whose concentrations were significantly higher in the urine samples of CT-infected women [28]. Fresno *et al.* showed urinary metabolomic fingerprinting after consumption of a probiotic strain in women with mastitis, the concentrations of three molecules, namely creatine, hippurate and trimethylamine N-oxide, were altered after 21 days of probiotic administration [29]. Capuani *et al.* found that probiotic treatment affected changes in non-alcoholic fatty liver disease (NAFLD) urinary metabolome mainly at level of valine, tyrosine, 3-aminoisobutyrate, pseudouridine, methylguanidine. Secondarily it altered at the level of 2-hydroxyisobutyrate through $^{1}$H-NMR approach [30].

These advantages mentioned above also apply to feces samples. The metabolic composition of fecal extracts can also provide a window for elucidating the complex metabolic interplay between mammals and their intestinal ecosystems, and these metabolite profiles can yield information on a range of gut diseases. Lin *et al.* used $^{1}$H-NMR based metabolomic approach to profile fecal metabolites of colorectal cancer (CRC) patients and healthy controls, CRC patients at various stages were clearly distinguished comparing to those in cancer free controls in terms of reduced levels of acetate, butyrate, propionate, glutamine, glucose and higher abundant of alanine, valine, succinate, proline, dimethylglycine, isoleucine, leucine, glutamate and lactate [31].

Serum is also considered as one of the widely used common biofluids for metabolic investigations. Serum is a readily accessible and informative biofluid, making it ideal for early detection of a wide range of diseases. Metabolite profiles of serum can be regarded as important indicators of physiological and pathological states and may aid understanding of the mechanism of disease occurrence and progression on the metabolic level, and provide information enabling identification of early and differential metabolic markers of disease. Analysis of these crucial metabolites in serum has become important in monitoring the state of biological organisms and is widely used for diagnosis of disease. Pedersen *et al.* found that the serum metabolome of insulin-resistant subjects is characterized with higher levels of branched-chain amino acids (BCAAs), which connect to a gut microbiome which can be produced a large abundant of BCAAs [32]. Liu *et al.* identified obesity-associated gut microbial species linked to changes in serum metabolome. In detail,

the abundance of *Bacteroides thetaiotaomicron*, a glutamate-fermenting commensal, was markedly decreased in obese individuals and was inversely correlated with serum glutamate concentration [33]. Maclntyre *et al.* found that serum metabolic profiles of chronic lymphocytic leukaemia (CLL) exhibited higher concentrations of pyruvate and glutamate and decreased concentrations of isoleucine compared with controls by means of $^{1}$H-NMR [34].

# *Chapter 2. Aims of the study*

Firstly, the design of robust SOPs for the investigation of the metabolome of human and animal biofluids by $^{1}$H-NMR was faced. In fact, extrapolating molecules concentrations from $^{1}$H-NMR spectra can be severely disturbed by shifts of the signals mainly caused by ionic strength and pH. The design of handy SOPs for different kinds of samples manipulation is therefore a key step to generate robust databases. Hence, the NMR-based SOP can be considered as the fundamental step for the next investigation, and obviously, as the first objective of the PhD thesis.

Secondly, the effect of food on animal metabolism was evaluated through an NMR-based metabolomic approach, particularly for arginine supplementation for broiler chickens and probiotic supplementation for horses.

Finally, I paid attention to the effect of food on human health, in terms of probiotic administration for the treatments for gastrointestinal diseases.

The present PhD project resulted in the publication of six peer-reviewed papers which were aimed at studying:

- Standard Operating Procedure (SOP) for NMR-based metabolomic investigations (Paper I and Paper II);
- The effect of food on the animal metabolism (Paper III and Paper IV);
- The effect of food on human health (Paper V and Paper VI).

The following sections of the PhD thesis are aimed at highlighting the main findings of each aim. In detail, chapter 3 summaries the NMR-based SOPs for urine, feces, serum and meat samples, as the fundamental step for following metabolomic investigations. The effects of food on the animal metabolism are described in the chapter 4. Finally, chapter 5 is dedicated to the introduction of the main results of the effects of food on human health from a metabolomic point of view.

# *Chapter 3. SOP for NMR-based Metabolomics Investigations*

This chapter is devoted to the experiments that aimed to set up $^{1}$H-NMR based SOPs specific for the study of serum, urine, feces and meat metabolome. For the purpose, yaks have been selected due to the limited information of their common biofluids and meat metabolomic profiles by means of $^{1}$H-NMR.

Yak (*Bos grunniens*) is considered as a very special species of ruminant. Because it represents one of the main sustaining food sources for the Tibetan people who live in Himalayas region, with an altitude variance between 2500 to 5500 m with mostly above the tree line and no frost-free periods. Therefore, its genetic evolution adaptive to the harsh conditions has led to larger lungs and heart, and a more abundant erythrocyte count, compared to the cattle (*Bos taurus*) [35], so as to a more efficient nitrogen and energy utilization [36,37]. Due to the above reasons, yak is regarded as an suitable model animal in order to study adaptation mechanisms to harsh conditions whereas a paucity of oxygen, low temperatures and energy sources. Its peculiar energy metabolic properties and gene expressions [38] have positive consequences also on the meat quality characteristics, specifically on tenderness, juiciness and leanness [39]. These appreciable properties seem to be mainly regulated by three muscles' physiological features. Firstly, yak meat shows, throughout all the maturation steps, an intense protease activity [40], causing a pronounced myofibrillar denaturation and fragmentation in correspondence to the Z-line [41]. In addition, the final pH of yak meat is peculiarly distant from the isoelectric point [42]. Moreover, yak shows a lower intramuscular fat content in comparison with beef.

Despite these peculiarities, yak has rarely been studied by means of an "omics" approach. One reason for the limited works may be the inconvenient accessibility of the geographical areas where the yaks are grazed, generally following traditional practices. Several works can be found in terms of yak milk [43–46] and yak genome [5,35,47], with just one work that simply described the characteristics of yak meat [48].

In this chapter, we would like to setup the NMR based SOPs for serum, urine, feces and meat by using yak samples. Furthermore, it was also possible for me to set the basis for the development of metabolomics investigations of yak's common biofluids and meat, by outlining their metabolome, as can be observed by $^{1}$H-NMR.

First of all, we created an NMR analysis solution with 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 10 mM in $D_2O$, as an NMR chemical-shift reference, and then buffered at pH 7.00 ± 0.02 by using 1 M phosphate buffer with 0.10 mL of $NaN_3$ 2 mM to avoid microbial proliferation.

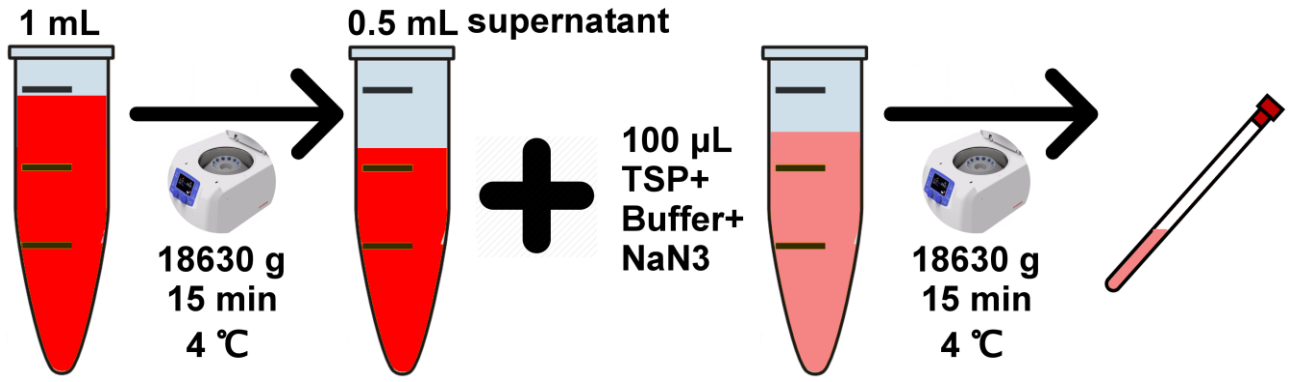


Figure 1. $^{1}$H-NMR based SOP for serum sample

We thawed and centrifuged 1 mL of serum original sample for 15 min at 18,630 g and 4 °C. And then 0.50 mL of supernatant was moved to a new Eppendorf tube by adding 0.10 mL NMR analysis solution, as shown in Figure 1. Finally, we centrifuged the obtained samples again at the above conditions prior to NMR analysis.

