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Mini-Review

Novel approaches for analysing gut microbes and dietary polyphenols: challenges and opportunities

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Polyphenols, ubiquitously present in the food we consume, may modify the gut microbial composition and/or activity, and moreover, may be converted by the colonic microbiota to bioactive compounds that influence host health. The polyphenol content of fruit and vegetables and derived products is implicated in some of the health benefits bestowed on eating fruit and vegetables. Elucidating the mechanisms behind polyphenol metabolism is an important step in understanding their health effects. Yet, this is no trivial assignment due to the diversity encountered in both polyphenols and the gut microbial composition, which is further confounded by the interactions with the host. Only a limited number of studies have investigated the impact of dietary polyphenols on the complex human gut microbiota and these were mainly focused on single polyphenol molecules and selected bacterial populations. Our knowledge of gut microbial genes and pathways for polyphenol bioconversion and interactions is poor. Application of specific *in vitro* or *in vivo* models mimicking the human gut environment is required to analyse these diverse interactions. A particular benefit can now be gained from next-generation analytical tools such as metagenomics and metatranscriptomics allowing a wider, more holistic approach to the analysis of polyphenol metabolism. Understanding the polyphenol–gut microbiota interactions and gut microbial bioconversion capacity will facilitate studies on bioavailability of polyphenols in the host, provide more insight into the health effects of polyphenols and potentially open avenues for modulation of polyphenol bioactivity for host health.

Introduction

The human intestine harbours a complex microbial ecosystem comprising a considerable metabolic versatility, using biological pathways that humans have not evolved (Qin *et al.*, 2010). This capacity of the gut microbiome, encoded by the collective genomes of the gut microbiota or gut metagenome, includes the metabolism of indigestible polyphenols derived from fruit and vegetables. Polyphenols are phytochemicals abundantly present in our diet in diverse products including tea, coffee, wine, fruit, vegetables and chocolate. Daily intake is estimated at 100–150 mg per day in Western populations (Manach *et al.*, 2004). Polyphenol consumption has been implicated to have diverse benefits such as improved gut health and reduced risk of coronary heart disease, amongst others (Fava *et al.*, 2006; Romier *et al.*, 2009). However, despite mechanistic evidence for these potential effects, in most cases they are far from being clearly proven.

Only a fraction of the known polyphenols is present in edible food crops and products, such as the anthocyanins colouring berries and tannins responsible for the specific astringent taste of wine and the bitterness of chocolate. Three predominant groups of dietary polyphenols are flavonoids, lignans and phenolic acids. In the case of flavonoids, monomers (catechins; present in tea) and polymers (proanthocyanidins; present in wine) can be distinguished. Moreover, nearly 80 % of the flavonoids occur as glycosides in plants and derived food products (Pérez-Jiménez *et al.*, 2010). While some flavonoids are ubiquitous, others are almost exclusively found in particular foods such as daidzein in soy.

Many studies on polyphenols to date have focused on the bioactivities of one specific molecule in aglycone form, often at supraphysiological doses, whereas foods contain complex, often poorly characterized mixtures with multiple additive or interfering activities (Steiner *et al.*, 2008). Furthermore, recent human studies indicate that plasma concentrations and urinary excretions of microbial metabolites can exceed those of host origin, especially for polyphenols that are not easily absorbed in the upper gastro-intestinal tract and persist to the colon (Manach *et al.*, 2009; van Dorsten *et al.*, 2010). The emerging consensus is that the gut microbiota may play a crucial role in the potential health benefits of polyphenols (Crozier *et al.*, 2009). This review addresses approaches for investigating this polyphenol–microbiota interaction, part of the

polyphenol–microbiota–host triangle. First, current knowledge on the bioconversion and bioavailability of polyphenols and their impact on the gut microbiota is outlined. Novel microbiomic approaches using emerging technologies in combination with appropriate models to study the microbial metabolic genes and pathways involved will be discussed.

Bioavailability of dietary polyphenols

Bioavailability (of polyphenols) encompasses dissolution and absorption, distribution to and disposition in target tissues, metabolism and excretion (Fig. 1). It is influenced by numerous, sometimes interrelated, factors, such as variation in food content, matrix and processing, but also genetic, microbial and dietary factors, as observed in human intervention trials (Bolca *et al.*, 2010; van Dorsten *et al.*, 2010). Despite the growing awareness of the poor bioavailability of native polyphenols, the full effects of intestinal digestion, phase I and II metabolism, and, in particular, microbial metabolism of most polyphenols are poorly characterized (Fig. 1).

