[Frontiers in Bioscience 14, 3074-3084, January 1, 2009]

Microbial functionality in the human intestinal tract

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1. ABSTRACT

The extent of metabolic interactions between symbiotic intestinal microbes and the human host, and their system-wide effects on the host physiology are beginning to be understood. The metabolic capacity encoded by the intestinal microbiome significantly extends that of the host, making many of man's physiological characteristics an outcome of a human-microbe co-metabolism. A detailed characterization of the composition and function of the gut microbial ecosystem is required to foster the understanding of its mechanisms and impact. The most recent research on the intestinal ecosystem is reviewed here, with specific attention to the ecological aspects including the anticipated effects of probiotics and prebiotics. Finally, the post-genomics approaches that advance discovering the functionality of intestinal bacteria are addressed.

2. INTRODUCTION

During recent years, it has become increasingly evident that our health status is to a large degree determined by human-microbe interactions, the major contributors being intestinal bacteria. A healthy adult provides a home to up to 10^{14} intestinal microbes, which are collectively termed the gastrointestinal (GI) microbiota. The recent advances in molecular methods, mainly based on the phylogenetic analyses of 16S rRNA gene, have revealed the remarkable diversity of this complex ecosystem. Results from recent sequencing studies suggest that members of less than ten bacterial divisions are represented, from which the Bacteroidetes (notably *Bacteroides* spp.), Firmicutes (low G+C content Gram-positive bacteria, notably *Clostridium* and *Bacillus* spp.) and Actinobacteria (high G+C Gram-positive bacteria,

notably *Bifidobacterium* spp.) account for over 90% of detected 16S sequences, while the estimations of the number of species varies from few hundreds to several thousands (1-6). However, there is no plateau in the observed number of phylotypes, necessitating and rationalizing the intensive ongoing and future attempts to characterize the diversity and metabolic potential of intestinal microbiota (7, 8).

The primary function of GI microbiota is thought to be the breakdown and conversion of food-derived nutrients and other ingested compounds. In addition, the GI microbiota has protective and trophic functions, which comprise regulatory effects of bacterial metabolites to human cells, affecting e.g. their proliferation and differentiation as well as specific immune responses (9, 10). The aim of this review is to highlight recent progress in the characterization of the metabolic functions of intestinal luminal microbiota detectable in fecal samples, supplemented with selected studied on animal models when data on humans does not yet exist. Additionally, the notable intra-individual variability of the human microbiota and the resulting methodological challenges will be discussed.

3. METABOLIC IMPACT OF GI MICROBIOTA

The intestinal microbiota has a major impact on bioavailability and bioactivity of diet-derived material; it consumes, stores and circulates nutrients effectively, and therefore makes an essential contribution to the human metabolism. When compared to the human genome, or to the average gene content of previously sequenced bacteria, the human gut microbiome (the collective genome of the microbiota) was shown to be enriched for metabolic genes central in the degradation of various diet- derived substrates and mucin, as well as in methanogenesis and bioconversion of beneficial compounds (11, 12), explaining the much wider range of nutritional resources utilizable by gut bacteria compared to the human host.

Since the physiology and microbial functionality of the small intestine has recently been thoroughly reviewed by Booijink *et al.* (13), we will discuss primarily the colonic microbial metabolism in this context. The digesta passing from the small intestine to the colon contains material indigestible to humans, mostly comprising of plant-derived fibers, but also of host-induced secretions such as digestive enzymes and mucin. The colonic microbial community, which feed on these 'leftovers', may attach to polymeric insoluble substrates, break them down to soluble components, ferment the monomers (mainly sugars and proteins) via sequential reactions, and finally get rid of the waste products including toxic gases, such as hydrogen sulphide and ammonia. These activities create complex food webs and stimulate cross-feeding among primary and secondary fermenters, methanogens and sulphate-reducing bacteria (14, 15). Details of the carbohydrate breakdown and fermentation by gut bacteria have recently been reviewed (16-18).

