# Challenges of metabolomics in human gut microbiota research

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## Abstract

The review highlights the role of *metabolomics* in studying human gut microbial metabolism. Microbial communities in our gut exert a multitude of functions with huge impact on human health and disease. Within the meta-omics discipline, gut microbiome is studied by (meta)genomics, (meta)transcriptomics, (meta)proteomics and metabolomics. The goal of metabolomics research applied to fecal samples is to perform their metabolic profiling, to quantify compounds and classes of interest, to characterize small molecules produced by gut microbes. Nuclear magnetic resonance spectroscopy and mass spectrometry are main technologies that are applied in fecal metabolomics. Metabolomics studies have been increasingly used in gut microbiota related research regarding health and disease with main focus on understanding inflammatory bowel diseases. The elucidated metabolites in this field are summarized in this review. We also addressed the main challenges of metabolomics in current and future gut microbiota research. The first challenge reflects the need of adequate analytical tools and pipelines, including sample handling, selection of appropriate equipment, and statistical evaluation to enable meaningful biological interpretation. The second challenge is related to the choice of the right animal model for studies on gut microbiota. We exemplified this using NMR spectroscopy for the investigation of spatial gut differences and cross-species comparison of fecal metabolite profiles. Finally, we present the problem of variability of human gut microbiota and metabolome that has important consequences on the concepts of personalized nutrition and medicine.

**Keywords:** metabolomics, gut microbiota, mass spectrometry, NMR, IBD

# Introduction

Gut microbiota plays a crucial role in human health and disease. Translational research with rodents demonstrated that gut microbiota can be involved in different physiological functions such as energy harvesting (Bäckhed et al., 2004), shaping and maintaining of the intestine function (Hooper and Gordon, 2001), or regulation of the host immune system (Kau et al., 2011). Furthermore, the microbial composition can influence the host response to pathogens and the predisposition to diseases (Vrieze et al., 2010). By possessing its own genome, all microbes in our gut undergo the same machinery of transcription, translation, and metabolism, as depicted in Fig. 1A (Qin et al., 2010). In order to conduct studies on human gut microbiota, fecal samples are mainly collected. Fecal genome, transcriptome, proteome, and metabolome can be potentially used to define specific members within the microbial ecosystem and investigate their functions by interpreting gene expression patterns and behavior of proteins and metabolites. Large-scale ‘omics’ studies are performed separately or in an integrated way in order to get a holistic overview of the processes taking place in a dynamic system (Fig. 1B). Omics studies regarding complex microbial communities and their interactions in a habitat, *e.g.* within a human intestine, are often accompanied by the prefix "meta" such as *metagenomics*, *metatranscriptomics*, *metaproteomics*, or *(meta)metabolomics* with the latter only rarely used in literature (Turnbaugh and Gordon, 2008; van Baarlen et al., 2013). Several studies attempted to combine these techniques or to integrate them on different levels of data processing and evaluation, thereby going beyond only taxonomic profiling (Daniel et al., 2013; Tong et al., 2014; Zhang et al., 2015b).

Metabolomics is defined as a comprehensive analysis of all metabolites in a biological system with their identification and quantification (Fiehn, 2002). To conduct a metabolomics study, spectroscopic or spectrometric techniques have been applied. Various biological matrices have been analyzed comprising urine, plasma, feces, or biopsies in order to monitor metabolites from host, microbes and their co-metabolism (Storr et al., 2013). Metabolomics on fecal samples for studying gut microbial metabolism is just a rising but promising field, since stool is an easy accessible and non-invasive matrix with metabolites originating from host, its gut microbiota, and food components (Marchesi et al., 2007). To obtain a quick overview of the published literature on studying microbial ecosystem using fecal samples via metagenomics, metaproteomics, metatranscriptomics or metabolomics, an ISI Web of Science search was conducted by the following queries: **TOPIC: (fec\* hum\* metabolom(or nom)\*)** for human fecal metabolomics; **TOPIC: (fec\* hum\* metatranscriptom\*)** for human fecal metatranscriptomics; **TOPIC: (fec\* hum\* metagenom\*)** for human fecal metagenomics; **TOPIC: (fec\* hum\* metaproteom\*)** for human fecal metaproteomics; **TOPIC: (fec\* hum\* 16S sequencing)** for 16S sequencing of genome present in human fecal samples. Next to the 16S sequencing, the omics area of metagenomics has the greatest number of publications followed by metabolomics whereas a relatively small number of publications is observed for metatranscriptomics and metaproteomics (Fig. 1C). Despite of their prevalence, a decreasing trend either for metabolomics, metagenomics or 16S sequencing can be seen after a certain time point (Fig. 1C). The majority of publications regarding metabolomics using fecal samples are focused on microbiota, health, disease related issues, and diet (Fig. 1D). In turn, disease related issues have their main focus on inflammatory bowel disease, cancer, infection and obesity (Fig. 1D).

