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Metabolomics of fecal samples: a practical consideration

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ABSTRACT

Background

Metabolic profiling is becoming increasingly popular to identify subtle metabolic variations induced by diet alterations and to characterize the metabolic impact of variations of the gut microbiota. In this context, fecal samples, that contain unabsorbed metabolites, offer a direct access to the outcome of diet - gut microbiota metabolic interactions. Hence, they are a useful addition to measure the ensemble of endogenous and microbial metabolites, also referred to as the hyperbolome.

Scope and Approach

Many reviews have focused on the metabolomics analysis of urine, plasma and tissue biopsies; yet the analysis of fecal samples presents some challenges that have received little attention. We propose here a short review of current practices and some practical considerations when analyzing fecal material using metabolic profiling of small polar molecules and lipidomics.

Key Findings and Conclusions

To allow for a complete coverage of the fecal metabolome, it is recommended to use a combination of analytical techniques that will measure both hydrophilic and hydrophobic metabolites. A clear set of guidelines to collect, prepare and analyse fecal material is urgently needed.

Highlights

1. Untargeted metabolic profiling of fecal material is robustly achieved using NMR-based metabolomics
2. Mass spectrometry is mostly used for targeted metabolic profiling of a class of molecules for deep coverage and high sensitivity
3. Lipidomics profiles are extremely complex as they contain a mixture of endogenous, diet-related and microbial lipids that may be of interest for bacterial identification

INTRODUCTION

The gut microbiota is a highly metabolically active community of micro-organisms inhabiting all niches along the intestine, that is now recognized as a critical regulator of its host homeostasis.(O'Hara & Shanahan, 2006) It has been estimated that the gut microbiota as a whole contains 100 times more genes than human cells, hence its potential to be a key metabolic player. It is therefore expected that modifying the gut microbial balance would induce a shift in the gut metabolic environment that can in turn affect our own metabolism.

The gut microbiota composition varies considerably over a lifetime.(Yatsunenko et al., 2012) It takes approximately two years to a newborn to acquire a stable GM population(Palmer, Bik, DiGiulio, & Relman, 2007) that will evolve through life under the pressure of various factors such as, for instance, diet, lifestyle and exposure to antibiotics, all commonly referred to as the 'exposome'. (Claesson et al., 2011; Claus & Swann, 2013; Lozupone, Stombaugh, Gordon, & Jansson, 2012) Later in life, a loss of microbial diversity is generally observed with senescence. (Biagi, Candela, Fairweather-Tait, Franceschi, & Brigidi, 2011; Claesson et al., 2011) As recently demonstrated, even perturbations of the circadian cycle have been observed to affect the balance of the gut microbial community. (Mukherji, Kobiita, Ye, & Chambon, 2013; Voigt et al., 2014)

Diet is the main factor influencing gut microbiota composition since it provides microorganisms with their main organic carbon source.(Flint, Duncan, Scott, & Louis, 2015) This connection was recently further evidenced by a study demonstrating that a drastic change of diet such as switching from vegetarian to carnivorous and inversely can profoundly reorient the GM ecosystem in a very short period of time.(David et al., 2015) Thus, along genetic and other

environmental factors, diet strongly contributes to the unique character of every individual's gut microbiota.

Bacteria have a high metabolic activity that generates a wide range of products such as organic acids, alcohols and gas that may become available for the host or other commensal bacteria for cross-feeding. This symbiotic activity shapes the gut metabolic environment. Complex carbohydrates, that cannot be digested in the upper gastrointestinal track and are a major source of carbon for colonic bacteria.(Scott, Duncan, & Flint, 2008) Their fermentation results in the production of short chain fatty acids (SCFAs: acetate, propionate, butyrate and valerate) that play an important role in human health.(Besten, van Eunen, Groen, & Venema, 2013) Other food components such as lipids and proteins can largely impact the composition of the gut microbiota and its metabolic activity.(Sonnenburg & Sonnenburg, 2014) Endogenous secretions such as the bile acids contained in bile are important regulators of the gut microbiota. Reciprocally, gut bacteria are known to extensively alter the structure of sterols that leads to the formation of secondary and tertiary bile acids.(Sayin et al., 2013) This is an example of a major gut microbiota-host interplay that contributes to regulating the absorption of dietary lipids during digestion. Hence, the diet-gut microbiota interaction plays a key role in the metabolic homeostasis of its host. It is therefore of utmost importance to understand the biological mechanisms that underlie this complex relationship. Systems biology approaches that study a system as a whole (e.g. a micro-organism within a host, the interactions occurring within a community of bacteria etc.) are increasingly popular to decipher these interactions. In particular, metabolic profiling techniques are tremendously useful to understand the metabolic pathways

regulated through the host-gut microbiota interaction. A variety of sample types ranging from biofluids to tissue biopsies can be analyzed to capture the systemic metabolic response to the exposome. Of particular interest, feces are easily accessible and provide a non-invasive window to study the outcome of the diet-gut microbiota-host interaction through the analysis of remaining unabsorbed metabolites. Yet, the analysis of fecal samples for metabolic profiling has received little attention. In this review, we will explore the dominant technologies that are commonly applied to assess the fecal metabolome and discuss about practical aspects that must be considered when dealing with this material.

NMR-based metabolomics of fecal samples

Metabolic profiling, also referred to as metabolomics, is mostly achieved using two analytical platforms: nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) to evaluate the metabolic composition of a chosen biological matrix. These techniques allow the simultaneous measurement of a wide range of metabolites in a sample, and when combined, offer a large coverage of the metabolome (i.e. the set of metabolites in a sample).