The first step upon ingestion is the release of polyphenols from their matrix. Deglycosylation of flavonoid glycosides, the cleavage of polymeric proanthocyanidins, as well as the hydrolysis of esterified phenolic acids are, for most compounds, a prerequisite for absorption through the intestinal barrier (Manach *et al.*, 2004). These hydrolyses can be performed from the small intestine onward by either brush border or microbial enzymes. Aglycones, monomers to trimers of flavonols, and some intact glycosides (anthocyanins) can rapidly be absorbed by enterocytes, most likely by passive diffusion. During intestinal absorption and passage in the liver, polyphenols are extensively glucuronidated and/or sulphated (phase II metabolism), whereas phase I metabolism (oxidation/reduction reactions) appears to be minor (Lampe, 2009). As a result, circulating polyphenols are mainly glucuronides. Polyphenol plasma curves often show a second peak, characteristic of enterohepatic circulation. Although faecal excretion is low, urinary recoveries of the parent polyphenols account for only a small fraction of the ingested doses. This indicates that a substantial part is transformed or degraded in the gut. Faecal incubation of anthocyanins, for example, results in a rapid

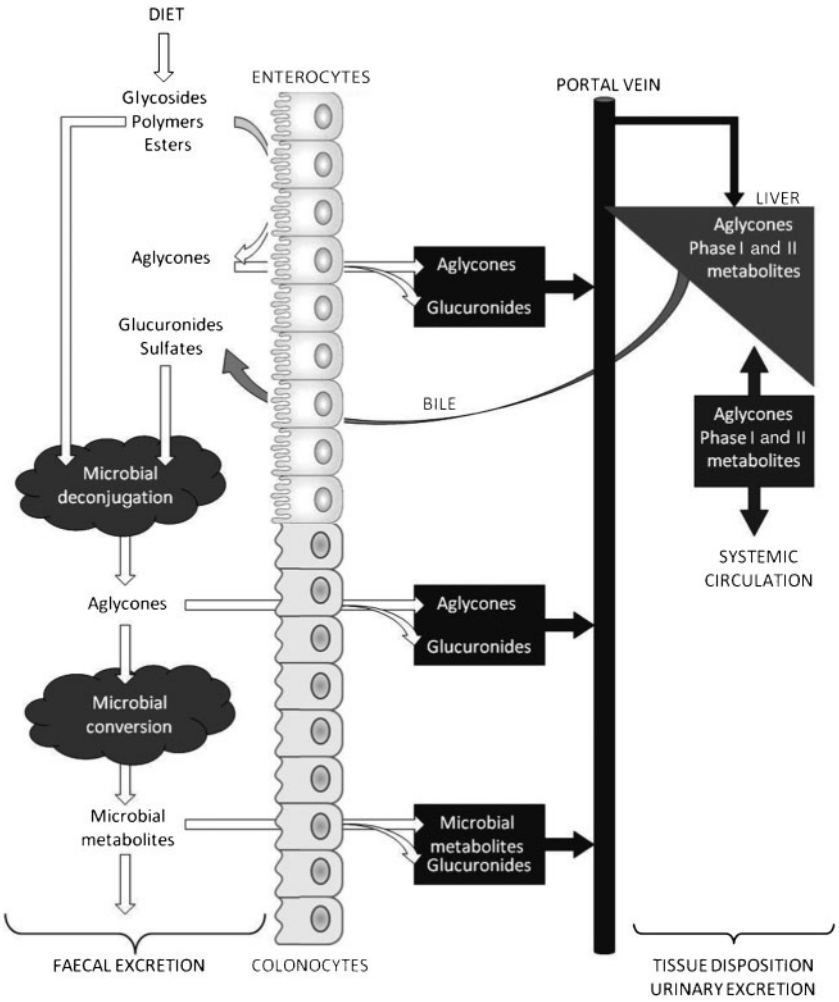

Fig. 1. Metabolic fate of dietary polyphenols. Partial absorption of polyphenols occurs in the small intestine, with modification by phase I and phase II enzymes, excretion with bile back into the small intestine, deconjugation by microbial enzymes and microbial conversion of the polyphenols in the colon, absorption of microbial metabolites or faecal excretion, deconjugation and excretion via bile or urine.

breakdown to their phenolic end products (Fleschhut *et al.*, 2006). This rapid breakdown of the parent polyphenols by the gut microbiota explains their apparent poor bioavailability in pharmacokinetic studies.