## Fecal metabolomics in inflammatory bowel disease

Inflammatory bowel disease (IBD) was found as one of the major diseases described in relation with metabolomics studies and gut microbiota (Fig.1). IBD is an idiopathic disease that mainly affects gastrointestinal tract. Two main forms, ulcerative colitis (UC) and Crohn’s disease (CD), have a complex etiology (Erickson et al., 2012). A disbalance of commensal microbiota is discussed in connection to IBD with a decrease of diversity and altered metagenome and metaproteome (Erickson et al., 2012; Kostic et al., 2014). For example, *Faecalibacterium prausnitzii*, which is a prominent short chain fatty acid (SCFA) producer in human gut, is decreased in IBD patients (Duncan et al., 2002; Miquel et al., 2015), which shows the connection between bacteria and metabolites. The state of art regarding the studies that performed non-targeted metabolomics in fecal samples of IBD patients is described in order to get an overview of altered metabolites (Table 1). In total, there are 9 studies that dealt with human and searched for IBD related metabolites (Ahmed et al., 2013; Bezabeh et al., 2009b; Bjerrum et al., 2015; Jansson et al., 2009; Le Gall et al., 2011; Lee T, 2016 accepted; Marchesi et al., 2007; Vanden Bussche et al., 2015; Vigsnaes et al., 2013). Two of the above mentioned works did not describe discriminative metabolites, and metabolomics was utilized as a classification/validation tool (Bezabeh et al., 2009b; Vanden Bussche et al., 2015). Different techniques such as nuclear magnetic resonance (NMR) spectroscopy, gas chromatography and liquid chromatography coupled to mass spectrometry (GC/MS and LC/MS, respectively), and direct infusion (DI) Fourier transform ion cyclotron mass spectrometry (FT-ICR/MS) were used to analyze metabolites (Table 1). All compounds that were described and shown to be significantly altered between control and IBD patients (categorized into UC and CD) are summarized in Table 1, with their respective Human Metabolome Database (HMDB) entry (Wishart et al., 2013). Studies from Ahmed *et al.*, Jansson *et al*. and Lee *et al.* described many metabolites that were altered in IBD patients using GC/MS to measure fecal volatile metabolites and FT-ICR/MS to measure higher molecular weight polar molecules (Ahmed et al., 2013; Jansson et al., 2009; Lee T, 2016 accepted). The most discriminative metabolites for IBD, mainly derived from NMR studies (3 in total), were alanine, isoleucine, leucine, lysine, valine, phenylalanine and butyrate. Metabolites described in IBD related studies using MS platform are diverse but it seems that long chain fatty acids could play an important role in the disease. We have to point out that metabolic patterns, frequently reported to be involved in IBD, are probably more unspecific and are rather a consequence of inflammation or altered gut microbiota than the cause. An increase of amino acids in fecal samples of IBD patients is explained as a malnutrition effect, since inflamed intestinal tissue has low absorption for nutrients (Ghishan and Kiela, 2014).

To mention an example on a specific metabolite, butyrate has been shown, among all compounds described in metabolomics studies, to be highly relevant in bacterial metabolism. Butyrate is a strict microbial metabolite, originating from the degradation of non-digestible carbohydrates that are fermented by bacteria (den Besten et al., 2013). *F. prausnitzii* is not the only species in human gut which produces butyrate. Other prominent butyrate producers primarily derive from *Firmicutes* phylum, including bacteria of *Roseburia* spp., and *Eubacterium rectale* within the *Clostridium* cluster XIVa and IV (Louis et al., 2010), whereas *Eubacterium rectale* is a dominant habitant in human subjects based on the butyryl-CoA:acetate CoA-transferase sequences (Louis et al., 2010). Butyrate is absorbed in the colonic lumen and used as an energy source for colonocytes (Bergman, 1990). It is difficult to conclude which bacterium is actively involved in the butyrate biosynthesis in each individual, since several species can be involved in the production of this metabolite. One possibility involves the subsequent measurement of absolute butyrate concentration (which is actually not reported in metabolomics studies summarized in Table 1) and observing the patterns of butyryl-CoA:acetate CoA-transferase (gene, mRNA, or proteins) in order to derive associations between butyrate concentration and its active producers for further questioning their role in disease.

In order to make the results, coming from different metabolomics experiments more comparable, it is important that the corresponding metabolites are subjected to further quantification analysis. Another promising way to measure microbial metabolism in human gut is to combine and integrate different techniques rather than to focus only on one method. For example, Shoaie *et al.* combined NMR and GC/MS metabolomics approaches in order to quantify amino acids and SCFAs in human fecal samples, plasma samples, and bacterial supernatants (Shoaie et al., 2015). However, it was done not in the context of IBD. Until now, given studies on IBD have applied either NMR or MS techniques for metabolome analysis, which defines *a priori* the metabolites you will look at.

**Analytical challenges in fecal metabolome analysis**

The detection of thousands of metabolites, which show an enormous variety of chemical polarities, is a challenging task. A well-established workflow, based on the integration of different analytical platforms, is the key for this task. Due to its high chemical diversity and complexity, a biological sample needs to be treated carefully relative to the study objective. Sample collection, sample preparation and the selection of the appropriate analytical tools are fundamental requirements for a robust sample handling. Within this review some aspects of a general metabolite analysis workflow are described, which includes precise study design, sample treatment, the instrumental setup followed by data analysis, and, as a final goal, biological interpretation of the results and data integration (Fig. 2).

Metabolomics can be subdivided into two different types of analysis: targeted analysis and non-targeted discovery oriented analysis (*i.e.* metabolite/metabolic profiling) (Dettmer et al., 2007). The targeted approach refers to the analysis of a specific class of compounds, *e.g.* amino acids, fatty acids, lipids, carbohydrates, or bile acids. Non-targeted analysis is defined as getting a rapid, global overview of the metabolic diversity of a sample by using technologies that enable to describe a large number of metabolites. The following section describes sample (pre)treatment and analytical techniques necessary for performing targeted and non-targeted metabolome analysis.

# Sample collection, storage and preparation

In the field of metabolomics, proper sample collection and storage conditions as well as adequate sample preparation are of high importance. Human fecal samples, necessary for DNA sequencing and metabolome analysis, are collected in special tubes and usually stored in range from -80°C to -20ºC prior to sample preparation (Bezabeh et al., 2009b; Deda et al., 2015; Jansson et al., 2009; Raman et al., 2013). Sample storage is a crucial and sensitive step. Freeze-thaw cycles need to be minimized to prevent possible metabolite degradation (Yin et al., 2013). For example, Comstock *et al.* investigated how continuous freezing and thawing of plasma and serum affects the concentration of cholesterol, micronutrients and hormones (Comstock et al., 2001). It was shown that no change of concentration was observed for previously mentioned metabolites within 3 freeze-thaw-cycles. For some hormones (estrone, estradiol, testosterone, and sex hormone-binding globulin) some variation in concentration was observed but the effect was relatively small. However, some metabolites were moderately influenced after 6-10 cycles. Pinto *et al.* reported changes in lipids, acetone, choline compounds, alanine, glucose, and pyruvate in human plasma subsequently within 4 freeze-thaw cycles (Pinto et al., 2014) Gautam *et al.* presented the stability of a chalcone derivative S001-469 through 3 freeze-thaw cycles in rat plasma, urine and feces (Gautam et al., 2014). Pappano *et al.* tested the hormone content in fecal samples from geladas (*Theropithecus gelada*) up to 4 freeze-thaw cycles (Pappano et al., 2010). No observable changes in hormone content was detected up to 3 cycles with significant increase only after the 4th cycle.