Untargeted metabolic profiling by ^{1}H NMR spectroscopy measures all metabolites with nonexchangeable protons that are present in a sample in a relatively high concentration (in the micromolar range). Because it is highly reproducible, is cost effective and usually requires only a few simple preparation steps, NMR-based metabolic profiling has been widely applied to the analysis of virtually all biological matrices, including feces. (Li et al., 2011; Martin et al., 2010; Saric et al., 2008)

In humans, ^{1}H NMR-based metabolic profiling of fecal material has been successfully applied to assess the impact of the composition of the gut microbiota on the gut metabolic environment in the context of ulcerative colitis (UC) and irritable bowel syndrome (IBS).(Le Gall et al., 2011) A similar approach was applied to monitor the gut microbial metabolic activity in elderly (Claesson et al., 2012). In this study, it was possible to cluster patients according to their community setting (length of hospital care) based on fecal water profiling. A recent study also demonstrated the possibility of evaluating independent bacterial contributions at a species level to the gut metabolic environment using this technique. (Le Roy et al., 2015). Applied to the monitoring of probiotic consumption, it was possible to detect faecal metabolic modifications in response to increased *Bifidobacterium* in the colon (Ndagijimana et al., 2009*)*

In animal models, profound reorientation of the gut microbial community induced by antibiotics in mice was associated to modifications of fecal metabolic profiles measured by the same technique. This was mainly associated to a modification in the fecal content in amino acids and SCFAs.(Yap et al., 2008). Similarly, Romick-Rosendale et al., (Romick-Rosendale et al., 2009) also showed a modification of murine fecal metabolic profiles in response to antibiotic treatments. NMR-based metabonomics analysis of fecal water also proved to be able to differentiate age groups in mice. (Calvani et al., 2014) Finally NMR-based metabonomics can be applied to nutrition (also referred to as nutrimetabonomics) (Claus & Swann, 2013) to access modification of the gut metabolic environment in response to diet modulation. As an example, a study by De Filippis et al., (De Filippis et al., 2015) used NMR-based metabonomics to evaluate the impact of a Mediterranean diet on gut microbiota metabolic activity.

The study demonstrated that following a Mediterranean diet improved the detection of SCFAs in fecal waters compared with a western diet. Similarly, fecal metabolic modifications have been observed in response to food supplementation investigated in *in vitro* gut models. (Frost et al., 2014).

Since fecal samples contain a complex mixture of metabolites, most NMR-based metabolic profiling studies use a selective NOESY experiment with water presaturation applied during recycle delay and mixing time to detect signals caused by small molecular weight molecules as well as some lipids. This is often referred to as the NOESYPR1D experiment [RD-90º-t_1-90º-t_M-90º-ACQ], where RD is the recycle delay, t_1 a short interval of about 3 µs, t_M the mixing time of approximately 100 ms and ACQ the FID acquisition period. Interestingly, a number of studies investigating fecal samples have also used a CPMG (Carr-Purcell-Meiboom-Gill) experiment, (Bjerrum et al., 2014; Li et al., 2011) which uses t_2 filtering to reduce signal resonance from large macromolecules. (Meiboom & Gill, 1958) However, this should be considered with care since the number of loops and length of echo time that must be optimized for each CPMG experiment would determine the signal/noise ratio, therefore preventing absolute quantification. This is not an issue when only relative quantifications are needed.

Preparation of fecal material for metabolomics studies

Recently, Deda et al. reviewed sample preparation methods for fecal samples for metabolomic analysis.(Deda, Gika, Wilson, & Theodoridis, 2015) They provided a comprehensive overview of fecal sample preparation for NMR, GC-MS and LC-MS analysis including some critical aspects and specific requirement of the different technologies. Therefore, we will not cover sample preparation in details here but

a summary of the protocols and methods used in previously published research papers are presented in Table 1. However, it is noteworthy that this review highlighted the lack of consensus about sample preparation for both metabolic profiling technologies. For sample extraction, it seems that a dilution of 1 volume of feces material for 2 volumes of PBS buffer is most commonly used. The buffer is generally composed of a mixture of H_2O and D_2O (minimum 10 %) in various amounts, with an adjusted pH of 7.4 and an internal standard to serve as NMR reference. The most common internal reference is 3-(trimethylsilyl)-2,2,3,3-propionate-d4 (TSP). Deda et al. discuss that TSP signal intensity can be affected by pH but so far, the only alternative is 2,2-dimethyl-2-silapentane-5-sulfo- nate-d6 (DSS). However, unlike TSP, DSS has multiple small resonances in addition to the main resonance at 0 ppm that may interfere with other signals and therefore it should be used at a very low concentration (0.01% would be recommended). Homogenization of fecal material can be done directly in the NMR buffer that will be used for NMR analysis, minimizing the number of sample processing steps that may alter metabolic profiling. However, it also appears that a water/methanol extraction tends to improve the overall recovery of fecal metabolites. Nevertheless it was also argued in a publication by Jacobs et al., (Jacobs et al., 2007) that methanol extracts were less representative of the real metabolic composition of the fecal water encounter in the colon and therefore of the metabolite pool that interacts with the intestinal membrane.

Mass spectrometry-based metabolomics of fecal samples

Untargeted metabolic profiling using MS-based techniques is more sensitive than NMR (in the nanomolar range) but often generates a large amount of unknown signals and as a consequence, these techniques have been mostly used for

targeted metabolomics, where the method is optimized to the detection of a specific class of samples. MS-based metabolic profiling is usually achieved using either gas chromatography (GC-MS) or liquid chromatography (LC-MS).

GC-MS based metabolomics

The group of Sébédio presented two GC-MS methods to analyze the metabolome of fecal water. In their first study they used an ethyl chloroformate derivatization (Gao et al., 2009). In fecal water samples of healthy subjects 73 compounds were identified and thereof 34 validated by reference standards. The second study applied trimethylsilylation and identified 133 compounds (including amino acids, carbohydrates short and long chain fatty acids and phenolics) in human fecal water and the majority validated by authentic standards (Gao, Pujos-Guillot, & Sébédio, 2010). In both studies several extraction conditions were tested and the highest recovery of metabolites detected for neutral and basic pH which was confirmed also by others (Deda et al., 2015). However, it has been suggested that increasing pH from 6 to 7 may decrease the loss of volatile SCFA during lyophilization (Gao, Pujos-Guillot, & Sébédio, 2010).

