Studying the Human Gut Microbiota in the Trans-Omics Era - Focus on Metagenomics and Metabonomics

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Abstract: The human gut microbiota comprises a diverse microbial consortium closely co-evolved with the human genome and diet. The importance of the gut microbiota in regulating human health and disease has however been largely overlooked due to the inaccessibility of the intestinal habitat, the complexity of the gut microbiota itself and the fact that many of its members resist cultivation and are in fact new to science. However, with the emergence of 16S rRNA molecular tools and "post-genomics" high resolution technologies for examining microorganisms as they occur in nature without the need for prior laboratory culture, this limited view of the gut microbiota is rapidly changing. This review will discuss the application of molecular microbiological tools to study the human gut microbiota in a culture independent manner. Genomics or metagenomics approaches have a tremendous capability to generate compositional data and to measure the metabolic potential encoded by the combined genomes of the gut microbiota. Another post-genomics approach, metabonomics, has the capacity to measure the metabolic kinetic or flux of metabolites through an ecosystem at a particular point in time or over a time course. Metabonomics thus derives data on the function of the gut microbiota *in situ* and how it responds to different environmental stimuli e.g. substrates like prebiotics, antibiotics and other drugs and in response to disease. Recently these two culture independent, high resolution approaches have been combined into a single "trans-genomic" approach which allows correlation of changes in metabolite profiles within human biofluids with microbiota compositional metagenomic data. Such approaches are providing novel insight into the composition, function and evolution of our gut microbiota.

Key Words: Metagenomics, metabonomics, microbiota, human, diet, prebiotic, fibre, intestine, gut.

INTRODUCTION

The completion and publication of the human genome [1,2] represented a major leap in our ability to study human biology. It provided a human genetic blueprint for identifying determinants of heritable diseases and the possibility of linking particular genotypes to disease risk [3]. It also heralded in a new era of biological investigation, the "post-genomics" era, providing many of the bioinformatic tools necessary for the emergence of transcriptomics, proteomics and metabolomics, and also creating the necessary scientific and commercial interest which has lead to technological leaps in terms of DNA sequencing capabilities. More recently, it has become apparent that this genetic blueprint does not tell the whole story of our individuality and other factors, environmental factors, play an important role in human health and disease. These extra-genomic influences may account for much of the individual variation in disease susceptibility, physiological responses to diet and in our response to pharmaceutical interventions. Epigenetics, which genes are "switched on or off" and our microbiome, principally our intestinal microbiota and its metabolic output, may be identified as two important contributors towards this extra-genomic environmental variation, and through their interaction with diet, life-style and xenobiotics (e.g. pharmaceuticals) they appear to impact greatly on human biology [4,5]. The advent of high through-put DNA sequencing has made possible the rapid characterisation of whole microbial genomes and even whole community genomes or "metagenomes" extracted directly from microbial consortia in environmental samples [6]. Metagenomics, defined as "the application of modern genomics techniques to the study of communities of microbial organisms directly in their natural environments, bypassing the need for isolation and lab cultivation of individual species" has given great insight into the composition and functioning of microbial communities in terrestrial and marine environments [7-9]. This approach is now being applied to study the human microbiome (the totality of the microbial community associated with the human body) with some revolutionary consequences on the perceived role of this microbiota in human biology and evolution [10-13]. This review will focus on the gut microbiota, probably the single largest component of the human microbiome. We will discuss how the study of this diverse and complex ecosystem is benefiting from the "post-genomic" advances in scientific technology and understanding.

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LIMITATIONS OF THE TRADITIONAL MICROBIOLOGICAL CULTURE BASED APPROACH

The human gastrointestinal tract is home to many hundreds of microorganisms which colonise to various degrees and in different ecological niches along the length of the alimentary canal, from mouth to anus. Host physiology plays an important role in nurturing these gastrointestinal microbial communities. Gastric acid limits bacterial numbers in the stomach, and in the large bowel, where more neutral conditions prevail and where the flow of digesta slows sufficiently, a sizable microbial consortia develops [14-16]. The adult colonic microbiota, in health, may be viewed as a climax microbial community, displaying remarkable compositional stability and maintaining a high degree of homeostasis and self-regulation, greatly impeding colonization by allochthonous microorganisms ingested by the host [17]. This homeostasis imparts a high degree of functional stability on the gut microbiota, ensuring for the host a steady supply of metabolites of microbial origin [12].

