Towards microbial fermentation metabolites as markers for health benefits of prebiotics

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Abstract

Available evidence on the bioactive, nutritional and putative detrimental properties of gut microbial metabolites has been evaluated to support a more integrated view of how prebiotics might affect host health throughout life. The present literature inventory targeted evidence for the physiological and nutritional effects of metabolites, for example, SCFA, the potential toxicity of other metabolites and attempted to determine normal concentration ranges. Furthermore, the biological relevance of more holistic approaches like faecal water toxicity assays and metabolomics and the limitations of faecal measurements were addressed. Existing literature indicates that protein fermentation metabolites (phenol, p-cresol, indole, ammonia), typically considered as potentially harmful, occur at concentration ranges in the colon such that no toxic effects are expected either locally or following systemic absorption. The endproducts of saccharolytic fermentation, SCFA, may have effects on colonic health, host physiology, immunity, lipid and protein metabolism and appetite control. However, measuring SCFA concentrations in faeces is insufficient to assess the dynamic processes of their nutrikinetics. Existing literature on the usefulness of faecal water toxicity measures as indicators of cancer risk seems limited. In conclusion, at present there is insufficient evidence to use changes in faecal bacterial metabolite concentrations as markers of prebiotic effectiveness. Integration of results from metabolomics and metagenomics holds promise for understanding the health implications of prebiotic microbiome modulation but adequate tools for data integration and interpretation are currently lacking. Similarly, studies measuring metabolite fluxes in different body compartments to provide a more accurate picture of their nutrikinetics are needed.

Key words: Microbial metabolites: Prebiotic health benefits: Metagenome: Nutrikinetics

Introduction

For a long time, the colon was considered as an organ that merely absorbs water and electrolytes and converts undigested food residues to drive their excretion without having important physiological functions. Nowadays, it has been generally recognised that the microbial ecosystem inhabiting the gut profoundly affects human physiology and health. The gut bacteria can be considered as a highly active metabolic organ that provides metabolic traits that complement those encoded within our own

Abbreviations: BCFA, branched-chain fatty acid; COX, cyclooxygenase; GPR, G protein-coupled receptor; IBD, inflammatory bowel disease; ILSI Europe, European branch of the International Life Sciences Institute; UC, ulcerative colitis.

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The metabolites produced by the gut bacteria are accessible to the host's cells and in this way influence physiological processes both locally in the intestine and systemically. They contribute to the metabolic phenotype of the host and hence may influence the risk of disease(3). Undigested carbohydrate and protein constitute the major substrates at the disposal of the microbiota for fermentation and result in the production of a range of well-established metabolites including SCFA, branched-chain fatty acids (BCFA), ammonia, amines, phenolic compounds and gases including hydrogen, methane and hydrogen sulfide. Other metabolic activities include the activation or inactivation of bioactive food components like isoflavonoids, flavonoids and plant lignans, the conversion of pro-drugs to drugs, the production of vitamins and the transformation of bile acids and xenobiotics(4,5). Although the fermentative and metabolic activity in the human intestine has been studied for many decades, it remains difficult to evaluate bacterial metabolism in the colon in vivo. Most studies have relied on analysis of the composition of faeces, in vitro incubation studies using faecal inocula or experimental animal models.

An imbalance in the composition of the microbiota has been increasingly associated with the occurrence of chronic or lifestyle-related diseases such as inflammatory bowel disease (IBD), obesity and type 2 diabetes as well as with certain autoimmune diseases such as type 1 diabetes, coeliac disease or allergic asthma(6). Therefore, manipulation of the microbiota has become a promising target for the improvement of host health. As diet is a major factor driving the composition and metabolism of the colonic microbiota, dietary interventions that modulate the supply of macronutrients (carbohydrates, proteins, fat) to the colon have been extensively investigated for this purpose. In particular prebiotics, defined as 'selectively fermented food ingredients that allow specific changes in the colon' at cell metabolism such as vitamins) were not included by-products of energy-yielding metabolism. Secondary metabolism that are essential for growth or that are the products of carbohydrate fermentation (acetic, propionic and butyric acid as well as lactic acid and succinic acid) and products of protein metabolism (ammonia, BCFA, phenol, amines, p-cresol, indole and hydrogen sulfide). In addition, metabolites of plant polyphenols have been included because of their putative health benefits and their bidirectional interaction with the intestinal microbiota.

**Metabolites produced by microbial fermentation**

The complex microbial ecosystem inhabiting the human intestinal tract produces a wide range of metabolites that interact with the host’s cells and in this way influence the physiological processes in the colon. In addition, the metabolites may be absorbed and influence the overall mammalian biochemistry, thereby eliciting systemic effects.

Table 1 provides an overview of the major bacterial compounds that can be found in the intestine.

From this list of compounds, we selected a subset of metabolites that were considered relevant to improved or decreased health. Most of these metabolites are so-called primary metabolites which comprise products of metabolism that are essential for growth or that are the by-products of energy-yielding metabolism. Secondary metabolites (products which do not have an obvious role in cell metabolism such as vitamins) were not included for further analysis. The metabolites reviewed here include products of carbohydrate fermentation (acetic, propionic and butyric acid as well as lactic acid and succinic acid) and products of protein metabolism (ammonia, BCFA, phenol, amines, p-cresol, indole and hydrogen sulfide). In addition, metabolites of plant polyphenols have been included because of their putative health benefits and their bidirectional interaction with the intestinal microbiota.

**Beneficial and harmful effects of relevant metabolites**

**Products of carbohydrate fermentation**

SCFA. SCFA are mainly produced in the colon by bacterial fermentation of carbohydrates that escaped digestion.
in the small intestine. They are saturated aliphatic organic acids consisting of one to six carbons of which acetate (C2), propionate (C3) and butyrate (C4) are the most abundant (≥95%) (10,11). SCFA production mainly occurs in the proximal part of the colon where the availability of substrates is most abundant. The majority of SCFA (up to 95%) are rapidly absorbed by the colonocytes resulting in decreasing concentrations from the proximal to distal colon. Only a minor fraction of SCFA (about 5%) is excreted in faeces (12).

Due to the inaccessibility of the human proximal colon for direct investigation and the rapid absorption of SCFA from the colonic lumen, it is extremely difficult to quantify SCFA production rates. Consequently, no systematic evaluation of normal ‘healthy’ production of SCFA is available. Assuming that 50–60 g carbohydrates reach the colon per d, the production of SCFA was estimated at 400–600 mmol/d (10). Most studies measure faecal SCFA which are the resultant of their production and absorption. Therefore, faecal SCFA rather indicate losses and do not adequately reflect in situ production rates. Table 2 provides an overview of reported values in the literature for total and individual faecal SCFA in adults. Faecal excretion of total SCFA ranges from 60 to 90 μmol/g and might be slightly higher in obese subjects (80–100 μmol/g). SCFA are also detectable in urine, but are the remnant of gut, liver and systemic metabolism and do not reflect colonic generation either. In addition, acetate not only originates from the gut but also from endogenous metabolism, in particular fatty acid oxidation and glucose and/or amino acid metabolism (13,14). Measurement of SCFA in plasma is similarly confounded. Stable isotope studies are required to reliably quantify colonic SCFA production as well as their metabolic fate in the host organism. The pattern and amounts of faecal SCFA change through the different stages in life. In early infancy, the predominant SCFA are acetate and lactate in breast-fed infants and acetate and propionate in (unsupplemented) formula-fed infants (15). In infants fed a formula supplemented with a mixture of galacto-oligosaccharides and fructo-oligosaccharides (9:1 ratio), faecal SCFA patterns were dominated by acetate, similarly as in breast-fed infants, with lower proportions of propionate and butyrate compared with the unsupplemented formula (16). The levels of propionate have been reported to increase in the months before weaning. Butyrate production increases in the

<table>
<thead>
<tr>
<th>Table 1. List of bacterial metabolites that may be found in the intestine</th>
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<tr>
<td><strong>Type of metabolite</strong></td>
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<tr>
<td>Metabolites derived from bacterial energy metabolism</td>
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<tr>
<td>Metabolites from bacterial cytosolic compartment or secondary metabolism (spilled over by excess production, efflux or upon cell lysis)</td>
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<tr>
<td>Gaseous metabolites</td>
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<td>Metabolites of fatty acid and lipid bioconversion</td>
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<td>Metabolites of the enterohepatic circulation</td>
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[48x325]in the small intestine. They are saturated aliphatic organic acids consisting of one to six carbons of which acetate (C2), propionate (C3) and butyrate (C4) are the most abundant (>95%) (10,11). SCFA production mainly occurs in the proximal part of the colon where the availability of substrates is most abundant. The majority of SCFA (up to 95%) are rapidly absorbed by the colonocytes resulting in decreasing concentrations from the proximal to distal colon. Only a minor fraction of SCFA (about 5%) is excreted in faeces (12).

