^{1}H-NMR spectroscopy to investigate the connection between food characteristics and health

Chenglin Zhu (email: chenglin.zhu2@unibo.it)
Dipartimento di Scienze e Tecnologie Agro-Alimentari, *Alma Mater Studiorum* - Università di Bologna
Corso di Dottorato: Scienze e Tecnologie Agrarie, Ambientali e Alimentari
Curriculum: Scienze e Biotecnologie degli Alimenti; Ciclo di dottorato: XXX; Anno di frequenza: II
Tutor: Luca Laghi

1. Stato dell'arte

Metabolomics is a powerful systems biology approach that aims to measure simultaneously all the low molecular weight metabolites 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 (Nicholson, Lindon, and Holmes 1999). 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 mammalian host and its resident gut microbiota. 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 (Baldassarre et al. 2018). Indeed, 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 (Gowda et al. 2008; Poli et al. 2005; Zhang et al. 2012).

Nuclear magnetic resonance (NMR) spectroscopy has a long tradition as a powerful platform in the hands of food scientists, with several applications related to food safety, traceability and authenticity. 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 (De Filippis et al. 2016; Siroli et al. 2017) or when the connection between food composition and technological treatment was investigated. This is because an NMR profile contains qualitative and quantitative information on hundreds of different small molecules present in a sample at 1mM concentration (Bertini et al. 2009). Moreover, NMR-based metabolomics 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 (Parolin et al. 2018).

The metabolic composition of human biofluids can provide important diagnostic and prognostic information. Among the biofluids most commonly analyzed in metabolomic studies, urine appears to be particularly useful. It is abundant, readily available, easily stored and collectable by simple, noninvasive techniques. Moreover, given its chemical complexity, urine is particularly rich in potential biomarkers of diseases. This makes it an ideal biofluid for detecting or monitoring disease processes. These advantages 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. In order to obtain useful pieces of information from the metabolome of biofluids, two key issues should be faced. Firstly, the extrapolation of molecules concentrations from NMR spectra can be severely hindered by shifts of the signals caused by factors like pH and ionic strength. The design of convenient SOP for samples manipulation seems therefore a key step to create robust databases. Secondly, it should be considered that the connection between metabolome and health status is obscured by molecules brought by diet. In this respect, such issue could be better dealt with, as a first approach, in simplified environments. They can be represented on one side by food interventions with remarkably high effects of metabolome, on the other side by biofluids poorly influenced by diet.

2. Bibliografia

Baldassarre ME, Di Mauro A, Tafuri S, Rizzo V, Gallone MS, Mastromarino P, Capobianco D, Laghi L, Zhu C, Capozza M and Laforgia N. (2018) Effectiveness and safety of a probiotic-mixture for the treatment of infantile colic: a double-blind, randomized, placebo-controlled clinical trial with fecal real-time PCR and NMR-based metabolomics analysis. Nutrients, 10(2), 195.

Bertini I, Calabro A, De Carli V, Luchinat C, Nepi S, Porfirio B, Renzi D, Saccenti E and Tenori L (2009) The metabonomic signature of celiac disease. J Proteome Res, 8(1): 170–77.

De Filippis F, Pellegrini N, Vannini L, B Jeffery I, La Storia A, Laghi L, I Serrazanetti D, Di Cagno R, Ferrocino I, Lazzi C, Turroni S, Cocolin L, Brigidi P, Neviani E, Gobbetti M, W OToole P and Ercolini D. (2016) High-level adherence to a mediterranean diet beneficially impacts the gut microbiota and associated metabolome. Gut, 65(11).

Gowda GA, Zhang S, Gu H, Asiago V, Shanaiah N and Raftery D (2008) Metabolomics-based methods for early

disease diagnostics. Expert rev mol diagn, 8(5): 617–33.
Nicholson, JK, Lindon JC and Holmes E (1999) "Metabonomics": Understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica, 29(11): 1181–89.
Parolin C, Foschi C, Laghi L, Zhu C, Banzola N, Gaspari V, Di Antuono A, Giordani B, Severginini M, Consolandi C, Salvo M, Cevenini R, Vitali and Marangoni A (2018) Insights into vaginal bacterial communities and metabolic profiles of chlamydia trachomatis infection: positioning between eubiosis and dysbiosis. Front Microbiol, 9: 600.
Poli D, Carbognani P, Corradi M, Goldoni M, Acampa O, Balbi B, Bianchi L, Rusca M and Mutti A (2005) Exhaled volatile organic compounds in patients with non-small cell lung cancer: cross sectional and nested short-term follow-up study. Resp Res, 6: 71.
Siroli L, Patrignani F, I Serrazanetti D, Parolin C, Palomino R, Vitali B and Lanciotti R (2017) Determination of antibacterial and technological properties of vaginal lactobacilli for their potential application in dairy products. Front Microbiol, 8: 166.
Zhang S, Liu L, Steffen D, Ye T and Raftery D (2012) Metabolic profiling of gender: headspace-SPME/GC–MS and 1H NMR analysis of urine. Metabolomics, 8(2): 323–34.