Selecting an extraction method is quite challenging and depends on the aim of the analysis and the choice of analytical technique. Furthermore, this step is crucial for obtaining a good metabolite extraction rate, *i.e.* to get a maximum number of quantifiable metabolites while preventing their possible physical and chemical alteration (Villas-Boas et al., 2005). Foremost, in order to stop the metabolism in a sample, immediate quenching is inalienable and can be achieved by the sudden change of pH or temperature. The quenching methods include addition of cold methanol, liquid nitrogen, perchloric or nitric acid. Usually, polar metabolites are extracted with pure organic solvents (*e.g.* methanol) or solvent mixtures (*e.g.* methanol-water). To extract lipophilic metabolites often non-polar solvents such as chloroform or hexane are used. Walker *et al.* showed in her study involving fecal samples from diabetic mice that, among four different extraction methods for non-targeted metabolomics experiment using DI FT-ICR/MS, the highest amount of observed metabolites is achieved via methanol extraction (Walker et al., 2014a). Cao *et al.* investigated methanol extracted fecal samples of liver cirrhosis and hepatocellular carcinoma patients (Cao et al., 2011). Gao *et al.* tested three different extraction solvents on human fecal water and revealed that deionized water is the best choice for metabolite analysis via GC/MS, especially for observing the signals corresponding to fatty and amino acids (Gao et al., 2009). For metabolome analysis, it needs to be ensured that proteins are removed. It can be achieved by centrifugation of the prepared mixture containing the fecal sample and the chosen solvent (Deda et al., 2015). The collected supernatant can be diluted, if necessary, and injected to the analytical system of choice.

Sometimes it is necessary to concentrate the sample after extraction procedure because the low amount of metabolites can be diluted in a relatively large volume of extraction solvent(Villas-Boas et al., 2005). Methods for concentrating the sample include, for example, solvent evaporation under vacuum or freeze-drying for removing water from aqueous solution (*i.e.* lyophilization). Solid-phase extraction (SPE) or solid-phase micro extraction (SPME), is another technique used to concentrate the sample, but is limited to certain metabolites since extraction of specific classes of compounds depends on the SPE trapping material. For targeted analysis the extraction method needs to be adjusted based on the type of compounds of interest. Müller *et al.* analyzed D-amino acids by ultra-high-performance liquid chromatography/mass spectrometry (UHPLC/MS) using biological samples comprising human urine, plasma, and serum, and mouse cecal content (Müller et al., 2014). Metabolites were extracted using methanol and further derivatization with a reagent o-phthalaldehyde/ isobuteryl-l-cysteine (OPA/IBLC) was applied. Cai *et al.* and Humbert *et al.* both reported methods for bile acid extraction procedure in fecal material, whereas Humbert *et al.* tested four different extraction methods for the analysis of bile acids (Cai et al., 2012; Humbert et al., 2012). They have shown that the extraction with isobuteryl-l-cysteine is the most efficient method to measure bile acids in fecal samples. For volatile fatty acids an extraction was performed by using a 6% phosphoric acid aqueous solution (1:2 m/V) and the resulting mixture was analyzed by headspace GC MS (Jiang et al., 2014).

# Analysis of human fecal samples

The interest for applying MS to metabolome analysis of biological samples has been increased within the last decades and, besides NMR, is nowadays the most important analytical method in the research (Zhang et al., 2012). The following section describes several ionization techniques necessary for producing charged molecules. Separation techniques that can be coupled with mass analyzers are described as well.