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Table 2. Faecal concentration of individual SCFA

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Reported measure</th>
<th>Acetic acid</th>
<th>Propionic acid</th>
<th>Butyric acid</th>
<th>Total SCFA</th>
<th>Unit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>10; 21–34 years</td>
<td>Mean (SD)</td>
<td>218 (99)</td>
<td>72 (37)</td>
<td>58.7 (54.5)</td>
<td>378 (188)</td>
<td>μmol/g dry weight</td>
<td>Whelan (2005)</td>
</tr>
<tr>
<td>20; 20–40 years</td>
<td>Mean (SEM)</td>
<td>320.3 (24.9)</td>
<td>97.3 (10.5)</td>
<td>93.8 (9.13)</td>
<td>511.4 (41.9)</td>
<td>μmol/g dry weight</td>
<td>Boler (2011)</td>
</tr>
<tr>
<td>13; 23–58 years</td>
<td>Median (IQR)</td>
<td>52 (14-2)</td>
<td>23.2 (13-6–37-3)</td>
<td>36.8 (5–128)</td>
<td>119.3 (64.5–197.0)</td>
<td>μmol/g dry weight</td>
<td>Lewis (1997)</td>
</tr>
<tr>
<td>27; 18–55 years</td>
<td>Mean (SEM)</td>
<td>35.8 (2.4)</td>
<td>11.4 (1.2)</td>
<td>10.0 (1.1)</td>
<td>61.1 (4.4)</td>
<td>μmol/g</td>
<td>Lecerf (2012)</td>
</tr>
<tr>
<td>12; 18–65 years</td>
<td>Mean (95% CI)</td>
<td>48</td>
<td>13.98</td>
<td>11.4 (1.2)</td>
<td>69.5 (61.3, 78.7)</td>
<td>μmol/g</td>
<td>Reimer (2012)</td>
</tr>
<tr>
<td>46; 31–66 years</td>
<td>Median (IQR)</td>
<td>43.7 (34.0–52.2)</td>
<td>13.1 (9.2–18.5)</td>
<td>8.8 (5.2–11.5)</td>
<td>91.8 (73.1–107.5)</td>
<td>μmol/g</td>
<td>Fernando (2010)</td>
</tr>
<tr>
<td>20; 22–55 years</td>
<td>Mean (SEM)</td>
<td>42.13 (3.84)</td>
<td>11.5 (1.19)</td>
<td>11.28 (1.42)</td>
<td>67.3 (6.22)</td>
<td>μmol/g</td>
<td>McOrist (2011)</td>
</tr>
<tr>
<td>8; 31–59 years</td>
<td>Mean (SD)</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>92.7 (33.9)</td>
<td>μmol/g</td>
<td>Nemoto (2012)</td>
</tr>
<tr>
<td>20; 23–28 years</td>
<td>Mean (SEM)</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>78.5 (6.4)</td>
<td>μmol/g</td>
<td>Tiihonen (2010)</td>
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<tr>
<td>30</td>
<td>Mean (SD)</td>
<td>50.5 (12.6)</td>
<td>13.6 (5.2)</td>
<td>14.1 (7.6)</td>
<td>84.6 (22.9)</td>
<td>μmol/l</td>
<td>McOrist (2008)</td>
</tr>
<tr>
<td>36</td>
<td>Median (IQR)</td>
<td>47.15 (3.80)</td>
<td>13.64 (1.34)</td>
<td>14.73 (1.47)</td>
<td>78.79 (6.19)</td>
<td>μmol/l</td>
<td>McOrist (2012)</td>
</tr>
<tr>
<td>30 overweight</td>
<td>Mean (SD)</td>
<td>56.0 (18.2)</td>
<td>18.3 (7.9)</td>
<td>18.5 (10.1)</td>
<td>98.7 (33.9)</td>
<td>μmol/l</td>
<td>Hylla (1998)</td>
</tr>
<tr>
<td>33 obese</td>
<td>Mean (SEM)</td>
<td>59.8 (18.3)</td>
<td>19.3 (8.7)</td>
<td>18.1 (10.0)</td>
<td>103.9 (34.3)</td>
<td>μmol/l</td>
<td>Schwitz (2010)</td>
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<tr>
<td>Obese subjects</td>
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<tr>
<td>91</td>
<td>Mean (SD)</td>
<td>58.5 (19.1)</td>
<td>17.6 (7.6)</td>
<td>18.3 (9.7)</td>
<td>102.3 (35.5)</td>
<td>mmol/l</td>
<td>Brinkworth (2009)</td>
</tr>
<tr>
<td>32; 20–65 years</td>
<td>Mean (SEM)</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>34 (6)</td>
<td>mmol/24 h</td>
<td>Benassi Evans (2010)</td>
</tr>
<tr>
<td>35 overweight</td>
<td>Mean (SD)</td>
<td>56.0 (18.2)</td>
<td>18.3 (7.9)</td>
<td>18.5 (10.1)</td>
<td>98.7 (33.9)</td>
<td>mmol/l</td>
<td>Schwitz (2010)</td>
</tr>
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</table>

IQR, interquartile range; NR, not reported.
second part of the first year of life when faecal lactate levels fall to negligible values (CA Edwards, unpublished results). By the age of 2 years the pattern becomes more similar to that observed in adults (17). Fig. 1 depicts the changes in SCFA from birth up to adulthood. In the elderly, the microbiota changes, with higher levels of Bacteroidetes (18), which is likely to affect SCFA production. Nevertheless, no differences were detected in SCFA levels in a group of French 68- to 89-year-olds compared with a group of 30- to 46-year-olds (19). In contrast, among participants in the pan-European project on the elderly gut microbiota (CROWNALIFE), elderly Europeans (76 ± 7.5 years; n 55) had lower concentrations of propionate, acetate and butyrate (by 30, 35 and 21 %, respectively) compared with younger adults (40 ± 9.7 years; n 53) (20). With these apparently contradicting results obtained in different studies, it remains to be established what the normal patterns of SCFA in faecal material are during different stages of life.

After uptake in the colonocytes, a considerable part of the SCFA is used as an energy source and is oxidised to carbon dioxide and ketone bodies (21). The fraction that is not consumed by the colonocytes is transported across the basolateral membrane and reaches the liver via the portal bloodstream. Acetate is used by the liver as a precursor for the synthesis of cholesterol and long-chain fatty acids (22). However, in individuals following a Western-type diet, high in refined carbohydrates, sugars and fatty acids and low in fibre, colonic acetate is likely to contribute only little to hepatic lipogenesis. A recent study in obese individuals even showed an inverse association between serum acetate levels and visceral adipose tissue (23). In mice, it was shown that acetate derived from colonic fermentation of fermentable carbohydrates crosses the blood–brain barrier and directly suppresses appetite through central hypothalamic mechanisms (24). Propionic acid is often the second most predominant SCFA and has received much attention for its potential roles in the reduction of lipogenesis, cholesterol synthesis inhibition, and more recently for its activation of G protein-coupled receptors (GPR) 41 and GPR43, release of satiety hormones and other metabolic and anti-inflammatory effects (25–27). Butyric acid has been studied for its ability to promote colonic healing in colitis (28) and its potential anti-cancer effects (29), including apoptosis stimulation (30), in part by inhibiting histone de-acetylase (31). It has also been shown to inhibit oxidative damage in cultured cancer cells (32) and it may improve gut barrier function (33). Recent studies in mice have shown that oral administration of propionate and butyrate, but not acetate, facilitates the extra-thymic de novo generation of anti-inflammatory regulatory T (Treg) cells. In contrast, rectal administration of acetate and propionate, but not butyrate, promoted accumulation of Treg cells, suggesting that butyrate promotes de novo generation but not colonic accumulation of Treg cells, whereas acetate has an opposite activity and propionate is capable of both (34). Furthermore, propionate and butyrate were shown to activate intestinal gluconeogenesis (IGN), which has beneficial effects on glucose and energy homeostasis, via complementary mechanisms. Whereas butyrate directly activates the IGN genes, propionate-mediated induction of IGN depends on a gut–brain communication axis involving the fatty acid receptor GPR41 (35). Those beneficial activities of SCFA produced in the intestine have been shown in different animal species including laboratory animals and production/farm animals (36–38). However, in human subjects the relevant body of evidence is limited mainly because SCFA are traditionally only measured in faeces or fasting blood samples.