Phua et al. presented a GC-TOF-MS analysis of feces after freeze drying followed by oximation and silylation (Phua, Koh, Cheah, Ho, & Chan, 2013). The authors argue that removal of a variable content of water increased the reproducibility of sample preparation. They identified 107 metabolites by matching with different mass spectra libraries. However, only a few analytes were confirmed by reference substances. This method was applied in detection of colorectal cancer (Phua et al., 2014). Main markers for CRC differentiation include decreased level of fructose, nicotinic acid and linoleic acid in CRC patients.

Using a similar methodology, Weir et al. analyzed the fecal metabolome in CRC patients (Weir et al., 2013). Metabolite identification was based on database matching. In agreement with Phua et al. they detected decreased linoleic acid in CRC patients compared to healthy controls. Moreover, reduced level of oleic and elaidic acids were found in CRC patients. In contrast to these fatty acids (FAs) myristic acid and several amino acids were increased in CRC patients. It is noteworthy that fatty acid identification does not differentiate double bond positions.

A very efficient approach to analyze volatile organic compounds (VOC) of feces is headspace solid-phase microextraction (SPME). Volatile metabolites are adsorbed to polymer coated fibers which are analyzed by GC-MS. Typically the analyte spectrum includes hydrocarbons, alcohols, aldehydes and organic acids (primarily short chain) and their esters. Dixon et al. tested different fibers to get a comprehensive coverage of VOCs (Dixon et al., 2011). Ahmed et al investigated fecal VOCs in patients with irritable bowel syndrome, active Crohn's disease, ulcerative colitis and healthy controls (Ahmed, Greenwood, de Lacy Costello, Ratcliffe, & Probert, 2013). They identified 240 metabolites which allowed a differentiation of patients with irritable bowel syndrome from patients with inflammatory bowel diseases and healthy controls.

LC-MS based metabolomics

In contrast to GC-based methods, LC-MS usually does not require metabolite derivatization but is restricted to analytes containing polar groups. Cao et al. used UPLC-MS/TOF-MS to analyze the fecal metabolome in patients with liver cirrhosis and hepatocellular carcinoma (HCC) (Cao et al., 2011). Fecal samples were homogenized, centrifuged and injected after filtration. Metabolic features

were analyzed by multivariate data analysis. Chenodeoxycholic acid, 7-ketolithocholic acid, urobilinogen, urobilin, lysophosphatidylcholine (LPC) 16:0 and 18:0 were found to discriminate between healthy controls and patients with liver cirrhosis and HCC. Whereas LPC species were found in increased levels, the other discriminatory markers were decreased in the patient samples. The identities of these markers were confirmed by comparison of chromatographic retention and product ion spectra with authentic standards.

A study by Jimenez-Girón investigated changes in the fecal metabolome related to the consumption of red wine (Jiménez-Girón et al., 2015). Feces samples were analyzed after mixing with saline solution, centrifugation and filtration by UHPLC-TOF-MS. Mass features were subjected to statistical analysis and 37 metabolites were found to be related to wine intake. Metabolite identification was performed by database searching and confirmation by authentic standards. This way 14 metabolites could be identified tentatively, 6 mass features match to standards (*m/z* and retention time).

The fecal metabolome of rats with chronic renal failure were analyzed by Zhao et al. (Zhao, Cheng, Wei, Bai, & Lin, 2012). Homogenized fecal samples were extracted with acetonitrile and analyzed by UPLC-Q-TOF-MS. Both polarities including fragment ions were recorded and used for identification and validation of mass features. Except an increase of adenine (used to induce kidney failure), 8 lipid metabolites were found decreased in rats with chronic renal failure.

An interesting approach to profile amine- and phenol-containing metabolites was presented recently by Su and colleagues (Su et al., 2015). Dried fecal samples are extracted sequentially with water and acetonitrile followed by derivatization with dansyl chloride. As an internal standard an aliquot of a

pooled fecal extract was added which was derivatized with ^{13}C-labelled dansyl chloride. Dansylation improves both LC separation efficiency and MS response of the compounds. 6200 peaks were detected in 237 different samples and 67 metabolites (mainly amino acids) were identified based on mass and retention time matching to a dansyl standard library.

In summary, both GC- and LC-based metabolomics studies are able to discover a number of differentially regulated metabolic features in fecal samples in various studies. However frequently, only a few of these features could be identified. Moreover, several studies did not prove the identity by authentic standards but only by matching of *m/z* values to database entries. In general, GC-MS based studies identify an increased number of metabolites which may partly reflect the superior chromatographic resolution and peak shape of GC compared to LC methods. This allows a more reliable extraction and comparison of metabolic features between different samples. Additionally, GC usually provides mass spectra generated by ionization-induced fragmentation that are useful for metabolite identification. A disadvantage of GC analyses is a more laborious sample preparation including the need of derivatization as a potential source of artifacts. Conversely, GC-MS clearly shows advantages compared to LC-MS in terms of deleterious matrix effects. So it is generally accepted that quantification of analytes by LC-MS requires internal standards, ideally stable isotope labeled for each analyte. Therefore a major source of errors of LC-MS metabolic profiling may be related to undiscovered matrix effects especially in heterogeneous sample material like feces. Consequently, in order to provide solid data metabolomics studies should validate their biomarkers by quantitative analysis

using authentic standards and internal standards especially when LC-MS is applied at least in a representative cohort.

Mass spectrometry-based targeted metabolic analysis

In contrast to untargeted analysis, targeted analysis is confined to a limited set of analytes. These methods are optimized for high analyte recoveries during sample preparation and a reproducible, accurate quantification of the target molecules. Most methods rely on internal standards (mandatory for LC-MS), calibration lines and method validation shows their performance.