Microbial metabolism of polyphenols

In general, the first step in polyphenol degradation involves the release of aglycones and oligomers by microbial glycosidases and esterases, which enhances their absorption. Secondly, microbial glucuronidases and sulphatases deconjugate the phase II metabolites extruded via the bile, thus enabling reuptake. Finally, several microbial transformations of the polyphenolic core, e.g. ring fission by *Eubacterium ramulus* (Clavel *et al.*, 2006), generate metabolites that may have altered bioactivities with respect to human health. Degradation of naringenin and most likely other polyphenols as well by *E. ramulus* putatively involves the enzymes calchone isomerase and phloretin hydrolase (Herles *et al.*, 2004; Schoefer *et al.*, 2004). Dehydroxylation and demethylation of both intermediates and end products can occur at various points in the pathway. Overall, this extensive microbial metabolism ultimately truncates the structural diversity of the native polyphenols to a limited number of mainly simple aromatic metabolites. The microbial conversion rate of polyphenols will also influence their bioavailability, as rapidly converted native as well as intermediate polyphenolic compounds are less available for absorption relative to their (phenolic) end products. The majority of these microbial metabolites undergo phase I/II metabolism during and after uptake through the intestinal epithelium and are detected as such in urine, serum and biopsies (Bolca *et al.*, 2010). As a consequence, the gut lumen, particularly of the ileum, is the only host site where relatively high concentrations of native polyphenols can be expected, whereas other target sites are mainly exposed to (conjugated) microbial degradation products. These conversions are characterized by a large inter-individual variation (Gross *et al.*, 2010), as also illustrated by the bioactivation of dietary phyto-oestrogens and consequent exposure to oestrogenically active metabolites (Bolca *et al.*, 2009).

Our current knowledge on the gut microbes, their enzymes and metabolic routes involved in polyphenol conversions, is limited to a few well-studied model compounds. For instance, the microbial conversion of the major green tea polyphenols catechin, epicatechin, epicatechin gallate and epigallocatechin gallate, results in 4-hydroxybenzoic acid and vanillic acid, which are both converted to hippuric acid derivatives upon absorption (Feng, 2006). To date, several specific gut microbes and microbial consortia have been identified that convert one or the other polyphenol, usually based on selective enrichment of faecal cultures (recently reviewed by Selma *et al.*, 2009). However, there is a mammoth task ahead if one considers the hundreds of different polyphenols and, so far, just a dozen bacterial polyphenol metabolism-related genes/proteins that have been identified with a further approximately 800 hypothetical genes/protein linked to polyphenols in general.

Modulation of gut microbiota by polyphenols

There are remarkably few studies investigating the influence of polyphenols on the composition and activity of the gut microbial community. This represents a significant gap in our understanding of the polyphenol–microbe interactions. Nevertheless, there is evidence from *in vitro*, animal and human studies that certain doses of selected polyphenols influence gut microbial populations, and while certain bacterial groups can be inhibited, others can thrive in the vacant niche of the ecosystem. For example, (+)-catechin significantly inhibited growth of *Clostridium histolyticum*, whereas growth of members of the *Clostridium coccoides–Eubacterium rectale* group and *Escherichia coli* was significantly enhanced and growth of *Bifidobacterium* and *Lactobacillus* spp. remained relatively unaffected (Tzounis *et al.*, 2008). Moreover, two rat studies showed an impact of a polyphenol-rich diet on particular gut bacterial populations. The *Clostridium leptum* cluster decreased significantly, while the *Bacteroides* group increased significantly when rats were given a tannin-rich diet (Smith *et al.*, 2005). Red-wine-polyphenols-treated rats had significantly lower levels of *Clostridium* spp. and higher levels of *Lactobacillus* spp. (Dolara *et al.*, 2005). These studies applied both conventional culturing as well as molecular methods such as fluorescence *in situ* hybridization and denaturing gradient gel electrophoresis, which essentially target either well-known groups or provide comparative profiles rather than direct microbial identification. Therefore, it would be valuable to perform more global diversity studies on gut microbial modulation by polyphenols with 'omics' technologies. These give a more in-depth, unbiased microbial analysis beyond the group level and involve multiple species, addressing whether or not all members of a bacterial group are equally affected by a polyphenol intervention.