3. Obiettivi

The work aims, in a stepwise approach, to investigate the relationship between probiotic assumption and health status and then to focus on the more complex connection between food processing and composition and in turn between food composition and possible consequences on health status.
The project of the doctoral thesis may be divided in the following activities, summarized by the Gantt diagram reported in Table 1:
A1) **Literature review about latest researchers related to investigating the relationship between food and health through a metabolomics approach**
A2) **Set up and apply specific NMR SOP (Standard operation procedures)**.
A3) **Metabolomics oriented experiments focusing on the relationship between probiotics assumption and health status**
A4) **Metabolomics oriented experiments focusing on the relationship between food processing and food composition and, in turn between food composition and health**
A5) **To write and publish the doctoral thesis, posters, scientific articles and oral presentations**

Table 1. Diagramma di Gantt dell'attività di ricerca del dottorato

Attività	Mese	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36
A1)	***Literature review and Experimental design***																		
	1) preliminary studies in metabolomics																		
	2) experiment design																		
A2)	***Set up and apply specific NMR SOP***																		
A3)	***Experiments focusing on probiotics assumption and health status***																		
	1) read papers to choose one or two kinds of probiotics																		
	2) assessments of the relationship between probiotics and health																		
A4)	***Experiments focusing on the food processing and health***																		
	1) assessments of the relationship between food processing and composition																		
	2) assessments of the relationship between food composition and health																		
	3) investigate the relationship through metabonomic approach																		
A5)	***Writing and publishing the doctoral thesis***																		

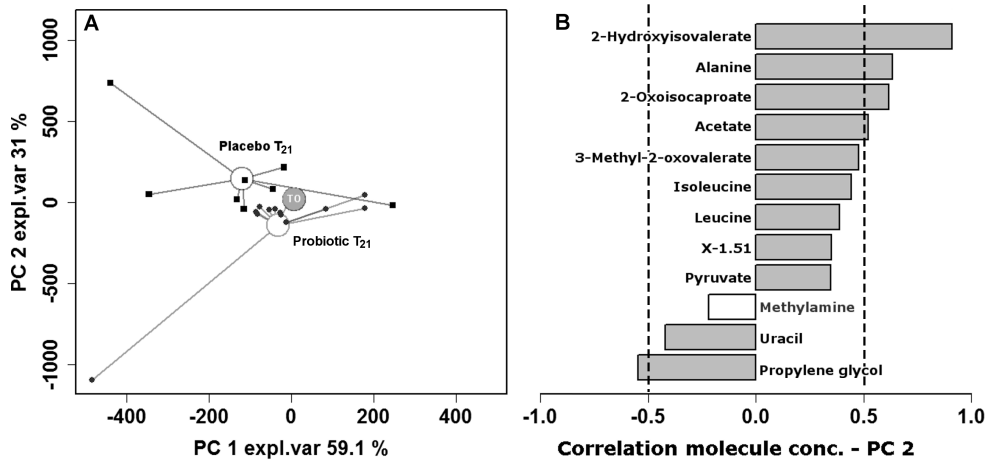
4. Stato di avanzamento della ricerca e principali risultati

A2 - During my first year, I focused on projects allowing me to set up NMR-based SOP designed to obtain information about human health status from biofluids metabolome. A group of works, dealing with women, children and horses urines allowed me to design a universally applicable SOP for urine metabolome investigation. In the work described in the paper "Urine metabolome in women with Chlamydia trachomatis infection" healthy or chlamydia affected women donated urines to look for biomarkers of this disease. As the selected women were allowed a free diet during the experiment, the obtained samples were perfectly tailored for the purpose.
A second group of work allowed me to set up SOP for feces related metabolomics works. One of them gave rise the communication by Tursi "Impact of treatments on fecal microbiota and fecal metabolic profiling in symptomatic uncomplicated diverticular disease of the colon". Other SOP were setup on serum samples from chicken and human beings, humor samples from pigs.
Women affected by Chlamydia described above donated also a sample of vaginal swab, so to find further biomarkers of Chlamydia. In the context of my research plan, this work served as a first insight into the relationship between

microbiota and metabolome in an environment that, from two key points of view, could be considered simpler than feces. On one side, previous works highlighted the connection between microbiota and most of the molecules that can be quantified by NMR. On the other side, diets habits that may act as strong confounding variables in the study of other biofluids play a minor role in vaginal environment.