In vitro fermentation studies with prebiotics or dietary fibre have consistently resulted in increased levels of SCFA. In contrast, several human prebiotic intervention studies failed to demonstrate increased faecal SCFA, most likely due to the rapid colonic absorption of the SCFA, preventing them from being excreted in faeces (39–44). The relative proportions of the SCFA vary between individuals and are particularly sensitive to the type of carbohydrate being fermented (45, 46). For example, the proportion of propionic acid production is increased during fermentation of
guar gum, long-chain arabinoxylans, oats and oat fractions (oat bran and β-glucan), pectin, pulses, wheat dextrin and pyrodextrins whereas oligofructose predominantly yields acetate. In contrast, the proportion of butyrate production increases with fermentation of starch and inulin-type fructans and often results from secondary fermentation of lactate and acetate, so-called cross-feeding between bacteria. Indeed, this type of microbial cross-feeding could be viewed as an important physiological function supporting microbiota homeostasis and species richness, which have both been associated with gut health. In view of the different effects of acetic, propionic and butyric acids, the relative proportions of the acids produced are probably as relevant as their total levels.

Several animal and human studies have recently demonstrated an association between the gut microbiota composition and obesity. Initial studies found with a higher Firmicutes:Bacteroidetes ratio in the obese than in normal-weight subjects (Table 2), which have more succinate in their caecum and faeces resulting in exacerbation of inflammation. In addition, succinate may be an ulcerogenic agent in the gut lumen, leading to mucosal damage and lower succinate levels in colonic tissues.

**Lactate and succinate.** Lactate and succinate are intermediates in the fermentation process of carbohydrates. In healthy conditions, they are further metabolised to acetate or butyrate and propionate, respectively, by cross-feeding species and do not substantially accumulate in the colonic lumen. Recent evidence suggests that succinate acts as a signal for inflammation. It stabilises the transcription factor hypoxia-inducible factor-1α (HIF-1α) in activated macrophages. When stabilised, HIF-1α up-regulates several genes including the inflammatory cytokine IL-1β, resulting in exacerbation of inflammation.

In addition, succinate acts as a ligand for GPR91, renamed SUNCRI. In the kidney, succinate-induced activation of GPR91 is reported to regulate the renin–angiotensin system and in dendritic cells, succinate signalling is required for enhanced antigen-presenting function. Increased levels of succinate have been linked to IBD as mice undergoing dextran sulfate sodium-induced colitis were shown to have more succinate in their caecum and faeces whereas in colonic tissue from dextran sulfate sodium-induced mice, succinate levels were lower than in control mice. Therefore, succinate may be an ulcerogenic agent in the gut lumen, leading to mucosal damage and lower succinate levels in colonic tissues.

Lactate has two optical isomers, which are L-lactate and D-lactate. L-Lactate is produced from pyruvate by the enzyme lactate dehydrogenase during normal anaerobic metabolism whereas D-lactate is produced by many commensal bacteria in the colon. Increased levels of D-lactate in plasma and urine have been demonstrated in IBD patients, as mice undergoing intestinal ischaemia short bowel and appendicitis are considered as a marker of dysbiosis and/or increased intestinal permeability. In faecal samples of IBD patients, mainly L-lactate levels are increased, suggesting a mucosal origin of lactate. As lactate is a potentially important co-substrate for many sulfate-reducing bacteria, increased colonic lactate levels may promote sulfide generation which is suspected of inhibiting the β-oxidation of butyrate in the colonocytes (see below).
Products of protein metabolism

Microbial products from protein metabolism include BCFA, ammonia, phenol, p-cresol, indole and hydrogen sulfide\(^{(101)}\). The toxic potential of these compounds is mainly derived from in vitro experiments, in which isolated cells or tissues are directly incubated with individual compounds, or from animal studies. The present review also encompasses the results of oral toxicity tests as a reasonable surrogate for assessing potential systemic effects. Minimally irritating concentrations were assessed as a marker of local effects and exceeded 1% for all compounds, which is well above the concentrations occurring in the colon. In human studies, there is little evidence for adverse effects of protein fermentation metabolites\(^{(102)}\).

In a recent study in healthy subjects, modulation of the degree of protein fermentation by changing dietary intake did not affect faecal water toxicity\(^{(103)}\).

An important determinant of the degree of proteolytic \(v\). saccharolytic fermentation is the nutrient availability and in particular the ratio of available carbohydrate to nitrogen\(^{(104,105)}\). Therefore, the production of protein degradation products can generally be reduced by increasing the amount of fermentable carbohydrate reaching the colon in the form of resistant starch\(^{(104)}\) or prebiotic oligosaccharides\(^{(106–112)}\). In contrast, faecal ammonia, phenol and p-cresol were not affected after 4 weeks consumption of the polyol isomalt (30 g/d)\(^{(113)}\).

**Phenol, p-cresol and indole.** The phenolic compounds phenol, p-cresol and indole are the major metabolites of bacterial fermentation of the aromatic amino acids tyrosine, phenylalanine and tryptophan. These metabolites are largely and rapidly absorbed by the colonic mucosa cells and are excreted in urine after sulfate or glucuronide conjugation in the mucosa or the liver\(^{(114)}\). In healthy subjects, these compounds do not accumulate in the body. Therefore, their urinary elimination is often considered as a reliable estimate of their production in the colon\(^{(105)}\).

Many studies have reported significant interindividual variation in the urinary excretion of p-cresol and phenol in healthy adults. Data on ranges in other age groups (children, elderly) are scarce. Reported mean or median values vary between 10 and 55 mg/d for p-cresol (Table 3) and between 4 and 7.5 mg/d for phenol (Table 4). In obese individuals, urinary p-cresol and phenol levels at baseline were considerably higher than those reported in normal-weight adults (94–9 mg/d and 150 g/d for p-cresol and phenol, respectively) and decreased upon weight loss\(^{(63)}\). The levels of urinary p-cresol may increase in the very old\(^{(115)}\).

Most studies on the urinary excretion of the indole metabolite indoxyl sulfate, also called indican, report values below 50 mg/d in healthy adults. In patients with liver cirrhosis\(^{(116)}\) and patients with diabetes\(^{(117)}\), excretion of indoxyl sulfate is higher (98.2 mg/d in cirrhosis, 65.7 mg/d in diabetics with neuropathy) and correlates with steatorrhoea. In a study in patients with bladder cancer there was no evidence that phenolic microbial metabolites had promoting or co-carcinogenic activity for the human urinary bladder, as the urinary excretion of p-cresol, phenol and indoxyl sulfate was not different in the patients as compared with controls\(^{(118)}\).

Faecal excretion of phenolic compounds is not often reported, but in available studies amounts of 5–8 mg/d for p-cresol and 0.25–0.66 mg/d for phenol have been found. Interestingly, faecal excretion of p-cresol was 4-fold higher in a group of hyperactive children as compared with control children\(^{(119)}\).

The effects of phenolic compounds on intestinal cells have been determined mainly in in vitro incubation studies. Viability of colonic epithelial cells isolated from human biopsies was decreased after exposure to 1.25 mM-phenol, a physiologically relevant concentration, whereas higher phenol concentrations (20 mM) were required to reduce viability of HT-29 cells\(^{(120)}\). Notably, cell cultures from ulcerative colitis (UC) patients showed similar sensitivity to phenol exposure as cell cultures from control subjects at all concentrations tested.

Several papers have reported a concentration-dependent increase in paracellular permeability and reduced epithelial barrier function after incubation with phenol (1 \(\mu\)M to 21 \(\mu\)M) of Caco-2-monolayers or SK-CO15 intestinal cells\(^{(121,122)}\). Enhanced permeability was already apparent at concentrations of phenol that did not cause cell death. Similarly, p-cresol altered endothelial barrier function in human umbilical vein endothelial cells. In chronic kidney disease patients, p-cresol is considered a uraemic toxin. It accumulates in serum and might participate in the endothelial dysfunction that is observed in such patients\(^{(123)}\).