An interesting aspect of the polyphenol–microbe interaction is the underlying mechanisms of action of polyphenols on bacterial cells. The antimicrobial action of different polyphenols is especially notable and several non-exclusive mechanisms have been proposed (Table 1). For example, polyphenols can bind to bacterial cell membranes in a dose-dependent manner, thereby disturbing membrane function and, consequently, inhibiting cell growth. Moreover, differences in antimicrobial activity of catechins might be due to differences in binding affinity to the actual lipid bilayer of the bacterial cell membrane (Sirk *et al.*, 2009). Another hypothesis leans towards the formation of polyphenols–metal ions complexes, which in turn would lead to iron deficiency in the gut and could, therefore, affect sensitive bacterial populations (Smith *et al.*, 2005). Overall, some mechanistic insights in polyphenol–microbiota interactions are being generated, but

Table 1. Proposed mechanisms of antimicrobial activity of polyphenols

Mechanism	Compound	References
Membrane interaction	Green tea polyphenols, epicatechin gallate	Kumazawa *et al.* (2004), Sirk *et al.* (2009), Stapleton *et al.* (2007)
DNA gyrase inhibition	Quercetin	Cushnie & Lamb (2005)
Metal sequestering	Tannins	Smith *et al.* (2005)
Enzyme inhibition	Epigallocatechin gallate	Navarro-Martínez *et al.* (2005)
Reactive oxygen generation	Epigallocatechin gallate	Arakawa *et al.* (2004)
Inhibition of virulence factors	Tea polyphenols, resveratrol	Evensen & Braun (2009), Wang *et al.* (2006)

further more detailed studies are required for a better understanding.

Models facilitate mechanistic studies

It is a particularly challenging task to study polyphenol–microbiota interactions and their relevance to human health. The high diversity of polyphenols, their bioavailabilities and the numerous molecular mechanisms by which they may trigger health responses, the large interindividual variability in both composition and activity of gut microbiota, all contribute to the complexity. Moreover, there is an inexhaustible list of interfering factors such as diet, polyphenol matrix and gut transit time. Understanding how these interact and contribute to the *in situ* exposure is crucial for the proper interpretation of biological responses, or the lack thereof, in observational and intervention studies. Furthermore, knowledge of the critical parameters involved will help to identify host subpopulations that may benefit or be at risk upon polyphenol exposure and to substantiate health claims. However, controlling and evaluating all, often interrelated, variables within one experimental setting is not feasible. Consequently, to get the whole picture, a multidisciplinary approach, combining both *in vitro* and *in vivo* studies, is the key to success.

Currently available *in vitro* intestinal models range from rather basic faecal incubations to multistage continuous culture systems (Macfarlane *et al.*, 1998; Minekus *et al.*, 1999; Molly *et al.*, 1993). These models lack a host compartment and, therefore, focus on the microbial aspect alone. Consequently, they are of great value as they allow for mechanistic studies with easy sampling and differentiation between fully controlled variables. Parallel faecal incubations are particularly suitable for assessment of the interindividual variation in microbial polyphenol metabolism (Gross *et al.*, 2010) or for comparison of different sources or doses of polyphenols (Bolca *et al.*, 2009). This is in contrast with human crossover studies which may be clouded by false washout phases between different doses of polyphenols due to adaptive metabolism of the gut microbes and by dietary background noise and variation. Longitudinal experiments with continuous culture model systems in turn, are preferred to study polyphenol-induced modulation of colon microbiota and the persistence of change.

In some cases, the use of animal models is necessary due to difficulties in accessing intestinal locations in humans. Inoculation of germ-free rodents with a human faecal microbiota, human-microbiota associated (HMA) animals, effectively mimics human microbial metabolism and thus provides a reliable model to study the human gut microbial ecosystem as well as metabolism (Hirayama & Itoh, 2005; Turnbaugh & Gordon, 2009). Some HMA rodent studies on catechin and isoflavone metabolism have been performed, but without investigating the effects on composition and activity of the microbial community itself (Bowey *et al.*, 2003; Lhoste *et al.*, 2003). Germ-free rodents inoculated with specific bacterial strains have been used to study polyphenol conversion, but appear to be of limited added value for studying microbial conversions as they have so far just confirmed *in vitro* results (Blaut *et al.*, 2003; Possemiers *et al.*, 2008).