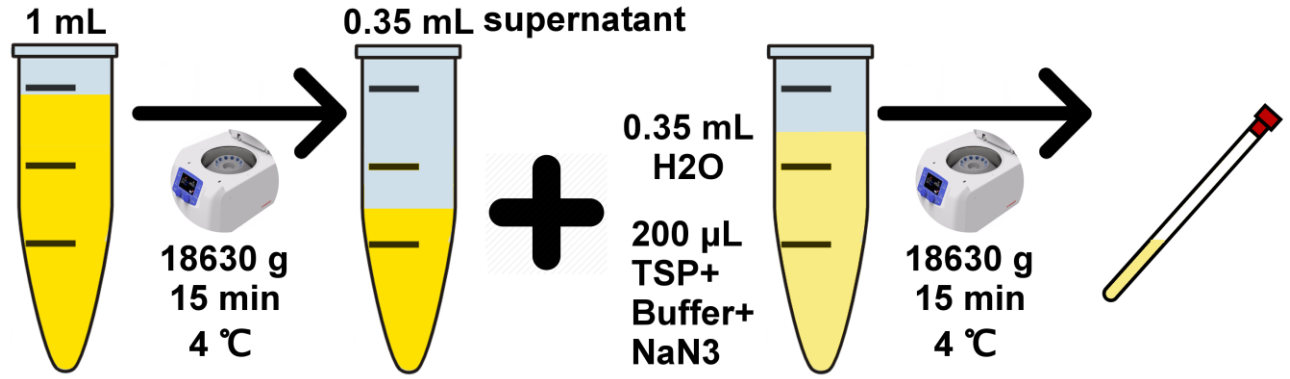


Figure 2. $^{1}$H-NMR based SOP for urine sample

We thawed and centrifuged original urine samples for 15 min at 18,630 g at 4 °C. And then 0.35 mL supernatant was added to 0.35 mL of bi-distilled water with 0.20 mL of NMR analysis solution, as shown in Figure 2. Finally, we centrifuged the obtained samples again at the above conditions prior to NMR analysis.

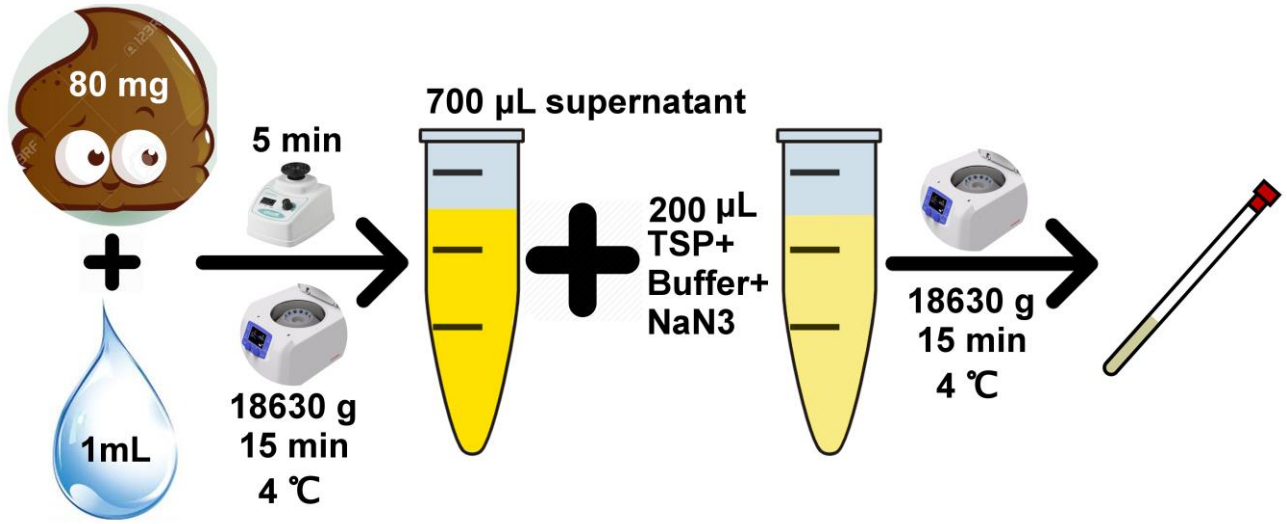


Figure 3. $^{1}$H-NMR based SOP for feces sample

Firstly, we vortex mixed 80 mg of stool with 1 mL of deionized water for 5 min and then we centrifuged the obtain samples for 15 min at 18,630 g and 4 °C. Secondly, we added 0.70 mL of supernatant to 0.20 mL of NMR analysis solution. Finally, we centrifuged the obtained samples again at the above conditions prior to NMR analysis.

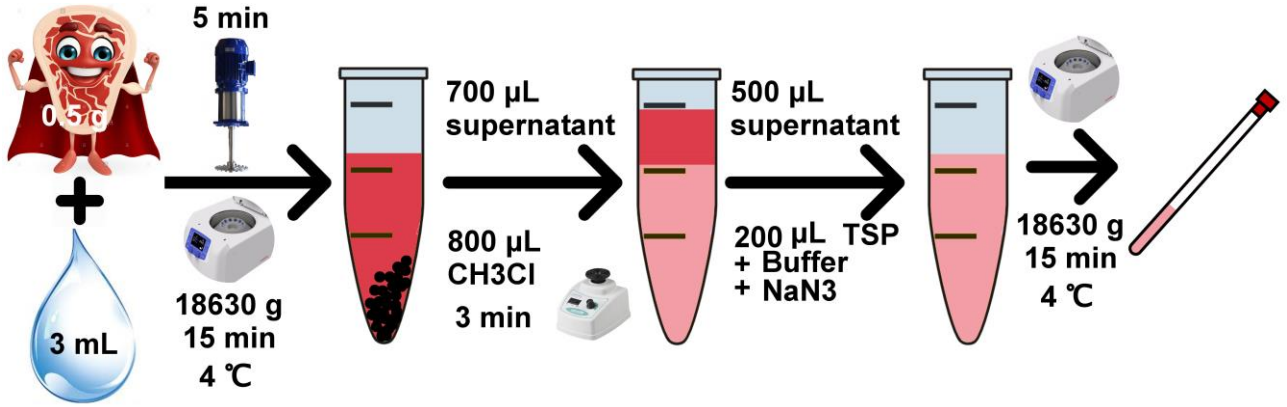


Figure 4. $^{1}$H-NMR based SOP for meat sample

We add 0.5 g meat to 3 mL distilled water, and then homogenized the mixture for 2 min by using a high-speed disperser (IKA, USA). One milliliter of the mixed sample was centrifuged at 18630 g and 4°C for 15 min. To get rid of fat substances from samples, 0.7 mL of supernatant were transferred to a new Eppendorf tube with 0.8 mL chloroform added before, vortex mixed for 3 min and centrifuged again. The supernatants (0.5 mL) were added to 0.2 mL of NMR solution. Finally, we centrifuged the obtained samples again at the above conditions prior to NMR analysis.

As a result, we were able to identify and quantify 56 molecules in yak serum, 49 in feces, 68 in urine and 53 in meat, as shown in Table 2 — nearly a twofold increase by comparing with

previous works on cattle [49,50], giving information about energy generation, protein digestion, diet or gut-microbial co-metabolism.