SCFA

An excellent example for targeted analysis of potential biomarkers is the quantitation of short chain fatty acids (SCFA). A recent study by Han et al. presented a LC-MS/MS method for SCFA quantification in human feces (Han, Lin, Sequeira, & Borchers, 2014). SFCAs are converted to 3-nitrophenylhydrozones (3NHPH) which are separated by reversed phase chromatography and detected in negative ion mode. The method covers 10 straight- and branched chain SCFAs. In an elegant way, this study introduced an internal standard for every analyte by conversion of a standard mixture with a $^{13}C_6$-labeled derivatization reagent. This method showed a high reproducibility and analysis of human fecal samples revealed an increased fraction of branched-chain SCFA in T2D patient compared the other analyzed samples.

In contrast to LC-MS/MS analysis, GC-MS may rely on a fewer number of internal standards since matrix effects are less pronounced. A study by Zheng et al. quantified SCFAs and branched-chain amino acids (BCAAs) in feces and other biological materials using D_3-caproic acid as internal standard (Zheng et al., 2013) after propyl chloroformate derivatization.

Analysis of sterols and bile acids in faeces

Sterols and bile acids belong to another class of analytes studied for a long time in feces by targeted analysis. Cholesterol is an essential component of all mammalian cell membranes and it is the precursor of steroid hormones and bile acids. There has been a long interest in the intestinal metabolism of cholesterol because the gut microbiome is highly involved in the balance between absorption, excretion and metabolism. Between 34-57% of dietary cholesterol is absorbed from the human intestine (Grundy et al., 1977). Fecal excretion of total neutral sterols has been reported to range between 350-900 mg/day, of which 20% is cholesterol. There are several primary sources of fecal cholesterol: unabsorbed from the diet, bile and intestinal epithelium. It is well-known that the luminal cholesterol can be metabolized by the gut microbiota. This cholesterol escaping intestinal absorption is degraded to coprostanol through reduction of the double bond at C-5. (Figure 1) Coprostanone is also produced in a lesser extent. (Eyssen et al., 1974; Lichtenstein, 1990; Gerard, 2013). In human feces, cholesterol derivatives have been reported in the following proportions: coprostanol 65%, cholesterol 20%, coprostanone 10 %. (Figure 1) Other minor derivatives include cholestanone, cholestanol and epicoprostanol.

Many attempts have been made to isolate bacteria capable of reducing cholesterol to coprostanol from human and animal faeces (Snog-Kjaer et al., 1956; Crowther et al., 1973). Certain anaerobic bacteria from human faeces are known to hydrogenate cholesterol in vitro. Cholesterol reduction by common intestinal bacteria such as Bifidobacterium, Clostridium, and Bacteriodes has also been reported and reviewed extensively (Gerard, 2013). In addition, reference values have been generated for fecal excretion of cholesterol and

353 coprostanol (Benno et al., 2005) to differentiate between high-, low- and non-
354 converters.

355 Bile acids are derived from cholesterol and are produced by every class of
356 vertebrate animals and show substantial diversity across species (Hofmann et
357 al., 2010). In bile, bile acids rapidly form mixed micelles with secreted
358 cholesterol and phospholipids. Bile acids enter the intestine as di- and
359 trihydroxylated acyl conjugates, in mammals with the amino acids taurine and
360 glycine (Hofmann, 2009). In the intestinal lumen, conjugated bile acids directly
361 affect the microbiota because they exert antimicrobial properties besides
362 stimulating enterocytes to secrete undefined antimicrobial compounds
363 (Hofmann et al., 2006). Conversely, gut bacteria structurally alter bile acids
364 through deconjugation of taurine, glycine and sulfate moieties and hydroxylation
365 of the sterol backbone.

366 During digestion, bile acids facilitate lipid absorption by stabilizing lipid micelles.
367 Since bile acids have various degrees of hydrophobicity, and therefore various
368 stabilizing properties, the bile acid composition of the bile is an important factor
369 that regulates fat absorption. The enterohepatic cycle of bile acids is a key
370 regulator of hepatic bile acid *de novo* synthesis and cholesterol excretion. Indeed,
371 bile acids undergo extensive reabsorption by active and passive routes so that
372 95 % of secreted bile acids are reabsorbed daily. Since deconjugated bile acids
373 are less polar, passive diffusion is reduced and it has been shown that active
374 deconjugation by gut bacteria increases the overall excretion of bile acids in
375 feces, hence the role of bacteria in regulating fecal cholesterol loss (Claus et al.,
376 2011; 2008; Kellogg, Knight, & Wostmann, 1970; Sayin et al., 2013). In human, a
377 limited number of commensal bacteria are capable of removing the 7-hydroxyl

group from di- and trihydroxy bile acids and 7-deoxy species are formed. The most common 7-deoxy bile acids are lithocholic and deoxycholic acid. Many excellent reviews exist in the recent literature covering bile acid function, signaling and therapeutic potential (Ridlon et al., 2006; Hofmann et al., 2008; 2009; Trauner et al., 2010; Hagey et al., 2013).

Sample Preparation and Instrumental Strategies for Targeted Metabolomics Analysis

SCFAs are volatile and therefore freeze drying of stool samples may result in lower recovery. Han et al. used homogenized samples and extracted SCFAs by addition of 50% aqueous acetonitrile (Han, Lin, Sequeira, & Borchers, 2014). These extracts were subjected directly to derivatization with 3-nitrophenylhydrazine HCl. Zheng et al. used 0.005 M aqueous NaOH to homogenize fecal samples at 4°C to protect the volatile SCFAs (Zheng et al., 2013). The homogenates were derivatized with propyl chloroformate and extracted with hexane extraction for GC-MS analysis.