Adherent bacteria are assumed to bind and break down substrates for themselves and for non-adherent fermentative bacteria. The primary colonizers of polymeric, insoluble substrates were shown to be metabolically distinct from the non-adherent ones, indicating metabolic specialization (19, 20). However, the predominant cultivable bacteria did not differ strikingly between the adherent and non-adherent fractions (20). In another study, species from genera *Bacteroides, Roseburia, Ruminococcus, Eubacterium* and *Bifidobacterium* were shown to be dominant in biofilms generated on carbohydrate substrates in a fermentor after fecal inoculum, with prominent substrate- and individual-specific differences in the composition of bacterial populations (19). Notably, the fecal inoculum was prepared using centrifugation to remove the coarse particulate matter, and concomitantly the bacteria bound to it, questioning the bacterial representativeness of the used inoculate. Recently, RNA based stable isotope probing combined with metabolic analyses was used to phylogenetically identify bacteria involved in the fermentation of starch in the human colon simulator (21). This study indicated species from genera *Ruminococcus, Prevotella, Bifidobacterium and Eubacterium* to participate in starch metabolism in a cross-feeding manner.

The proportion of substrate adherent bacteria in human feces has not been thoroughly addressed; about 5% was reported to be strongly bound and require surfactant treatment for release (20). Substrate-adherent bacteria are likely to be important for gut ecology because of their ability to colonize on incoming food particles, thereby permanently establish themselves in the colon, and effectively control the downstream niches via the bioavailabity of fermentable substrates. The distribution between adherent and free-living bacteria is likely to vary considerably relative to substrate availability (diet, mucus secretion) and between individuals. Current knowledge on the structure and function of microbial biofilms on different surfaces through the whole GI track has recently been reviewed (22).

Not only the amount and quality of substrates in the diet, but also their metabolic by- and end products regulate the colonic ecosystem. Methanogens consume hydrogen and possibly other fermentation products in a competitive manner with sulphate reducing bacteria (SRB), which are considered harmful for humans due to their toxic metabolites (23, 24). The structure and activity of the colonic cellulolytic microbial community were found to differ greatly according to the methane-excreting status of the volunteers so that there was a clear positive correlation between cellylolytic and methanogenic communities (25). This suggests a feedback regulation system that shapes the community upstream in the food chain. Intestinal methanogens possess other interesting features as well. One view is that methanogens are benefical because of their presumed competition with potentially harmful SRB. Populations with low colon cancer have high rate of methanogenesis (24), suggesting that methanogens may be important decontaminators of the colon. However, recently

an anti-methanogen therapy was suggested as a potential option to reduce energy harvest and thereby obesity (26).

The prevalence of methanogens among a human population is less than 50% of population, while they typically constitute up to 10% of carriers' total microbiota (6, 27). Similarly, the genus *Prevotella* was detected only in half of the studied 46 Japanese individuals, but being at predominant level (average of 10^9 bacteria per g feces) in carriers (28). The function of *Prevotella* spp. remains enigmatic while that of the methanogens has been well established. Remarkably, the distribution of the methanogens seems to be non-random although there is contradictory data regarding gender-dependent prevalence of methane excretion. In adolescent twins, 63% of females were postive compared to 37% of men (27), while another recent study reported no significant difference between sexes (29). Both hydrogen disposal pathways can coexist in the same individuals, and their preference is likely to be controlled through substrate availability and local colonic conditions like pH (30 and references therein). The causes and effects of pronounced intra-individual differences on hydrogen disposal strategies dominated either by methanogens or SRB remain unknown. Altogether, the accumulation of metabolites, whose analysis is facilitated with the emerging metabolomics technology (see below), can induce deviations to the microbiota homeostasis and act as indicators of its dysbiosis.

