Chapter 5
Can We Use Metabolomics to Understand Changes to Gut Microbiota Populations and Function? A Nutritional Perspective

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Abstract Food is an integral part of human life, and the composition of our diet is an important determinant of our health and well-being. Food is also the main source of energy and nutrients for the gut microbiota, the 100 trillion cells that coexist inside us. The impact of macronutrients (protein, fat, carbohydrates, and fiber) and specific non-nutrient food components (polyphenols) will be reviewed in the context of gut microbial function and interaction with the host. Colonic microbiota provides diverse enzymatic activities differing from our own, which lead to the production of metabolites essential for key metabolic functions, including carbohydrate and amino acid metabolism. Certain gut metabolites are specific to microbial activity and confer functionalities beyond energy production, such as signalling cascades across cells, tissues, and organs. Metabolomics has proven to be a useful tool to measure the effects of food on the gut microbiota and its interaction with host metabolism.

Keywords Nutrition • Gut • Metabolomics • Digestion • Phase II metabolism • Food • One carbon metabolism • Polyphenols • Fiber • Microbiota • Diet • Fat • Protein • Carbohydrate • Choline • Short-chain fatty acids • Phenolic and phenyl metabolites • Indole metabolites • Hippurate • *p*-Cresol sulfate • Trimethylamine oxide • Metabolism • Metabolomics • Colon • Intestine • Butyrate • Gut-liver • Gut-brain • Pathways • Chocolate • Whole-grain cereals • Carnitine • Branched-chain fatty acids • Prebiotics

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5.1 Introduction

The sum of all small molecules in a system (i.e. the metabolome) not only reflects the metabolic response of the subject of interest but also the organisms living in symbiosis with the subject – in the case of humans, the gut microbiota is an example. The gut microbiota produces thousands of metabolites through their reproduction, interaction with other microorganisms, the host and with partially digested food. Many of these metabolites are specific for microbial metabolism, and cannot be synthesized by mammalian enzymes. These specific microbial metabolites can be absorbed from the gut, adding to the diversity of the metabolome, and at the same time providing a window into the interaction between host, food and gut microbiota. In this chapter, we examine what dietary components are known to have an impact on gut microbial metabolism, which biochemical classes of gut metabolites are produced from different diets, and how metabolomics can be a powerful tool to measure the effect of food on the gut microbiota, and its interaction with mammalian metabolism.

5.2 Colonic Digestion

The large intestine is a digestive organ where dietary substrates not absorbed in the small intestine, are further broken down by anaerobic bacteria (Fig. 5.1). The major substrates for colonic fermentation include carbohydrates that have escaped digestion in the upper gut (mainly dietary fibers: resistant starch and non-starch polysaccharide such as celluloses, pectins and gums, and non-digestible oligosaccharides). The main products of carbohydrate fermentation are short-chain fatty acids (SCFAs), such as butyrate, propionate, and acetate, which are then absorbed and used as an energy source. In Western diets, SCFAs contribute less than 10 % to the total energy obtained from food, although in some cases this value can be up to 30 % [1]. Bacteria well adapted for fermenting carbohydrate come from the Prevotella and Xylanibacter genuses [2, 3].

In addition, residual amounts of protein (such as elastin, collagen and albumin), peptides and amino acids can also reach the colon. Proteolytic bacteria in human feces are predominantly Bacteroides and Propionibacterium, with lesser numbers of the genera Streptococcus, Clostridium, Bacillus and Staphylococcus [4]. Low levels of the amino acid fermentation products ammonia and branched-chain fatty acids (BCFA) are found in ileal contents indicating that little amino acid fermentation occurs in the small intestine, underlining the importance of the gut microbiota for producing these compounds. Protein fermentation leads not only to the production of BCFAs but also to relatively low amounts of a variety of products, such as branched-chain amino acids (BCAAs), phenols, and amines which are both absorbed into the host as well as excreted.

The proportion of carbohydrates to protein in the colon has been estimated as 3–4:1. Regional differences occur in the gut, where the right (proximal) gut has a

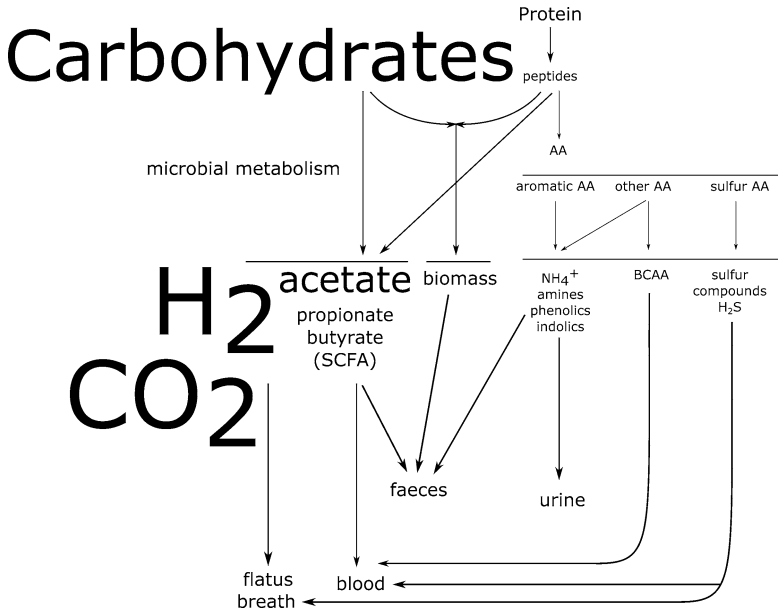

Fig. 5.1 Fermentation in the large bowel (size of compounds' font indicate approximate relative concentration)

higher saccharolytic activity while the left (distal) is more proteolytic. These fermentation processes provide the nutrients required for gut microbiota biomass growth while at the same time producing waste products hydrogen, carbon dioxide, ammonia, and methane which are excreted via flatus. More complex chemical structures such as polyphenols or alkaloids, mostly from plant foods, are also degraded during colonic fermentation [1].