Table 2. Molecules quantified by means of $^{1}$H-NMR in yak serum, urine, feces and meat.

| Molecules | ppm | Functional group | Multiplicity | Molecule source * | Groups |
|---|---|---|---|---|---|
| 1,3-Dihydroxyacetone | 4.4122 | $CH_2$ | s | D | Serum, Meat |
| 2,6-Dihydroxybenzoate | 6.4221 | CH-2 | d | D | Urine |
| 2-Aminobutyrate | 0.9648 | $CH_3$ | t | P | Serum |
| 2-Hydroxy-3-methylvalerate | 0.9310 | $CH_3$-9 | d | P | Urine |
| 2-Hydroxybutyrate | 0.8859 | $CH_3$ | t | P | Serum |
| 2-Hydroxyisobutyrate | 1.3478 | $CH_3$ | s | D | Urine |
| 2-Hydroxyisovalerate | 0.8244 | $CH_3$ | d | P | Urine |
| 2-Hydroxyvalerate | 0.8970 | $CH_3$ | t | E | Urine |
| 2-Oxoisocaproate | 2.6038 | $CH_2$ | d | P | Feces |
| 2-Oxovalerate | 0.9037 | $CH_3$ | t | D | Meat |
| 3-Hydroxybutyrate | 1.1863 | $CH_3$ | d | E | Serum, Urine, Feces |
| 3-Hydroxyisobutyrate | 1.0503 | $CH_3$ | d | P | Serum, Urine |
| 3-Hydroxyisovalerate | 1.2585 | $CH_3$ | s | P | Urine |
| 3-Hydroxyphenylacetate | 3.4718 | $CH_2$ | s | P | Urine |
| 3-Indoxylsulfate | 7.6894 | CH-6 | d | D, P | Urine |
| 3-Methyl-2-oxovalerate | 1.1042 | CH3-9 | d | P | Urine |
| 3-Methylglutarate | 0.9095 | $CH_3$ | d | P | Urine |
| 3-Methylhistidine | 7.1366 | CH-5 | s | P | Serum |
| 3-Phenylpropionate | 2.8659 | $CH_2$-7 | m | p | Urine, Feces |
| 4-Hydroxybutyrate | 2.2051 | $CH_2$-3 | m | P | Meat |
| 4-Hydroxyphenylacetate | 6.8582 | CH-3 | d | D | Urine, Feces |
| Acetate | 1.9071 | $CH_3$ | s | P | Serum, Urine, Feces, Meat |
| Acetoacetate | 2.2688 | $CH_3$ | s | E | Serum, Feces, Meat |
| Acetoin | 1.3583 | $CH_3$-3 | d | E | Feces, Meat |
| Acetone | 2.2178 | $CH_3$ | s | E | Serum, Feces |
| Adenosine | 8.3436 | CH-7 | s | P | Urine |
| Alanine | 1.4675 | $CH_3$ | d | P | Serum, Urine, Feces, Meat |
| Allantoin | 5.3643 | CH | s | P, M | Urine |
| Anserine | 8.3554 | CH-3 | s | D | Meat |
| Arabinose | 4.5038 | CH-2 | d | M, E | Serum |
| Arginine | 1.9229 | $CH_2$-6 | m | P | Serum |
| Ascorbate | 4.5072 | CH | d | D, E | Urine |
| Asparagine | 2.9282 | $CH_2$ | dd | P | Serum |
| Aspartate | 2.7842 | $CH_2$ | dd | P | Serum, Feces |
| *beta*-Alanine | 2.5558 | $CH_2$-4 | t | P | Serum, Urine, Meat |
| Betaine | 3.8894 | $CH_2$ | s | P | Serum, Urine, Meat |
| Butyrate | 0.8818 | $CH_3$ | t | E | Feces |
| Carnitine | 3.2208 | $CH_3$ | s | E | Serum, Feces, Meat |

| | | | | | |
|---|---|---|---|---|---|
| Carnosine | 8.3087 | CH-3 | s | D | Meat |
| Choline | 3.1888 | $CH_3$ | s | E, P | Serum, Feces, Meat |
| *cis*-Aconitate | 3.1089 | $CH_2$ | d | E | Urine |
| Citrate | 2.5093 | $CH_2$ | d | P | Serum, Urine, Meat |
| Creatine | 3.0222 | $CH_2$ | s | P | Serum, Urine, Feces, Meat |
| Creatinine | 3.0325 | $CH_3$ | s | P | Serum, Urine, Meat |
| Dimethyl sulfone | 3.1391 | $CH_3$ | s | D, M | Serum, Urine, Feces, Meat |
| Dimethylamine | 2.7117 | $CH_3$ | s | P | Serum, Urine |
| Dimethylglycine | 2.9124 | $CH_3$ | s | E, P | Serum, Urine, Meat |
| Ethanol | 1.1699 | $CH_3$ | t | E, M | Serum, Urine, Feces |
| Ethylmalonate | 1.7091 | $CH_2$ | m | E | Urine |
| Formate | 8.4446 | CH | s | E | Serum, Urine, Feces, Meat |
| Fucose | 1.2024 | $CH_3$ | d | E | Urine, Feces |
| Fumarate | 6.5090 | CH-5 | s | E | Serum, Feces, Meat |
| Galactarate | 4.2597 | CH | s | E | Urine |
| Galactose | 4.5825 | CH-2 | d | E | Feces |
| Gluconate | 4.1362 | CH-9 | d | E | Urine |
| Glucose | 3.2233 | CH-2 | dd | D, E | Serum, Urine, Feces, Meat |
| Glucose-1-phosphate | 5.4459 | CH | dd | D | Meat |
| Glutamate | 2.0606 | $CH_2$-6 | m | P | Serum, Feces, Meat |
| Glutamine | 2.4430 | CH | m | P | Serum, Urine, Meat |
| Glutarate | 2.1540 | $CH_2$ | t | E, P | Urine, Feces |
| Glutathione | 2.9438 | $CH_2$ | m | P | Meat |
| Glycerol | 3.5664 | $CH_2$ | dd | E | Serum, Feces, Meat |
| Glycine | 3.5533 | $CH_2$ | s | P | Serum, Urine, Feces, Meat |
| Guanidoacetate | 3.7782 | $CH_2$ | s | P | Urine, Meat |
| Guanosine | 5.8929 | CH-2 | d | P | Urine |
| Hippurate | 3.9607 | $CH_2$ | d | P | Urine |
| Hypoxanthine | 8.1981 | CH | s | M | Meat |
| IMP | 8.3404 | CH | s | M | Meat |
| Inosine | 4.4293 | CH | s | M | Meat |
| Isobutyrate | 1.0473 | $CH_3$ | d | M | Urine, Feces |
| Isocaproate | 1.4133 | $CH_2$-5 | m | E | Urine |
| Isoleucine | 1.0020 | $CH_3$-9 | d | P | Serum, Urine, Feces, Meat |
| Isovalerate | 0.9030 | $CH_3$ | d | P | Feces |
| Lactate | 4.1059 | CH | dd | E | Serum, Urine, Feces, Meat |
| Leucine | 0.9356 | $CH_3$-9 | d | P | Serum, Feces, Meat |
| Lysine | 3.0077 | $CH_2$-9 | t | P | Serum |
| Malate | 2.3517 | $CH_2$ | dd | E | Meat |
| Malonate | 3.1199 | $CH_2$ | s | D, E | Urine, Meat |
| Mannose | 5.1767 | CH-6 | d | E | Serum, Meat |
| Methanol | 3.3481 | $CH_3$ | s | E | Serum, Urine, Feces, Meat |
| Methionine | 2.6178 | $CH_2$-3 | t | P | Serum, Feces, Meat |
| Methylamine | 2.5878 | $CH_3$ | s | D | Urine, Feces |

| | | | | | |
|---|---|---|---|---|---|
| Methylsuccinate | 1.0742 | $CH_3$ | d | P, E | Serum, Urine |
| myo-Inositol | 4.0585 | CH-2 | t | E | Serum, Urine, Meat |
| N-Acetylglucosamine | 5.1989 | CH | d | E, P | Feces |
| N-Carbamoylaspartate | 2.6698 | $CH_2$ | dd | P | Serum |
| Niacinamide | 8.7017 | CH | m | M | Meat |
| N-Methylhydantoin | 2.9129 | $CH_3$ | s | P | Feces |
| N-Phenylacetylglycine | 3.6605 | $CH_2$-11 | s | P | Urine, Feces |
| O-Acetylcarnitine | 2.5157 | $CH_2$ | dd | P | Meat |
| O-Acetylcholine | 3.2081 | $CH_3$ | s | E | Feces |
| O-Phosphocholine | 3.1977 | $CH_3$ | s | E | Feces |
| Ornithine | 3.0440 | $CH_2$-8 | t | P | Serum |
| *p*-Cresol | 6.8170 | CH-2 | d | P | Urine |
| Phenylacetate | 7.2826 | CH-4 | m | P | Feces |
| Phenylalanine | 7.3669 | CH-4 | t | P | Serum, Feces, Meat |
| Proline | 1.9998 | $CH_2$-3 | m | P | Serum, Meat |
| Propionate | 1.0292 | $CH_3$ | t | P | Feces |
| Propylene glycol | 1.1227 | $CH_3$ | d | E | Serum, Urine |
| Pyruvate | 2.3573 | $CH_3$ | s | E | Serum, Urine, Meat |
| Sarcosine | 2.7262 | $CH_3$ | s | P | Serum, Urine |
| Serine | 3.9555 | $CH_2$ | dd | P | Serum, Feces |
| Serotonin | 3.3023 | $CH_2$ | t | P | Urine |
| Succinate | 2.3933 | $CH_2$ | s | P, E | Serum, Urine, Feces |
| Tartrate | 4.3311 | CH | s | D | Urine |
| Taurine | 3.2368 | $CH_2$-6 | t | P | Urine, Meat |
| Threonine | 3.5838 | CH-4 | d | P | Serum, Feces, Meat |
| Tiglylglycine | 6.4968 | CH | m | P | Urine |
| *trans*-Aconitate | 6.5814 | CH | s | E | Urine |
| Trehalose | 5.1837 | CH | s | E | Feces |
| Trigonelline | 4.4165 | $CH_3$ | s | D | Urine |
| Trimethylamine | 2.8226 | $CH_3$ | s | D | Serum, Urine, Meat |
| Trimethylamine N-oxide | 3.2589 | $CH_3$ | s | D | Urine |
| Tyrosine | 7.1776 | CH-3 | d | P | Serum, Urine, Feces, Meat |
| UMP | 5.9755 | CH | s | M | Meat |
| Uracil | 5.7903 | CH-5 | d | P | Serum, Feces |
| Uridine | 5.9079 | CH-2 | d | P | Serum, Meat |
| Valerate | 0.8756 | $CH_3$ | t | D | Feces |
| Valine | 1.0206 | $CH_3$-7 | d | P | Serum, Urine, Feces, Meat |
| Xanthine | 7.9336 | CH | s | M | Meat |
| Xanthosine | 5.8460 | CH-2 | d | P | Urine |
| Xylose | 4.5781 | CH-2 | d | E | Urine |