A3 – Some of the works described in point A2 allowed me to explore the relationship between probiotics and biofluids metabolome. Among them, the work described in Baldassare et al applied a probiotic-mixture for the treatment of infantile colic. The infantile colic research project aims to evaluate how probiotic supplementation could affect the fecal molecular profile, for the destination, we focused on the molecules whose concentration varied relating to the investigated treatments by calculating the differences between before and after, then compared by means of Mann-Whitney U test. To highlight the underlying trends characterizing the samples, a robust Principal Component Analysis (rPCA) was built on the molecules concentration, centered and scaled to unity variance, according to Hubert.

As a result, fifty-nine molecules could be quantified pertaining to the chemical groups of amino acids, short chain fatty acids, organic acids and monomeric carbohydrates. The evolution of the fecal metabolome differed for 12 molecules. Interestingly, 10 out of 12 molecules showed opposite trends for the two groups, with acetate and methylamine representing the only exceptions. Consequently, samples at T21 constituted of two distinct groups according to the treatment, as it could be inferred also form the statistical significance ($p < 0.01$) of the intragroup/intergroup samples distance in the 12 dimensions space. To have an overview of such findings, we calculated an rPCA model on the centered and scaled concentrations of these molecules (Figure 1). To consider the paired structure of the experiment, we subtracted the concentration of the molecules at timepoint T0 from every timepoint. This is why, in the scoreplot (Figure 1A), the two groups at T0 appear superimposed and with scores along PC 1 and PC 2 very close to 0. The median scores of both groups at T21 appear at negative values along PC 1. Such PC, therefore, allows us to focus on the changes occurring to the metabolome of the infants upon growing. Along PC 2, contrastingly, samples from the placebo group appear at positive scores, while samples from the probiotic group appear separated from the previous ($p < 0.01$), with negative scores. PC 2, therefore, gives a holistic view of the different responses of infants, to the two treatments. From this perspective, it is of importance to notice that along PC 2, samples at T21 appear differently from samples at T0 only for infants treated with probiotics ($p < 0.01$). The molecules that mostly contributed to the trends (Figure 1B) were 2-hydroxyisovalerate, alanine and 2-oxoisocaproate, increasing only in subjects treated with the placebo. Acetate increased mainly in subjects treated with the placebo and propylene glycol, as well as increasing in subjects treated with probiotics. In the present investigation, propylene glycol appeared as the clearest biomarker of subjects supplemented with probiotics. Propylene glycol is normally found in newborn feces and, interestingly, a higher concentration has been found in the feces of breast-fed infants in comparison with formula-fed ones, which suggesting a beneficial effect for this molecule.



5. Elenco delle pubblicazioni prodotte nell'ambito dell'attività di dottorato

Foschi C, Laghi L, D'Antuono A, Gaspari V, Zhu C, Dellarosa N, Salvo M and Marangoni A (2018) Urine metabolome in women with Chlamydia trachomatis infection. PLoS ONE 13(3): e0194827.

Parolin C, Foschi C, Laghi L, Zhu C, Banzola N, Gaspari V, D'Antuono A, Giordani B, Severgnini M, Consolandi C, Salvo M, Cevenini R, Vitali B and Marangoni A (2018) Insights into vaginal bacterial communities and metabolic profiles of chlamydia trachomatis infection: positioning between eubiosis and dysbiosis. Front Microbiol. 9:600.

Baldassarre M. E, Di Mauro A, Tafuri S, Rizzo V, Gallone MS, Mastromarino P, Capobianco D, Laghi L, Zhu C, Capozza M and Laforgia N (2018) Effectiveness and safety of a probiotic-mixture for the treatment of infantile colic: a double-blind, randomized, placebo-controlled clinical trial with fecal real-time PCR and NMR-based metabolomics analysis. Nutrients, 10(2), 195.

Tursi A, Mastromarino P, Capobianco D, Elisei W, Laghi L, Zhu C, Picchio M, Giorgetti G, Fabiocchi F, Di Mario F and Brandimarte G (2018) Impact of treatments on fecal microbiota and fecal metabolic profiling in symptomatic uncomplicated diverticular disease of the colon. Digestive and Liver Disease, 50(2), pp. 75-76.