Extensive overviews of state-of–the-art methods to analyze fecal steroids can be found in the literature, e.g. (Story & Furumoto, 1991; Perwaiz et al., 2002; Griffiths & Sjövall, 2010) . The first and often referred methods for fecal steroid and bile acid analysis were a combination of thin-layer chromatography and gas-liquid chromatography published 1965 by Grundy, Miettinen and co-workers (Grundy et al., 1965; Miettinen et al., 1965). Sample pretreatment included homogenization, saponification and liquid/liquid extraction followed by thin-layer chromatography and trimethylsilylation. Individual components are then quantitatively measured by gas-liquid chromatography equipped with a flame ionization detector. Bile acids underwent a methylation step before thin-layer

chromatography. This approach was applied in many studies for the analysis of endogeneous and exogeneous (labelled) compounds.(Spritz et al., 1965; Grundy et al., 1969). Evrard and Janssen suggested a method for bile acid analysis which took advantage of a different extraction scheme and an alternate derivatization (Evrard & Janssen, 1968). The method is based on heating in presence of acetic acid and extraction with toluene. Quantification was done as methylketone derivatives. In the 1980s methods with subfraction steps have been proposed to isolate taurine and glycine conjugated and sulphated bile acids (Setchell et al., 1983; Owen et al., 1984) using GC analysis. Nowadays, the most common strategies to analyze sterol profiles including cholesterol, coprostanol and coprostanone in faeces are based on GC-MS. The sample preparation protocol includes repeated sampling for better representativeness, a dilution step, hydrolyzation of esterified sterols, extraction with a mixture of hexane and ethanol and derivatization (Lutjohann et al., 1993; Midtvedt et al., 1990; Andrasi et al., 2011). For example, Korpela et al. suggested a very detailed protocol which included a 72 h sampling of faeces, methanol-chloroform extraction, separation of free and esterified sterols with a Lipidex-5000 column, saponification, separation of hydroxylated and oxo-forms by a second column, and trimethylsilyl derivatization followed by GCMS (Korpela, 1982). LC-MS and MS/MS have also been widely exploited for bile acid analysis of human urine, plasma/serum, bile and also feces (Perwaiz et al., 2002; Hagio et al., 2009; Griffiths & Sjövall, 2010). Quantification is best performed by addition of isotope labeled internal standards. These should be added as early as possible in the analytical process so as to account for analyte loss during sample preparation.

Bile acids can usually be extracted from fluids and tissues with ethanol, methanol or acetonitrile. An ethanol fraction may be followed by extraction with a less polar solvent, such as chloroform, to recover less polar bile acid derivatives (e.g., the fatty acid esters) and bile acids remaining in the lipophilic residue (Griffiths et al., 2010). Batta et al. compared different methods to extract fecal bile acids and sterols (Batta et al., 1999) to suggest a simplified method of extraction. However, this is based on the assumption that fecal bile acids are unconjugated (Setchell et al., 1983), which is not a valid assumption for many clinical conditions, especially when subjects have been exposed to oral antibiotic treatments that have affected the gut microbial ecosystem.

Lipidomics

Parallel to metabolomics, lipidomics emerged during the past decade as a specialized discipline. Today virtually a full quantitative coverage of the lipidome is possible by mass spectrometric methods (Wenk, 2010).

In contrast to global metabolomics, lipidomic analysis mostly relies on lipid extracts prepared by extraction with apolar solvents like chloroform (Bligh & Dyer, 1959) or MTBE (Matyash, et al., 2008). The complexity of these extracts is greatly reduced compared to protein precipitates frequently applied for untargeted metabolomics as polar analytes are removed.

Up to now there are only a few studies of the fecal lipidome. Gregory et al. compared different extraction methods for lipidomics profiling by LC-HR-MS (high resolution MS) (Gregory et al., 2013). Stools of preterm infants were homogenized in water and lipophilic metabolites were extracted using either dichloromethane or a MTBE/hexafluoroisopropanol mixture. Additionally, the effect of pressure cycling on the extraction of lipid species was investigated.

Polar species like lyso-lipids showed a higher response after MTBE extraction whereas increased responses were observed after dichloromethane extraction for more hydrophobic species. The effect of pressure cycling does not show a consistent increase of the lipid species response for both extraction methods. Analysis by reversed phase chromatography couple to HR-MS was performed in both positive and negative ion modes. 304 lipid species were identified by unique monoisotopic m/z and retention time including 29 phosphatidylethanolamine (PE), 22 phosphatidylcholine (PC), 14 phosphatidylglycerol (PG), 88 triacylglycerol, 19 diacylglycerol and interestingly 50 ceramide species.

Recently, Davis and colleagues analyzed stools from mice fed a high fat or control diet with or without induction of colitis-associated tumors (Davies et al., 2014). Feces was homogenized by repeated freeze-thawing, minced to powder and extracted with a modified Bligh and Dyer method. Samples were quantified by shotgun lipidomics. However, mass spectra displayed very low intensities. The selection of dioleoyl species of PE, PS, PI as internal standards has to be considered as a potential source of error for quantification since these species may be present in feces (PE and PI 36:2 were detected by Gregory et al. (Gregory et al., 2013)). A general problem of this study and also a number of metabolomics studies is the annotation of lipid species. Davis et al. showed detailed annotations including even double bond positions of the fatty acyls. Such structural differences usually may not be resolved by standard lipidomic or metabolomics methods. Most methods determine the number of carbons and number of double bonds with the acyl chains. Therefore, it is recommended to annotate only structural details which are resolved by the analysis (Liebisch et

al., 2013). Moreover, common shorthand nomenclature provides a standard for reporting and searching of lipid species including deposition in and retrieval from databases.

Figure 2 presents the result of a lipidomic analysis of a fecal sample using a method that has been applied to a variety of sample types such as plasma (Sigruener et al., 2014), lipoproteins (Scherer, Böttcher, & Liebisch, 2011), cells (Leidl, Liebisch, Richter, & Schmitz, 2008), cell culture (Binder, Liebisch, Langmann, & Schmitz, 2006) and tissues (Hebel et al., 2015). Fecal samples were homogenized including bead-based grinding and subjected to liquid extraction according to Bligh and Dyer (Bligh & Dyer, 1959). Analysis by flow injection ESI-MS/MS using lipid class specific head group scans revealed huge differences between the individual samples. For example, we could find substantial concentrations of PG in some samples whereas other samples showed only minor PG content (Figure 2). Similar observations were made for other lipid classes. Additionally, the method of homogenization and extraction may greatly influence lipid species recovery. So sample preparation may determine whether analysis of lipids is confined to “easily accessible” lipids or includes also “hardly extractable” bacterial lipid. Bacterial lipids could be of particular interest since they are used as chemotaxonomic parameter to classify and identify bacterial (Busse, Denner, & Lubitz, 1996). However, bacterial lipids also increase the complexity of the fecal lipidome. So a number of additional fatty acids usually not or only present at low concentrations in mammalian cells are found in bacteria such as branched chain, cyclopropane and hydroxyl fatty acids. In summary, an accurate analysis of the fecal lipidome poses a great challenge especially due to its high complexity and high variability.