The European Food Safety Authority recently evaluated the toxicity of phenol following oral administration (http://www.efsa.europa.eu/en/efsajournal/doc/3189.pdf) and established a tolerable daily intake (TDI) of 0.5 mg/kg body weight per d. For a 75 kg individual, the TDI amounts to 37.5 mg/d, which is about 5-fold higher than the amount of phenol generated in the colon (7.5 mg/d) (assuming that urinary excretion rates reflect colonic generation rates). On repeat-dose administration to non-pregnant rats and mice, no consistent effects were seen at doses \(\geq\) 250 mg/kg body weight per d.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) reviewed the oral toxicity of p-cresol in 2011. The systemic toxicity of p-cresol was evaluated in a 2-year study in rats following dietary administration as a 60:40 mixture of \(m\)–\(p\)-cresol. (http://www.inchem.org/documents/jecfa/jecmono/v64ej01.pdf). A no observed adverse effect level of 230 mg/kg body weight per d was identified, based on increased incidence of renal tubule adenomas in male rats at 720 mg/kg body weight per d. Effects seen in other studies (nasal sinuses, forestomach) were attributed to the local irritancy of p-cresol and did not reflect its
<table>
<thead>
<tr>
<th>Biofluid</th>
<th>Subjects (n); age</th>
<th>Reported measure</th>
<th>p-Cresol excretion Unit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>11; 35 ± 10 years</td>
<td>Mean (SEM)</td>
<td>454 (92)*</td>
<td>Birkett (1996) (104)</td>
</tr>
<tr>
<td></td>
<td>27; median 25 (IQR 23–29) years</td>
<td>Median (IQR)</td>
<td>168 (93–3–304) and 208 (114–288)</td>
<td>Damen (2012) (107)</td>
</tr>
<tr>
<td></td>
<td>9; range 19–69 years</td>
<td>Mean (SEM)</td>
<td>408 (271·3)</td>
<td>Ling (1992) (245)</td>
</tr>
<tr>
<td></td>
<td>11; 39 ± 11 years</td>
<td>Median (IQR)</td>
<td>532 (250–659)† p-cresyl sulfate</td>
<td>Patel (2012) (246)</td>
</tr>
<tr>
<td></td>
<td>32; range 47–95 years</td>
<td>Mean (SD)</td>
<td>510 (358)*</td>
<td>Renwick (1988) (118)</td>
</tr>
<tr>
<td></td>
<td>10; range 22–45 years</td>
<td>Mean (SD)</td>
<td>248 (99)*</td>
<td>De Preter (2004) (247)</td>
</tr>
<tr>
<td></td>
<td>9; range 22–45 years</td>
<td>Median (IQR)</td>
<td>315 (206)*</td>
<td>De Preter (2007) (248)</td>
</tr>
<tr>
<td></td>
<td>15; 23 ± 1 years</td>
<td>Mean (SD)</td>
<td>164 (101)*</td>
<td>De Preter (2007) (249)</td>
</tr>
<tr>
<td></td>
<td>15; 25 ± 1 years</td>
<td>Mean (SD)</td>
<td>187 (96)*</td>
<td>De Preter (2007) (249)</td>
</tr>
<tr>
<td></td>
<td>10; 21 ± 1 years</td>
<td>Median (IQR)</td>
<td>196 (168–322)*</td>
<td>De Preter (2007) (249)</td>
</tr>
<tr>
<td></td>
<td>20; median 23 (IQR 21–24) years</td>
<td>Median (IQR)</td>
<td>297 (194–437)</td>
<td>Cloetens (2010) (108)</td>
</tr>
<tr>
<td></td>
<td>20; range 19–41 years</td>
<td>Median (IQR)</td>
<td>297 (239–349)</td>
<td>Windey (2012) (103)</td>
</tr>
<tr>
<td></td>
<td>19; range 21–53 years</td>
<td>Mean (SEM)</td>
<td>218 (58)</td>
<td>Gostner (2006) (113)</td>
</tr>
<tr>
<td>Faeces</td>
<td>11; range 3–11 years</td>
<td>Mean (SEM)</td>
<td>0·54 (0·29)</td>
<td>Adams (1985) (119)</td>
</tr>
<tr>
<td></td>
<td>11; 35 ± 10 years</td>
<td>Mean (SEM)</td>
<td>0·60 (0·07)*</td>
<td>Benassi-Evans (2010) (244)</td>
</tr>
<tr>
<td></td>
<td>112; range 0–1 years</td>
<td>Mean (SD)</td>
<td>0·14 (0·14)</td>
<td>Boler (2011) (111)</td>
</tr>
<tr>
<td></td>
<td>15; 23 ± 1 years</td>
<td>Mean (SD)</td>
<td>124 (36)*</td>
<td>Benassi-Evans (2010) (244)</td>
</tr>
<tr>
<td></td>
<td>15; 22 ± 1 years</td>
<td>Mean (SD)</td>
<td>161 (53)*</td>
<td>Benassi-Evans (2010) (244)</td>
</tr>
<tr>
<td></td>
<td>15; 23 ± 1 years</td>
<td>Mean (SD)</td>
<td>101 (43)*</td>
<td>Benassi-Evans (2010) (244)</td>
</tr>
<tr>
<td></td>
<td>16; range 23–66 years</td>
<td>Mean (SEM)</td>
<td>58·86 (7·3)</td>
<td>Benassi-Evans (2010) (244)</td>
</tr>
<tr>
<td></td>
<td>20; range 18–24 years</td>
<td>Mean (SEM)</td>
<td>0·52 (0·05)*</td>
<td>Benassi-Evans (2010) (244)</td>
</tr>
<tr>
<td></td>
<td>19; range 21–53 years</td>
<td>Mean (SEM)</td>
<td>0·36 (0·04)*</td>
<td>Benassi-Evans (2010) (244)</td>
</tr>
<tr>
<td></td>
<td>21; range 21–28 years</td>
<td>Mean (SEM)</td>
<td>1·5 (0·20)</td>
<td>Benassi-Evans (2010) (244)</td>
</tr>
<tr>
<td>Obese</td>
<td>91, range 24–64 years</td>
<td>Mean (SD)</td>
<td>879 (00) and 524 (259)*</td>
<td>Brinkworth (2009) (63)</td>
</tr>
<tr>
<td>Urine</td>
<td>33, range 20–65 years</td>
<td>Mean (SD)</td>
<td>0·54*</td>
<td>Brinkworth (2009) (63)</td>
</tr>
<tr>
<td>Faeces</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IQR, interquartile range.

* Calculated from reported values in mg/d using a molecular mass value for p-cresol of 108.

† Calculated from reported values in mg/d using a molecular mass value for p-cresyl sulfate of 188.
Table 4. Reported excretion of phenol in urine and faeces

<table>
<thead>
<tr>
<th>Biofluid</th>
<th>Subjects (n); age</th>
<th>Reported measure</th>
<th>Phenol excretion</th>
<th>Unit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>11; 35 ± 10 years</td>
<td>Mean (SEM)</td>
<td>53 (0)</td>
<td>μmol/d</td>
<td>Birkett (1996)³⁰⁴</td>
</tr>
<tr>
<td>27; median 25 (IQR 23–29) years</td>
<td>Median (IQR)</td>
<td>47 (37–66) and 43 (35–59)</td>
<td>μmol/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9; range 19–69 years</td>
<td>Mean (SEM)</td>
<td>73 (45)</td>
<td>μmol/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32; range 47–95 years</td>
<td>Mean (SD)</td>
<td>67 (91)</td>
<td>μmol/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15, 23 ± 1 years</td>
<td>Mean (SD)</td>
<td>45 (21)</td>
<td>μmol/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15; 22 ± 1 years</td>
<td>Mean (SD)</td>
<td>42 (26)</td>
<td>μmol/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15; 23 ± 1 years</td>
<td>Mean (SD)</td>
<td>46 (22)</td>
<td>μmol/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20; median 23 (IQR 21–24) years</td>
<td>Median (IQR)</td>
<td>55 (31–76)</td>
<td>μmol/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Faeces</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11; range 3–11 years</td>
<td>Mean (SEM)</td>
<td>115 (32)</td>
<td>μmol/d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11; 35 ± 10 years</td>
<td>Mean (SEM)</td>
<td>0.083 (0.034)</td>
<td>μmol/g faeces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>112; range 0–1 years</td>
<td>Mean (SD)</td>
<td>0.58 (0.84)</td>
<td>μmol/g faeces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15, 23 ± 1 years</td>
<td>Mean (SD)</td>
<td>8.4 (7.1)</td>
<td>μmol/g faeces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15; 22 ± 1 years</td>
<td>Mean (SD)</td>
<td>21.0 (16.5)</td>
<td>μmol/g faeces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16; range 23–66 years</td>
<td>Mean (SEM)</td>
<td>0.07 (0.13)</td>
<td>μmol/g faeces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20; range 18–24 years</td>
<td>Mean (SEM)</td>
<td>0.0003 (0)</td>
<td>μmol/g dry weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19; range 21–53 years</td>
<td>Mean (SD)</td>
<td>0.058 (0.015)</td>
<td>μmol/g faeces</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obese</td>
<td>33, range 24–66 years</td>
<td>Mean (SD)</td>
<td>158 (119) and 159 (184)</td>
<td>μmol/d</td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td>0.037</td>
<td>μmol/g faeces</td>
<td>Benassi-Evans (2010)³⁴⁴</td>
</tr>
</tbody>
</table>