** D stands for dietary metabolites, P stands for protein and amino acid metabolism, E stands for energy metabolism and M stands for gut microbial co-metabolism* [51]

**Yak – Serum** Among the quantified molecules in yak serum, lactate is the most abundant metabolite. In cattle, its presence has mainly produced by ruminal microflora [52]. Its abundance

together with pyruvate in yak serum seems play a particular role, which is a direct consequence linked to the low oxygen levels adaptation [53–55]. In fact, hypoxia triggers a alteration towards anaerobic energy generation connected to an active withdrawal of pyruvate from the Krebs cycle to lactate production [56–58].

**Yak – Feces** Fecal metabolome can open a window for investigating the complex metabolic interactions between mammals and their intestinal ecosystem. In addition, the metabolomic profiles can grant information on a variance of gut conditions [58]. It's worth to notice that, adjusting the total concentration of the molecules identified in the feces samples equal to 100, three short chain fatty acids (SCFAs) weighted 75.17%, namely acetate, butyrate and propionate. In cattle rumen, SCFAs are mainly attributed by the microbial fermentation of fiber polysaccharides, which may be regarded as an important role linked to the efficient absorbing energy from plants tissues [59]. Zhang *et al*. who compared animals that were genetically adapted and non-adapted to high altitudes, was recently found this efficiency was likely to be mediated by ruminal microbiome selection [60].

**Yak – Urine** Creatinine was the most abundant molecule quantified by $^{1}$H-NMR in yak urine. It is synthesized linked to the absorption of creatine phosphate by the muscles, and then released to serum, finally cleared by kidneys. In the studies of cattle, creatinine has been found to be proportional to muscle activity, as a particular reference to heart and respiration rates [61], but its concentration is higher in yak compared to cattle [35]. Hippurate and phenylacetylglycine were the second and third most abundant molecules quantified in yak urine. According to Kaur *et al.*, these molecules are considered as urinary metabolomic biomarkers linked to hypobaric hypoxia [62]. Koundal *et al.* setup a mouse model to investigate the variations of mice urinary metabolomics in response to hypobaric hypoxia. TCA and taurine metabolism were addressed as important pathways which might be altered by hypobaric hypoxia-induced pathophysiology. Such evidences are in accordance with our findings. Hippurate and phenylacetylglycine are classified as molecules linking to gut microflora metabolism and those lower concentrations indicating decreased gut microflora also has been demonstrated. In ruminants, the purine derivative allantoin is considered as a candidate of nitrogen clearance, which is produced by uric acid through uricase. In adaption of the harsh forage conditions, yaks expel through urine a lower level of purine derivatives [36], with mechanisms related to nitrogen recycling, which are likely connected to the low level of degradation of nucleic acids through rumen microbiota [63].

**Yak – Meat** Yak meat samples were collected from *longissimus thoracis* (LT), exemplificative of muscle rich in white fibers, from *trapezius* (TP) exemplificative of muscle rich in red fibers, and *triceps brachii* (TB) and *biceps femoris* (BF) considered as intermediate.

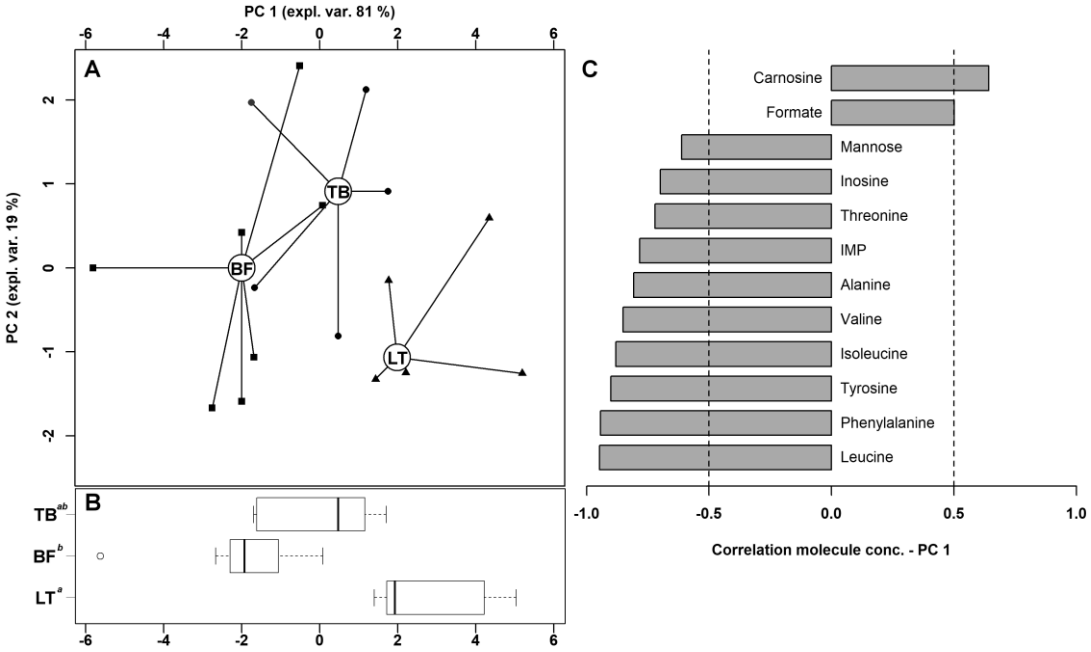


Figure 5. rPCA model built on the concentration of the molecules which showed a statistically significant difference among different groups. In the scoreplot (A), samples from the three groups are represented with triangles (LT), circles (TB) and squares (BF). The empty circles represent the median values of each group. Boxplot (B) summarized the position of the samples along PC1. The loading plot (C) shows the correlation between each molecule concentration and its importance over PC 1. (Figure 2 in Paper II)

A total of 53 molecules was identified and quantified in yak raw meat. Due to the limited number of TP samples, only LT, TB and BF groups were involved in the univariate analysis. Among them, twelve of them were found to be differently concentrated in relation to muscle type through ANOVA followed by Tukey HSD test. On the basis of the above molecules, an rPCA model was setup to have an overall view of the data, as shown in Figure 5. LT, compared to BF, had higher concentrations of carnosine and formate and lower concentrations of mannose, inosine, threonine, IMP, alanine, valine, isoleucine, tyrosine, phenylalanine and leucine.

LT is characterized by a peculiarly high number of white, fast-twitching glycolytic fibers. TP, at the opposite, harbors a higher number of slow-twitching oxidative fibers, while TP brachii and BF can be considered as intermediate between the two [64–66]. Carnosine content is known to reflect the ratio between glycolytic and oxidative fibers, being higher in white muscles, where it grants a high buffering capacity, compensating for the lactic acid accumulation connected to the anaerobic metabolism [67]. The concentration of this histidine-containing dipeptide we measured was coherent with such findings, being in LT muscle 42% higher than TB and 24% higher than BF. The comparison of the trends between TP and LT seems a further confirmation, with the former

characterized by higher concentration than the latter, in agreement with the observations in beef by Aristoy et al. [68]. From this point of view, some discrepancies in the literature must be evidenced. Mora et al. [69], for example, found in pigs similar carnosine concentrations in LT and BF, likely in connection with peculiarities connected to species and environment. Environment is expected to play a key role in the physiological differences between yak and other species or cattle breeds, with yak perfectly adapted to graze in steep slopes and harsh environment.