MS analysis can be performed via direct infusion or combined with an on-line separation technique, *e.g.* gas chromatography, capillary electrophoresis, or liquid chromatography. Based on the study objective and/or the compounds of interest, the right ionization technique needs to be selected. Electrospray ionization (ESI) is the most utilized technique to ionize polar and ionic compounds, whereas atmospheric pressure chemical ionization (APCI) is used for ionization of less polar and neutral molecules (Villas-Boas et al., 2005). Several types of mass analyzers are commonly used in metabolomics, each having its own limits on accuracy, sensitivity, specificity, and resolution: ion trap, single- (Q) and triple (QQQ) quadrupole mass spectrometer, time-of-flight (ToF) mass spectrometer, an orbitrap, Fourier transform ion cyclotron resonance mass spectrometer. Often mass spectrometers include a quadrupole collision cell to select ions of a specific m/z value for further analysis (*e.g.* MS/MS). DI MS is recommendable for high-throughput studies involving a large amount of samples and has an advantage of short analyzing time. Application of DI ESI FT-ICR/MS for metabolome analysis offers high resolution and high mass accuracy allowing the detection of thousands of metabolites simultaneously (Forcisi et al., 2015). It can be used both as targeted and non-targeted approach. The high mass accuracy of the instrument allows us to assign an elemental composition to m/z values corresponding to mass peaks (Breitling et al., 2006).Coupling MS to an LC system offers the possibility to analyze polar, non-polar as well as neutral compounds in a complex biological matrix separately. Commonly used ionization techniques in LC/MS systems are ESI or APCI, which are suitable for a wide mass range (Villas-Boas et al., 2005). LC/MS is an outstanding technique because of its resolving power, sensitivity, specificity, and the possibility to extract additional information about analyzed metabolites from retention time domain (Forcisi et al., 2015). Two main branches of LC approaches, commonly used in combination with MS analysis, include high- and ultrahigh performance liquid chromatography (HPLC and UHPLC, respectively). The advantages of UHPLC over HPLC are shorter analyzing time, higher resolution, better efficiency and sensitivity, and reduced sample and solvent volume necessary for the analysis. GC/MS is another coupling that is widely used in metabolome analysis because of its separation efficiency. However, the use of this approach is limited to volatile compounds and, therefore, often requires an additional preparation step consisting of metabolite derivatization (*e.g.* by silylation or alkylation/esterification) (Villas-Boas et al., 2005). The choice of mass analyzer for GC/MS depends on the desired level of mass accuracy and commonly used systems include coupling with Q or ToF MS. Extensive application of LC/MSn, offers an opportunity to identify target metabolites within a complex sample not only by the information regarding monoisotopic mass alone but can provide some hints about the structure of a metabolite (Villas-Boas et al., 2005). Several MSn modes can be applied such as product ion scan, precursor ion scan, neutral loss scan and multiple reaction monitoring (MRM). In the precursor scan, a chosen ion of interest collides with an inert gas (*e.g.* Ar or N2) in a collision cell (*i.e.* collision induced dissociation) and gives the raise to product ions, which are responsible for the appearance of a characteristic fragmentation pattern in a spectrum. Walker *et al.* presented a data dependent MS/MS approach for identification of taurine- and sulfate conjugated fatty acids in feces of diabetic mice using an UHPLC Q-ToF/MS system(Walker et al., 2014a). Examples on studying fecal metabolome include the application of non-targeted metabolomics to differentiate breast-fed versus formula-fed infants and using GC/MS and LC/MS/MS analysis to identify various changing metabolites due to in vitro batch culture fermentation (Chow et al., 2014).

# Data processing and statistical evaluation

A recently published review listed the most frequently used platforms for handling the data originating from metabolomics studies differentiating by the type of analysis (*e.g.* preprocessing, statistical interpretation, network analysis) or the type of the analytical technique used (*e.g.* LC/MS, GC/MS, NMR, MS/MS) (Misra and van der Hooft, 2016). Data analysis and interpretation greatly depend on the preprocessing that, in turn, can appear to be the most time-consuming step. It needs to be planned carefully, sometimes depending on the analytical technique used. This step can be very challenging for data sets originating from metabolomics studies because of the enormous number of different metabolites detected in a sample (Wu and Li, 2016). Preprocessing step includes data filtering (*e.g.* elimination of chemical and instrumental noise), spectral alignment in order to produce a data matrix, normalization (*i.e.* a table row operation), and scaling (*i.e.* a table column operation). Performing these operations ensures the comparability (Craig et al., 2006) between the samples and allows the determination of differences of metabolite concentrations within a sample set (Wu and Li, 2016). Commonly used scaling methods are pareto scaling, autoscaling and vast scaling as described in detail in van den Berg *et al.* (van den Berg et al., 2006). It should be pointed out that handling LC/MS data is tricky since preprocessing involves two or more dimensions. The corresponding data often has retention time drifting as well as peak intensity drift effects within the same experimental setup, which both can lead to false statistical results. Therefore, a retention time alignment, and an intensity drift correction is recommended as described in Misra and van der Hooft (Misra and van der Hooft, 2016).

After data preprocessing, several statistical tools can be used in order to find discriminative features within the sample set (Worley and Powers, 2013). Data analysis involves application of different univariate and multivariate methods that can be of parametric (*e.g.* Student t-test, multivariate linear regression) or non-parametric (*e.g.* Mann-Whitney test, random forests) nature. The methods can be also divided into unsupervised techniques (*i.e.* methods where labeling of the samples is not involved into calculations, *e.g.* principal component analysis (PCA), hierarchical cluster analysis (HCA)) and supervised techniques (*i.e.* methods where calculations involve the information regarding sample labels, *e.g.* linear discriminant analysis (LDA), k-nearest neighbor (kNN)). A supervised multivariate technique, partial least squares discriminant analysis (PLS-DA), has been shown to be a particularly useful tool in metabolomics studies. It is frequently used due to its simplicity and the ability to identify potential biomarkers. Nevertheless, the field of metabolomics has a huge capacity for developing and applying new valuable statistical and computational methods.

Although MS based non-targeted metabolomics is a powerful tool for global screening of all small-molecule metabolites within a biological system, identification of metabolite signals present in spectra represent one of the key challenges (Naz et al., 2014). This task can be partly accomplished by finding matches in compound and spectral databases such as HMDB (Wishart et al., 2013), METLIN (Smith et al., 2005), or ChemSpider (Pence and Williams, 2010). In order to describe the biological system more vividly, metabolic pathway databases such as KEGG (Kanehisa et al., 2008) or MetaCyc (Caspi et al., 2008) are frequently used. To simplify and optimize the task of annotating spectral peaks as potential metabolites and mapping the identified compounds onto metabolic pathways, the MassTRIX web server was developed (Wagele et al., 2012). However, even after database search, a large amount of metabolites, possibly responsible for phenotype classification, remain unknown (Walker et al., 2014b).