IQR, interquartile range. *Calculated from reported values in mg/kg using a molecular mass value for phenol of 94.
The toxic potential of hydrogen sulfide on colonic cells has been extensively investigated. Sulfide influences oxidative metabolism of colonic epithelial cells by inhibiting cytochrome oxidase activity which catalyses the reduction of oxygen to water\(^{132,133}\). Several lines of evidence also suggest a role of hydrogen sulfide in the aetiology and/or risk of relapse of UC. In experimental animal models, a pathological condition similar to UC can be induced using undigestible sulfates in the form of dextran sulfate or risk of relapse of UC. In experimental animal models, a pathological condition similar to UC can be induced using undigestible sulfates in the form of dextran sulfate or as a regulator of colonic Na\(^+\) absorption\(^{128} \) but as a high sulfur or sulfate intake was associated with increased likelihood of relapse in UC patients\(^{130}\). Exposure of non-transformed rat intestinal crypt cells (IEC-18 cells) to sodium hydrogen sulfide (50 \(\mu\)M) caused acute hypoxia and promoted early cell-cycle entry with an associated up-regulation of genes coding for proteins related to proliferative activity\(^{139}\). A series of in vitro experiments by Attene-Ramos et al.\(^{140}\) revealed that hydrogen sulfide provokes genomic DNA damage in colonic cancer cells (HT-29 cells) at concentrations of 250 \(\mu\)M. No cellular metabolism was required for sulfide to induce genotoxicity and co-incubation with a radical scavenger reduced DNA damage induced by hydrogen sulfide, suggesting a radical-mediated mechanism\(^{141}\). In non-transformed human intestinal epithelial cells, the expression of genes involved in cell-cycle progression, inflammation and DNA repair response was modulated by sulfide. In particular, expression of the cyclo-oxygenase (COX)-2 gene, which is elevated in most human colorectal cancers (CRC), was significantly up-regulated\(^{142}\). Overexpression of COX-2 may play a decisive role in promoting CRC initiation or progression through the stimulation of angiogenesis, inhibition of apoptosis and increasing the proliferation in intestinal epithelial cells\(^{143}\).

In contrast to those reports on harmful effects, hydrogen sulfide is now also known to be a systemic signalling molecule. It is endogenously produced in micromolar concentrations from cysteine by the action of cystathionine \(\gamma\)-lyase and cystathionine \(\beta\)-synthase. At these low concentrations, it has been proposed that hydrogen sulfide is involved in neuromodulation of chloride secretion, in controlling ileum contractility and in nociception from the large intestine\(^{144}\). Blachier et al. proposed as a working hypothesis that any imbalance between levels of free sulfide in the large intestine and the capacity of epithelial cells to metabolise it will result in a loss of normal oxidative cell capacity\(^{144}\).

Information on the effects of oral exposure to hydrogen sulfide is very limited. The US Environmental Protection Agency (EPA) established a reference dose on the basis of effects observed in pigs, but subsequently withdrew this as it was concluded that the effect was irreproducible (http://www.epa.gov/iris/subst/0061.htm). Although the US EPA has established an inhalation reference concentration for hydrogen sulfide, this is based on local effects and hence is not suitable for assessing the systemic toxicity of the compound.

**Branched-chain fatty acids.** The BCFA isobutyrate, 2-methylbutyrate and isovalerate are produced by bacterial fermentation of valine, isoleucine and leucine, respectively. These BCFA constitute approximately 5–10 % of the total SCFA\(^{145}\). Similar to other protein fermentation metabolites, faecal BCFA concentrations are reduced after prebiotic intake\(^{106,146,147}\). In an in vitro incubation experiment with five different epithelial cell lines; minimal concentrations of isovalerate to induce cytotoxicity were lower than the concentrations produced by intestinal bacteria and both isovalerate and isobutyrate were able to induce apoptosis\(^{148}\). Several in vitro studies indicate that BCFA affect the exchange of ions in the colon and may act as a regulator of colonic Na\(^+\) absorption\(^{126}\) but little information is available regarding other effects of BCFA on colonic epithelial cells. Therefore, faecal

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**Table 5. Reported excretion of ammonia in faeces**

<table>
<thead>
<tr>
<th>Population</th>
<th>Subjects (n); age</th>
<th>Reported measure</th>
<th>Ammonia excretion</th>
<th>Unit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11; 35 ± 10 years</td>
<td>Mean (SEM)</td>
<td>23.3 (1.5)</td>
<td>(\mu)mol/g faeces</td>
<td>Birkett (1996)(^{104})</td>
<td></td>
</tr>
<tr>
<td>112; range 0–1 years</td>
<td>Mean (SD)</td>
<td>7.7 (7.1)</td>
<td>(\mu)mol/g faeces</td>
<td>Heavey (2003)(^{251})</td>
<td></td>
</tr>
<tr>
<td>16; range 23–66 years</td>
<td>Mean (SEM)</td>
<td>14.1 (1.7)</td>
<td>(\mu)mol/g faeces</td>
<td>Clarke (2011)(^{252})</td>
<td></td>
</tr>
<tr>
<td>12; range 27–49 years</td>
<td>Mean (SD)</td>
<td>5.1 (2.5)</td>
<td>(\mu)mol/g faeces</td>
<td>Slavin (2011)(^{41})</td>
<td></td>
</tr>
<tr>
<td>9; 59 ± 3 years</td>
<td>Mean (SD)</td>
<td>2.9 (0.5)</td>
<td>(\mu)mol/g faeces</td>
<td>Bianchi (2010)(^{253})</td>
<td></td>
</tr>
<tr>
<td>46; 31–66 years</td>
<td>Mean (95 % CI)</td>
<td>14.6 (12.6, 16.7)</td>
<td>(\mu)mol/kg faeces</td>
<td>McO'Nest (2011)(^{254})</td>
<td></td>
</tr>
<tr>
<td>20; range 20–55 years</td>
<td>Mean (SEM)</td>
<td>22.6 (1.6)</td>
<td>(\mu)mol/g faeces</td>
<td>Tihonen (2010)(^{127})</td>
<td></td>
</tr>
<tr>
<td>8; range 21–60 years</td>
<td>Mean (SD)</td>
<td>35.2 (7.9)*</td>
<td>(\mu)mol/g faeces</td>
<td>Shinohara (2010)(^{110})</td>
<td></td>
</tr>
<tr>
<td>21; range 21–28 years</td>
<td>Mean (SEM)</td>
<td>137.6 (7.82)</td>
<td>(\mu)mol/g dry weight</td>
<td>Boler (2011)(^{111})</td>
<td></td>
</tr>
<tr>
<td>36; range 22–67 years</td>
<td>Median (IQR)</td>
<td>24.6 (16.5–30.0)</td>
<td>(\mu)mol/g faeces</td>
<td>Nemoto (2012)(^{245})</td>
<td></td>
</tr>
<tr>
<td>Obese</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19; range 20–55 years</td>
<td>Mean (SEM)</td>
<td>26.6 (2.2)</td>
<td>(\mu)mol/g faeces</td>
<td>Tihonen (2010)(^{127})</td>
<td></td>
</tr>
<tr>
<td>121; 24–64 years</td>
<td>Mean (SD)</td>
<td>26.5 (9.2)</td>
<td>(\mu)mol/g faeces</td>
<td>Brinkworth (2009)(^{253})</td>
<td></td>
</tr>
</tbody>
</table>

IQR, interquartile range.

*Calculated from reported values in mg/d using a molecular mass value for ammonia of 17.
concentrations of BCFA are considered only as markers for bacterial protein fermentation rather than markers of colonic health\(^{(114)}\).

**Plant polyphenolic compounds/catabolites**

While the physiological relevance of polyphenol catabolites derived from the gut microbiota is currently understudied, it is recognised that the majority of plant bioactive compounds must first be rendered biologically available, often through degradation and hydrolysis by the gut microbiota before being absorbed by the host, and that microbial metabolic transformation can make an impact on polyphenol biological activity. While certain classes of phytochemical are broken down to unique catabolites, many different classes of polyphenol give rise to common small phenolic compounds. However, we currently do not know the physiological relevance of many of these compounds, or even the habitual or 'normal' concentration ranges of these phenolic acids, or how they respond to diet. For a few of these compounds, for example, the phyto-oestrogens equol, enterolactone and enterodiol, and the uro lithins, specific health effects have been suggested\(^{(150)}\). Table 6 provides an overview of the microbial catabolites of common plant polyphenols and their putative health effects. For an up-to-date review on plant polyphenols catabolites and their putative health effects, see Del Rio et al.\(^{(150)}\) and Dall'Asta et al.\(^{(151)}\).

Furthermore, the interaction between polyphenols and the microbiota is bi-directional. Recent evidence has shown that a number of polyphenols and their metabolites cause a selective stress or stimulus to some micro-organisms and influence other metabolic pathways like the production of SCFA\(^{(152,153)}\). In addition, in vitro incubation of faecal samples with quercetin-3-O-ruteno side (rutin) in the present of glucose as a carbon source showed a significant increase in deglycosylation of rutin and catabolism of quercetin, suggesting that prebiotic intervention might modify the bacterial metabolism of plant polyphenols\(^{(154)}\).