Compared to well-studied and abundant carbohydrate metabolism by intestinal bacteria, much less is known about the bacterial protein degradation. Dietary protein and proteinaceous human secretions are used as substrates by intestinal bacteria mainly in the distal colon in contrast to the fermentation of carbohydrates occurring in the proximal part of colon (31). The true diversity of predominant proteolytic species is currently not known, since this issue has not yet been addressed in the era of molecular methods. In contrast, there are a small number of recent mice studies demonstrating a contribution of the intestinal microbiota in the fat metabolism. The work by Cani *et al.* (32) suggests that *Bifidobacterium* spp. have potential for reducing the impact of high-fat food on the occurrence of metabolic diseases, while Martin *et al.* (33) postulate that *Lactobacillus* spp. affect bile acid metabolism and consequently the adsorption and storage of fat to the body. Both studies are discussed in more detail in section 5.3. Several high impact publications from the Gordon lab support a link between gut microbiota and obesity, explained by the differences of the fermentative capacity of our intestinal microbiota and further, by its regulatory effects to the consumption and storage of energy (3, 34 and references therein).

Although the basic 'household' functions of intestinal microbiota are fairly universal among individuals, a part of the metabolic traits manifested by microbiota appear to be segregated within the population. We are starting to find correlations between the differences on microbiota composition and the resultant differences at the functional level, such as the metabolic phenotype. When fecal inocula from three volunteers were used to study the *in vitro* conversion of isotopically labelled lactate, the major operating metabolic pathways were found to differ significantly between individuals (35). Similarly, the bacterial metabolism of beneficial dietary phytoestrogens improving their bioavailability and –activity in the human body was found to be individual-specific (36). It is likely that in-depth metabolic studies will reveal the unity and diversity in the biochemical reactions present in one's individual GI tract.

4. EFFECT OF DIET ON GI MICROBIOTA

Although the diet can be considered as one major contributor to composition of the intestinal microbiota, its notable resilience against environmental perturbations is manifested through the intra-individual temporal stability. Therefore, the consumption of probiotic bacteria, their preferred substrates (prebiotics) or combinations of both (synbiotics) are likely to have minor effect on the GI community structure. Based on the ecological background, a single bacterial strain is not likely to undermine the established community, where selection pressures from the host and the bacterial ecosystem fortify the stability of the microbiota. Indeed, the global impact of ingested probiotics on the composition and diversity of established colonic microbiota is limited (37, 38). In a recent study, yogurt intake was shown to shape the intestinal microbiota irrespective of the type the consumed yoghurt (either containing probiotic or fermentative *Lactobacillus* strains) and suggested that yogurt exerted a prebiotic-like rather than a probiotic effect (37). The most prominent yogurt-induced changes recorded were the decrease of *Bacteroides* spp. and increase of *C. coccoides/E. rectale* group as well as highly individual-specific effects in bifidobacteria. The general scarcity of probiotic-induced changes in bacterial community structure might, however, partially reflect the difficulty to select the phylogenetic resolution for analyses. Global analysis is required to get an overview of the community structure, but is likely to mask any subtle changes at species level, which may still be interesting. The analyses of selected genera or species may, in turn, overlook the unpredicted overall differences. We are presently using phylogenetic microarray analysis as an approach to acquire a global picture of the potential effects of probiotic intervention on the intestinal microbiota as has recently been reported in a double-blind placebo-controlled study of a probiotic mixture alleviating symptoms of irritable bowel syndrome (39). In summary, a combination of holistic and reductionistic approaches is required to define the actual effects of diet on shaping the GI microbiota.

Compared to probiotics, the effect of prebiotics is likely to be more pronounced, since diet is a major contributor to the spectrum of intestinal niches. The metabolic consequences of prebiotic intake, mainly mediated by the microbiota, are much better documented than the effects on the microbiota composition itself (40). The amount of carbohydrate intake has been shown to correlate positively with the abundance of butyrate-forming *Roseburia/Eubacterium rectale* group and butyrate concentration in feces (41). This indicates that the diet,

Table 1. An overview of human gut metagenome reports and major ongoing efforts

Number and nationality of individuals	Subject health status	Sequence (Mb)	Predicted proteins (thousands)	Reference
2 Americans	Healthy	78	50	12
6[1] French 6[1] French	CD patients Healthy	<1 (2000)[2]	-	51
13 Japanese	Healthy	727	660	11
European project MetaHIT	Healthy and diseased	(>1000)[2, 3]	-	52