Univariate analysis showed that the muscle type had a profound effect on the amino acids overall profile, with significant effects on the concentration of 7 amino acids of the 14 measured. Moreover, enrichment analysis showed that all the mostly altered pathways were related to amino acids metabolism. A similarly profound effect of muscle type on amino acids profile has been observed also for cattle [70], with consequences on important characteristics for meat quality, like oxidative stability.

The fact that each of the 7 amino acids showed a negative correlation with PC 1 scores suggests that the main reason for such trend is linked to the use of the muscles, more intense for legs and neck. The concentration of the each of them was statistically different between BF and LT. Generally, TB showed intermediate values. An exception was represented by alanine, differently concentrated between TB and LT. Alanine is a non-essential amino acid, highly concentrated in muscles, where it serves as one of the major energy sources [71]. Goldstein et al. [72] noted that the release of alanine by muscle is a metabolic consequence of both the initial transamination reaction and the conversion of the keto acid into pyruvate, via the normal oxidation pathway for the amino acids. In vivo, alanine can be produced from the breakdown of carnosine [73], what could explain the inverse relationship between the concentration of alanine and carnosine we noticed in the present investigation.

IMP pertains to the molecules deriving from purines, often produced by ATP hydrolysis during the aging process of beef samples [74]. Dannert et al. [75] found that the concentration of IMP is not significantly different among the LT, BF and semimembranosus muscles of pork carcasses sampled 48 hours post-mortem. At the opposite, we found in yak that the concentration of IMP was significantly higher in BF than in LT. Such discrepancy suggests that the degradation phenomena occurring during muscles transformation into meat are likely to be, in yak, strongly muscle specific [76].

# *Chapter 4. The effect of food on animal metabolism*

This chapter shows the main results of two experiments aimed to investigate the effect of food on animal metabolism, particularly for arginine supplementation for chickens and probiotic supplementation for horses.

**Arginine supplementation for chickens**

Formulating diets with sufficient amino acid characteristics is a key step to better exploit the genetic potential of modern broilers characterized by rapid growth. In addition, amino acid nutrition plays a central role in animal health, the welfare and quality of poultry products, and the environmental impact and sustainability of the poultry industry [77]. To improve feed efficiency and to increase breast meat production, modern broilers have been selected for decades [78]. This selection process, especially for amino acids and proteins, has produced significant changes in their body composition and nutritional requirements.

Arginine plays several crucial roles in variate metabolic, pathophysiological and immunological processes in poultry as systematic reviewed by Fernandes and Murakami [79], Khajali and Wideman [80] and Fouad et al. [81]. Due to the shortage of a functional urea cycle [82], broilers cannot synthesize endogenous L-arginine, and therefore it is considered as essential amino acids. In fact, chickens rely on a dietary source of arginine, so they should provide adequate amounts of arginine in their diet. A specific relationship between arginine and lysine in the diet has been identified, and any deficiency or excess will negatively affect plasma and muscle amino acid concentrations, which will affect growth performance [83]. However, considering the important functions of arginine mentioned above, it should be questioned whether the current status of Arg: Lys ratio in the diet is sufficient to meet the needs of modern broilers. Lack or excess of arginine can have serious adverse effects on animal health, welfare and productivity, and the economic and environmental sustainability of the poultry industry. There is also little scientific information on the effect of different Arg: Lys ratios on broiler diets on meat quality and onset of dysmyosinosis. Therefore, this trial aimed to determine the effect of dietary arginine supplementation on plasma and muscle metabolomics profiles of modern broilers.

$^{1}$H-NMR spectra were obtained from plasma samples and 62 molecules were quantified. Arginine birds (ARG) showed significantly higher levels of plasma arginine and leucine, whereas plasma proline, glutamate, adenosine and acetoacetate were more abundant in control birds (CON). In order to get an overview of the molecules with the most differences between the groups, these 6 molecules were used as the basis for the rPCA model, as shown in Figure 6. In parallel with plasma, a $^{1}$H-NMR spectrum muscle sample was obtained from the breast. Of a total of 37 quantitative molecules, 4 metabolites showed significantly different concentrations between the CON and ARG groups. The breast muscle of the ARG group showed higher levels of arginine and acetate, while lower levels of acetone and inosine. The rPCA model obtained using these four molecules is shown in Figure 7.

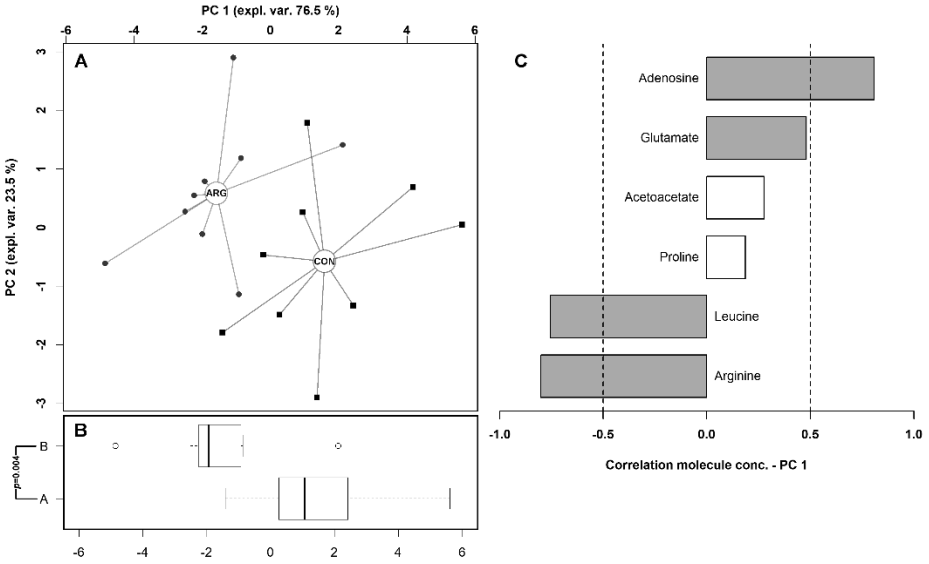


Figure 6. rPCA model on plasma molecules selected by univariate analysis showing differential trend between CON and ARG groups. In the scoreplot (A), samples obtaines from chickens with and without treatment are represented with circles and squares circles respectively. The empty circles represent the median values of various groups. Boxplot (B) summarizes the position of the subjects along PC1. Loading-plot (C) shows the correlation between each molecule concentration and its importance along PC 1. Highly significant correlations ($p < 0.05$) are highlighted with grey bars. (Figure 2 in Paper III)

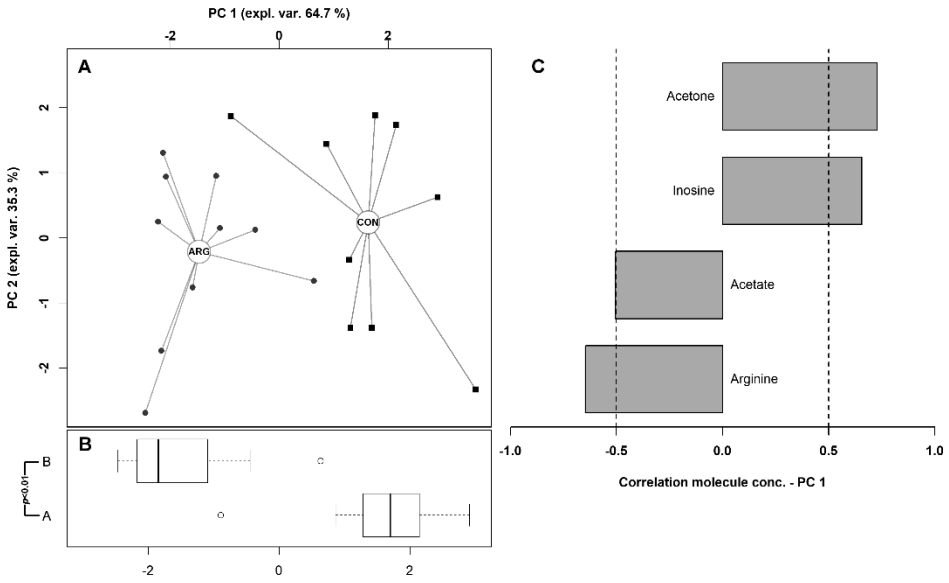


Figure 7. rPCA model on breast molecules selected by univariate analysis showing differential trend between CON and ARG groups. In the scoreplot (A), samples obtaines from chickens with and without treatment are represented with circles and squares circles respectively. The empty circles represent the median values of various groups. Boxplot (B) summarizes the position of the subjects along PC1. Loading-plot (C) shows the correlation between each molecule concentration and its

importance along PC 1. Highly significant correlations ($p < 0.05$) are highlighted with grey bars. (Figure 3 in Paper III)