Analytical platforms and data integration

Many of the insights gained into the interaction of polyphenols with gut microbiota are based on experiments with a single polyphenol and/or individual micro-organisms. At present, next-generation sequencing platforms, high-throughput proteomics and metabolomics provide the opportunity to explore the taxonomic, protein-coding gene or expression diversity by applying more comprehensive and less biased measurements to all systems involved, i.e. diet, microbiota and host. Each technique offers specific insights (Fig. 2) and in combination with novel pattern recognition techniques they can accelerate the research in this field (van Velzen *et al.*, 2009).

Phylogenetic analyses were the first to benefit from the high-throughput sequencing revolution, enabling (partial) 16S rRNA gene sequencing on metagenomic samples. Simultaneously, and based on the increased sequence data available, 16S rRNA-based microarrays have been developed for diversity analysis of the gut or other environments (DeSantis *et al.*, 2007; Rajilić-Stojanović *et al.*, 2009). Both methods are still equally applicable. Direct metagenomic sequencing is currently generating a qualified understanding of the metabolic potential embedded in selected gene pools of the gut microbiota. Moreover, the metagenomic sequencing data offer a solid basis for post-genomic research strategies. Post-genomic functional screening of metagenomic libraries

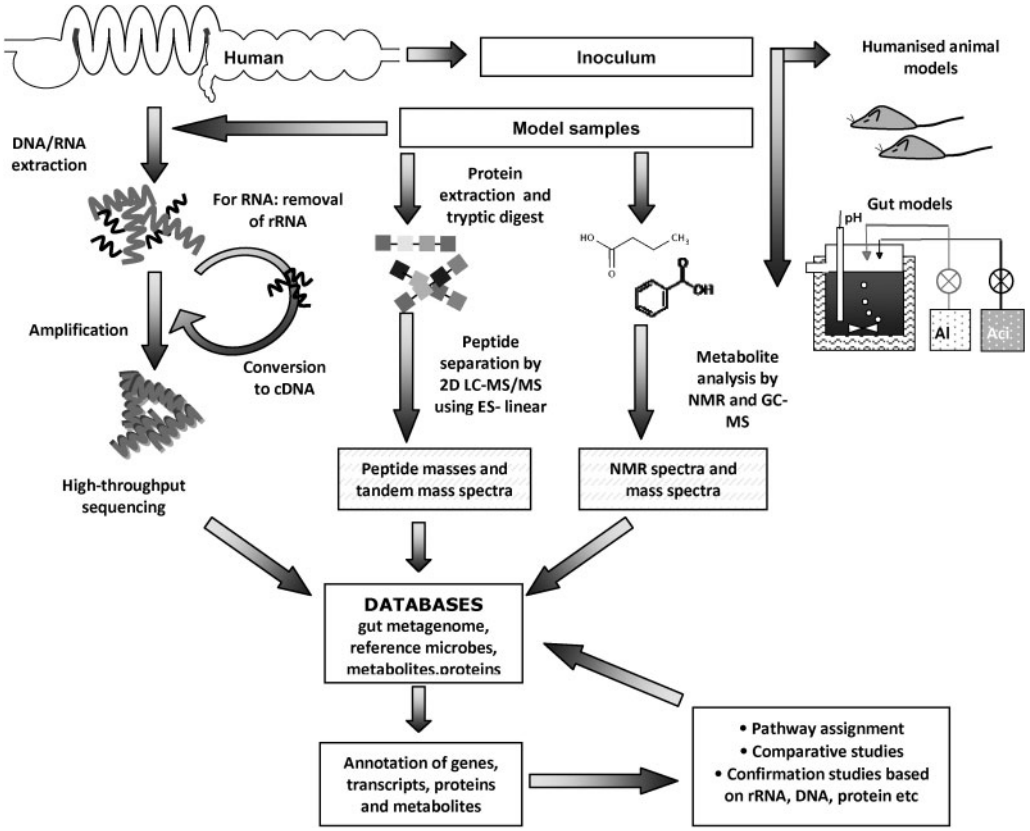

Fig. 2. Schematic representation of functional microbiome approaches for investigation of the effects of polyphenols on the human gut microbiota, including metabolic analyses, metatranscriptomics and shotgun metaproteomics. The latter two protocols follow methods presented previously (Turnbaugh & Gordon, 2009; Verberkmoes *et al.*, 2009). LC-MS/MS, liquid chromatography–tandem mass spectrometry; ES, electrospray ionization.

