THE HUMAN INTESTINAL MICROBIOTA complements our physiology with functions that we have not had to develop on our own. In fact, the intestinal microbiota have a metabolic capacity that is comparable to that of the liver (83). The human colon contains an extremely complex and dynamic microbial ecosystem with high densities of living bacteria in concentrations of $10^{11}$-$10^{12}$ cells/g of luminal contents belonging to more than 1,000 different species. In healthy adults, 80% of the identified fecal microbiota can be classified into three dominant phyla: Firmicutes, Bacteroidetes, and Actinobacteria, but there is substantial variation in the species composition between individuals (30, 101).

The intestinal microbiota plays an important role in human physiology. For example, the intestinal microbiota is responsible for the further metabolism and energy harvest from nondigested nutrients, is involved in the synthesis of vitamins such as B and K and metabolism of polyphenols, provides colonization resistance toward potential pathogens, is involved in the metabolism of bile acids, and stimulates the immune function of the host (74, 83).

Molecular approaches, mainly based on the 16S ribosomal RNA gene, have revolutionized the field of gut microbial ecology. Nowadays, the uncultured and complex microbial communities can be characterized with greater sensitivity by using high-throughput technologies, such as pyrosequencing (5) and phylogenetic microarrays (79), compared with former molecular fingerprinting methods, such as PCR-denaturing gradient gel electrophoresis (DGGE). Complementary quantitative technologies, such as fluorescence in situ hybridization (FISH) and real-time quantitative PCR, can be used to confirm shifts in particular groups or species (113).

In recent years an increasing amount of literature has demonstrated that several diseases are related to alterations in the intestinal microbiota (known as dysbiosis), such as irritable bowel syndrome (54), inflammatory bowel disease (93), diabetes (55), atopic diseases (76), cancer (85), and obesity (7). For example, a reduction in the abundance and diversity of Firmicutes is frequently associated with inflammatory bowel disease and irritable bowel syndrome (105, 113). These studies have mainly shown that differences in the composition of the intestinal microbiota are associated with disease.

**Functional Capacity of the Microbiota**

The human microbiota is characterized by a significant degree of functional redundancy, meaning that different bacteria can perform similar functions and metabolize the same substrate (60, 64). Therefore, not only the composition but also
the functional capacity of the intestinal microbiota is highly important regarding the clinical end points.

Next-generation pyrosequencing can be applied to further evaluate the functional capacity of the colonic microbiota by creating a catalog of the genetic potential of the microbiota. However, it has to be realized that the detection of genes in a metagenomic library does not necessarily mean that these are functionally important (113). To gain better insight into the activity and functionality of the intestinal microbiota, other meta-“omics” approaches can be applied that use RNA, proteins, and metabolites as targets. In this review, we summarize the different approaches that have been used to quantify and characterize the metabolites produced by the microbiota, which yield information on the actual end products of metabolism. Colonic microbial fermentation results in the production of large amounts of different end products. The type and amount of these fermentation-derived metabolites largely depend on the composition of the microbiota, transit time, and the substrates available for fermentation (95). Some of these end products have been shown to be protective to the colonic epithelium, and others have proved to be proinflammatory or procarcinogenic metabolites (3, 48). By using knowledge of these specific metabolites, a hypothesis-driven targeted approach can be applied to evaluate changes in colonic metabolism following dietary interventions or during different disease states, for example, through quantification of selected metabolites from carbohydrate and protein fermentation, secondary bile acids, or colonic enzymes. On the other hand, a top-down approach can be followed by applying metabolite fingerprinting techniques based on $^1$H-nuclear magnetic resonance (NMR) or mass spectrometric analysis. By following this approach, novel metabolites and mechanisms can be identified that are involved in health and disease. This is, however, not an easy task, since the signals first have to be identified and their metabolic roles elucidated.

For analyses of metabolites as end products of intestinal metabolism in humans, we mainly rely on fecal samples or on breath (for example, hydrogen, methane, and carbon dioxide), urine, and plasma samples due to the relative inaccessibility of the colon to sample at different locations (Fig. 1) (50). In these human studies, an elegant solution to study metabolic pathways in vivo is the application of stable isotope tracers.