NMR has also been used for structural analyses and quantification of lipid species including lipoproteins (Bou Khalil *et al.*, 2010; Sander *et al.*, 2013; AlaKorpela *et al.*, 1996; Fernando *et al.*, 2010). ^{31}P-NMR is an attractive method to investigate phospholipid molecules due to the fact that all phospholipids have at least one phosphorous nucleus and this NMR active isotope occurs at a natural abundance of 100% and has got a high gyromagnetic ratio. Therefore, high resolution ^{31}P-NMR spectroscopy has successfully been employed to determine the phospholipid composition of tissues and body fluids. Comprehensive reviews with many applications can be found in the literature (Schiller & Arnold, 2002; Schiller *et al.*, 2007). However, we could not identify any study that applied NMR spectroscopy for lipid investigations in fecal samples.

Final considerations

It is noteworthy that although extremely useful and widely used, as illustrated by this review, fecal materials reliably reflect the microbial activity of the distal colon, which is moderately representative of the rest of the gastro-intestinal tract. For example, although it is commonly accepted that SCFAs measured in feces are an indicator of colonic production by gut bacteria, it is important to remember that these metabolites are quickly absorbed by the intestinal membrane and an increased detection in feces may also reflect a poorer absorption. To overcome this issue, metabolic profiling techniques can be applied on luminal content collected in various sections of the gastro-intestinal track, although this implies a more invasive sample collection. Fecal water profiling by NMR spectroscopy has been widely referenced for numerous animal models such as rodents(Romick-Rosendale et al., 2009) and horses(Escalona et al., 2014) as well as humans,(Jacobs et al., 2008) providing a database for future

530 investigations. This is also extremely useful for similar evaluations performed on
531 fecal waters derived from *in vitro* batch cultures that simulate digestion by the
532 gut microbiota. Even if *in vitro* models are not a perfect representation of the
533 host-gut microbiota interplay, they provide a valuable overview of the microbial
534 activity in the gut in controlled conditions. For instance, metabolic profiling of
535 samples derived from such *in vitro* gut models has been recently applied to
536 compare the impact of diet on human and baboon gut microbial activity.(Frost,
537 Walton, Swann, & Psichas, 2014)
538 Another important consideration when analyzing feces metabolome, and
539 particularly fecal bile acids, is the irregularity of bile secretion and the
540 inhomogeneity of fecal samples as carefully studied by Setchell et al., who
541 demonstrated that this was strongly correlated with diet patterns (Setchell et al.,
542 1987). This is particularly relevant to human studies since humans tend to have
543 a defined regular feeding pattern with set time and number of meals per day
544 (unlike rodents that tend to feed all night, and from time to time during the day).
545 As a consequence, it is recommended to analyze aliquots of thoroughly
546 homogenized 4–5 day collections of feces.
547 Finally, since one of the main factors influencing the gut metabolic environment
548 is the microbiome, it is important to assess the microbial composition of the fecal
549 material when possible, using 16S rRNA sequencing or metagenomics when
550 possible, although these methods are not fully quantitative. Such metabolic
551 associations with gut bacteria should also be interpreted carefully as it is not
552 always possible to differentiate the host from the bacterial metabolic activity.
553 This is particularly true for amino acids that can be released by dead host cells or
554 be derived from protein digestion by host and bacterial enzymes. Typical

bacterial metabolites include SCFAs, some organic acids such as formate and by-products of protein degradation such as indole. However, many fecal metabolites derive from host-bacterial co-metabolism, which is typically the case for secondary and tertiary bile acids.

To summarize, the measurement of the fecal metabolome is becoming increasingly popular as it provides an easy estimate of the diet-gut microbiota-host metabolic interaction. However, there is a need for establishing clear guidelines for fecal sample collection, preparation and analysis for metabolic profiling. Both NMR and MS-based metabolic profiling are complementary techniques and none of them to date is able to holistically assess the fecal metabolome. Instead, it is recommended that a combination of methods is used to extend the metabolic coverage.

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FIGURE CAPTIONS

Figure 1: Estimated proportion of fecal cholesterol derivatives in humans.

Figure 2: Neutral loss (NL) of 189 of lipid extracts prepared from fecal samples. Suspensions of human fecal samples in water/methanol (1/1) were subjected to bead-based homogenization. Homogenates were extracted according to Bligh and Dyer (Bligh & DYER, 1959). Crude lipid extracts were analyzed by direct flow injection analysis as described previously (Matyash et al., 2008). Displayed are NL 189 spectra, specific for phosphatidylglycerol (PG), of three different samples normalized to the highest intensity.

Table 1: Summary of published studies applying metabolomics to study fecal samples using NMR and MS platforms. Summary of protocols and main outcomes are included.