As mentioned earlier, the ultra-high mass accuracy of FT-ICR/MS allows putative chemical formulae to be assigned to spectral peaks. As a consequence, the connections between metabolite pairs can be established since they may be related by clearly defined mass differences (Breitling et al., 2006). A reconstructed network, based on real biochemical transformations, enables to create hypotheses regarding the behavior of biological system and to attribute putative formulae more precisely since the ability of correct assignment diminishes as m/z value of a metabolite ion increases. This idea was further developed (Tziotis et al., 2011) and applied in other studies (Moritz et al., 2015; Walker et al., 2014b). It was also shown in an experiment using reverse phase UHPLC off-line coupled to FT-ICR/MS that metabolites in the reconstructed network cluster according to their retention time (Forcisi et al., 2013). Thereby, the signals corresponding to unknown metabolites are not only given a chemical formula but also can be contextualized with respect to their biological function. The strategy of using mass difference networks for formula assignment was also implemented for data obtained by UHPLC coupled to ToF/MS that has lower accuracy than FT-ICR/MS (Forcisi et al., 2015). It was shown that annotation of isomers separated by LC is possible and the arrangement of metabolites in the network clearly shows a RT dependent pattern. These findings open up new perspectives in the context of metabolite identification since much of the information can be retrieved from the network analysis where the position of a metabolite corresponds to its nature (Fig. 3).

**NMR spectroscopy for the investigation of spatial gut differences and cross-species comparison of fecal metabolite profiles**

NMR spectroscopy generally profits from the minimal sample preparation and lack of inter-metabolite suppression effects, which enables a parallel investigation of different sample matrices and the quantitative nature of the analysis. One drawback of this technique however lies in the relatively low sensitivity of metabolite detection, which can only partly be overcome by the use of high-field magnets and cryo-probes. Another drawback is the overlap of signals from different metabolites that can be addressed by acquisition of two-dimensional experiments. In contrast to biofluids such as plasma and urine, where no extraction is necessary and only addition of buffer solution is sufficient, analysis of feces or gut luminal content need removal of undigested material, dead bacteria and other solid particles. While MS analysis uses various extraction methods, as discussed in the previous chapter, fecal extracts for NMR acquisition are mainly prepared with water or methanol (Jacobs et al., 2008). Aqueous extraction focusses on small and hydrophilic molecules such as amino acids, glucose, glycerol, trimethylamine; methanol extraction focusses on lipophilic substances such as lipids, cholate, small phenolic acids and N-acetyl compounds (Jacobs et al., 2008; Lamichhane et al., 2015). SCFA and bile acids can be detected in both extracts (Jacobs et al., 2008). However, as extraction of lipids leads to substantial signal overlap across large parts of the NMR spectrum in the methanol extract, aqueous extracts are generally the technique of choice. Lamichhane *et al.* studied the effect of sample to extraction solvent ratios and sonication on the metabolite profile and concluded an optimal recovery and reproducibility with a feces weight to solvent volume ratio of 1:2 (Lamichhane et al., 2015). Even though it reduces possible dilution of metabolites with buffer solvents, lyophilization is generally not recommended as it may lead to loss of volatile metabolites (Saric et al., 2008). After metabolite extraction, NMR buffer solution containing phosphate buffered (pH 7.4) lock substance D2O and the internal standard 3-(trimethylsilyl)propionicacid-*d4* sodium salt (TSP) is added to the fecal water extract.

In order to obtain an overview of metabolites present in the sample, generally 1-dimensional NMR spectrum is acquired with a standard pulse sequence including water presaturation to suppress signals from water molecules (Beckonert et al., 2007). Identification of metabolites is achieved with comparison of chemical shifts to information available in chemical databases such as HMDB (Wishart et al., 2013), Chenomx NMR Suite (Chenomx, Canada), and literature. In addition, a series of 2D NMR spectra are needed for metabolite assignment (Everett, 2015). Furthermore, 2D experiments offer a much reduced signal overlap and therefore increase the number of elucidated metabolites in a sample. The tradeoff here is the need for increased acquisition time and need for additional considerations towards quantification of the signal intensities (Giraudeau, 2014), while spectra from 1D experiments can be directly submitted to multivariate statistical analysis for non-targeted analysis (Trygg et al., 2007) or integration of selected resonances can be used for targeted analysis (Lamichhane et al., 2014; Michail et al., 2015).

The influences on the fecal metabolome are manifold. Most investigations have been carried out in rodent models aiming to characterize and quantify the contribution of age (Calvani et al., 2014; Saric et al., 2008), drug administration (Coen et al., 2012; Romick-Rosendale et al., 2009) and the metabolic description of different diseases (Bezabeh et al., 2009b; Trezzi et al., 2015). Research in humans is up to date limited to different diseases such as cancer (Bezabeh et al., 2009a; Monleon et al., 2009) celiac disease (Di Cagno et al., 2011), non-alcoholic fatty liver disease (Michail et al., 2015), ulcerative colitis and Crohn’s disease (Bjerrum et al., 2015; Le Gall et al., 2011) and to the characterization of dietary effects on the fecal metabolome. Food items, *e.g.* cheese and milk, (Zheng et al., 2015), and specific dietary components (*e.g.* polydextrose fibre (Lamichhane et al., 2014), dietary glucosinulates (Combourieu et al., 2001), food-associated carcinogens (Vanhaecke et al., 2006), fructans (Dewulf et al., 2013) and grape juice extract (Jacobs et al., 2008)) generate their imprint on the metabolite composition of feces, and can be used for food biomarker detection, thereby supporting discussion of beneficial or adverse effects on the host. Dietary influences might also play a strong role when looking at the microbiota composition of humans and primates in regards to type of residence (Claesson et al., 2012), geographical origin (Yatsunenko et al., 2012), and seasonal differences (Gomez et al., 2016). Within the gastrointestinal tract, microbiota compositional differences were observed between mucus and lumen (Turnbaugh et al., 2009), and longitudinally from cecum to colon and feces (Eckburg et al., 2005). Lavelle *et al.* and Zhang *et al.* reported spatial variation of distinct bacteria in different regions of the colon (Lavelle et al., 2015) and distal gut including ileum (Zhang et al., 2014), while a large part of the detected variation was due to different bacterial assembly between the volunteers. We have reported spatial variation of gut luminal metabolites in mice that directly link to microbial activity such as digestion of carbohydrates and fermentation of protein in the cecum and re-absorption processes in the colon (Heinzmann and Schmitt-Kopplin, 2015). These metabolites were also detected in gut tissues (Martin et al., 2009). Such metabolic variation is affected by the microbiota but might also directly promote the feedback for growth of selected bacteria along the gut and trigger signaling pathways for the microbiome and the host (Delzenne et al., 2011).