In metabolomic studies (see below) many of the compounds encountered relate to microbial catabolites of polyphenols that escape absorption in the small intestine\(^{(155,156)}\). These may become key markers of colonic bacterial activity.

**Emerging metabolites**

A range of amino acids and related molecules, including tryptophan, γ-aminobutyric acid, γ-hydroxybutyric acid, or biogenic amines and also host–microbiota co-metabolic metabolites including catecholamines like dopamine and noradrenaline, and bile acids have the potential to make an impact both in a beneficial or harmful way with the host depending on concentration and chemical profile\(^{(157)}\). Recent scientific interest has been focused on microbiota production of cell-signalling molecules and neurotransmitters which through the gut–liver–brain axis appear to regulate a number of diverse physiological functions including energy intake and expenditure, brain development and cognitive function and mood\(^{(158,159)}\). However, by and large, human data are scarce and it needs to be evaluated whether prebiotic intervention might affect these signalling pathways.

**Factors that influence fermentation**

The mechanisms controlling the metabolic activities of the colonic microbiota are only partly understood.

First of all, the type and quantity of dietary carbohydrate entering the colon have a dramatic impact on SCFA production. Many factors make an impact on the digestibility of carbohydrate in foods, not least their intrinsic chemical structure or biological availability in low-processed plant-derived foods. However, other food macromolecules ingested at the same meal, for example, red wine polyphenols or fat, and the load of complex carbohydrates like starch, can also determine the amount that reaches the colon\(^{(160,161)}\). Similarly, the extent of carbohydrate fermentation by the gut microbiota may be affected by other food components, for example, complex polyphenols, which may have antibacterial activity\(^{(162)}\).

Besides the diet, the specific phylogenetic and functional composition of the gut microbiota is influenced by a range of factors including host genetics, immunological factors and environmental factors including use of drugs such as antibiotics\(^{(163)}\). The large-intestinal microbiota has a strongly individual composition in human subjects and exhibits a remarkable compositional stability over time\(^{(164)}\), but is also amenable to dietary modulation\(^{(165)}\). Whereas some bacterial species are able to degrade a wide variety of substrates, other bacteria are nutritionally highly specialised\(^{(166)}\).

In addition, key geographic differences exist in bacteria and metabolites in different populations in different countries and regions, for example, in North and South China\(^{(166)}\), different countries in Europe\(^{(163)}\) and different populations (Afro-, Caucasian and Native-Americans) in the USA\(^{(167)}\). Some of these differences may be due to diet and lifestyle but genetic background may also be involved. These differences are important to take into account when investigating the relationship between bacterial metabolism and disease.

Finally, fermentation patterns are also determined by colonic transit times\(^{(168)}\). In a study by Cummings et al. a significant correlation was observed between colonic transit times and urinary excretion rates of phenols\(^{(169)}\). With shorter transit times, turnover rate is faster and microbial growth is more efficient, resulting in a greater mass of bacteria\(^{(170)}\). Similarly, in in vitro fermentation experiments with faecal inocula from volunteers with pharmacologically modified transit times, reduction of transit time was
<table>
<thead>
<tr>
<th>Plant polyphenol</th>
<th>Microbial catabolite</th>
<th>Possible health effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-(3-Hydroxyphenyl)propionic acid</td>
<td>Antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilisation</td>
<td>Roowi (2010)(256)</td>
</tr>
<tr>
<td>(-)-Epigallocatechin</td>
<td>4-Hydroxyphenylacetic acid</td>
<td>Antimicrobial/antimycotic activity in vitro</td>
<td>Roowi (2010)(256)</td>
</tr>
<tr>
<td>(-)-Epigallocatechin-3-O-gallate</td>
<td>Pyrocathecol</td>
<td></td>
<td>Ko (2009)(256)</td>
</tr>
<tr>
<td></td>
<td>Pyrogallol</td>
<td>Antibacterial activity (especially against Gram-negative enterobacteria)</td>
<td>Roowi (2010)(256)</td>
</tr>
<tr>
<td></td>
<td>4-Hydroxyphenylacetic acid</td>
<td>An acetylcholinesterase inhibition greater than gallic acid parent inhibition of Vibrio spp. quorum sensing</td>
<td>Ni (2008)(258)</td>
</tr>
<tr>
<td>Quercetin</td>
<td>2-(3,4-Dihydroxyphenyl)acetic acid</td>
<td></td>
<td>Selma (2009)(263)</td>
</tr>
<tr>
<td></td>
<td>3,4-Dihydroxybenzoic acid</td>
<td></td>
<td>Larrosa (2006)(264)</td>
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<td></td>
<td>Phloroglucin</td>
<td></td>
<td>Selma (2009)(263)</td>
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<td>Kaempferol</td>
<td>2-(4-Hydroxyphenyl)acetic acid</td>
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<td>Naringenin</td>
<td>3-(3-Hydroxyphenyl)propionic acid</td>
<td>Antimicrobial activity against Gram-negative enterobacteria via outer membrane destabilisation</td>
<td>Selma (2009)(263)</td>
</tr>
<tr>
<td></td>
<td>Phloroglucin</td>
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<td>Isoxanthohumol</td>
<td>8-Prenylnaringen</td>
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<td>Catechin and epicatechin</td>
<td>3-(3-Hydroxyphenyl)propionic acid</td>
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<td>5-(3',4'-Dihydroxyphenyl)-γ-valerolactone</td>
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<td>2,3-Dihydroxyphenoxyl 3-(3',4'-dihydroxyphenyl) proionic acid</td>
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associated with increased production of SCFA and increased disappearance of substrate\(^{(171)}\). Although fermentation of carbohydrate occurs mainly in the proximal colon, a mixture of fermentable and less fermentable carbohydrates in the diet can push fermentation further around the colon and thus increase SCFA also in the more distal parts of the colon\(^{(172–175)}\). The European Food Safety Authority has recently approved two health claims for wheat bran in relation to two beneficial physiological effects, namely an increase in faecal bulk and a reduction of intestinal transit time. Whether increased SCFA production is responsible for the increased transit time in humans remains to be studied. In animal models, SCFA inhibit peristaltic contractile activity, however, only at concentrations above a physiological threshold\(^{(176)}\). In addition, the motor effects of SCFA may differ between species as intracolonic infusion of a 100 mM-SCFA solution did not modify transit in two healthy human subjects\(^{(177)}\). In a recent study in ten volunteers, infusion of a 100 mM-SCFA solution did not affect the phasic or tonic motor activity of the colon or the number of high-amplitude-propagated contractions\(^{(178)}\).

### Gaps and limitations

**Where to measure: choice of the biomatrix**

A major obstacle in the evaluation of intestinal bacterial metabolism *in vivo* in human subjects is the inability to directly sample at the site of production. Therefore, much information has been obtained from analysis of faecal samples and supportive data from *in vitro* and experimental models. However, information on the activity of the intestinal microbiota can be derived from analysis of various biological samples including faecal samples, serum or plasma and urine samples. Zhao *et al.*\(^{(174)}\) nicely showed that a modification of the intestinal microbiota in mice resulted in altered metabolite patterns in faeces. Administration of non-absorbable antibiotics resulted in increased levels of *Bacteroides* and *Enterococcus* species and was accompanied by a reduction in the overall fermentation of indigestible carbohydrates with lower levels of SCFA, lower levels of many amino acids and a disturbance of bile acid metabolism\(^{(174)}\). In contrast, the faecal metabolome in horses was shown not to be representative of the colonic metabolome\(^{(179)}\), which reflects the different patterns of bacterial metabolism and the absorption of products in different parts of the colon. Similarly, the impact of microbiota activity is reflected in the levels of several serum metabolites\(^{(180)}\). Interestingly, the host responds to many of those metabolites with phase II metabolism comparable with the response to drugs, as many metabolites are sulfated, glycine conjugated or glucuronidated to facilitate urinary excretion.

In recent years, many research efforts have focused on the mechanisms by which the SCFA acetate, propionate and butyrate affect host physiology. Nevertheless, reliable
and quantifiable methodologies have rarely been employed to measure the relative SCFA production for different fibres in human subjects, or to quantify their relative contribution to circulating SCFA pools, for example, using stable isotope tracking or pharmaco- or nutri-kinetic approaches\(^{(181)}\). In addition, very few studies have examined the time course of SCFA production, absorption and utilisation after prebiotic intervention or examined the impact of other food components, underly host disease or gut microbiota composition and genetic potential on these processes. Therefore it has been difficult to convincingly prove in human subjects a role for colonic SCFA produced from prebiotics in the key physiological processes proven to be regulated by SCFA in animal models. There is a critical need for multidisciplinary studies to address these questions and take that final mechanistic step from animal model to human physiology and health.