In terms of metabolomic investigations, rPCA models reported different levels of plasma and muscle molecules between groups (Figure 6 and 7, respectively) showing a clear separation between different dietary supplementation of arginine. Following these findings, L-arginine supplementation in the diet can increase its concentration in plasma and pectoralis major muscles, indicating that L-arginine is able to be absorbed by the intestinal epithelium effectively and to enter the systemic circulation and reach surrounding tissues such as the pectoral muscles. Arginine in the diet is absorbed in the small intestine through sodium-dependent and non-dependent mechanisms, the latter showing greater efficacy [84]. As most of the arginase activity happens in the kidney [82], a large amount of arginine diet may have passed the brush margin and then entered the system circulation with limited degradation. Arginine could participate protein synthesis and cell proliferation in muscles [81]. In addition, a higher level of leucine in plasma in birds taken the arginine-supplemented diet. Its higher concentration in plasma has been linked to a greater protein synthesis in pig skeletal muscle [85]. Similarly, Baeza *et al.* [86] found a positive correlation between Pectoralis major weight and histidine level in plasma, which, in the current study, was higher in ARG birds. Taken together, these results indicate that dietary arginine supplements may improve the anabolic process of breast muscles through enhanced protein synthesis, which deserves further study. In addition, arginine dietary supplements seem to regulate energy and protein metabolism. The two ketone bodies, acetoacetate and acetone, show lower concentrations in ARG plasma and pectoral muscle, respectively. Ketone bodies can be recruited from the blood circulation through surrounding tissues, including breast muscles, and catabolized to produce energy. Therefore, these findings may indicate increased ketone body utilization in peripheral tissues in response to dietary arginine supplementation. Fouad *et al.* [81] found that dietary arginine supplementation is able to alter chicken body fat deposition. Indeed, Fouad *et al.* [87] associated the lower abdominal fat deposition coherent to the dietary arginine supplementation to both the increasing level of expression of genes involved in fatty acid β-oxidation and to the reduced fatty acid synthase gene expression in heart and liver, respectively. A potential role of the dietary arginine supplementation on fat and energy metabolism has been previously reported also in meat-type ducks [88]. Proline and glutamate, both of them come from arginine metabolism [79,81], also showed lower levels in ARG birds plasma. In mammals, glutamate has been found to be included in a few physiological aspects, such as biosynthesis of neurotransmitters and other amino acids, acid-base balance, cell proliferation, gene expression and immune function [89]. Proline is included in pivotal biological functions related to cell metabolism, including regulating gene transcription and cell differentiation, clearing oxidants,

protein synthesis and structure, cell signaling and bioenergetics [90]. However, in special metabolic cases (e.g., nutritional or metabolic stress), glutamate can be involved in gluconeogenesis in kidney [89] or entering the Krebs cycle [91]. Similarly, proline metabolism is able to produce electrons which can be involved in the mitochondrial electron transport chain to produce ATP [92,93]. Otherwise, proline is also able to degraded to produce α-ketoglutarate, an intermediate of the Krebs cycle [91]. Therefore, it is possible to hypothesize that the lower plasma levels of proline and glutamate in ARG birds may because of an increased recruitment and utilization of these amino acids in peripheral tissues (possibly the skeletal muscle,) to supply energy precursor for cells. Inosine can be produced through ATP degradation, which is able to be converted to hypoxanthine and finally, be released into the blood circulation [94,95]. Higher plasma concentration of hypoxanthine in ARG birds compared to control group suggesting that muscle ATP could be energy supplementation through its catabolism within cells. Although there is still limited knowledge of the molecular mechanism, increasing acetate concentration in the pectoral muscle suggests that the pathway, namely acetate acetate-valproate pathway, is activated to elevate muscle cell development by means of triterpenoids and/or steroids. Finally, the adenosine concentration in the plasma of poultry fed a supplementary diet was reduced. In mammals, endothelial cells and cardiomyocytes release adenosine in plasma in response to ischemia, hypoxia or oxidative stress [96,97]. L-arginine has also been reported to have critical antioxidant properties [98]. Therefore, the decrease in plasma adenosine may be related to the potential effects of arginine on the oxidative and hypoxic conditions that may occur in the breast muscle of fast-growing broilers.

**Probiotic supplementation for horses**

Probiotic supplementation for promoting exercise and training performance, as well as good health has more attentions in recent years in sports community [99,100]. More than 700 human controlled and randomized studies have been performed with probiotics already in 2011 [101], mainly relating to gastrointestinal performances but also included metabolic, inflammatory, allergic and respiratory conditions. In fact, probiotic supplementation was found to modulate cytokines production, increase plasma antioxidant, reduce gut permeability and decrease incidence and severity of respiratory diseases in human athletes [100,102–106]. Unfortunately, these studies have been criticized as suggesting that the spectrum of discrete biomarkers in physiology, immunity and health is too rich. In addition, these studies are generally considered to lack a practical perspective, including transformation results or clinical benefits that can be applied by athletes and coaches [99]. Probiotics is regarded as a way for health status improving, and in turn performance, has successfully applied also on horses, with positive results [107–110]. Unfortunately, the ambiguity of the

results also seems to influence the choice of formulations for horses, mainly due to tightly controlled quality of commercial over-the-counter products and inconsistent strain and dose selection [111]. Moreover, many formulations increasingly administered for horses have been originally designed for humans. This means that any evidence obtained from humans may not apply directly to horses. It is particularly true for the genera most commonly used in human probiotic formulations (Lactobacillus, Bifidobacterium, and Enterococci). The presence of genera in the horse microbiota is lacky acknowledged [111–114]. For at least three main reasons, more research is urgently needed on specific probiotics for horses. First, the safety and no side effects of probiotic formulations designed initially for humans must be evaluated. The first safety assessment will reduce the need to bypass specific horse counterparts, reducing manufacturing costs. Second, it still has to be proven whether "human" probiotic strains can colonize in the horse's intestine. This will confirm the possibility of utilizing bacterial strains selected based on their probiotic properties rather than source [115]; third, it must be demonstrated whether and to what extent probiotics affect horse training performance. This piece of information is much more limited for horses than for humans [111]. In the present trial, we investigated the possibility to improve the training performance of Standardbred horses by using highly concentrated multispecies probiotic formulation specifically applied for humans. We measured lactate concentration in horse blood, which is considered as a translational outcome largely applied for performance. From a metabolomics perspective, we combined this observation with the study of urine to give the body a holistic understanding of the response to exercise [116].

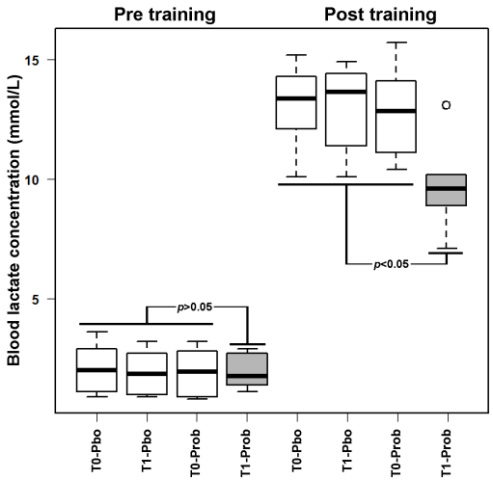


Figure 8. Blood lactate concentration in horses before (T0) or after (T1) the administration of probiotics (Prob) and placebo (Pbo), and before and after training. White boxes showed samples collected at T0 and after placebo treatment as constituting a single group. Grey boxes showed samples obtained after probiotic treatment. The above boxes were compared by nonparametric univariate analysis test. (Figure 2 in Paper IV)

Before all training sessions, a similar blood lactate concentration was found. Post-training, every horse but those at T1 treated with placebo showed again a similar value of lactate. On contrast, horses at T1 under probiotics treatment showed significantly lower post-training a blood lactate concentration than the other groups observed post training (Figure 8).