Comparing urine from different animals and human, the overall profile looks quite distinct with many metabolites not occurring in the other species. End-products in feces are largely comparable: SCFAs as products of indigestible carbohydrate fermentation, amino acids, fermentation products of proteins and amino acids (*e.g.* 5-aminovalerate, phenol, cadaverine, putrescine), fermentation products from lipid oxidation such as branched-chain fatty acids, simple carbohydrates, choline metabolites and ethanol. However, comparing the overall quantities of different metabolites, distinct differences are noticeable (Fig. 4).

Concentration of SCFAs and amino acids are different between human and mice (Figs. 4A and 4B). Furthermore, the human fecal profile shows higher glucose levels, a greater variation of different sugars and larger quantities of choline metabolites. Citric acid cycle metabolites, fumarate and malic acid, were also only seen in human profiles. Food putrefaction products, *e.g.* from digestion of polyphenols as evidenced by the appearance of 3-hydroyphenylpropionate (mHPP), are highly diet-dependent (Rechner et al., 2004) and only observed in humans (Figs. 3C and 3D). On the other hand, mice excrete with feces more bile acids and apparently more macromolecules (putatively assigned to lipids and proteins) suggesting a less complete fermentation of host-indigestible material compared to humans. It is especially the concentration of SCFAs and amino acids that allow a link between the abundance and functional properties of bacteria in the gut (Shoaie et al., 2015). Therefore, the translatability of rodent model research to human health and disease is limited. Even though animal models offer powerful tools to investigate principles of metabolic pathways and interplay between host and microbiota, it is of utmost importance to turn to animal models having closer proximity to humans in terms of gut physiology, immunology and nutrition. The pig has been proposed as an excellent non-primate model, especially in terms of its gut physiology and omnivorous diet (Merrifield et al., 2011; Miller and Ullrey, 1987). Furthermore, taking into account the size of the animal, it offers the possibility to sample larger biofluid and tissue quantities and allows invasive investigation of the gut lumen in order to trace complex interactions between host and microbiota regarding nutritional interventions and disease etiology, while limiting the large variation and exogenous influences generally observed in humans.

**Variability of human gut microbiota and metabolic phenotyping**

The biological complexity of a human organism is enormous considering the immense amount of molecules that perform their functions inside and outside cells (Naylor and Chen, 2010; Zhang et al., 2015a). In addition, approximately 70% of the cells represent various symbiotic bacteria inhabiting primarily our gut. This fact emphasizes the importance of human individuality in the context of developing personalized treatment that would focus on predictive and preventive medicine rather than symptomology. This development is becoming possible due to the progress in systems biology tools and technologies.

Considerable attention is paid to connect human gut microbiota composition as well as its dynamics to the host metabolism because of a constant interplay between them (Dorrestein et al., 2014). Recent studies (Lagkouvardos et al., 2015; Le Gall et al., 2011) showed the existence of individual-specific patterns present both in gut microbiota and metabolome that can be relatively stable over time indicating the importance of understanding the afore-mentioned interaction within a subject.

Although there is possible evidence towards the existence of a common core of microorganisms in the gut, the inter-individual differences seem to be much larger than intra-individual suggesting functional redundancy of the ecosystem (Faith et al., 2013; Jalanka-Tuovinen et al., 2011; Martinez et al., 2013b; Wu et al., 2011). The diversity between subjects and the stability within them can be explained by many reasons such as heritability, genetics, diet, lifestyle, environment, the resilience of microbial community (Lozupone et al., 2012). The chosen taxonomic scale influences as well the results of temporal stability that decreases from the phylum level to the species level. Additional difficulty arises due to the connection of the composition of the gut microbiota to the age of the host when the most stable states are observed in adulthood (O'Toole and Claesson, 2010). It is worth noting that longitudinal stability of the gut ecosystem can be seen not only on compositional level but also on genomic level. This implies that phylogenetically unrelated taxa can have similar functions, underlying that host physiological states can be associated to functional components rather than compositional (Franzosa et al., 2015; Schloissnig et al., 2013).

Estimation of different sources of variation in metabolomics studies showed that special attention should be taken when planning the experiments with longitudinal or crossover study design (Nicholson et al., 2011; Sampson et al., 2013). NMR-based non-targeted metabolomics experiments on urine human samples showed a clear clustering of data points belonging to same subjects (Assfalg et al., 2008; Bernini et al., 2009; Heinzmann et al., 2012). It should be pointed out that in the work of Assfalg *et al.* the sampling was carried out over a period of 3 months with only 12 metabolites sufficient for donor identification. Bernini *et al.* reported the up to 3-year relative stability of human urine metabolome even when sudden or monotonous changes in some of the metabolites apparently affected by gut microbiota were observed. A mass spectrometric study investigating blood samples revealed persistence of individual metabolic phenotypes even 7 years after initial sampling. Highly conserved metabolites were mainly responsible for this behavior (Yousri et al., 2014). Although long-term stability of these metabolites was significantly correlated with heritability, specific traits could be addressed to other reasons such as some of the metabolites belonging to androsterone pathway that can be explained by gender. Clustering of data points corresponding to distinct individuals were observed as well in metabolomics studies involving fecal samples (Jacobs et al., 2008) but, comparing with microbial data, the latter showed better separation of subjects (Lagkouvardos et al., 2015; Le Gall et al., 2011).