**When to measure: snapshot analysis**

Intestinal microbial fermentation is a dynamic process influenced by a wide range of factors (see above). Therefore, the nature and concentration of metabolites produced by the microbiota is context-dependent and the levels of each metabolite are a result of metabolic fluxes of highly variable rates, which are not adequately represented in steady-state metabolite profiles. The rapid uptake and conversion of metabolic intermediates, as well as their removal from the intestinal lumen through host absorption creates a highly dynamic system, which is strongly discrepant with the methodologies for analyses that can only provide a single time point quantification or ‘snapshot’. At present, very little is known about the short- or long-term variation in metabolite concentrations produced by the gut bacteria. The dynamic analysis of metabolic conversions within the microbiota may be further unravelled through the application of stable isotope-labelled nutrients\(^{(182)}\) that, in combination with metabolic modelling (for example, including the use of meta-transcriptome or meta-proteome datasets), may enable the determination of metabolic fluxes in the microbiota and the host mucosa.

**More holistic approach**

**Functional analysis of faecal water**

Prebiotics can affect the levels of many compounds in the gastrointestinal tract. These may be constitutively produced in the body, such as bile acids, or may be bacterial metabolites, such as SCFA. This complicates assessment of the potential effects of prebiotics on human health based on evaluation of individual substances. Hence, to supplement such assessment, a more holistic approach to the effects of prebiotics on the biological activity of the gastrointestinal milieu is required. One approach that might be useful for this purpose is the functional analysis of faecal water (for a review, see Osswald et al.\(^{(183)}\)). This should provide an integrated measure of the overall contribution of the compounds present to a defined biological endpoint, such as genotoxicity.

Faecal water has a number of potential advantages in such studies. It is non-invasive, it can reflect the effects of diet directly, it samples the initial target compartment, the gastrointestinal tract, it provides a measure of the total activity of what was present in the distal colon, it can provide repeated measurements over time, and subjects can serve as their own controls. Disadvantages include the practicalities of sample collection and the reluctance of some subjects to provide samples, the complexity of the biofluid may interfere in the assessment of some endpoints and it may not always be truly representative of the biological compartment of interest, because of modulation of intestinal contents before faecal excretion.

Perhaps the endpoint most widely assessed using faecal water is genotoxicity. A number of assays have been used for this purpose, including the Ames Salmonella test and SOS Chromo test for bacterial mutagenicity\(^{(184–187)}\). Over the past 15 years, those bacterial mutagenicity assays have been almost completely replaced by assays using mammalian cells as targets\(^{(187)}\). The Comet assay for DNA strand breaks in enterocyte cells (Caco-2, HT-29 and Hep G2 (liver derived)) has been commonly used to assess the genotoxic potential of faecal water. The considerable inter- and intra-individual variability between samples and individuals, possibly reflecting the effects of dietary variation, constitutes a major limitation. Use of a controlled dietary regimen for 9 d was of insufficient length to reduce interindividual variability, suggesting that changing the genotoxic potential of the gut microbiota may require much longer\(^{(188)}\). There is no ready means for normalisation of sample activity, equivalent, for example, to creatinine in urine. Efforts to use wet or dry weight of the stool have met with little success. However, the method of preparation of faecal water has little effect on the biological measurement that facilitates comparison across studies\(^{(103)}\).

The toxicity of faecal water has been assessed using a number of endpoints. Cytotoxicity has been measured by colorimetric cell viability assays based on the cleavage of a tetrazolium salt (for example, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or water-soluble tetrazolium salt 1 (WST-1)) in the mitochondria of living cells to a coloured formazan derivative\(^{(189,190)}\). Changes in barrier function are assessed from impairment of tight junctions using transepithelial resistance\(^{(191,192)}\) or phenol red leakage\(^{(193,194)}\) in Caco-2 cells, and invasive potential of HTC116 cells using fluorescence-activated cell sorting (FACS) analysis\(^{(191)}\). In addition, the lytic activity of faecal water to erythrocytes has been quantified as a parameter of cytotoxicity and was significantly correlated to colon cell proliferation\(^{(195)}\). Finally, the effects of faecal water on apoptosis rates in the human colon-derived cell lines HT-29 and FHC (fetal human cells)
have been evaluated\textsuperscript{190}. A number of apoptotic hallmarks were measured: changes in cell morphology, DNA fragmentation, FACS analysis of DNA strand breaks assessed using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, and poly(ADP-ribose) polymerase cleavage.

In addition, several assays have been used to measure the effects of faecal water on cell proliferation. Using two human colon carcinoma cell lines, HT-29 and HCT 116, faecal water or their lipid extracts were found to activate activator protein-1 (AP-1), a transcription factor associated with the promotion of neoplastic transformation\textsuperscript{197}, and to induce COX-2 promoter activity, which has been implicated in colon carcinogenesis\textsuperscript{198}. Faecal water also inhibited cell cycle progression in HT-29 cells and down-regulated the gene expression of proliferating cell nuclear antigen, a protein essential for replication\textsuperscript{199}.

Results on the effects of oligofructose and/or inulin treatment on the genotoxicity of faecal water in rats have been equivocal\textsuperscript{200}. In a study in rats treated with azoxymethane, DNA damage assessed using the Comet assay was reduced with faecal samples obtained after 4 or more months of prebiotic treatment. Evidence from this study suggested that the antigenotoxic effects of prebiotics occur rapidly and that azoxymethane-induced tumour development increases the genotoxicity of faecal water\textsuperscript{201}.

Relatively few studies to date have evaluated the effects of prebiotic administration on the biological effects of faecal water samples from human subjects. Feeding male smokers and non-smokers either plain sourdough bread, bread supplemented with prebiotics (inulin, linseed and soya flours) or bread additionally supplemented with antioxidants resulted, in the non-smoker group, in about 50% reduction in DNA strand breaks induced by faecal water with both the control and test breads\textsuperscript{202}. In smokers, the control and test breads reduced faecal water genotoxicity only in those with the glutathione S-transferase genotype GSTM1*0. Administration of polydextrose (8 g/d for 3 weeks) reduced faecal water genotoxicity using the Comet assay in thirty-three healthy subjects in a double-blind placebo controlled cross-over study\textsuperscript{244}. Similarly, supplementation of the diet with konjac glucomannan (4·5 g/d) for 4 weeks in thirty healthy subjects significantly reduced faecal water genotoxicity\textsuperscript{203}. In contrast, no changes in faecal water genotoxicity were observed after 4 weeks treatment with galacto-oligosaccharides (4 g/d) in a population above 50 years\textsuperscript{204}.

Measurement of biological activity of faecal water samples is an attractive means of linking changes in the colonic contents with health outcomes. However, there are a number of potential limitations to this methodology. Standardisation of the assay protocols in terms of target cells and sample preparation is mandated to allow comparison of data between studies. To date there have been few studies using this approach in the evaluation of prebiotics.

**Metabolomics**

Metabolomics offers an alternative and holistic approach to understanding the interaction between the human gut microbiome and host metabolism as well as to identify possible biomarkers of gut health. Metabolic studies allow simultaneous evaluation of a wide range of metabolites by a top-down approach bypassing the need for an *a priori* hypothesis. Several analytical platforms allow detection, identification and quantification of different ranges of molecules, including \(^1\)H-NMR, LC-MSMS and GC-MS\textsuperscript{205,206}. However, due to the chemical diversity and different physico-chemical properties of the metabolites and the large dynamic range of metabolite concentrations in different biological samples, it is virtually impossible to measure the complete metabolome. In addition, the term ‘metabolome’ can refer to faeces\textsuperscript{205}, urine or plasma\textsuperscript{207}. By selecting a specific analytical platform and a biofluid in which metabolites will be measured, the metabolome will be reduced to those specific conditions. Metabolome approaches can be non-targeted so that any compounds are considered in a pattern, or targeted where more specific types of molecules are identified and sometimes quantified. In general, however, the strategy is to discover patterns of metabolites which are associated with disease states such as cancer\textsuperscript{208–210}, the metabolic syndrome\textsuperscript{211}, obesity\textsuperscript{212}, CVD, diabetes\textsuperscript{213}, gut disease such as UC\textsuperscript{205}, irritable bowel syndrome\textsuperscript{214}, peptic ulcer\textsuperscript{215} and the impact of changes in body weight or diet\textsuperscript{150,211,216,217}.