Clone libraries of PCR amplified 16S rRNA genes are not included. [1]microbiome DNA from six individuals was pooled into single libraries of healthy and CD (Crohn's Disease) patients, [2]size of (planned) libraries is indicated - no metagenome sequences reported yet. [3]For further information see http://locus.jouy.inra.fr/metahit/index.php

more specifically the supply of non-digestible carbohydrates, regulates the fermentative population in the colon. However, the targeted attempts to increase a population of beneficial bacteria inherently affect also other bacteria in the same food web, sharing or competing for the same nutritional resources. Therefore, the positive or negative overall impact as well as the temporal duration of pro- and prebiotic effects on the intestinal ecosystem is hard to predict, and remain to be experimentally addressed.

In conclusion, there is accumulating data to support the idea that the mechanistic basis of most pro- and prebiotic effects rely on their regulatory metabolic effects in the system rather than via direct changes in the host's GI microbiota composition (42). This is in line with animal studies indicating that even if the diet is kept constant, stress and other factors regulating e.g. endocrine secretion affect the actual metabolic phenotype of the organism. Recent findings on the metabolic alterations after probiotic intake are discussed below (section 5.3.).

5. FROM DIVERSITY TO FUNCTIONAL GENOMICS

Bacterial populations of the gut are markedly stable in time within individuals, but highly diverse and individual-specific (3, 28, 43). However, a high inter-individual diversity applies only for species and low taxonomic levels, hence; at higher taxonomic levels all studied humans are found to share a uniform microbiota (6, 12, 44). The observed inter-individual similarity at high taxonomic levels most likely reflects the restricted amount and overlap of niches within the intestinal microbiota, and functional uniformity of its basal population (5, 45, 46). A functional core that is at least partly shared between individuals, and comprises sustainable microbes with household functions, is likely to be selected for by the host (5, 8). Functional redundancy is observed at the community level and ensures the stable maintenance of central metabolic functions even if the community composition shifts. This can be illustrated by the rapid changes in butyrate-producing population (predominantly belonging to Clostridial cluster XIVa) in the feces from three individuals that showed little phylogenetic overlap to each other, and also differed markedly from the follow-up samples taken one year later (47). At a bacterial level, stability within an ecosystem is assured through its capacity to switch substrates according to nutrient availability. This is most typically enabled by a large and adaptive genome, and, to unknown extent, through mobile genetic elements (11, 48). Both strategies are involved in the acquisition of a set of alternative glycan substrates by *Bacteroides* spp., the prominent members of the suggested functional core (49, 50).

Given the large inter-individual variation and different methodologies used in different laboratories, separate microbiota studies tend to end up with variable or even contradictory data. This is evident from the dissection of present metagenomic (Table 1) and 16S rRNA library studies. Variable amounts of *Bifidobacterium* spp. detected in fecal samples serve as an representative example. Clone libraries from seven Chinese subjects contained only 0.2% Actinobacterial 16S rRNA sequences, identical to the score for mucosal and stool samples (taken one month after colonoscopy and bowel cleansing) from two Americans (6). The results are in line with another American study, where 16S rRNA-based microarray quantification of bifidobacteria suggested their proportion to be less than 1% (53). However, the first comprehensive metagenomic study of two American individuals revealed bifidobacterial sequences to constitute 4% and 23% of total microbiota, respectively (12). This strongly suggests that Americans, as most likely Europeans and Asians as well, do carry highly variable but considerable amounts of bifidobacteria, which tend to get under presented in molecular studies due to their challenging lysis and high G+C content affecting PCR efficiency. Furthermore, the American metagenomic study did not retrieve any 16S rRNA sequences from *Bacteroides* spp. and in total, retrieved 2-3 times less (depending on individual) predicted genes than the Japanese metagenomic study (11, 12). The Japanese study, in turn, retrieved surprisingly few Clostridial sequences; in other metagenomic analyses Clostridial sequences have accounted up to 70% of all phylotypes (6, 12). All these examples highlight the importance and inherent impact of sampling, DNA extraction and cloning to the outcome of the experiment aiming to characterize the intestinal microbial diversity. To promote the collective accumulation of high-value data out of ongoing and future research, technical and biological variation should be separable. Despite some methodological imperfections, each metagenomic investigation has given valuable insight to the diversity and functional potential of the intestinal microbiota (Table 1; 13). More light to the underlying reasons for the large intra-individual variations in the abundance of intestinal bacteria and other related issues are anticipated from the forthcoming cohort studies (7, 8, 52).