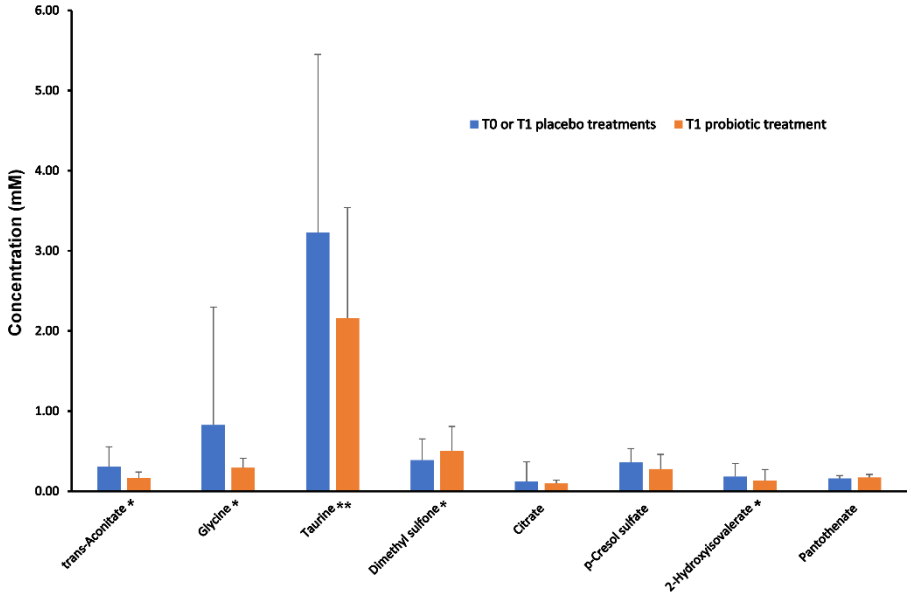


Figure 9. Concentration (mmol/L) of the molecules which concentration pre-training varied because of probiotic supplementation. Due to readability, only comparisons with a *p*-value lower than 0.1 are reported. The concentration of *p*-cresol sulfate and 2-hydroxyisovalerate was multiplied by 10 for the same reasons. * $p<0.05$.

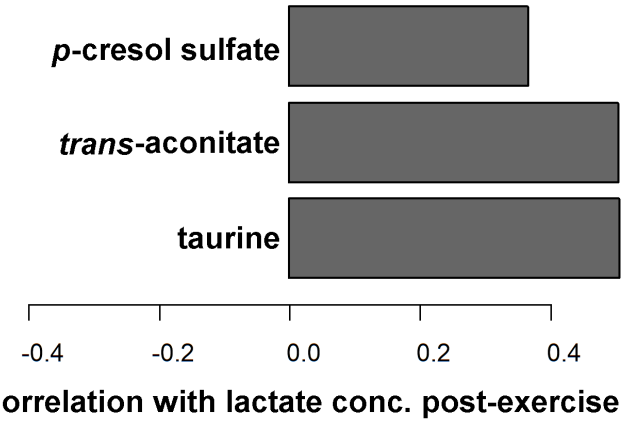


Figure 10. Significant correlations ($p < 0.05$) with lactate concentration registered post-exercise of the significantly different molecules.

The metabolomic investigations of urine before exercise were likely to support that energy source related molecules were modified. In particular, *trans*-aconitate concentration was found to be

significantly modified by the probiotic administration and correlated with lactate concentration in blood after exercise. *Trans*-aconitate is endogenously originated through cis-aconitate, as the TCA cycle's intermediates [117]. Human similar study has confirmed that urine *trans*-aconitate concentration can be modified by exercise [118]. In the current trail, we found the trend of citrate was consistent with the one of *trans*-aconitate. Both of the molecules can provide information about Krebs cycle efficiency. In fact, the level of citrate in urine can be regarded as an indirect biomarker reflecting horses training status. Indeed, the concentration in mitochondria of citrate synthase enzyme can be modulate by endurance training [119], which in turn modifies the levels of TCA intermediates. In addition, human studies confirm that trained subjects excrete less citrate through urine [120]. The trend evidenced for blood triglycerides concentration is likely to give a different insight of the same phenomenon. Actually, the decreasing blood triglycerides concentration under probiotics supplementation could also be attributed to the accumulation of SCFAs, which can pass from the lumen into the bloodstream regulating the fatty acids synthesis balance [121–123]. In addition, experimental exercise mice and other laboratory animals have also confirmed the similar effect of Lactobacillus plantarum [122,124], which could not be supressed occurring in horses. Among the molecules quantified by urine metabolomics, the concentrations of sulfone containing metabolites, namely taurine, *p*-cresol sulfate, and dimethyl sulfone were found to be modified by probiotic administration. In addition, taurine and *p*-cresol sulfate were found positively correlated to blood lactate concentration after exercise, as shown in Figure 8. Therefore, it's likely to suggest that sulfur metabolism may play a role in the highlighted modifications of energy metabolism. Taurine is reported to play a pivotal role in skeletal muscle development [125], its high level excretion in urine has even been related to disuse-related muscle atrophy. *P*-cresol sulfate probably links to body inflammatory status, therefore its low concentration in urine seems to be positive. *P*-cresol sulfate excreting through urine entirely is provided by colon bacteria catabolism of food components [126]. Particularly, in inflammatory bowel disease, its production has been found to be associated with disordered bacterial colonization [127]. Dimethyl sulfone has been reported to protect horses from systemic inflammation linked to exercise injuries [128], likely by exerting a cleaning effect on reacting oxygen substances, therefore it may even be employed as a food additive in horse feed.

# *Chapter 5. The effect of food on human health*

This chapter shows two experiments aimed to investigate the effect of food on human health, particularly for the treatment of gastrointestinal diseases by probiotics administration.

**Probiotics administration for the treatment of infantile colic**

Infantile colic is a self-limited and benign process with the characterization of paroxysms of inconsolable crying. Approximately 10% to 40% of infants are affected by infantile colic around worldwide [129] associated with repeated physician visits and significant parental frustration [130]. Despite decades of research, as a functional gastrointestinal disorder, it's still unclear [131]. Consequently, several treatments have been applied to alleviate this issue [132]. Preclinical data and recent studies have suggested that new-borns intestinal microbiota changing can affect brain signaling systems linked to pain, playing a pathogenetic role of infantile colic [133]. Because of this reason, probiotics dietary supplementation has been recommended as a possible preventive [134] or therapeutic measure [135], by modifying perception centrally. Safety and effects of probiotics are highly strain-specific [136,137]. Despite data not being solid, till now, only Lactobacillus reuteri DSM 17938 (L. reuteri) is likely to elevate colic treatment success in meta-analysis studies [138,139] and systematic reviews [140,141]. L. reuteri did not cause an increasing level of D-lactic acid more than two weeks, while D-lactic acidosis in infants under the administration of lactic acid bacteria-containing products is still a question mark in prolonged period [140]. In pediatric patients, other probiotics, which have a good safety profile, have been evaluated for functional gastrointestinal disorders. The multi-strain high-concentrated probiotic mixture has been proposed for the treatment of symptoms in irritable bowel syndrome (IBS) children [142].