**Types of Fermentation**

The colonic microbiota ferment endogenous host-derived substrates such as mucus, pancreatic enzymes, and exfoliated epithelial cells, as well as dietary components that escape digestion in the upper gastrointestinal tract. Two main types of colonic microbial fermentation can be distinguished, including saccharolytic fermentation of carbohydrate and proteolytic fermentation of protein (Fig. 2). In the proximal part of the colon, mainly saccharolytic fermentation takes place, since most microorganisms preferentially ferment carbohydrates and switch to protein fermentation when carbohydrate sources are depleted (75). The main products of carbohydrate metabolism are short-chain fatty acids (SCFA), mainly acetate, propionate, and butyrate, which have been shown to contribute to colonic health. SCFA provide energy to the colonic epithelial cells, decrease luminal pH, and improve mineral absorption. Furthermore, butyrate has been shown to possess an anti-inflammatory and anticarcinogenic potential (46, 63). Besides their contribution to gut health and maintenance, SCFA may provide further benefits for the systemic metabolism. For example, it has been shown that acetate and propionate affect hepatic lipid metabolism. Acetate is the primary substrate for cholesterol synthesis, whereas propionate can inhibit cholesterol synthesis (27, 110). Since the different SCFA may show distinct effects, not only the amount of SCFA produced but also their ratio is of importance with regard to these health effects. Furthermore, SCFA stimulate increased plasma levels of satiety hormones such as peptide YY (PYY), leptin, and glucagon-like peptide-1 (GLP-1) and may attenuate insulin resistance (1, 34). These effects may occur due to the fact that SCFA are ligands for G protein-coupled receptors (GPR) 41 and 43, expressed on adipocytes, enteroendocrine L-cells, and immune cells (88). In addition, recent studies have linked SCFA activation of GPR 43 to the suppression of colon cancer (94, 96). Proteolytic fermentation also leads not only to the production of SCFA (lower amounts than produced from carbohydrates) and branched-chain fatty acids but also to potentially toxic metabolites such as phenolic compounds, sulfur-containing compounds, amines, and ammonia (Fig. 2) (48). The toxicity of these protein fermentation metabolites has mainly been established in vitro studies (6, 11, 58) and animal studies (4, 97). However, human epidemiological studies do not consistently support an association between protein intake and colorectal cancer (2) and inflammatory bowel disease (47). Unfortunately, actual protein fermentation metabolites were not determined in these studies. The different metabolites of protein fermentation and their potential involvement in colorectal cancer have previously been reviewed (48).
Targeted Approach

Quantification of carbohydrate fermentation. The beneficial effects of saccharolytic fermentation have mainly been ascribed to the production of SCFA (68, 91). Several studies have evaluated SCFA concentrations and profiles in patients with different diseases. For example, a lower butyrate-to-acetate ratio has been found in colonic luminal content of patients with adenomatous polyps or colon cancer compared with healthy controls (107), and increased amounts of SCFA were found in fecal samples from obese compared with lean individuals (89). Dietary interventions with prebiotics, such as inulin and oligofructose, defined as “nondigestible food ingredients that stimulate the growth and/or activity of bacteria in the digestive system which are beneficial to the health of the host,” aim to increase and prolong the saccharolytic fermentation toward the distal colon and thereby aim to reduce proteolytic fermentation (38) (Fig. 3). Increased SCFA production after addition of different prebiotics to the diet has previously been demonstrated by measuring fecal or plasma concentrations of SCFA (52, 73). However, the in situ production of SCFA is difficult to determine, since more than 95% of the produced SCFA are absorbed and metabolized by the host depending on the gastrointestinal transit time (59). To gain more insight into the actual SCFA production over time, the use of stable isotope tracers can be considered. A recent study with 13C-labeled barley has evaluated the kinetics of SCFA appearance in the systemic circulation in healthy volunteers. In this study, the pattern of SCFA appearing in the systemic circulation was different after consumption of a meal with dietary fiber (nonstarch polysaccharides) combined with resistant starch compared to a meal with dietary fiber alone (102). Further studies are necessary to address the significance of these different SCFA profiles with regard to health benefits. In another study, stable isotope tracers were applied to determine the production rate of acetate during colonic fermentation of lactulose in humans. Healthy volunteers received a primed continuous infusion of [1-13C]acetate followed by the ingestion of lactulose. The colonic acetate production was calculated from the reduction in the plasma [13C]acetate enrichment as a result of the colonic fermentation of lactulose (77). With the use of a dynamic in vitro model of the large intestine, the fermentation of 13C-labeled starch was evaluated by determination of the label incorporation into the microbial biomass and metabolites using stable isotope probing and NMR analysis (53). From the labeling pattern of microbial metabolites, it was concluded that cross-feeding between Ruminoccus bromii and Eubacterium rectale occurred, wherein R. bromii produced acetate, which was subsequently converted to butyrate by E. rectale.