directly identifies metagenomic fragments encoding a metabolic function (Beloqui *et al.*, 2006). A high-throughput assay is critical for this. We investigated the possibility of identifying gut microbial genes for catechin bioconversion using clones from a metagenomic library (Manichanh *et al.*, 2006) by measuring catechin conversion by HPLC. However, several challenges confounded our efforts to optimize assay conditions; in particular, differential catechin depletion occurred without actual conversion, likely due to the interaction of the catechins with the bacterial membrane or possibly through binding to cellular proteins. In addition, metagenomic library screening is probably most successful for functions encoded by a single gene or operon, while (certain) polyphenol conversions may require a consortium of microbes, which limits its applications, unless multiple screening assays are combined.

Metatranscriptomics and metaproteomics are being applied to the gut microbial community and provide functional analysis of the gut microbial species beyond a mere phylogenetic description. While metatranscriptomics has the potential to reveal the nearly instantaneous responses to environmental fluctuations, metaproteomics more directly reflects the immediate catalytic potential of a microbial community. In the context of polyphenol–microbiota interactions, such approaches can be adopted to identify genes and micro-organisms involved in polyphenol (in)activation and conversion, to reconstruct metabolic pathways, and to monitor how microbial communities adjust their metabolic activities upon polyphenol administration. Metatranscriptomic analyses enable the detection of changes in gene expression over time or under various physiological/environmental conditions, which will help to link community structure with function, and genotypes with phenotypes (Turnbaugh & Gordon, 2009). For the human gut, this is facilitated by a catalogue of non-redundant human intestinal microbial genes, indicating that individuals share about 38 % of their gene pool, identifying what is called the ‘minimal gut genome’ and ‘minimal gut metagenome’ (Qin *et al.*, 2010). We assume that this catalogue also harbours some of the genes involved in polyphenol conversion, enabling us to identify genes encoding polyphenol metabolism by comparative sequence analysis of metatranscriptomes before and after a polyphenolic intervention. The application of shotgun metaproteomics to human distal gut microbial samples has

been demonstrated (Verberkmoes *et al.*, 2009). Interestingly, human proteins related to innate immunity and inflammation response were also identified amongst the microbial proteins highlighting the interplay between gut microbes and host. Eventually, environmental proteomics and proteogenomics of the gut ecosystem may be applied to link environmental changes, including polyphenol interventions, to protein level changes, as has been done for a less complex microbial community (Denef *et al.*, 2010). The role of metabolomics will also increase in terms of identification of microbial polyphenol metabolites, monitoring not only shifts in polyphenol levels and their respective metabolites upon interaction with gut microbes but also changes in general microbial metabolites, such as short chain fatty acids, as a result of polyphenol exposure.

We are currently investigating these approaches to study the effects of dietary polyphenol mixes on the human gut microbiota both *in vitro* (Gross *et al.*, 2010) and *in vivo* (van Duynhoven *et al.*, 2010). The influence of polyphenol extracts from black tea on the intestinal microbiota was assessed in a continuous gut model (Fig. 3a). Quantitative PCR showed the inhibitory effect of tea polyphenols on the microbial community, which was more pronounced upon continuous feeding than after a single bolus. Phylogenetic information could also be directly correlated with metabolite data *in vivo*, as was demonstrated for a small human study in which several microbial species were found to correlate to urinary metabolites, some of which appear in polyphenol bioconversion pathways (Li *et al.*, 2008). In a human nutrikinetic study with 20 individuals consuming a bolus dose of tea polyphenols, we found correlations between the microbial diversity, as measured with the Human Intestinal Tract Chip (HITChip) and nutrikinetic phenotypes. Specifically, the *Actinobacteria* and *Clostridium* clusters correlated with degradation of catechin-type structures (van Duynhoven *et al.*, 2010). Ultimately, using the actual genomic diversity rather than microbial diversity for correlation is expected to give a more detailed insight into metabolome–microbiome interactions. *In vitro* gut models challenged with polyphenols are a feasible means for obtaining initial data on the impact of polyphenols on phylogeny and functionality (Fig. 3b), especially as they are amenable for use to isolate quality mRNA for metatranscriptomics. Overall, the initial exposure to polyphenols will likely upregulate polyphenol-induced stress mechanisms while longer term exposure should also induce polyphenol-resistance and bioconversion genes. These preliminary data will facilitate identification of biomarkers/targets for subsequent application in human faecal samples upon dietary interventions (Fig. 2). Functions may be tentatively assigned to the ORFs/genes identified based on homology comparisons and correlation to metabolite data, and eventually confirmed by biochemical tests.