In conjunction with the metagenomics approach, sequencing and annotation of isolated keystone species is equally important in improving our understanding on species metabolic versatility and adaptability. Additionally, reference genomes are required for the interpretation of

metagenomic data. However, the knowledge of the microbiome as such is only indicative of potential activity, presuming the translation of the genetic code and diversity into consequent functional attributes. Historically, the functional studies of intestinal microbiota have been carried out by determining indirect parameters such as bacterial counts, enzymatic activities and survival through the GI tract. The actual, and specifically measurable functionality of microbial community, or even single species, has recently become possible due to the holistic, high-throughput post-genomic technologies, notably transcriptomics, proteomics and metabolomics. The principles and methodologies of these omics platforms are covered elsewhere (54). Recent advances on the elucidation of gut microbiota-related functionality via omics technologies will be presented here.

5.1. Transcriptomics

Expression profiling, or transcriptomics, can be used to determine genome-wide changes at the transcriptional level, thereby characterizing the genes responsible for the phenotypic response to a given condition or stimulus. There are no community level transcriptional analyses from human intestinal commensals reported yet and not many from the separate species either. Genomic microarray was used for the whole-genome transcription profiling of *Roseburia inulinivorans* (55). Upregulated genes were involved in the utilization of provided substrates, as well as in quorum-sensing. Transcriptional responses of bifidobacteria present in feces of breast- and formula-fed infants were analysed using mixed-species genomic microarray (56). Total RNA was extracted and hybridized to microarrays containing cloned inserts of libraries covering several bifidobacterial species from the intestinal track. Significant transcriptional differences were detected between the groups; a major contributing group consisted of genes involved in carbohydrate metabolism. This is likely to reflect the substrate-driven induction by a variety of oligosaccharides present in the breast milk. The effect of prebiotic intervention on adult bifidobacterial transcriptome was also analysed using the same bifidobacterial array (56). Substantial metabolic activity was detected; however, gene expression patterns combined from all subjects did not differ significantly before and after prebiotic intake, although three out of four individuals showed significant changes at individual level. The global gene expression of a *Lactobacillus plantarum* strain was analysed from the mucosal samples using a genomic microarray (57). Ingested bacteria were observed to be metabolically active in the colon and genes predicted to participate in energy metabolism and survival in the intestinal track showed significant transcriptional response.

Expression analysis has been utilized in the characterization of probiotic strains also in animal models. The relative expression levels of selected *L. plantarum* genes were increased up to 350-fold in the mouse intestine when compared to levels observed during the growth in a laboratory medium (58). Moreover, expression of several genes was spatially confined to either the small intestine or colon. An analogous independent observation on transcriptional differences *in vivo* and *in vitro* and between different intestinal compartments was done with another *Lactobacillus* strain, *L. johnsonii* strain NCC533 (59). Remarkably, a total of 44% of the NCC533 genes were not detectably transcribed under any of the investigated conditions. The most recent study from the same authors exemplifies the application of genotyping and expression microarrays on the identification of strain-specific genes responsible for phenotypic differences (60).

The identification and functional analysis of single reporter genes provides an in-depth analysis of the activity of interest (61). Real-time reverse transcriptase (RT) PCR was utilized recently to demonstrate transcriptional activity of a transcarboxylase gene encoding *Propionibacterium freudenreichii*-specific fermentation enzyme during the transit through the human GI tract (62). It is expected that these and other studies, including comparative analyses based on metagenomic data, will help to find sets of (candidate) biomarker genes that reflect the GI tract functionality.