Quantification of protein fermentation. The degree of proteolytic fermentation can be determined by quantification of typical end products of protein fermentation (Fig. 2) (48). Some of these metabolites are reused as nitrogen source for bacterial growth, whereas others will be taken up by colonoocytes and transported into the bloodstream. For instance, phenols and indoles are breakdown products of the aromatic acids tyrosine and tryptophan, respectively. Generally, once these compounds are produced, they enter the hepatic circulation to be detoxified in the liver and eventually excreted in urine. Since p-cresol and phenol are unique bacterial metabolites from protein fermentation that are not produced by human enzymes, these metabolites have been frequently used to assess the degree of proteolytic fermentation (25, 37). Urinary levels of p-cresol and phenol have shown to be increased during high
protein intake (37) and decreased after oral supplementation with oligofructose-enriched inulin (OF-IN) (25).

In hemodialysis patients, p-cresol generation and p-cresyl sulfate serum concentrations were lowered after oral OF-IN administration (69). As renal function declines in these patients, substances that are either excreted or metabolized by the kidney accumulate in the circulation, resulting in increased levels of numerous molecules in blood (33). A number of these retained solutes originate from colonic bacterial protein metabolism (33). In addition, small intestinal assimilation of proteins is impaired in renal failure (10), resulting in an increased availability of proteins for fermentation in the colon (31). Accumulation of the protein fermentation metabolites in serum has been suggested to alter endothelial function (32, 70) (31). Accumulation of the protein fermentation metabolites in serum has been suggested to alter endothelial function (32, 70) and has been associated with increased mortality in hemodialysis patients (9). As a consequence, a dietary strategy with OF-IN that contributes to a lower generation of protein fermentation metabolites might constitute a significant improvement in the treatment of these patients. In addition to p-cresol and phenol, other protein fermentation metabolites, such as sulfur-containing metabolites, were decreased in fecal samples after incubation with OF-IN in vitro (22). Increased concentrations of sulfides have been associated with the pathogenesis of ulcerative colitis (UC). Hydrogen sulfide (H2S) has been found to provoke genomic DNA damage in colonic cancer cells (HT-29 cells) in concentrations similar to those found in the human colon (6). In addition to inducing DNA damage, sulfide impairs the oxidation of butyrate, the major energy substrate in colonocytes, by inhibition of cytochrome c oxidase (81). These observations are the basis for Roediger’s “energy deficiency” hypothesis as a cause of UC (67, 82). Different studies have reported increased luminal concentrations of sulfide as well as high numbers of sulfide-reducing bacteria in patients with UC (84). This patient group may therefore also benefit from dietary interventions with prebiotics.

Colonic ammonia metabolism. To evaluate the ammonia metabolism in the colon, the stable isotope-labeled biomarker lactose[15N,15N]ureide has been validated (20, 36) (Fig. 4). Human enzymes are not able to hydrolyze the bond between the sugar moiety and urea, whereas this bond can be split by bacterial enzymes when it reaches the colon. Released [15N,15N]urea is rapidly hydrolyzed to [15N]ammonia by ubiquitous bacterial urease. As such, lactose[15N,15N]ureide is used as a vehicle to introduce a known amount of 15N, in the form of ammonia, to the colon. The formed [15N]ammonia can be either taken up by the colonic microbiota, followed by fecal excretion, or absorbed through the mucosa and renally excreted after conversion to [15N]urea in the liver. When microbial activity was stimulated after intake of a prebiotic, the urinary 15N excretion decreased, whereas the fecal 15N excretion increased (20, 36). In general, fermentable carbohydrates stimulate bacterial proliferation, which leads to incorporation of nitrogen (from ammonia and other sources) into bacterial cells and consequent excretion in feces (25, 26, 108). Thus a shift of nitrogen excretion from urine to feces can be explained by increased bacterial protein synthesis and a subsequent decrease in colonic absorption of nitrogen in the form of ammonia. The removal of ammonia from the colonic lumen might be considered a health benefit, since it may prevent ammonia from damaging colonocytes (87) and increased systemic levels of ammonia cause neurotoxic effects (106). Accumulation of ammonia in the bloodstream is associated with hepatic encephalopathy. Lactulose is frequently utilized in the treatment to reduce ammonia levels in these patients (90, 106).