Additional information that could support the existence of a stable composition of gut microbiota and metabolic phenotype within individuals are the results obtained from our current study which aim is to investigate the impact of whole grain diet on gut microbial ecology and metabolome. The cross-over study design lasted for 14 weeks (Martinez et al., 2013a) and included 4 time points corresponding to different diets. Fecal and plasma samples were collected at the baseline and at the end of each treatment period. In addition to microbial analysis described in the afore-mentioned publication, fecal and plasma samples were investigated through non-targeted metabolomics analysis via DI FT-ICR/MS (Forcisi et al., 2015; Walker et al., 2014b). Acquired spectra were aligned and resulting data matrices were subjected to multivariate statistical analysis. Primary results obtained by hierarchical cluster analysis of the data corresponding to operational taxonomic unit counts and to fecal metabolome showed that the leaves related to the same individuals tend to cluster together (Fig. 5). Such a picture was not observed for the plasma samples as a primary outcome, suggesting for the necessity of a deeper analysis. Revealing connections between gut microbiota composition and metabolic phenotype within individuals could help to come closer to understand intrinsic processes that lead to similar functionality. Inspired by our findings, we are currently focused on searching the connections between the individual microbiota and the corresponding metabolic phenotype.

The variability and the complexity of a human organism result in different responses at both the individual and population level whereas current advances in systems biology could help to overcome these difficulties leading personalized treatment development (Naylor and Chen, 2010). This can be considered as a key goal of a modern medicine since everyone responds individually to same therapeutic intervention. Examination of gut microbiota composition in association with metabolic phenotyping could serve as a fingerprint of a current condition of a host in order to impact prevention, diagnosis and treatment of a disease (Holmes et al., 2008).

**Outlook**

Genomic era was marked with a discovery that every human can be uniquely identified by the information coded in the genome. With a rapid development in technologies, especially in the omics research, it has become possible to show that this uniqueness can be observed (in a less robust way comparing to genome) on the level of human gut microbiota composition and human metabolome. However, as a population, most of us respond similarly to certain stimulus implying that there are stable functional and dynamical patterns, and their disturbance can lead to a disease. Perhaps, at some part research should pay attention to the less variable part of signals in order to reveal potential biomarkers of a disease, although personalized approach would be more appropriate. Since the “extended” genome interacts with the host, its metabolism should be studied in a complex manner by combination and integration of several methods and techniques in order to reveal crucial interactions in the system and come closer to its understanding. Metabolomics has a great potential in studying the functions in complex systems and expanding knowledge on gut microbial metabolism. Some examples regarding IBD related research are shown in this review. Current state of art in metabolomics allows detection of thousands of metabolites in a sample and analytical techniques constantly develop. In order to study the gut microbiota, special attention is paid to fecal samples to model the functions of the microbial community and the impact on host due to alternations in its composition. Combining several analytical platforms used in metabolomics is crucial for this task since the maximal information regarding the properties of detected metabolites has to be retrieved. The identified compounds can then be projected onto biochemical pathways to reveal and explain intersections between host and microbial metabolism. Although understanding the human complexity is an extremely attractive perspective, more invasive research has to be carried out using animal models that are physiologically translatable to humans. Therefore, it is highly important to consider animal models that can have certain level of similarity to human organism and allow tracking synergetic interactions within the host. Overall, studying gut microbiota has been becoming increasingly appealing field of research since it significantly affects our health status through its composition that depends on genetic or environmental factors (Fig. 6). Metabolomics represents one of the approaches shown to be effective in this investigation and common challenges of metabolome analysis were described in this review.