The bottle neck remaining is no longer the ability to generate data, but the ability to process these data efficiently in a coherent manner. Though diversity analysis tools are now integrated into pipelines such as QIIME (Caporaso *et al.*, 2010), processing these large datasets remains a challenge. Integrating the disparate information gained by these novel analytical platforms in a systems biology approach may lead to workable, testable hypotheses for the impact of polyphenols on the microbial composition and human health, and to the development of fundamental understanding of these complex systems (Steiner *et al.*, 2008; van Duynhoven *et al.*, 2010).

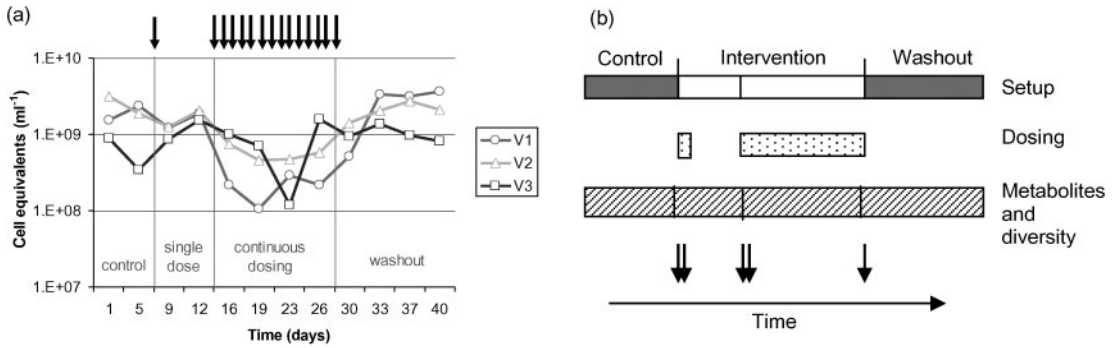

Fig. 3. (a) Total bacterial counts as measured by SYBR-green-based qPCR (Nadkarni *et al.*, 2002) in a SHIME system fed (arrows) with 1000 mg tea polyphenols administered as either a single bolus or part of the daily feeding. Vessels mimicking the ascending (○), transverse (△) and descending (□) colon sections are indicated. (b) General experimental design for studying polyphenol microbiota interactions using an *in vitro* gut model. This includes periods for stabilization of the ecosystem (control), single and/or a continuous dosing of polyphenols to the model (intervention) and a washout phase to determine the persistence of change. Samples are withdrawn at least daily to measure microbial diversity (e.g. HITChip or 16S rRNA gene sequencing) and polyphenol metabolite (NMR, GC-MS) analysis and for the more detailed metatranscriptomic analysis at key time points (arrows) during the intervention period.

Conclusions

Key challenges of studying polyphenol metabolism in a dietary background are the variability in the composition and activity of the gut microbial community, as well as the diversity in polyphenolic mixes, combined with their variable and typically low abundance in the diet. Though many effects of polyphenols on gut microbes were revealed using conventional approaches, their exact mechanisms of action have not yet been fully established. The emerging high-throughput meta-genomic, -transcriptomic and -proteomic, and high-throughput metabolomic technologies will accelerate the elucidation of the species/consortia and pathways involved in polyphenol degradation and resistance. Moreover, holistic approaches will provide a superior description of the complexity of polyphenol–microbiota interactions and more comprehensive insights into their physiological relevance. Using various *in vitro* and animal models for easily accessible, high quality samples in combination with the advanced analytical techniques has the potential to continue to facilitate understanding of the metabolic processes. Application of these technologies to human faecal samples requires further investigation to determine how these samples reflect metabolism inside the gut. Once suitable targets have been identified in more controlled environments, the step linking the microbial metabolism to host metabolism can be made. In addition, as gut microbial metabolism and host metabolism are intimately linked through exchange of metabolites, metabolomic analyses can capture information on the actual exposure and response of the host, for example, by analysing metabolites in plasma and urine in response to a polyphenol intervention (van Dorsten *et al.*, 2010). Knowledge of the gut microbial genes/pathways and cellular mechanisms of action of polyphenols on the human gut microbial ecosystem will allow us to better assess the fate of polyphenols and, ultimately, enhance/improve our understanding of the impact of polyphenols on host health.

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