Secondary bile acids. Bile acids are natural amphipathic detergents that assist the emulsification and absorption of lipids and fat-soluble vitamins. The human liver synthesizes the primary bile acids, cholic acid and Chenodeoxycholic acid. Primary bile acids are secreted in bile from the gallbladder into the small intestine during digestion. They are then actively absorbed in the ileum and returned to the liver via the portal vein (Fig. 5). About 5% of bile salts escape this enterohepatic circulation and enter the colon, where they are subject to bacterial biotransformation reactions. When the primary bile acids reach the colon, they are deconjugated and successively undergo other enzymatic reactions, the most predominant being the dehydroxylation by bacterial 7α-dehydroxylase to form the secondary bile acids, primarily deoxycholic (DCA) and lithocholic acids (LCA) (8). Secondary bile acids have been hypothesized to be cocarcinogenic and tumor promoters (18, 112). It has been reported that patients with adenomatous polyps have a higher concentration of fecal bile acids and total secondary bile acids compared with control subjects (49). Furthermore, the ratios of the primary bile acids and their secondary bile acids are significantly lower in cancer patients compared with controls (17, 49). However, a large meta-analysis of 20 clinical trials and a total number of 1,226 individuals showed no difference between the fecal concentrations of secondary bile acids (DCA and LCA) in colorectal cancer patients compared with controls (98). It has been shown that dietary interventions are able to modulate fecal bile acid concentrations. For example, Boutron-Ruault et al. (15) have shown that ingestion of short-chain fructooligosaccharides decreased fecal concentrations of LCA. This decrease may be
related to the increased production of SCFA, since SCFA decrease the colonic pH. A low pH inhibits dehydroxylation of bile acids and, consequently, conversion to secondary bile acids (19). This hypothesis is supported by the negative correlation between butyrate and acetate production and bile acid metabolism (100).

**Bacterial enzyme activity.** Several bacterial enzyme activities have been studied in relation to their effect on host health. Bacterial β-glucosidase and β-glucuronidase hydrolyze the glycosidic bond of glycosides and glucuronide conjugates, respectively, to release aglycones. Glycosides in the gut originate from the plant material in the diet. Glucuronide conjugates are endogenously produced compounds that are metabolized in the liver and conjugated to glucuronic acid before being excreted by the liver via the bile into the small intestine (86). Since some aglycones have been reported to be potentially toxic or carcinogenic, elevated activity of these bacterial enzymes is hypothesized to be a risk factor for colon cancer (39, 43). On the other hand, anticarcinogenic effects of flavonoid aglycones also have been reported (29). Other examples of bacterial enzymes are azoreductase, nitrate reductase, nitroreductase, and sulfatases (39). Azoreductase leads to the production of amines, whereas nitrate reductase converts nitrate into nitrite and nitroreductase is involved in the formation of heterocyclic and aromatic nitro compounds (66). Sulfatases are involved in the degradation of mucosal glycans, such as colonic mucins (14). Activities of these bacterial enzymes are related to the composition of the microbiota. For example, lactobacilli and bifidobacteria produce low levels of β-glucuronidase but also low levels of azoreductase and nitroreductase, whereas strict anaerobes (Bacteroides sp., Eubacterium sp., and Clostridium sp.) produce high levels of these enzymes (14, 71). A number of animal and human intervention studies have investigated the ability of pre- and probiotics to modulate these bacterial enzyme activities in the colon. However, these studies have obtained conflicting results (23, 40–42, 45, 62, 65, 66, 92), and their significance for host health is often difficult to determine (23, 65).

**Top-Down Approach/Metabolomics**

New techniques such as metabolomics allow evaluation of the colonic metabolism by a top-down approach, bypassing the need for an a priori hypothesis. Metabolomics has been defined as “the quantitative measurement of the multiparametric metabolic responses of a living system to pathophysiological stimuli or genetic modification” (72).

Metabolomic analysis requires analytical technologies that are capable of detecting and quantifying the large number of metabolites in biological samples, such as feces (24, 35, 37, 103), intact tissue (12, 16), breath (80), blood (111), and urine (109). Analyses of these multiple biological samples complement each other, since they yield different information with regard to the origin of the metabolites. Certain breath and urinary metabolites result from cometabolism by the host and the intestinal microbiota, whereas fecal metabolites are primarily derived from the intestinal microbiota (Fig. 1).