The human gut microbiota has evolved closely with the human race over the millennia, providing functional activities not encoded by the human genome and allowing its host to derive energy from otherwise inaccessible substrates locked into plant structural and storage polysaccharides, and mediating many co-metabolic processes such as deconjugation of bile acids [13, 18]. The fact that this gut microbiota is largely fermentative, converting un-digested carbohydrate and protein into short chain fatty acids (SCFA), has long been appreciated but only now is the complex array of mutualistic, symbiotic and cross-feeding interactions between the gut microbiota in SCFA metabolism been recognised [19, 20]. Until relatively recently, the colon was viewed as a retention tank for waste material and water absorption was considered its most important contribution to systemic biological function. An important contributor towards this lack of understanding was the inaccessibility of the gut and the recalcitrance of its mainly anaerobic gut microbiota to cultivation under laboratory conditions [21]. Traditionally microbiology is carried out using pure culture, where single strains of microorganisms are cultivated on nutrient media under environmental (temperature, pH, substrate availability, atmosphere) conditions conducive to their growth. However, many bacteria in the gut, an estimated 70-80% of organisms present, will not grow under laboratory conditions in pure culture [21, 22]. For those microorganisms which may be cultivated, traditional microbiological culture techniques face an additional problem in that many phylogenetically diverse bacteria share phenotypic traits and can grow under the same conditions and on the same nutrient media, even in the presence of selective supplements [23]. To differentiate between these different bacteria, sub-culturing followed by biochemical and genetic characterisation is required before the microorganism may be identified with any certainty. This process is time consuming and costly. The traditional microbiological culture approach is therefore not well suited to study mixed microbial consortia, such as the human gut microbiota. The recognition of the 16S rRNA gene as a universal, phylogenetically relevant molecular chronometer has allowed the development of a number of molecular tools which greatly facilitate the study of the gut microbiota in a culture independent manner [23-25]. Repositories of phylogenetic information such as the Ribosome Database Project (http://rdp.cme.msu.edu/) currently hold approaching 700,000 16S rRNA gene sequences [25]. However, many of these 16S rRNA gene sequences were derived from direct cloning of environmental DNA and do not correspond to previously cultured bacteria. Comparisons may be drawn between the physiology and ecological functioning of these novel phylotypes and their closest culturable relatives but such functional inferences are limited, since many core metabolic functions may be shared even between distantly related bacteria whilst traits enabling occupation of a particular ecological niche may be strain specific and not shared with close relatives. This situation is further complicated in the gut, where high species richness and high cell density combine to render the colonic environment a hot bed of microbial promiscuity facilitating horizontal gene transfer between even distantly related bacteria. Many of the genes carried by highly transmissible genetic elements within the gut microbiota encode ecologically important functional genes, involved in substrate metabolism, drug resistance or antimicrobial production [26, 27]. Thus despite the growing number of novel 16S rRNA species identified, we know little about how these bacteria behave in their natural environments. Technological advances in the post-genomics era are now providing the tools necessary to study these bacteria directly within their environmental habitats in a culture independent manner.

GUT MICROBIOTA COMMUNITY LEVEL PHYLOGENETIC ANALYSIS

Molecular techniques, based around the phylogenetic information encoded by the bacterial 16S rRNA gene, have provided a new insight into the composition and species richness of the human gut microbiota [23, 28]. By directly isolating bacterial DNA from intestinal samples, the microbial species composition may be determined upon separation and DNA sequencing of the 16S rRNA genes present. One approach to achieve this is by using PCR to amplify the 16S rRNA genes present in the total DNA recovered from an environmental sample (the metagenome) and creating a clone library of these amplified 16S rRNA gene fragments. The 16S rRNA carried in these clones can then be sequenced and the relative abundance of each 16S rRNA species recovered from the original sample established. Using this approach a detailed picture of the composition of the human gut microbiota has emerged. Suau *et al.* [21] showed that the vast majority, 95%, of 16S rRNA species recovered from a single adult faecal sample fell within one of three phylogenetic groupings: the *Bacteroides* group, the *Clostridium coccoides-Eubacterium rectale* group, and the *C. leptum* group. The *C. leptum* and *C. coccoides-Eu. rectale* groups include many genera previously described as important members of the gut microbiota including species of *Eubacterium*, *Ruminococcus*, *Butyrivibrio* and *Faecalibacterium prausnitzii*. Recent studies using this approach have shown that the two most abundant bacterial phyla found in the healthy human large intestine are the Gram negative *Bacteroidetes* and the Gram positive, low GC% *Firmicutes* [16, 22, 29]. *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* phyla are relatively less abundant [16, 22] but include bacterial genera which play important roles in gastrointesti-

nal health, including the enterobacteria, bifidobacteria and *Atopobium*/*Collinsella* group. Moreover, the bacterial community in the stomach and jejunum was shown to be different from that in the distal ileum, ascending colon and rectum, and the major phylogenetic groups were similar within the distal ileum and rectum [16, 30]. Suau *et al.* [21] also showed that a major proportion, up to 70% of the 16S rRNA species present in this faecal microbiota belonged to novel phylogenetic lineages all be it within the three dominant groupings. Only 24% of clones corresponded to previously identified bacterial phylotypes. More recently, Gill *et al.* [10] using a direct high-through put sequencing of metagenomic DNA present in faecal samples collected from two American individuals found that 22.2% of phylotypes were novel and 83.3% of phylotypes corresponded to previously uncultured bacterial species.