**Table 1**. A summary of metabolites that were reported to be involved in inflammatory bowel disease (categorized according to Crohn‘s disease or ulcerative colitis form)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **metabolites associated with Crohn's disease** | | | | |
| **study and**  **the instrument used** | **increased in the control group** | **HMDB**  **entry** | **decreased in control the group** | **HMDB**  **entry** |
|
| NMR  (Marchesi et al., 2007) | alanine | HMDB00161 | butyrate | HMDB00039 |
| isoleucine | HMDB00172 | acetate | HMDB00042 |
| leucine | HMDB00687 | methylamine | HMDB00164 |
| lysine | HMDB00182 | trimethylamine | HMDB00906 |
| valine | HMDB00883 |  |  |
| GC/MS  (Ahmed et al., 2013) | heptanal | HMDB31475 | butyrate | HMDB00039 |
| propanal | HMDB03366 | propionate | HMDB00237 |
| pentanal | HMDB31206 |  |  |
| 2-heptanone, 6-methyl | - |  |  |
| S-methyl 3-methylbutanethioate | HMDB39843 |  |  |
| 2-piperidinone | HMDB11749 |  |  |
| NMR  (Bjerrum et al., 2015) | isoleucine | HMDB00172 |  |  |
| leucine | HMDB00687 |  |  |
| valine | HMDB00883 |  |  |
| lysine | HMDB00182 |  |  |
| alanine | HMDB00161 |  |  |
| tyrosine | HMDB00158 |  |  |
| phenylalanine | HMDB00159 |  |  |
| glycine | HMDB00123 |  |  |
| FT-ICR/MS  (Jansson et al., 2009) | 2-carboxy-2,3-dihydro-5,6-dihydroxyindole | HMDB04067 | prostaglandin f2alpha | HMDB01139 |
| 4-hydroxyphenyl-acetylglycine | HMDB00735 | 2,3-dinor-8-iso-prostaglandin F2alpha | HMDB00735 |
| (Z)-4-hydroxyphenylacetaldehyde oxime | - | prostaglandin F1-alpha | HMDB02685 |
| tyrosine | HMDB00158 | prostaglandin E2-alpha | HMDB01220 |
| tryptophan | HMDB00929 |  |  |
| phenylalanine | HMDB00159 |  |  |
| glycocholic acid | HMDB00138 |  |  |
| taurocholic acid | HMDB00036 |  |  |
| cholic acid | HMDB00619 |  |  |
| glycochenodeoxycholic acid | HMDB00637 |  |  |
| oleic acid | HMDB00207 |  |  |
| stearic acid | HMDB00827 |  |  |
| palmitic acid | HMDB00220 |  |  |
| arachidonic acid | HMDB01043 |  |  |
| linoleic acid | HMDB00673 |  |  |
| linolenic acid | HMDB01388 |  |  |
| hydroxyphenyllactic acid | HMDB00755 |  |  |
| FT-ICR/MS  (Lee T, 2016 accepted) | C18:0 | HMDB00827 | pyridoxate | HMDB00017 |
| LPA(dm16:0e) | HMDB11154 | methylcytidine | HMDB00982 |
| LPA(16:0e) | HMDB11144 | C15:0 | HMDB00826 |
| ceramide phosphate | - | C16:2 | HMDB00477 |
|  |  | methylcitrate | HMDB00379 |
|  |  | retinylglucuronide | HMDB10340 |
|  |  | gamma-Glu-Gln | HMDB11738 |
|  |  | Gln-Pro | HMDB28805 |
|  |  | Asn-Hpro | HMDB28732 |
| **metabolites associated with ulcerative colitis** | | | | |
| NMR  (Marchesi et al., 2007) | glutamate | HMDB00148 | butyrate | HMDB00039 |
| lysine | HMDB00182 | methylamine | HMDB00164 |
|  |  | trimethylamine | HMDB00906 |
| GC/MS  (Ahmed et al., 2013) | 1-propanol, 2 methyl- | HMDB06006 |  |  |
| undecane | HMDB31445 |  |  |
| methoxy-phenyl-oxime | - |  |  |
| NMR  (Bjerrum et al., 2015) | isoleucine | HMDB00172 | butyrate | HMDB00039 |
| leucine | HMDB00687 | propionic acid | HMDB00237 |
| valine | HMDB00883 |  |  |
| lysine | HMDB00182 |  |  |
| alanine | HMDB00161 |  |  |
| lactic acid | HMDB00190 |  |  |
| FT-ICR-MS  (Jansson et al., 2009) | deoxycholic acid | HMDB00626 | tryptophan | HMDB00929 |
| oleic acid | HMDB00207 | phenylalanine | HMDB00159 |
| linoleic acid | HMDB00673 | hydroxyphenyllactic acid | HMDB00755 |
| aminosalicylic acid | HMDB14378 |  |  |
| salicyluric acid | HMDB00840 |  |  |
| NMR  (Le Gall et al., 2011) | taurine | HMDB00251 | 2-methylbutyrate | HMDB02176 |
| cadaverine | HMDB02322 |  |  |
| choline | HMDB00097 |  |  |
| glucose | HMDB00122 |  |  |
| FT-ICR/MS  (Lee T, 2016 accepted) |  |  | cervonoyl ethanolamide | HMDB13627 |
|  |  | tridecanoate | HMDB00910 |
|  |  | oxotetradecanoate | HMDB10730 |
|  |  | (NH-CH2-S)-sulfanyloctanoate | HMDB13639 |
|  |  | undecanedionate | HMDB00888 |
|  |  | sebacate | HMDB00792 |
|  |  | dodecanedioate | HMDB00623 |
|  |  | LysoPE(16:0) | HMDB11503 |
|  |  | LysoPE(15:0) | HMDB11502 |
|  |  | LysoPE(14:0) | HMDB11500 |
|  |  | carboxytocopherol | HMDB12798 |
|  |  | (CH3-CO-NH)-5-methoxykynurenamine | HMDB04259 |
|  |  | Tyr-HPro | HMDB29106 |
| all amino acids are assumed to be L amino acids | | | | |

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**Figure legends**

**Fig. 1**. A meta-omics overview on studying functional properties of human gut microbiota. A) Human gut microbiota owns its own genome, transcriptome, proteome and metabolome that can be investigated independently or in combination with other omics disciplines. B) Existing fields of metagenomics, metatranscriptomics, metaproteomics and metabolomics, with some typical representatives. C) Emerging of meta-omics field is expressed through the number of published literature for each year starting from the year 2000. D) Fecal metabolomics has been dealing with topics related to microbiota, disease, health, and diet, outlined by the number of publications found for each topic. The disease related issues are further represented by eight different disease categories.

**Fig. 2**. Metabolome analysis workflow from sample collection to biological interpretation

**Fig. 3**. Unraveling metabolite structure by analyzing a mass difference network where structural information on metabolites is coded via fusion of data obtained by DI FT-ICR/MS and LC/MS. The network can include additional information coming from databases or statistical analysis.

**Fig. 4**. Overview of 1D-1H metabolite profiles and 2D-1H-13C-HSQC metabolite profiles of aqueous fecal extracts from mouse (A, C) and human (B, D) origin. (A, B) 1D NMR overview of metabolites such as SCFAs for quantitative comparison between species with higher abundance of SCFAs and many amino acids in the human sample. Spectra normalization (total area and probabilistic quotient normalization) retrieved congruent results. (C, D) 2D NMR analysis reduces overlap of metabolites and depicts presence/absence of differences between mouse and human fecal profiles, *e.g.* mHPP in human feces and the high abundance of macromolecules in mouse feces. Abbreviations: but=butyrate, prop=propionate, mHPP=3-hydroxyphenylpropionate, DMA=dimethylamine, BCAA=branched-chain amino acids, u.a. unassigned, Leu=leucine, Val=valine, Ile=isoleucine, C5/C6-FA=C5/C6-fatty acids, TMA=trimethylamine, Thr=threonine, \*putatively assigned to macromolecular background from lipids and proteins.

**Fig. 5**. Results obtained by applying hierarchical cluster analysis using Euclidean distance metric and Ward’s method to datasets corresponding to A) fecal microbiota; B) fecal metabolome.

**Fig. 6**. Various factors, influencing the host through the commensal gut microbiota, can lead to either healthy status or a disease.