Beyond its role in digestion and absorption, the large intestine contributes to health in a variety of ways: as a physical barrier preventing invasion of pathogenic bacteria and stimulating immune function and as a site for biosynthesis of vitamins and metabolism of xenobiotics.

5.3 Gut Modulation by Foods and Diet

5.3.1 How Do Different Foods Alter Gut Microbiota and Their Metabolism?

There is little controversy in the idea of using food or food ingredients to alter both gut microbiota populations and gut microbiota metabolism. Directly or indirectly, food is the main source of energy and nutrients for the gut microbiota and evolutionary pressure to adapt to the gastrointestinal environment and a major determinant of which

microbial genes are upregulated. Technological developments over the past two decades in the areas of genetic sequencing, to determine the gut microbiome from fecal samples without the need for culture techniques, have led to a rapid explosion of our understanding of the importance of the gut microbiota and how it changes with changing diet. An example of this are pre- and probiotics, where fermentable fiber sources or live bacterial cultures (often in dairy products), are given with the aim to positively alter the gut microbiota.

There is still discussion about what constitutes an "ideal" gut microbiota population, though favorable changes to gut microbiota are generally described towards bacterial genus or species that succeed when carbohydrate is the abundant energy source, while "negative" bacterial species are those that are well adapted to fermenting protein. Arguably, quantifying the population of different bacterial families or species provides little direct information about actual gut bacterial metabolism: many can switch between proteolytic and saccharolytic metabolism. It may be that the end products of microbial metabolism are able to help build the best possible picture of how gut microbiota are collectively responding to different diets or conditions. Some metabolites of dietary substrates are well known and are summarized in Table 5.1.

5.3.2 Microbial Metabolism and a Carbohydrate-Rich Diet

Carbohydrates are an important energy source for both humans and our gut microbiota. They are found in foods in several different forms, including monosaccharides (e.g., glucose), disaccharides (e.g., sucrose or lactose), starch, and a range of different types of dietary fiber, carbohydrates not broken down by human digestive enzymes, but are often fermentable by gut microbiota. While traditional diets are generally rich in complex carbohydrates (e.g., starch) and high in diverse forms of fiber, in "Western" pattern diets, simple sugars (e.g., glucose and sucrose) dominate the carbohydrate fraction of the diet, with low diversity in the small amount of fiber present [2]. It is likely that the difference between traditional and Western dietary patterns also leads to an impact on the gut microbiota and intestinal milieu leading to an increased risk of gastrointestinal disorders including large bowel cancer, gall stones, and Crohn's disease. Diet intervention studies high in refined sugar have found an altered gut metabolism, increased mouth-to-cecum transit time, and increased production of secondary bile acids [5].

One of the main sources of dietary fibers is cereal-based foods. Cereal grains that have the bran and germ fractions removed (refined or "white" flour) are also largely depleted in dietary fiber. Whole grains are cereal grains that still have all the three grain components in their correct proportions (bran, germ, and endosperm) and are generally rich in both insoluble and soluble fibers [6]. Soluble dietary fibers are by definition water soluble and tend to be highly fermentable by the gut microbiota, producing SCFAs. SCFAs appear to have a wide range of roles, including as an energy source and for reducing gut inflammation [2]. Diets high in fiber such as

Table 5.1 Common gut microbial metabolites of dietary substrates detected using metabolomics (urine, feces, blood). oxidation is typical of phase i metabolism and glycine, glutamine and sulfate conjugations are typical of phase ii metabolism occurring in the liver. glycine or glutamine conjugation is pathway- and species-specific (differences may occur between mice, rats and humans)

Microbial metabolite	Dietary precursors	Specific bacterial species	References
Phenolic, benzoyl, and phenyl derivatives	Phenolic compounds; flavonoids; protein (phenylalanine, tyrosine)	*Lactobacillus*	[34, 43, 44, 75–77]
Hippurate		*Clostridium difficile*	
Cinnamoyl (glycine)		*Clostridium scatologenes*	
		Proteus vulgaris	
Phenol (sulfate)		*E. coli*	[77]
		Clostridium bifermentans	
		Clostridium specticum	
		Bacteroids fragilis	
		Bifidobacterium longum	
p-Hydroxyphenylacetate		*Clostridium difficile*	[77]
		Bacteroides ovatus	
		Bifidobacterium bifidum	
		Bifidobacterium adolescentis	
		Bifidobacterium infantis	
		Bifidobacterium longum	
		Bifidobacterium pseudolongum	
p-Hydroxyphenylpropionate		*Clostridium bifermentans*	[77]
		Clostridium paraputrificum	
		Clostridium specticum	
		Bacteroides thetaiotaomicron	
		Bifidobacterium infantis	
p-Cresol (sulfate)		*Clostridium difficile*	[77]
		Clostridium paraputrificum	
		Clostridium perfringens	
		Bacteroids fragilis	
		Bacteroides thetaiotaomicron	
		Bifidobacterium bifidum	
		Bifidobacterium adolescentis	
		Bifidobacterium infantis	
		Bifidobacterium pseudolongum	
		Bacteroides thetaiotaomicron	

(continued)

Table 5.1 (continued)

Microbial metabolite	Dietary precursors	Specific bacterial species	References
Phenylacetate (glycine)		*Clostridium bifermentans*	[77]
		Clostridium difficile	
		Bacteroids fragilis	
		Bacteroides ovatus	
Phenylpropionate (glycine)		*Clostridium difficile*	[77]
		Peptostreptococcus asaccharolyticus	
		Bacteroides ovatus	
Phenyllactate (glycine)		*Clostridium perfringens*	[77]
		Bacteroides ovatus	
		Bifidobacterium longum	
Indole derivatives	Protein (tryptophan)	*E. coli*	[43, 44, 65, 78]
Tryptamine		*Clostridium bifermentans Proteus vulgaris*	
Serotonin		*Paracolobactrum coliforme*	
		Achromobacter liquefaciens	
		Bacteroides spp	
		Clostridia	
Indole (sulfate)		*E. coli*	[77]
		Bacteroides ovatus	
Indole pyruvate		*E. coli*	[77]
		Clostridium perfringens	
		Peptostreptococcus asaccharolyticus	
		Bacteroides ovatus	
		Bifidobacterium bifidum	
		Bifidobacterium adolescentis	
		Bifidobacterium infantis	
		Bifidobacterium pseudolongum	
Indole lactate		*E. coli*	[77]
		Clostridium perfringens	
		Peptostreptococcus asaccharolyticus	
		Bacteroides ovatus	
		Bacteroides thetaiotaomicron	
		Bifidobacterium bifidum	
		Bifidobacterium adolescentis	
		Bifidobacterium infantis	
		Bifidobacterium longum	
		Bifidobacterium pseudolongum	