5.2. Proteomic profiling

Cellular processes are mostly carried out by proteins that are involved in the actual functions rather than being intermediates in the biosynthesis, such as mRNA. The holistic analysis of proteins produced not by single species but rather by the complement of the active bacterial population in the gut is know as metaproteomics. This is an emerging approach with massive potential to untangle the physiological processes determining the phenotypic outcome of the community. However, progress in this territory has been limited by the complex nature of fecal samples and the substantial methodological limitations rising from it. Markedly, the recent advances in analytical technologies (reviewed in 63) have shifted the impending bottle necks of proteomic analyses from analytical part to pre-analytics (requiring reproducible and unbiased sample processing) and post-analytics (data analysis; identification of proteins, assigning them to species and linking of biological functions to gene sequences). Indeed, for proteomic analyses of microbial communities, one has to extract proteins in a reproducible way from a sample being complex in its microbial composition, having a high dynamic range of proteins and containing compounds interfering in protein analysis (64). For the time being, the challenges of analyzing the multitude of fecal proteins are represented by the existence of a single publication describing proteome analysis of fecal samples (65). This study did not cover the whole complexity of the intestinal microbiota, since baby feces with relatively low bacterial diversity were analysed. The majority of the obtained peptide sequences could not be identified due to the insufficient reference genomes in public databases, emphasizing the acute demand for updated custom databases supporting proteomic analyses of intestinal proteins. We anticipate that the first reports on adult fecal proteomes will be published in the near future.

It should be noted that the proteins found in feces are not only derived from the intestinal bacteria, but also from the food and the host, making the peptide assignment to parent proteins and further to host organisms challenging

but highly informative due to the holistic view into the symbiotic co-metabolism of the GI track. Most of the essential proteins participating in the metabolism, i.e. those utilized in the attachment to and break down of substrates, as well as those related to the interaction between the host and other bacteria are not intracellular but rather secreted or remain cell surface bound. These and related aspects should be noted when considering measures aiming at reducing the complexity in the samples and the resultant data, so that acquisition of reliable and high-quality information can be focused on the microbial component of the fecal protein ensemble.

The progress in proteomic characterization of the isolated members of intestinal inhabitants has been reviewed recently (66). In addition, the proteomic response to a particular growth substrate has been described for *Lactococcus* (67) and *Fusobacterium* in mice (68). These results are in line with the metabolic adaptation of bifidobacteria monitored at transcriptional level (see above). It can be concluded that proteomics holds a panoply of promises towards the molecular characterization of microbiota functionality and the mechanisms how it responses to perturbations. For the full swing of proteomic analyses, however, the improved coverage of intestinal microbiome sequences is required to enable the identification and functional annotation of retrieved proteins.

5.3. Metabolomic profiling

The advent of high-throughput metabolomics has enabled the global analysis of the products of intestinal microbiota metabolism (69). Metabolites have a high information value due to their direct link to cellular phenotype and function. Moreover, their identification is not dependent on the coverage of genomic databases. Several recent studies have proven the potential of metabolomic analyses of fecal extracts on the elucidation of the mammalian-microbial co-metabolism activity in the gut lumen. The metabolic profiles of fecal water obtained from healthy controls and subjects having either inflammatory bowel disease (70) or colon cancer (71) were significantly different, the most prominent feature of patients' profiles being a depletion of SCFAs or an increase of amino acids, respectively. Based on the analysis of dominant microbiota in fecal samples, persons either having colon cancer or being at high risk of developing it (adenomatous polyposis patients) were characterized by decreased temporal instability and interestingly, increased diversity of *C. coccoides* and *C. leptum* subgroups (71). This work emphasizes the concept of atypically high species diversity in the GI tract, which is associated with a disease state - this issue is seldomly addressed, but deserves to be taken into consideration as an indicator for the absence of gut homeostasis.

Concurrently with fecal analyses, metabolomics of other sample materials have confirmed and capitalized on the fact that the impact of intestinal bacterial activity is not limited to the gut, but reaches organs and tissues well beyond it (33, 42, 44, 72-74). This is explained by the fact that many of bacterial metabolites are absorbed into the blood, and thereafter enter the circulatory system. A recent proof-of-principle study on transgenomic methodology cabable of combining microbiota composition to human fecal and urinary metabolic phenotypes provides significant progress towards the characterization of system-level microbial contribution to host metabolism (44). Multivariate statistics was used to identify associations between the microbiota structure and co-incidental metabolites, and species from genera *Bacteroides, Clostridium* and *Bifidobacterium* were shown to be functionally active and associated in variable metabolic interactions.