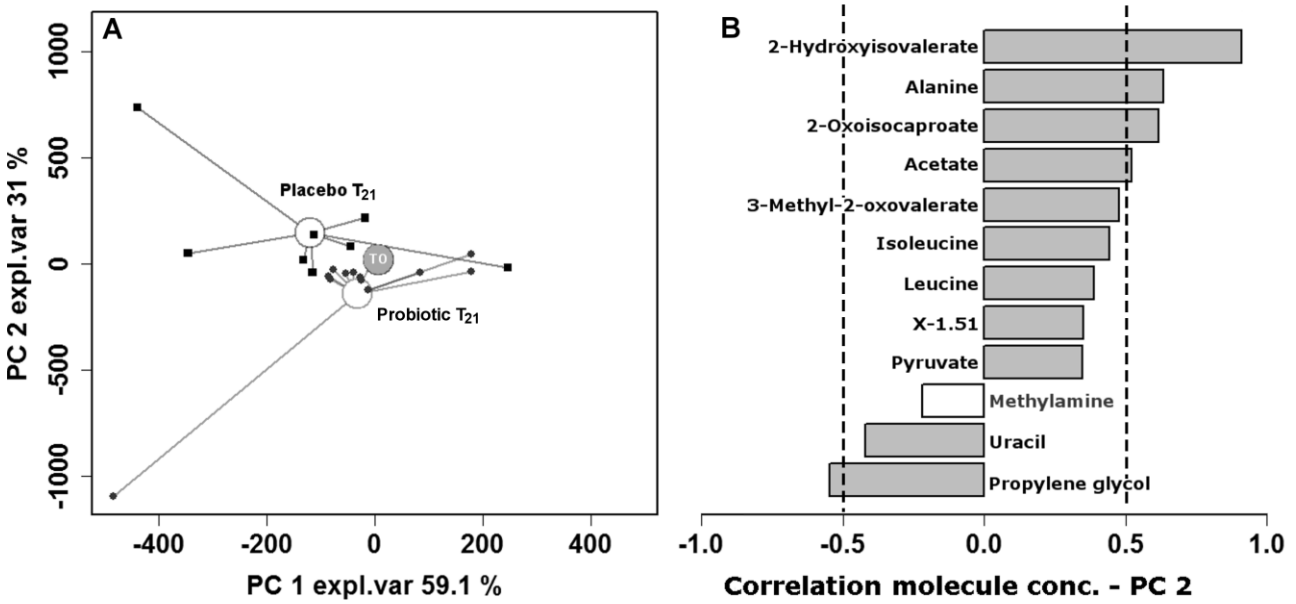


Figure 11. rPCA model built on the basis of molecules whose concentration significantly altered by different treatment. In scoreplot A, samples from individuals treated with the probiotics and the placebo are represented with circles and squares, respectively. The empty circles showed the median value of each group. Barplot B described the correlation between molecule concentrations

and their importance along PC 2. Statistically significant correlations were highlighted by grey bars ($p < 0.05$). (Figure 3 in Paper V)

A total of 59 molecules was characterized pertaining to the chemical groups of organic acids, monomeric carbohydrates, amino acids and short chain fatty acids. In order to find out the potential biomarkers of the effects of probiotic treatment, for each molecule we calculated the T21–T0 difference on an subject basis, and then we compared the differences through a two-tailed Mann-Whitney U test. Among all the quantified molecules in feces samples, 12 molecules showed statistically significant between the two groups, namely 2-hydroxyisovalerate, alanine, 2-oxoisocaproate, acetate, 3-methyl-2-oxovalerate, isoleucine, leucine, pyruvate, methylamine, uracil, propylene glycol and one peak without proper name. Ten out of twelve molecules showed opposite trends between the two groups, with the exceptions of acetate and methylamine.

To have an overview of such data, we calculated an rPCA model on the concentrations of the above molecules, as shown in Figure 11. Taking into consideration of the paired structure of the trail, we subtracted the molecule concentrations at timepoint - T0 from every timepoint. Due to this reason, in the scoreplot of rPCA model (Figure 11A), the two groups appeared superimposed at T0 with scores very close to 0 in both PCs. Along PC1, the median scores of both groups showed opposite trends at T21. Therefore, such PC allowed us to focus on the changes due to the infants growing from a metabolomic point of view. On the contrast, along PC 2, samples in the placebo group appeared at positive scores, while probiotic group samples appeared significantly separated from the previous ($p < 0.01$), with negative scores. Consequently, PC 2 seems provided a holistic insight of the different responses of infants under the two treatments, evidenced by a molecule-by-molecule basis. From this point, it is worth to notice that samples at T21 differenced from samples at T0 only for infants under probiotics treatment ($p < 0.01$) along PC 2. As shown in Figure 11B, the molecules that contributed mostly to the trends were alanine, 2-hydroxyisovalerate and 2-oxoisocaproate, increasing only in subjects under the placebo treatment. However, propylene glycol showed an increasing trend in subjects under probiotics trentment.

In the current study, it seems that propylene glycol could be considered as the clearest biomarker of individuals under probiotics administration. Propylene glycol is commonly found in new-born feces. Interestingly, a higher level of propylene glycol has been reported in the feces of breast-fed infants comparing to formula-fed infants, which suggesting a beneficial effect of such molecule [143].

**Probiotics administration for the treatment of Symptomatic uncomplicated diverticular disease (SUDD)**

Colon diverticulosis is regarded as the most common anatomic alteration found by colonoscopy [144]. Its symptomatic form, namely symptomatic uncomplicated diverticular disease (SUDD), affects one-fifth of the people with diverticulosis [144]. Although the pathogenesis is not completely clear, it has been suggested that a alteration of gut microbiota proportion is a proper etiopathogenetic factor in diverticular disease [145]. In fact, recent studies indicated that the significantly higher diversity of the Proteobacteria phylum was found in fecal samples of patients compared to controls [146], however, some Lactobacillaceae, Clostridium clusters and Fusobacterium were reduced in symptomatic versus asymptomatic patients [147]. In addition, a significant increase level of Akkermansia muciniphila in asymptomatic and symptomatic patients comparing to healthy controls has been found [148].

Till now, a few treatments are currently proposed in order to treat symptoms in SUDD patients. Particularly, pharmacological treatments, such as mesalazine and rifaximin or fibers and probiotics supplements, share some features linked to the ability to change the anti-inflammatory activity and intestinal microbiota. However, there is limited knowledge of whether those treatments are also able to influence fecal microbiota in connect to metabolome in SUDD patients.

In the current experiment, 65 molecules could be characterized, and 16 of them showed statistically significant between T0 and T1. As many as 6 amino acids and the non-proteinogenic amino acid taurine showed significantly different in this comparison. Interestingly, all the significant molecules were found to increase along time. Moreover, these trends were also confirmed by the T0-T2 and T0-T3 comparisons. The level of formate in fecal samples was found to increase after treatments, both immediately and in the long term. On contrast, valerate and its derivative 2-hydroxy-3-methylvalerate showed the opposite trend.

As a consequence, there was an overall trend of fecal metabolome in those patients showing in those of symptoms and fecal microbiota. We found that fecal metabolome was clearly modified after SUDD treatment, with a trend towards restoration during the experimental period. A few concentrations of molecules significantly changed during the follow-up. The presence of ethanol has been connected to yeast overgrowth and bacteria synthesis by the Embden-Meyerhof pathway at the same time [149]. Formate and some aminoacids, among which valine, have been considered as substrates for microrganisms fermentation, leading to the production of valerate [150]. The increase we noticed in concentration of formate and 6 amino acids, paralleled by the decreased concentration of valerate, probably to suggest that the conversion of the former in SCFAs is reduced by each of the treatments underwent. It is worth to note that the increased consumption of fibers is commonly

recognized to be linked to an increase of SCFAs [151]. The opposite trend we find in the context is therefore coherent with the observation that we here face a different phenomenon, specific to the microbiota associated with SUDD. In order to identify such mechanism, it is interesting to investigate that SCFAs production from peptides has been found to be depleted, in in-vitro systems, by the increase of several saccharides [152]. SCFA probably plays therefore a mainstay role in the complex metabolic pathway characterizing SUDD patients, as hypothesized in the previous paper [153] but still not clearly confirmed. Going into detail about the effect of each treatment on the modifications of fecal metabolome, we found that mesalazine, probiotic mixture VivoMixx® and fibers could modify it deeply in the short term, with a return to the initial levels during the follow-up. On the contrary, rifaximin was not able to modify significantly fecal metabolome in those patients, but the mechanism of this phenomenon is unknown.

# *Chapter 6. Conclusions*

This PhD thesis has set up NMR-based SOPs for serum, urine, feces and meat, which helped providing highly replicable and reproducible procedures for the further researches. The works devoted to set up SOPs were found also useful in the field of comparative metabolomics, because they contributed to enrich open access metabolomic database. Based on this first step, the effects of food on animal metabolism could be evaluated by means of $^{1}$H-NMR, as the second section of the PhD thesis describes. NMR analytical platform resulted as particularly informative in revealing the consequences of probiotic supplementation on animal metabolism. This prompted us to extend this kind of observations to the connection between food and human health, with a specific emphasis on probiotics administration.

In conclusion, being downstream of genome, transcriptome and proteome, metabolome of a host is considered as the best representation of its phenotype. For example, even though the microbiota was statistically insignificant before and after the treatment of probiotic for infantile colic, we were able to investigate the molecule concentrations variance through a metabolomic approach. Among the platforms for metabolomic investigations, $^{1}$H-NMR is confirmed as a useful tool to provide traceable, reproducible and interoperable quantitative information, which can monitor the variances under different treatments from a metabolomic point of view.

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