(continued)

Table 5.1 (continued)

Microbial metabolite	Dietary precursors	Specific bacterial species	References
Indole-3-acetate (glutamine)		*E. coli*	[77]
		Clostridium difficile	
		Clostridium paraputrificum	
		Clostridium perfringens	
		Peptostreptococcus asaccharolyticus	
		Bacteroides fragilis	
		Bacteroides thetaiotaomicron	
		Bifidobacterium pseudolongum	
		Bifidobacterium longum	
Indole-3-propionate		*Clostridium paraputrificum*	[77]
		Peptostreptococcus asaccharolyticus	
		Bacteroides fragilis	
		Bifidobacterium longum	
		Bifidobacterium bifidum	
		Bifidobacterium adolescentis	
		Bifidobacterium infantis	
		Bifidobacterium pseudolongum	
Choline metabolites	Choline	*Bacteroides fragilis*	[15, 79]
Methylamine	Carnitine	*Clostridium perfringens*	
Dimethylamine		*Faecalibacterium prausnitzii*	
Trimethylamine (-*N*-oxide)			
Flavones		*Lactobacillus mucosae*	[43, 80]
Equol (sulfate)	Flavonoids	*Enterococcus faecium*	
Methyl equol (sulfate)		*Finegoldia magna*	
Short-chain fatty acids		*Bifidobacterium*	[81]
Acetate	Glucose and starch	*Propionibacterium*	
Propionate	Polysaccharides	*Lactobacilllus*	
Butyrate	Fiber	*Clostridium*	
Branched-chain fatty acids	Protein (branched-chain amino acids: leucine, isoleucine, valine)	*Bacteroides ruminicola*	[82]
Isobutyrate		*Megasphaera elsdenii*	
Isovalerate			

those rich in whole grains can alter gut microbiota populations [7–9] and gut microbiota metabolism [10, 11]. Of the few metabolomics studies that compared intake of whole grains with refined grains, one rat study found that urinary hippurate was increased on a whole grain diet, along with the aromatic amino acids phenylalanine, tryptophan, and tyrosine [12]. This finding was not replicated in human urine samples after a whole grain intervention, though other biomarkers of gut microbiota activity were decreased on a whole grain diet, including 4-hydroxyphenylacetate, a microbial metabolic product of aromatic amino acid metabolism and trimethylamine, a microbial metabolic product of choline and carnitine [11]. As in many areas of nutritional science, results on the impact of whole grains on gut microbiota are variable, with some studies not finding any changes to gut microbial species measured [13]. This variation in gut microbial response to an admittedly broad and heterogeneous food group may explain in part some of the variation in results between intervention studies, an area that will be further explored as more advanced microbial sequencing techniques become routine [9].

5.3.3 Fat-Rich Diet Interactions with Gut Microbiota

High-fat diets are frequently used in metabolic studies for testing diet-induced metabolic syndrome (increased risk of developing cardiovascular disease and diabetes), especially in rodent models. A direct relationship has been established between high-fat feeding and metabolic disorders, where altered gut flora is causal in inducing gut permeability, increasing lipopolysaccharide (LPS) absorption, and inflammation [14, 15]. Given this association between diet and gut microflora, specific strategies for modifying gut microbiota may be a useful means to reduce the impact of high-fat feeding on the occurrence of metabolic diseases. However, as these results mainly stem from rodent models, where high-fat diets represent a far greater proportion of energy intake than would normally be found among humans, caution is required until definitive clinical studies are performed.

5.3.4 Choline Metabolism: An Interaction Node Between Diet, Host, and Gut Microbiota?

Recent studies from a cohort of non-Caucasians based in Cleveland, United States, have highlighted that gut microbial metabolism of specific dietary components may result in toxic metabolites that lead to cardiovascular disease. Using LC-MS metabolomics, Wang et al. found that high plasma concentrations of a microbial metabolite of choline, trimethylamine (TMA), was related to cardiovascular disease risk, concluding that whether the gut microbiota converted choline into TMA or not was a key modifiable risk factor for development of cardiovascular disease [16]. The active molecule mediating increased disease risk was identified as trimethylamine oxide

(TMAO), a toxic metabolite of liver metabolism of TMA. Choline, and its related metabolite, TMA, and betaine (a downstream mammalian metabolite of choline) were related to cardiovascular disease in this cohort. These metabolomics results were cross validated, and biomarkers confirmed in mouse models of cardiovascular disease, though do not fully explain other findings with the same biomarkers. For example, a comprehensive study on the sources of TMA in humans found that very little choline was converted into TMA [17]. While this could be explained by differences in gut microbiota, the intake of fish and shellfish led to extremely high production of TMA [17], with some fish species leading to an excretion of over 4,000 μmol of TMA and TMAO in urine over 8 h. Meat, eggs, and dairy products conversely did not lead to more TMA and TMAO excretion compared to a control diet [17]. If these results in urine are reflected in plasma, any increase in TMA due to nonoptimal gut microbiota metabolism of choline from fatty foods would be "drowned" out by that due to fish intake. Thus, TMAO being a biomarker for cardiovascular disease risk would be at odds with a high intake of fish being associated with a decrease in cardiovascular disease risk [18–20], which would suggest that TMAO is not a good biomarker of cardiovascular disease in populations where fish intake is common. Similarly, the finding that elevated betaine may be associated with cardiovascular disease risk goes against other work finding that betaine is substantially associated with a decreased risk of cardiovascular disease risk factors [21]. The same study however also found that plasma choline was associated with risk factors for cardiovascular disease [21]. Betaine is one of the main phytochemicals present in whole grain wheat [22], and fasting betaine concentrations can be increased on a whole grain-rich diet [8], and both oral choline and betaine lead to decreased circulating homocysteine [23, 24], a cholesterol-independent risk factor for cardiovascular disease. In the context of these other findings, it is possible that elevated choline and TMAO are biomarkers of cardiovascular disease risk in this population, if fish intake is low.