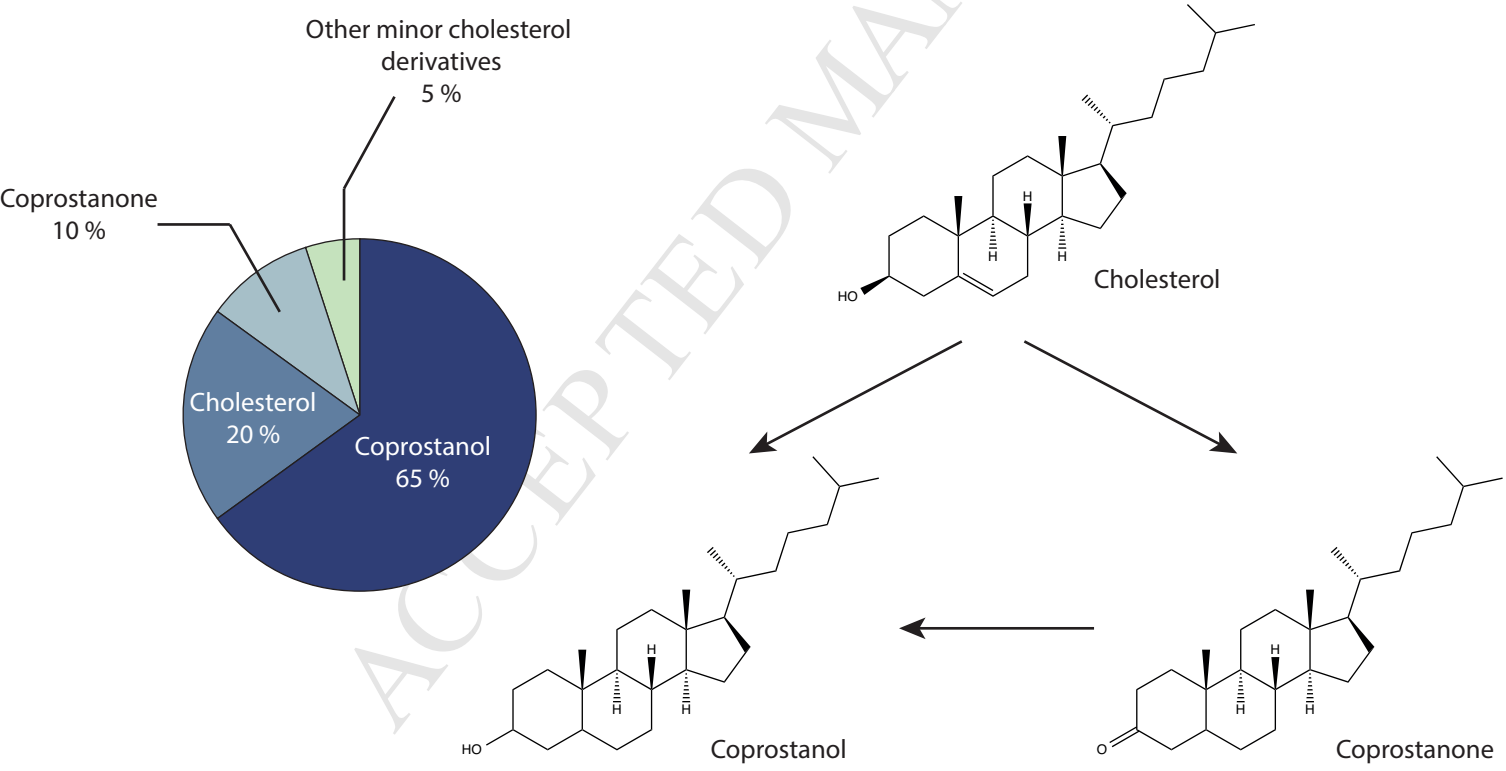
Key: GC-FID: Gas chromatography- Flame ionization detector; GC-MS: Gas chromatography-Mass spectrometry; LC-MS: Liquid chromatography-Mass spectrometry; UPLC-MS: Ultra-Performance Liquid Chromatography-Mass spectrometry

Analytical technique	Study aim	Sample preparation overview	Material / buffer	Measurement	Main results	References
NMR 400 MHz	Aging in mice	1:4 (wN/Av) feces in deuterated PBS + two filtration step	60 µL deuterated PBS containing 2 mM TSP in 600 µL of extract	TOCSY & HSQC	Aging induces ↑ 4-hydroxyphenylacetate, histidine, formate, succinate and ↓ α-ketoisocaproate, α-ketoisovalerate, -hydroxybutyrate, bile salts, isoleucine, methionine	Calvani et al., 2014
	Colorectal cancer human	1:3 (wN/Av) feces in deuterated PBS + vortexing and centrifugation	50 µL TSP (4 mM) in D2O added in 500 µL supernatant	N/A	N/A	Bezabeh et al. 2009
NMR 500 MHz	Experimental optimisation, mice	1:10 mg.µL−1 feces-to-buffer ratio, tissuelyser	N/A	COSY, TOCSY, HSQC, HMBC	Identification of 40 metabolites	Wu el al., 2010
NMR 600 MHz	Ulcetative colitis activity, humans	1:2 (WN/Av) feces to PBS, vortexing, cenrifugation and filtration	4 µL of D2ON/A500 µM TSP with 40 µL of fecal extract	CPMG	Active UC induces ↑ BCAAs, lysine, alanine, taurine	Bjerrum et al. 2014
	Antiobiotic treatment (gentamicine, ceftriaxone), mice	1:10 feces to PBS, freeze–thaw treatment, tissuelysr, centrifugation	30% D2O, 0.002% TSP, 0.03% of Na3N (wN/Av)	COSY, TOCSY, JRES, HSQC, HMBC	Antibiotic induces ↑ oligosacharides. phenolic acids and ↓ SCFAs, uracil, hypoxanthine	Zhao et al., 2013
	Age, rat	1:10 feces to PBS, vortexing, freeze–thaw, tissuelyser, centrifugation	0.1 M K2HPO4N/ANaH2PO4, pH = 7.4, containing 10% D2O, 0.58 mM TSP	COSY, TOCSY, HSQC, HMBC, DOSY	Aging induces ↓ arabinose, xylose, galactose, arabinoxylan, propionate and inosine and ↑ taurine, xylose, arabinose, galactose, arabinoxylans	Tian et al., 2012
	Infection *Schistosoma*	2 fecal pellets homogenized in	PBS containing 0.01% TSP	CPMG, COSY, TOCSY	Infection induces ↑ 5-Aminovalerate, SCFAs	Li et al., 2011