The systemic effect of dietary substances on metabolomic phenotype has recently been addressed as well. A study using humanized mice suggests that probiotics may affect multiple organs in mammalian metabolism (33). Mice colonized with seven bacterial species originating from human baby microbiota revealed large scale and strain-specific metabolic consequences following *L. paracasei* or *L. rhamnosus* intake, including changes in amino acid and lipid metabolism. Surprisingly, metabolite comparisons of fecal samples were less discriminative between the control and probiotic groups; the most pronounced differences were detected in plasma and liver. Likewise, no metabolite changes could be detected in human feces after the intake of grape juice extract (75). In contrast, microbial modulation of dietary flavonoids could be traced in the urinary metabolic profiles, highlighting the versatility of mammalian-microbial co-metabolism, and the potential of urine to carry indirect information on the gut microbial metabolic activity (74). In this study, fecal extracts were not analysed for comparison. Interestingly, this work proposes that urinary metabolomic profiles reflect not only the recent dietary intake, but also behavioural dietary preferences interconnected with the gut microbiota activity, possibly related to long term health effects of an individual (74).

There are indications that an individual-specific metabolomic signature is based mainly on quantitative differences and ratios of several metabolites rather than large-scale qualitative differences in the composition of metabolite pool (75, 76), although Saric *et al.* found SCFAs and certain amino acids in similar relative concentrations between all analysed human samples (n=12; 77). The application of metabolomic data from animal models to humans must be done with reservation, since the metabolic profiles were reported to be highly species-specific between humans, mice and rats, mice and humans being the most distinct groups (77). Taking together, the highlighted recent reports give us a flavour of the full potential of metabolomics in the investigation of physiology and function of intestinal microbiota. The full descriptive potential of metabolic signatures will come into play when the invariable 'household' metabolites can be used to normalize the samples, allowing focused analysis of the conditional, e.g. food- and intestinal bacteria driven metabolic differences due to the individual difference and e.g. dietary interventions (78). Moreover, the correlation of metabolic alterations to the quantitative and qualitative

deviations in the microbiota composition will be most informative.

6. SUMMARY AND PERSPECTIVE

Despite the significant recent advances in the accumulation of genomic data, we are only at the very beginning of understanding human intestinal microbiota, and its enormous metabolic potential remains to be characterized. At least one quarter of microbiome genes recovered from 13 individuals could not be assembled into any contigs and remain unidentified (11). Orphan sequences are likely to originate from rare and individual-specific organisms, which may be functionally interesting but repeatedly remain poorly covered in clone libraries. Correlation between abundance and gene expression by functional genomics will hopefully shed light into this issue.

Very high-throughput sequencing possibilities will accelerate the accumulation of sequence information compared to the rate so far (79, 80). In addition to community-level approach, sequencing of individual species is required to assemble the intestinal microbiome. Recently introduced concept of functional metagenomics is an advanced approach to identify the key functional microbiota members and their metabolic connections to host physiology (44). Sampling of several individuals for the isolation of selected species, followed by comparative genomics, will expand the information of single genomes to the complete gene pool (pan-genome) of the isolated phenotypes (8, 26). This should be carried out query-specifically i.e. focusing on keystone species and their central metabolic genes. Moreover, the comparison of multiple genes, in addition to the 16S rRNA gene, provides a prospective strategy to targeted phylogenetic grouping of functionally related organisms (81). Analysis of a single gene can enhance the detection of phylogenetically heterogeneous but functionally related groups, and additionally estimate the amount of respective functional activity within a fecal community. Such approach was utilized recently for the Clostridial butyryl-coenzyme A transferase (82). Taking together, both reductionistic and holistic approaches, preferably carried out in parallel, are required to identify new functional genes and in the end, to link the genetic and functional diversity together.