In a follow-up study using stable isotope-labelled phosphatidylcholine, the role of gut microbiota in the formation of TMAO from choline was clearly established, along with choline being the main source of circulating betaine [25]. So in this population, elevated betaine probably comes from a high intake of choline, rather than a high intake of betaine-containing foods. The complementary analysis of food records and use of dietary biomarkers of intake (e.g., alkylresorcinols for whole grains [26] or omega-3 fatty acids for fish intake [27, 28]) along with gut microbiota measurements and metabolomics may be instructive for determining if elevated concentrations of these biomarkers are related to disease risk or diet.

A second study by the same group hypothesized that another TMA, L-carnitine, may also be a risk factor for cardiovascular disease as it can also be metabolized by gut microbiota to TMAO [29]. Carnitine, which is a major component of red meat and is conditionally essential for fatty acid transport for mitochondria, was found to lead to increased TMAO production that depended on gut microbiota. Of interest for metabolomic methodology is that in the initial screening of the same cohort where choline was suggested to be a risk factor for CVD [16], carnitine was only found to be a significant risk factor if correction for multiplicity was not used in the statistical analysis. While statistical considerations are important, the possibility

that associations of interest may be lost when using stringent tests should not be overlooked. A recent study comparing a whole grain diet to a refined grain diet found that urinary excretion of carnitine and acetylcarnitine was reduced when consuming whole grains, along with a decrease in the TMAO precursor TMA [11], in a study where gut microbial populations were also altered due to the whole grain intervention [8]. It is clear from this work on precursors of TMAO that while there may be several complexities in assigning biomarkers to disease risk that are also derived from diet, the one carbon metabolism pathway and phospholipid metabolism are likely key areas of interaction between diet, physiology, gut microbiota, and cardiovascular disease.

5.3.5 *Protein-Rich Diets and Gut Microbiota*

While human protein digestion and amino acid absorption are efficient, some proteins and free amino acids still reach the colon and are associated to increased production of potentially toxic substances such as volatile sulfur compounds, ammonia, and *p*-cresol [30, 31]. Experimental evidence from animal models and in vitro data shows that dietary proteins can influence cancer expression. Increased dietary protein consumption can cause increased colonic DNA damage and thinning of the colonic mucosal barrier [32]. Production of microbial metabolites from amino acids can be reduced by dietary fiber (via increasing the proportion of carbohydrate reaching the colon), as carbohydrate appears to be a preferred substrate for most gut microbiota species [32].

The molar ratios of the BCFAs isovalerate and isobutyrate, compounds resulting from the bacterial fermentation of valine and leucine, were found to be increased relative to total fecal SCFAs with high-protein diets. A marked increase in fecal nitrogenous organic compounds (NOC) amounts was also found when subjects consumed high-protein diets. NOCs are carcinogens in vitro; although the toxicological significance of increased fecal NOCs is uncertain, NOCs, at concentrations present in the colonic lumen, contribute to DNA damage in the colon and rectum and possibly to increased risk of human cancer [33]. Broadly speaking, much evidence suggests that interaction between protein and amino acids is negative for the host, though secondary roles of these metabolites on gene signalling and immune function have not been researched.

5.3.6 *Interaction of a Polyphenol-Rich Diet with the Gut Microbiota*

While not a nutrient in the strict sense, polyphenols, or at least polyphenol-rich foods may also lead to a change in gut microbiota metabolism, notable examples being coffee and chocolate [34]. This metabolic interaction may lead to many

downstream effects and it has even been suggested that there is a link between preference for chocolate and gut microbiota, depending on how cocoa polyphenols are metabolized [35]. While conceptually it makes sense that people who regularly consume chocolates have gut microbiota more readily adapted to metabolizing cocoa polyphenols compared to those who avoid chocolate, it remains an intriguing question as to whether there are wider effects beyond gut microbial metabolism and into the realm of "gut-host interactions." Certainly recent studies in both humans [36] and rodents [37] clearly demonstrate that cocoa polyphenols can alter gut microbiota populations. In vitro colon model studies find that cocoa polyphenols increase butyric acid production and formation of 3-hydroxyphenylpropionic acid from cocoa flavanols [38]. Consumption of dark chocolates also increases 3-hydroxyphenylpropionic acid and hippurate excretion in urine [35]. As will be addressed below, phenolic compounds may also be metabolized into hippuric acid [39], and this convergence with amino acid metabolism may lead to some problems in interpreting metabolomics data relating to polyphenols and amino acid intake. To be confident of identifying changes in gut microbial metabolism, several related changes may need to be identified, preferably with concurrent changes to gut microbiota populations.

While it is clear that there is a relationship between diet, gut microbiota, and certain metabolites resulting from gut microbial metabolism, the link between gut microbial metabolites and systemic effects remains largely unclear. Are they simply markers, or also mediators? A number of dietary phenolic compounds act as signalling molecules for regulating various metabolic cascades [40], though no data exists on whether of the common aromatic metabolites identified as being produced by gut microbiota have any role in influencing gene expression.

There is much that remains to be studied in terms of the diet and gut microbiota – protein-rich or sulfur-rich diets have received relatively little attention compared to high-fat diets or different sources of carbohydrates and prebiotics. Beyond specific pre- and probiotics, several different food groups are also known to have an effect on the gut microbiota, with consequent possible effects on gut microbial metabolism, though to date whether these effects have long-term effects on the host is less clear. This is further complicated by the apparent resistance of gut microbial populations to long-term change, as demonstrated by fecal transplantation studies, where host populations frequently revert towards pretransplantation levels [41].