Ideally, the sample preparation for metabolomic analysis should be minimal to enable the quantification of as many metabolites as possible. However, no single analytical technique can measure all the metabolites present in a particular sample due to the diverse chemical properties of metabolites and the limitations of each analytical technique (57). Commonly used analytical platforms are mass spectrometry (MS) (28) and NMR (13). Multivariate statistical techniques are required to determine which metabolites differ between samples from different groups. Commonly used multivariate discriminant tests in metabolomic studies include principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA). PCA may be considered an unsupervised classification method, since the variation in the data is analyzed without a priori designation of samples into their classes. In contrast, PLS-DA is considered a supervised classification method, because the samples are designated into their classes for comparison. These different platforms to detect hundreds, or even thousands, of metabolites in one single analysis can be used to evaluate the differences in metabolite profiles between different diseases (51, 109) or different stages of disease (56).
and to evaluate the effect of different (dietary) interventions (44, 57).

Williams et al. (109) used NMR spectroscopy to evaluate urinary metabolites in patients with UC and Crohn’s disease compared with controls. PCA analysis revealed that each of the three groups clustered together and had different principal components, indicating different metabolite patterns in patients with the two distinct inflammatory bowel diseases and controls. After further analysis of the individual metabolites, hippurate levels were found to be lowest in Crohn’s disease patients, whereas formate levels were higher and p-cresol sulfate levels were lower in Crohn’s disease patients compared with UC patients and controls, reflecting inherent differences in intestinal microbes between cohorts (109). It remains to be determined whether these metabolites play a causative role in the development of inflammatory bowel diseases or are just innocent bystanders associated with the disease. Metabolic profiling of fecal samples using gas chromatography-MS (GC-MS) also demonstrated that the patterns of volatile organic compounds from feces of patients with UC, C. difficile, and C. jejuni infection were each significantly different (35). In another study in which metabolite patterns in fecal slurries were studied using GC-MS, incubation with OF-IN resulted in increased SCFA levels as expected. However, a more discriminatory factor for the clustering of the fecal slurries incubated with different doses of OF-IN was an increase in the concentration and number of esters (22). Increased acid production might be the origin of a higher presence of esters. The relevance of esters to health is yet unknown. Future studies are warranted and may also have to consider esters as a marker of saccharolytic activity. Incubation of fecal slurries with OF-IN was furthermore associated with a reduction in sulfur-containing compounds and phenols in a dose- and time-dependent way (22). Analysis of patterns of volatile organic compounds in exhaled breath has been proved to be useful to discriminate patients with chronic obstructive pulmonary disease (COPD) from healthy controls (99) and to discriminate asthmatic children from healthy subjects (21).

Microbial Metabolites Relevant to Health?

The top-down evaluation of microbial metabolites may lead to new insights into which components could be associated with different diseases and (dietary) interventions. Another future challenge remains to associate which microbial species or consortia are responsible for the production of distinct types of metabolites. Several recent studies have used a “trans-genomic” approach to link gut microbiome and metabolic phenotype variation (61, 104). These approaches can offer new possibilities for future interventions. However, more knowledge on the clinical effects of a decrease or increase in certain metabolites and microbial species is warranted, because detected associations may not always be relevant. It remains unknown whether changes in metabolites are a cause or a consequence of the disease. Large prospective cohort studies are warranted to evaluate whether there exists a causal relationship between the production of different metabolites and the different diseases, their clinical features, or disease progression.

Metabolic profiles can also be used for diagnosis of different diseases or classification of patients. This is a promising noninvasive, rapid, and relatively inexpensive diagnostic tool. Before introduction into the clinical practice, the specificity and sensitivity needs to be further evaluated (35, 78).

Conclusions

Changes in colonic metabolism can be evaluated following a targeted approach or a top-down approach. The targeted approach may include quantification of known metabolites from carbohydrate fermentation (SCFA) or protein fermentation (such as p-cresol, phenol, ammonia, or H2S), levels of secondary bile acids, or bacterial enzyme activities. All these “biomarkers” have been identified in epidemiological or intervention studies and have been related to different pathologies, such as colorectal cancer, inflammatory bowel disease, or metabolic syndrome. However, at present, these biomarkers have been insufficiently validated in large prospective trials. With the emergence of metabolomic analyses, new insight into metabolic pathways in relation to health and disease will evolve. Furthermore, these specific metabolic profiles can function as a diagnostic tool for the identification of several diseases, such as ulcerative colitis and Crohn’s disease. Until now, the described biomarkers of bacterially mediated colonic metabolism can only be regarded as intermediate end points. An important challenge for current and future research remains to relate changes in bacterial metabolism to human health.

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