Upon collection and storage of microbiome data incorporation of corresponding metadata (nationality of subjects, sample handling and storage conditions, DNA/protein/metabolite extraction method, used primers etc.) would be highly recommended to facilitate the integration and comparison of data coming from different sources. Unintentional selective sampling should be avoided. As an example, substrate-adherent bacteria are lost if coarse fecal material is removed prior to bacterial lysis, partly explaining the underestimation of certain bacteria e.g. in clone libraries. The controlled enrichment of desired populations may, in turn, be used to reduce the high complexity of samples, facilitating both genomic and functional characterization of mixed populations (83).

High intra- and inter-individual variations are a challenge not only to the analytical part, but to a great extent also to the data analysis and retrieval of biologically relevant information. At this point in time, the apparent indefinite variations of the microbiota composition are likely to mask the possible diagnostic effects. While there is evidence for more than 1000 species to be found in the human GI tract (see above), each single individual carries approximately 100-400 predominant species-level phylotypes (6, 44, 84). A total of 69% of the intestinal phylotype 16S rRNA sequences that were stored in public databases by 2006 appeared to be subject-specific (1). This makes a definition of an average or typical intestinal microbiota at the moment impossible. Identification of the bacteria contributing most to the observed dissimilarity between individuals will be crucial, and even approximate reference limits for the normal microbiota in terms of species prevalence and abundance would help enormously the definition of abnormal microbiota composition. This would further facilitate the search for candidate indicator bacteria capable to discriminating the individuals, effects of diet or other variables to be tested. We postulate the existence of so called *sentinel species* that are functionally irreplaceable and contrast with species that are free to fluctuate. The latter group includes the occasional, tourist-like inhabitants of the human bowel. If this concept is applicable, it could provide avenues for defining human health and well-being by identifying these sentinel species.

An unbiased and maximal coverage of microbiome is highly desirable, not least to due to the dependence of proteomics pipeline on the reference genomes. High level of functional redundancy in the intestinal ecosystem is likely to give special features to the interpretation of community proteomics and metabolomics data. On one hand, the overlapping metabolic activities encoded on genomes are conditional, e.g. dependent on the nature of substrates, and therefore not likely to be manifested at their full scale on protein and metabolite level. On the other hand, the fact that communities with different compositions can be functionally similar, exemplified with the sharing of same substrates between different families and genera, inevitably complicates the unambiguous assignment of functional attributes to respective genomes.

In conclusion, a limited amount of direct data regarding metabolic processes involved in the functionality of intestinal microbiota is currently available, but the prolific nature of the data accumulation on the systems components (genes, proteins, metabolites) makes this era very intriguing to the scientific community elucidating the ways and scales the intestinal microbiota impacts our life. Post-genomic strategies combined with multivariate pattern-recognition techniques and other bioinformatics tools are required to integrate and interpret the acquired information into exploitable wealth of knowledge, which will be indispensable in providing functional dimensions to the genomic datasets. They will drive our understanding on the metabolism, bacteria-host and bacteria-bacteria interactions and other types of bacterial activities related to human health and physiological processes far beyond the

potential provided by the sole presence/absence information of different intestinal bacteria.

The anticipated progress related to the mechanistic explanations of functionality will in turn pave the way for the avantgardenist future strategies for the development of gut microbiota-targeted therapies, potential strategies ranging form the restoration of metabolic homeostasis to treatment of neurological disorders (85, 86). At the moment biomarkers for healthy or diseased gut are scare; some of the intestinal bacteria and their expression products are likely to become one in the near future. Comparative fingerprinting techniques able to classify individuals according to the characteristics of their GI microbiota are going to be complemented with the ambitious endeavour to identify the discriminative components. Application of several post-genomics strategies to the same sample, accompanied by sequence-based identification of its bacterial community, will provide the synergism that opens completely new dimensions for the possibilities to relate bacterial community to its *in vivo* activity.

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Abbreviations: GI: gastrointestinal, SRB: sulphate reducing bacteria, SCFAs short chain fatty acids, PCR polymerase chain reaction.

Key Words: Intestinal Microbiota, Metagenomics, Functional Post-Genomics, Review

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