5.4 Nutritional Metabolomics: A Methodology Well Suited for Understanding the Effects of Food on Gut Microbiota

Metabolomics is the study of multiple metabolites (small molecules, generally <1,500 Da) in response to different stimuli or conditions and generally involves the measuring of several to hundreds of metabolites or thousands of features in a metabolic profile [42]. This is followed by multivariate analysis to determine what metabolite(s) best explain(s) the research question. Metabolomics is

complementary to other omics such as genomics and proteomics. So while genetics is often seen as a "blueprint," genetic disposition is often not reflected in phenotype. As metabolites are the end product of genotype differences, they reflect how a system is responding to different stimuli. Simplistically, metabolites can be seen as the result of genotype + epigenetic modification + posttranslational modification of proteins + interaction with the environment. Chapters 2 and 3 elaborate on general metabolomics methodologies and data modelling.

5.4.1 Metabolomics Methods to Study Gut Activity Effects on Metabolism

Metabolomics is mainly based on two technologies: nuclear magnetic resonance (NMR) and mass spectrometry (MS). Among a wide variety of applications, it has been used in characterizing the metabolic fingerprint of mammalian hosts under conditions designed to alter the microbial communities in the gastrointestinal tract. While a wealth of studies have found associations between metabolic patterns and diseases to (deregulated) gut microbiota, the full biochemical characterization of the gut microbial activity is yet to be defined. To define the metabolome of the gut microbiome, Wikoff et al. [43] used an untargeted MS-based strategy to compare plasma extracts of germ-free mice to conventional mice. Indole-containing metabolites, phenylated-organic acids, and phase II metabolites of these (sulfated and glucuronidated species) were found in conventional mice and either absent or poorly represented in germ-free animals. The absence of phase II metabolites in germ-free mice suggests a direct impact of the gut microflora on the drug metabolism capacity of the host, where interplay between gut (microbes) and liver (mammal) takes place.

Other strategies to investigate the function of the metabolite influenced by the gut microbiota have included urinary and fecal MS-based profiling of metabolites from Wistar rats exposed to a broad-spectrum β-lactam antibiotic (imipenem/cilastatin sodium) and were compared before and after exposure [44]. An apparent metabolic switchover is observed within 24 h of antibiotic exposure and affecting a wide range of central metabolic pathways (mainly amino acid metabolism, but also organic acid metabolism, oligopeptides, carbohydrate metabolism, purine and pyrimidine metabolism, and the TCA cycle). Benzene- and indole-containing substances, including tryptophan and hippuric acid, were dramatically reduced by the antibiotic treatment.

These two studies [43, 44], using different strategies to remove the influence of the gut microbiota, lead to consistent results on the chemical nature of metabolites produced by gut microbiota activity. The fact that different biological matrices were used for metabolomics analysis (plasma [43] and urine and feces [44]) suggests comparable effects in all systemic biofluids, at least in rodents.

While most metabolomics studies have focused on metabolite analysis of plasma, urine, and fecal water, there are other potential methods for understanding gut microbiotal metabolism that to date have not had widespread use. These include

headspace GC-MS analysis of volatile organic compounds from feces [45], which can measure up to 90 compounds present in the fecal headspace. Breath analysis may also be a fruitful area for understanding gut microbiota activity; several breath analyses are already used in nutrition to measure gut microbiota activity including breath hydrogen to monitor gut fermentation and the urea breath test for *Helicobacter pylori* infection [46]. Gasses produced by gut bacterial fermentation not only exit via flatus but can also be measured in breath – especially those that are active in the stomach, as is the case with *H. pylori*. The lactose breath test is another practical example, where lactose tolerant people can break down lactose before reaching the intestine, while in lactose-intolerant people, lactose reaches the intestine and is rapidly fermented, leading to the production of hydrogen gas. Hydrogen is normally present in low concentrations in breath, so any spike in breath hydrogen is clearly linked to lactose intolerance. The same concept is also used for measuring the fermentation of dietary fibers by gut microbiota in clinical trials [47].

5.5 Metabolites of Gut Activity

The ensemble of bacterial species in the gut can modulate metabolic reactions essential to the host's metabolism and health. There are a set of metabolites that consistently directly or indirectly stand out in association studies on diseases such as obesity, insulin resistance, type II diabetes, cancer, cardiovascular disease, chronic systemic inflammation, and autism and related neurological conditions [15]. These metabolites include SCFAs, bile acids, choline metabolites, phenolic, benzoyl, and phenyl derivatives and indole derivatives (Figs. 5.1 and 5.2). Given the range of conditions where these metabolites may be involved, there is now little doubt about the contribution of the gut microbiota to host metabolism and maintenance.

5.5.1 Short-Chain Fatty Acids

Possibly the best known examples of gut microbiota metabolites are the SCFAs acetate, propionate, and butyrate, which result from bacterial fermentation of carbohydrates. These are water-soluble and readily absorbed respiratory fuels used by the colonic epithelial cells (colonocytes) produced by anaerobic bacteria. Luminal fatty acids are the preferred fuels of colonocytes and the order of preference is SCFAs > ketone bodies > amino acid > glucose [48]. Butyrate is the preferred source of energy for colonic epithelial cells. Butyrate is transported into colonocytes, enters the mitochondria, and undergoes β oxidation to acetyl-CoA, which enters the TCA cycle resulting in the reduction of NAD^+ to NADH. NADH enters the electron transport chain culminating in ATP production with CO_2 as a by-product [49]. Butyrate is associated with a decreased risk of colon cancer proliferation, modulation of