	mansoni, mice	700 µL PBS, sonication, centrifugation			(propionate)	
	IBS and UC, human	1:50 (wN/Av) feces to PBS, centrifugation, filtration	deuterated PBS containing 1 mM TSP	COSY, HSQC, HMBC	UC induces ↑ taurine and cadaverine, IBS induces ↑ bile acids and ↓ BCFAs	Le Gall et al., 2011
	ProN/Aprebiotics, mice	1:12 (wN/Av) mashed feces to PBS, centrifugation	60 µL DSS (5 mM) in D2O added in 600 µL supernatant	TOCSY,HMBC,HSQC	Prebiotic induces ↓ threonine, alanine, glutamate, glutamine, aspartate, lysine, lycine, butyrate, uracil, hypoxanthine and ↑ monosaccharides, glucose, trimethylamine. Pre and probiotic ↓ trimethylamine and ↑ acetate, butyrate, glutamine	Hong et al., 2010
	Colorectal cancer, human	1:2 feces to distilled water, homogenization, freezing, thawing, centrifugation	100 µL D2O to 500 µL fecal water	TOCSY	Cancer induces↓ acetate, butyrate	Monleon et al., 2009
	Grape juce and wine extract consumption, human	1:20 (wN/Av) feces to cold D2O or CD3OD, vortexing, centrifugation	D2O or CD3OD containing 1 mM TSP	CPMG	Grape juce consumption + wine induces ↓ isobutyrate	Jacobs et al., 2008
	Effect of Species, storage, lyophilization, sonication, filatrtiona nd homognisation	N/A	N/A	COSY, TOCSY, HSQC, HMBC	Storage: ↑ alanine, glutamate, threonine, aspartic acid, BCAAs, glucose. Lyophilization: ↑ BCAAs and ↓ succinate, SCFAs. Sonication: ↑ uracil, glucose and ↓ SCFAs	Saric et al., 2008
	UC, human	1:2 (wN/Av) feces to PBS, vortexing, filtration, centrifugation	200 µL buffer (10% D2O & 0.01% TSP) in 400 µL fecal water	COSY, TOCSY	UC induces ↓ acetate, butyrate, methylamine, TMA and ↑ isoleucine, leucine, lusine	Marchesi et al., 2007

NMR 700 MHz	*Lactobacillus*	1:2 (wN/Av) feces to detarated PBS, tissue lyser, centrifugation	Fecal water extracted in 9:1 D2ON/AH2O and 0.05 % TSP	Noesy	*L. helveticus* induces ↑ butyrate, lactate and incresed *Lactobacillus* level induces ↑ phenylalanine, tyrosine, lysine, lactate, propionate, valine, leucine, isoleucine, butyrate, acetate.	Le Roy et al., 2015
NMR 850 MHz	Baytrill treatment, mice	1:2 (wN/Av) feces to PBS, vortexing, centrifugation	200 µL buffer 10% D2O and 0.01% TSP in 400 µL fecal water	CPMG	Treament induces ↓ alanine, butyrate, isoleucine, leucine, propionate, threonine, valine and ↑ urea	Romick-Rosendale et al., 2009
GC-MS	Colorectal cancer human	oximation and silylation	lyophilized human feces	N/A	Cancer patients↓ butyrate, poly and monounsaturated fatty acids, ursodeoxycholic acid and ↑ acetate, amino acids	Weir et al., 2013
	VOCs irritable bowel syndrome, active Crohn's disease, ulcerative colitis	SPME	human feces	N/A	240 metabolites; esters of short chain fatty acids, cyclohexanecarboxylic acid associated with irritable bowel syndrome	Ahmed, Greenwood, de Lacy Costello, Ratcliffe, & Probert, 2013
	technical paper	trimethylsilylation	human fecal water	N/A	133 compounds structurally confirmed; 33 quantified	Gao, Pujos-Guillot, & Sébédio, 2010
	technical paper	ethyl chloroformate derivatization	human fecal water	N/A	73 compounds identified; 34 validated by reference standards	Gao et al., 2009
GC-TOF-MS	technical paper	oximation and silylation	lyophilized human feces	N/A	107 metabolites matched with mass spectra libraries, influence of blood on fecal metabolome	Phua, Koh, Cheah, Ho, & Chan, 2013
	Colorectal cancer human	oximation and silylation	lyophilized human feces	N/A	fecal metabolomic profiles of patients clearly differ from healthy subjects	Phua et al., 2014
GC-MS, GC-FID	VOCs, technical paper	SPME	human feces	N/A	evaluation of eight different commercially available SPME	Dixon et al., 2011

					fibers	
UPLC-MSN/ATOF-MS	liver cirrhosis, hepatocellular carcinoma	homogenization, centrifugation, filtration	human feces	N/A	Cancer patients ↓ chenodeoxycholic acid, 7-ketolithocholic acid, urobilinogen, urobilin and ↑ lysophosphatidylcholine (LPC) 16:0 and 18:0	Cao et al., 2011
UHPLC-TOF-MS	Effect of consumption of red wine	mixing with saline solution, centrifugation, filtration	human feces	N/A	37 metabolites related to wine intake	Jiménez-Girón et al., 2015
UPLC-Q-TOF-MS	chronic renal failure	homogenization, extraction with acetonitrile	rat feces	N/A	renal failure ↑ chenodeoxychrolic acid, palmitic acid, adenine, phytosphingosine, monoglycerol 24:1, 12-hydroxy-3-oxochola-dienic acid, lysophosphatidylethanolamine 18:2 and 16:0 and ↓ 7-ketolithocholic acid	Zhao, Cheng, Wei, Bai, & Lin, 2012
LC-MS, LC-UV	technical paper	Dried fecal samples extraction with water and acetonitrile, derivatization with dansyl chloride	human feces	N/A	67 metabolites (mainly amino acids) identified	Su et al., 2015



Minoor fecal cholesterol derivatives