Although broad-spectrum metabolomics is shedding new light on the metabolites derived from the gut microbiota at an unprecedented resolution, compound quantification has been found time-consuming and has not been a priority in many metabolomics protocols. However, there is still a place for accurate, reproducible and targeted analytical chemistry approaches to quantify selected panels of health-relevant metabolites present in various body biofluids including faeces. Accurate quantification is particularly important when determining subtle changes in metabolite levels in response to dietary interventions which rarely block or ‘turn-off’ pathways or metabolic activities as drugs do but rather modulate the production rate of metabolites. Advances in mass spectroscopy instrumentation and methodology have allowed the development of ‘targeted metabolomics’ approaches where there are panels of specific metabolites, often of related physiological relevance, as an example metabolites related to diabetes or dyslipidaemia, can be accurately and reproducibly quantified in blood and urine\textsuperscript{218}.

Application of metabolomic analysis in the evaluation of putative health benefits of prebiotics might provide additional evidence or elucidate the molecular bases of their actions. Martin *et al.* used a model of mice colonised with a human baby’s microbiota to evaluate the impact of prebiotic galactosyl-oligosaccharides on the metabolic...
changes in ten biofluids/compartments. Prebiotic administration significantly reduced the lipids in the liver and kidneys and altered the transmethylation metabolic pathways (homocysteine–betaine)\(^{(219)}\). So far, only a few intervention studies in human subjects applied metabolomics to evaluate the impact of a synbiotic\(^{(220–222)}\). Recently, a metabolomic approach was applied in a placebo-controlled trial in patients with Crohn’s disease who received a dietary intervention with oligofructose-enriched inulin (OF-IN) \((2 \times 10 \, \text{g/d})\) for 4 weeks\(^{(223)}\). Faecal butyrate levels were up-regulated to the levels found in faecal samples of healthy controls after OF-IN intake. In view of the immunomodulatory and anti-inflammatory properties of butyrate, these observations might encourage follow-up studies in Crohn’s disease with prebiotics.

**Link to metagenome analysis**

Meta-omics technologies enable the simultaneous, ecosystem-wide readout of phylogenetic composition and function, providing a blueprint of the microbiota’s functional potential. Metagenomics employs established technologies that are fuelled by the continuous advances made in sequencing technologies, allowing the cost- and time-effective creation of functional catalogues of the human intestinal microbiota in individuals\(^{(2)}\), which have confirmed a substantially distinct microbial community composition in the large \(v\) the small intestine\(^{(224)}\). It is far from trivial to translate the genetic repertoire or the metagenome of the microbiota into its actual in situ activity, and there is a strong requirement for the further development of functional metagenomic approaches, including metatranscriptomics and metaproteomics. Finally, meta-metabolomics/metabonomics approaches (on faecal water, blood or urine) allow monitoring the pool of metabolites produced by the microbiota in the intestinal lumen (faecal water metabolomes), and their impact on the host’s systemic biochemistry (urine and blood metabolomes).

A main bottleneck in these approaches is the biological interpretation of these data and their integration to decipher the underlying interactions. All the techniques mentioned above yield massive datasets with large proportions of unknown features (unannotated genes, unidentified spectra, etc.). In addition, all data are fragmented and include the assignment of incomplete spectra to fragmented gene sequences and partially sequenced species, which is a challenging problem even for metatranscriptomics and metaproteomics. Also the integration of metabolomic profiles and intestinal metagenome composition remains challenging, which is also a consequence of the ‘snapshot characteristics’ of the datasets generated.

Fig. 2 shows a schematic presentation of the future needs to analyse the functional capacities of the microbiota.

**Fig. 2.** Schematic presentation of the future needs for the functional analysis of the microbiota. Metagenome mapping of metatranscriptome and metaproteome data can rely on established methodologies (darker arrows), but the integration to these (functional) metagenome data with the meta-metabolome is far from trivial and in need of methodology development (lighter arrows). OTU, operational taxonomic units. A colour version of this figure can be found online at http://www.journals.cambridge.org/nrr

Bioinformatic processing and interpretation of large-scale metabolic datasets are hampered by the inherent broad scope of the current metabolic databases (for example, KEGG (Kyoto Encyclopedia of Genes and Genomes), etc.). These generic metabolic maps contain pathways that are not present in the gut ecosystem (for example, reactions requiring molecular oxygen as a substrate) and at the same time lack gut-specific pathways, which leads to a loss of both sensitivity and specificity in meta-omics data interpretation. This situation implies that there is a great need for gut-specific, specialised and curated databases, combined with dedicated software and visualisation tools to facilitate the effective interpretation of meta-omics datasets. These bioinformatic environments are under construction (J Raes, personal communication) and will be of great value for the metabolic deciphering of microbiota adaptations to dietary or pre-/pro-/symbiotic interventions. The detailed inference of metabolic potential and activity, when combined with species-function mapping and reconstruction of metabolic interactions between microbial groups, including the reconstruction of syntrophic chains and metabolite exchange\(^{(225)}\), will open the way towards novel strategies in mathematical modelling of the metabolic processes taking place in the intestine.

The challenges of data integration become even more pronounced when the intestine meta-omics data are to be connected to parameters that relate to the host’s physiology. Some of these host analyses can encompass high-resolution measurements like blood or urine metabolite profiles, blood transcriptomes, or peripheral blood analyte patterns (cytokines, chemokines, hormones, etc.).
Connecting these host measurements to meta-omics data is far from trivial, for example, because for many metabolites detected in blood or urine it is uncertain whether they are of intestinal origin. As a consequence, current studies usually limit themselves to the descriptive analysis of metabolic potential and/or activity across patient cohorts, in which the integration is commonly limited to the detection of correlated entities within the datasets that can be identified by multivariate statistics. Only in a few cases were the identified correlations explained through biological context and network biology reconstructions that explain the molecular relationships between the observed correlations. The latter process commonly requires a time-consuming and largely manual sifting of results combined with massive literature mining to decipher the biological context of the observed correlations. Systems biology mathematical frameworks that accelerate the conversion of correlation-based mining to comprehensive, hypothesis-generating biological interpretation, could accelerate the progress of the meta-omics field and its relevance in human health and disease. However, these computational frameworks are still in their infancy, and will require a substantial amount of validation before they can reliably be applied to effectively mine the complex multivariate datasets obtained through meta-omics and high-resolution host analyses.

Conclusions

Currently, there is insufficient evidence to use changes in levels of individual bacterial metabolites as markers in the assessment of prebiotic effectivity. Several in vitro and experimental animal studies indicate that protein fermentation metabolites including ammonia, phenol, p-cresol, indole or hydrogen sulfide intrinsically affect epithelial cellular metabolism and barrier function. However, there is no evidence from human studies that a reduction in faecal excretion of those metabolites contributes to health. Possibly, the impact of protein fermentation is overshadowed by other dietary or lifestyle factors. Although SCFA are generally recognised as markers of carbohydrate rather than protein fermentation in the colon and are therefore commonly considered as beneficial to health, a number of critical questions need to be answered before their concentrations can serve as biomarkers.

In particular, the lack of reliable concentration ranges defining the ‘normal’ or healthy state for these different metabolites in faeces and other biofluids, and the fact that steady-state metabolite concentrations or profiles do not take into account the rapid absorption and/or conversion of the metabolites, hampers the routine application of those techniques to human dietary interventions where microbiota modulation is an objective. There is an urgent need for dynamic, nutrikinetic-type studies, for example, with stable isotopes, to determine and quantify the pathway of microbial metabolites into the different body compartments. Functional analysis of faecal water toxicity has been proposed as a more holistic approach to link changes in colonic content to health outcomes but suffers from some practical considerations and the limited validation of this biomarker towards the end point of colorectal cancer.

Despite the challenges encountered in the integration of the different levels of quantitative analyses of the intestinal system through meta-omics and the corresponding host-specific parameters, the available meta-omics and other high-resolution analytical methods enable the determination of correlated multivariate signatures that can place potential metabolic or health markers in their context, thereby enhancing their value as markers in health and disease or in therapy efficacy evaluation. Of course, these meta-omics must first consider the prevailing ‘meta-data’ which govern nutrient concentrations within human biofluids, not least dietary intake, a difficult parameter to measure and control in free-living subjects. However, these developments may significantly refine our views of concepts like the bandwidth of health\(^{\text{(226)}}\) that postulate that multiple molecular solutions for a healthy functioning mucosa and/or microbiota exist. The multivariate signatures mentioned may enable appropriate population stratification for the more effective application of specific nutritional interventions in subpopulations that are predictably more responsive to a certain treatment. Meta-omic stratification of the human population is illustrated by the distinction of three ‘metagenomic enterotypes’ that are characterised by elevated community sizes of the Bacteroidetes, Prevotella and ruminococci\(^{\text{(227)}}\). Taken together, the deciphering of detailed and specific mechanisms of interaction in the host–microbe-metabolic interplay are a challenge for the future, but hold great promise for rationalised nutritional health improvement and/or even disease therapy in stratified population cohorts.

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