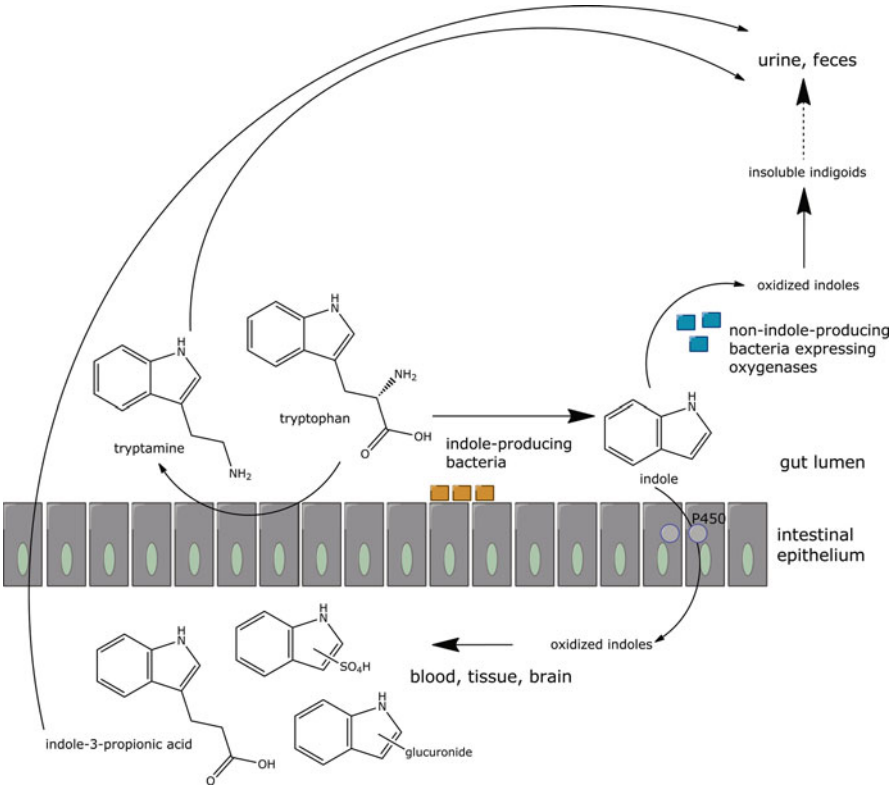

Fig. 5.2 Proposed indole signalling and metabolism in the intestine (inspired by [43, 44, 65])

inflammation, and an increase in satiety [50–54]. Absorbed acetate and propionate are delivered to hepatocytes, which consume most of the propionate for gluconeogenesis. Although acetate can be used for lipogenesis in colonocytes, hepatocytes and adipocytes are the principal sites for de novo lipogenesis, at least in rodents. SCFAs also act as signalling molecules. Propionate, acetate, and to a lesser extent butyrate and pentanoate are ligands for at least two G protein-coupled receptors (GPCRs), Gpr41 and Gpr43. Both GPCRs are broadly expressed, including in the distal small intestine, colon, and adipocytes. SCFAs (C1–C6), which are ligands for Gpr41, stimulate expression of leptin in mouse-cultured adipocytes [55]. Leptin is a polypeptide hormone with pleiotropic effects on appetite and energy metabolism. This possible link between fiber intake, gut microbiota, and satiety has opened up a new area of possibilities for nutrition research. Clostridia are saccharolytic and amino acid fermenting species and are able to produce the three main SCFAs in the colon. Many other bacteria such as Bacteroides, Eubacteria, and Propionibacteria are known for producing specific SCFA from various substrate sources [4, 49]. Most SCFAs are produced from the fermentation of carbohydrates, with smaller amounts by microbial protein degradation in the large intestine. In addition to providing energy for the colon, SCFAs are important energy sources for muscle, kidneys, heart, and brain.

5.5.2 *Phenolic and Phenyl Metabolites*

Hippuric acid is a conjugate of glycine with benzoic acid and is a common end product of metabolism excreted in urine. Dietary sources of protein and polyphenols (fruits, vegetables, coffee, tea, chocolate) ultimately lead to the degradation into quinic acid and benzoic acid by colonic microflora which are then oxidized to hippurate by hepatic mitochondrial function, in a CoA-dependent fashion [34, 39, 56]. Therefore, diets rich in protein and polyphenols lead to increased excretion of hippurate and other phenol-related metabolites such as *p*-cresol, phenol, *p-hydroxybenzoic* acid, and *p*-hydroxyphenylacetic acid [34, 57]. *p-Hydroxyphenylacetic* acid has been identified as an intermediate of *p*-cresol production from tyrosine and is elevated in a variety of conditions [34, 57]. Hippurate is possibly the most recognized gut microbial co-metabolite and has been associated with a variety of conditions or disease status, such as obesity, high blood pressure, Crohn's disease and ulcerative colitis, autism (decreased excretion), type I and II diabetes, and anxiety (increased excretion) [39]. As an example of the relative amount of hippurate precursors, Table 5.2 summarizes different foods and their potential to form hippurate. The amount of potential hippurate from coffee and tea is far greater than the other types of food listed, most notably dark chocolate, also considered a major source of polyphenols in the diet. Aromatic amino acids are also potential sources of hippurate, though the amount of these actually reaching the large intestine is potentially lower than for polyphenols. Caution is needed with the interpretation that elevated hippuric acid is mainly due to polyphenol-rich foods in the diet, as aromatic amino acid-rich foods such as meat and fish could lead to elevated levels in cases of protein malabsorption.

p-Cresol-sulfate is an abundant compound in urine that is obtained from protein fermentation in the human gut, derived from tyrosine and phenylalanine metabolism. Gut bacteria [57, 58] such as the pathogen *Clostridium difficile* [59] are able to convert tyrosine into *p*-cresol. In humans, *p*-cresol is almost completely sulfonated into *p*-cresol sulfate by SULT1A1 (human cytosolic sulfotransferase) which is an enzyme able to sulfonate various substrates, including xenobiotics [60]. High amounts of *p*-cresol in urine are found in adult celiac disease patients [57]. *p*-Cresol can exert a variety of effects such as activation of leukocyte free radical production [61] and blocking the conversion of dopamine into noradrenaline [62]. It is argued that given the high amount of *p*-cresol produced in the body, depending on the diet and eventual modulation of gut bacterial composition, impaired sulfation and events thereof (drug metabolism) might take place, depending on the individual [60]. In addition to *p*-cresol, phenol is also produced in the gut, mostly attributed to aerobic bacteria (*E. coli*, *Proteus* sp, *S. faecalis*, *Staphylococcus albus*), while *p*-cresol is produced by anaerobic bacteria [58]. As anaerobic bacteria outnumber aerobic bacteria in the gastrointestinal tract, it is expected that there is greater excretion of *p*-cresol than phenol.

Phenylacetylglutamine is derived from β-phenylethylamine formed in the large bowel by decarboxylation of phenylalanine released by bacterial proteolysis of

Table 5.2 Relative amounts of aromatic amino acids and common phenolic precursors for hippurate in different foods. Several of these precursors may be metabolized through different pathways, so this table should only be considered as a relative indication of the hippuric acid potential of different foods

	Phenylalanine (g/100 g)	Tyrosine (g/100 g)	Glycine (g/100 g)	Chlorogenic acids (mg/100 g)	Caffeic acid (mg/100 g)	Catechin and epicatechin (mg/100 g)	Total hippurate mole equivalents (mmol/100 g)	Total glycine equivalents (mmol/100 g)	References
Milk chocolate	0.34	0.23	0.17	–	–	67	3.56	2.26	[83–85]
Dark chocolate	0.2	0.074	0.18	–	–	200	2.31	2.40	[83–85]
Fish (smoked salmon)	0.86	0.72	0.99	–	–	–	9.18	13.19	[83]
Fish (smoked herring)	0.82	0.73	0.92	–	–	–	8.99	12.26	[83]
Beef (rump steak)	0.75	0.62	1.1	–	–	–	7.96	14.65	[83]
Chicken (breast meat)	0.74	0.61	1.1	–	–	–	7.85	14.65	[83]

Refined wheat	0.51	0.22	0.35	–	–	–	4.30	4.66	[83]
Whole grain wheat	0.52	0.29	0.48	–	–	–	4.75	6.39	[83]
Refined rice	0.45	0.24	0.38	–	–	–	4.05	5.06	[83]
Brown rice	0.48	0.26	0.41	–	0.3	6	4.36	5.46	[83, 86]
Coffee (soluble, dry)	0.24	0.16	0.42	8,800	1,740	–	36.83	5.59	[83, 87, 88]
Tea (black, leaves)	Trace	Trace	Trace	100	56.8	3,700	13.34	0.00	[87, 89]
Tea (green, leaves)	Trace	Trace	Trace	150	52	5,960	21.24	0.00	[87, 89]

Trace: Trace amounts found, but close to methodological limit of detection
–: No data found, probably not present in foodstuff

unabsorbed protein residues [63]. Indoxyl sulfate and phenylacetylglutamine have been found in higher concentrations in the plasma of diabetic individuals compared to nondiabetics. Abnormal urinary excretion of phenylacetylglutamine, hippurate, and hydroxyhippurates has been reported in autistic children [64] (see Chap. 16).

5.5.3 Indole Metabolites

Copious amounts of indole are produced by the human body and ultimately excreted in urine, in the form of indoxyl sulfate. Indole has been thus associated to gut microbial activity and is produced by tryptophanase (TnA) that reversibly converts tryptophan into indole, pyruvate, and ammonia [65]. The elimination of tryptophan, instead of indole, in urine can be associated to altered microbial activity in the gut. Over 85 bacterial species are known as indole-producing bacteria [65] and in the gut, indole is a signalling molecule recognized by intestinal epithelial cells and is used to strengthen the host cell barrier, maintain controlled inflammation, and increase resistance to pathogen colonization [66]. It is not known if *E. coli* is able to degrade indole, but many non-indole-producing bacteria encode various oxygenases that can modify or degrade indole, producing indole derivatives, such as indigoid compounds [43, 65]. Independent from gut microbial activity, indole can be further modified into sulfated, glucuronidated, and fatty acid-conjugated species, such as indoxyl sulfate, indoxyl glucuronide, and indole-3-propionic acid (IPA) and indole-2-acetic acid (IAA) [43] (Fig. 5.2). Indole has been compared to the known autoinducer-2 (AI-2), a furanosyl borate diester, from the family of signalling molecules used in quorum sensing, although it is still unclear how the roles of two molecules are connected to each other [65].

Tryptamine is another metabolite in the tryptophan metabolism that is decarboxylated by mammalian L-tryptophan decarboxylyase from tryptophan, as well as degraded into indole-3-acetaldehyde by gut bacteria. Low tryptamine levels in urine have been detected in patients with severe depression [67], while high levels of this molecule in urine and feces have been found in antibiotic-treated subjects [44]. Abnormal tryptophan metabolism is indicated in cognitive disorders, such as depression, and 5-hydroxy-L-tryptophan has been used clinically for decades to increase serotonin production in the brain [68].

IAA is a known phytohormone, which belongs to the auxin class of compounds that regulates cell growth and development. Diverse bacterial strains produce IAA, especially endophytes, which signal biofilm formation. In the gut, indoles have been described to lead to biofilm formation [65] and regulation of virulence in vitro and in vivo [69] and specifically IAA has been identified as a marker of gut activity [44] and enhancer of cellular defense [70]. Thus, it could be speculated that the indole class of compounds may act as inter-kingdom signalling molecules regulating mammalian, bacterial, and plant signalling.

IPA is a potent antioxidant and neuroprotective molecule. IPA completely protected primary neurons and neuroblastoma cells against oxidative damage and

death caused by exposure to Alzheimer β-amyloid protein, by inhibition of superoxide dismutase, or by treatment with hydrogen peroxide [71]. The capacity of IPA to scavenge hydroxyl radicals exceeded that of melatonin, an indoleamine considered to be the most potent naturally occurring scavenger of free radicals [71].

In addition, 6-hydroxymelatonin, an oxidation product of melatonin was also identified as a marker of gut activity, as well as other known neurotransmitters such as DOPA, dopamine, norepinephrine, and epinephrine which play important roles in the brain [44]. Several stress mechanisms have been correlated to alteration of bacterial composition of the gastrointestinal tract (GI), altering epithelial cell function, motility, and mucus secretion. Upon stress, norepinephrine is released into the GI tract, potentially altering gut microbial composition and function [72]. These findings are evidence of the strong association of microbial-mammalian metabolism to the gut-brain axis.

5.5.4 Choline Metabolites

Eggs, milk, liver, red meat, poultry, shell fish, and fish are natural sources of phosphatidylcholine and choline. As described earlier, microbial conversion of dietary phosphatidylcholine and choline (or betaine) leads to the production of TMA in the gut which is oxidized in the liver to TMAO by the hepatic flavin monooxygenase (FMO) family of enzymes, FMO3. A study on mice has shown that dietary supplementation with choline, TMAO, or betaine was found to promote upregulation of multiple receptors linked to atherosclerosis [16]. Increased levels of TMAO were also associated with nonalcoholic fatty liver disease [73].

5.6 Gut-Liver Interplay

A portion of the products of colonic fermentation are absorbed by the colonocytes and via specific pathways lead to the production of ATP, while others undergo biotransformation in the liver entering phase I and phase II types of metabolism (Fig. 5.3). Especially for phenyl, phenol, and indole derivatives, sulfation, glucuronidation, and glycine conjugation occur and have been described [43]. Most commonly these metabolites are more water soluble and increased polarity of conjugates may limit passive partitioning into cells, thus increasing excretion.

Biotransformation capability of the host is dependent on several factors, including age, gender, genetic variability, nutrition, disease, exposure to other chemicals that can inhibit or induce enzymes, and dose levels. For instance, the elderly shows decreased biotransformation capabilities and gender may also influence the efficiency of biotransformation for specific metabolites or xenobiotics, as this is usually limited to hormone-related differences in the oxidizing cytochrome P-450 enzymes. This area is especially deserving of attention as we seek to further our understanding of what role a dynamic gut microbiota may play in the host aging process.

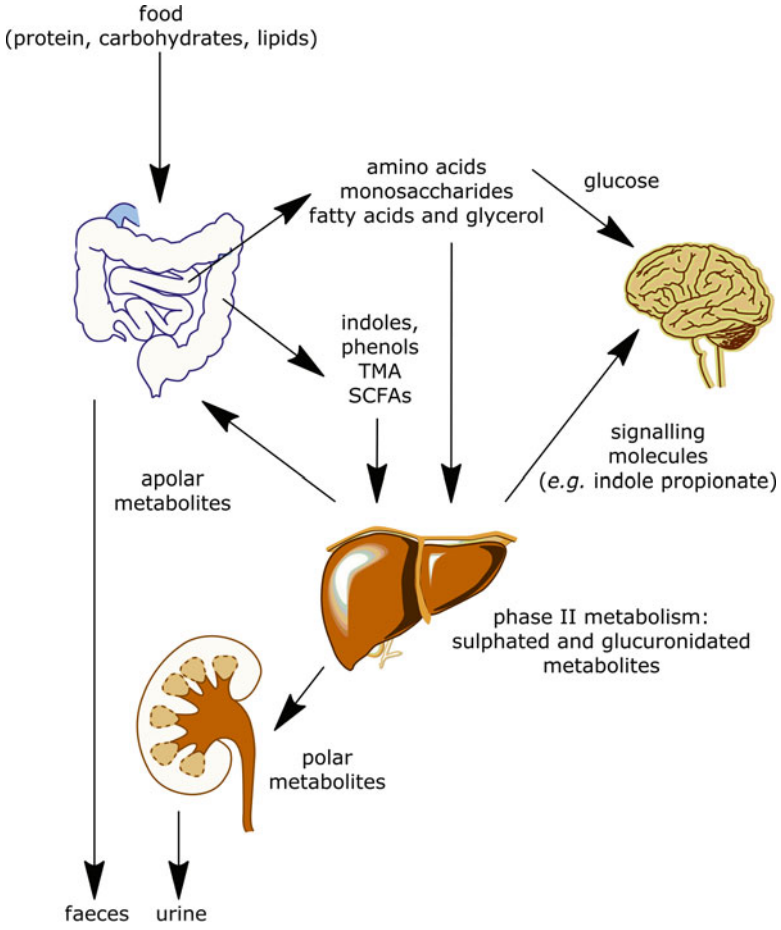

Fig. 5.3 Overview of the interaction between host organs and gut microbiota

5.7 Future Directions

Gut microbiota has relevance for human health and disease beyond the gastrointestinal tract, appearing to have a systemic impact on human metabolism, through interaction with multiple organs. The gut microflora has proven causality in the induction of some metabolic disorders (see Chap. 12), and therapies that target the gut microbiota are at the forefront of nutrition research. Modulation of the gut microbiota is potentially attainable by altering dietary habits; however, we are still far away from understanding either general effects of macronutrients or specific effects of ingredients on gut microbiotal metabolism. There are cautionary tales too – while it is tempting to propose a role for gut microbiota in all observed health benefits related to food, this is not always the case. In one study, cereal fiber changed

gut microbiota composition, but there was no association between these changes and an observed improvement in insulin sensitivity [74]. It is possible that more focus is needed on microbial metabolism, rather than population shifts, an area where metabolomics may be a particularly useful methodology for helping to find answers.

While metabolomics has been instrumental as an exploratory tool to fuel ideas and propose novel hypotheses, we believe that strategies to monitor the gut microbial metabolome will be crucial to define gut activity and its impact on metabolism. To achieve this, targeted metabolomics methods should be implemented to follow the different classes of gut microbial metabolites in health and disease. The quantitation of metabolites will becoming increasingly important to define the kinetics of metabolic fluxes, and to determine mechanisms of action and their association with functionality.

Studying the potential activity of the gut cannot be deduced by solely looking at fecal samples, as fecal transit can vary considerably (12–120 h) and bacterial gradients in the colon exist and thus fecal samples may only be a poor approximation of metabolism along the colon. Gut microbiota metabolites seem to be not only products of digestion, and therefore simpler molecules to be either taken up as energy or discarded by the host, but also signalling functions are being unraveled that prospect a more complex interplay between microbiota and host. It is clear that in terms of our knowledge on the relationship between the diet and nutrition of the host, and our gut microbiota, we are at the beginning of an area that will have a profound impact on our current understanding of human nutrition.

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