COMMUNITY FINGER-PRINTING BY PCR-DGGE

Complementary to direct sequencing of 16S rRNA pools recovered form environmental samples, a profile or fingerprint of the gut microbiota may be obtained upon separation of 16S rRNA amplicons using denaturing gradient gel electrophoresis (DGGE) or similar techniques (e.g. temperature gradient gel electrophoresis, TGGE and temporal temperature gradient gel electrophoresis, TTGE) [31]. This approach capitalizes on the fact that double stranded DNA denatures at different rates across a gradient of denaturant (e.g. urea, or temperature), depending on its DNA sequence and will thus migrate differentially along a denaturing gradient on an electrophoresis gel. The advantage of this approach is that it generates a snap-shot of gut microbiota composition at the time of sampling, it is a rapid and cost effective method, and as such can be used to track fluctuations within the gut microbiota over time or in response to environmental stimuli such as substrate supply, prebiotic or probiotic ingestion, antibiotics or in the presence of disease states. Differential bands within the DGGE 16S rRNA gene fragment profiles can be excised and sequenced for phylogenetic positioning in order to identify which bacteria respond to environmental change. Zoetendal *et al.* [32] used this approach to show that individuals harbour a unique intestinal microbiota, and that the species composition of the adult gut microbiota is remarkably stable over extended periods of time. These authors also compared gut microbial species composition of genetically and non-genetically related family members [33]. Gut microbiota composition of spouses, who were living in the same environment, showed less similarity in their TGGE profile than siblings who showed increased similarity in species make-up. Surprisingly, even though the gut microbiota profiles of identical twins showed a high degree of similarity, they were none the less distinct, and unique, indicating that acquisition and successional development of the human gut microbiota may be susceptible to both genetic and environmental influences [33]. A particular advantage of these approaches is that by using PCR primers targeted at different phylogenetic levels, the composition of particular taxonomic groups of bacteria within the gut microbiota may be established and monitored over time in response to different environmental perturbations. Tannock *et al.* [34] recently employed PCR-DGGE to follow the compositional changes that occur within the human gut microbiota upon ingestion of prebiotic functional foods.

LIMITATIONS OF PCR BASED TECHNIQUES

The molecular approaches described above for determining the composition of the gut microbiota, with the exception of direct sequencing techniques, rely on PCR to amplify 16S rRNA species from a pool of 16S rRNA genes recovered from the gut microbiota. This approach has a number of now well recognized limitations, and bias towards recovery of particular 16S rRNA species may be introduced at different stages. The method used to isolate bacterial DNA from faeces or mucosal specimens may select for a certain fraction of the gut microbiota since bacteria with different cell wall conformations display different susceptibilities to cell disruption and thus recovery of their DNA. PCR is a competitive reaction governed by melting and renaturation efficiencies of the target DNA sequences. PCR based approaches may therefore select for particular pools of 16S rRNA based on ease of melting and re-naturation rather than initial proportions of target DNA in the original sample. Bacteria with high GC content in their DNA, and thus high double stranded DNA melting temperatures, such as the bifidobacteria, consequently are often under-represented in 16S rRNA gene libraries derived from competitive PCR reactions [35]. Finally, cloning of the amplified 16S rRNA gene fragments may introduce its own selection since different cloning vectors and hosts show bias towards certain DNA sequences [36, 37]. PCR based approaches therefore have tremendous power to generate a snap-shot of gut microbiota composition, allowing the identification of which species of bacteria are present within a microbial community and can be used to monitor changes in the species richness over time in response to different environmental stimuli. However, the limitations of these approaches must be recognized particularly when enumerating different bacterial groups within an ecosystem.

MOLECULAR CHARACTERIZATION OF THE GUT MICROBIOTA *IN SITU*

Fluorescence *in situ* hybridization (FISH) using 16S rRNA targeted probes coupled with epifluorescence microscopy or flow-cytometry, provides a direct method for counting specific bacterial populations in environmental samples without the need for microbiological culture [38]. The advantage of this approach is that phylogenetically related bacteria (at differing phylogenetic levels ranging from kingdom or phylum levels to species level depending on probe design) may be enumerated directly in environmental samples. For example, the ability of prebiotic food ingredients to stimulate the growth of particular members of the human gut microbiota has been established using a panel of FISH probes in human intervention studies [39, 40]. The FISH technique does have some limitations, in particular variability in permeabilization of target cells and the need for prior 16S rRNA gene information for probe design, but it does allow the estimation of relative bacterial population levels within mixed microbial consortia without the bias introduced by DNA recovery and subsequent PCR and cloning [41]. FISH gives an accurate picture of relative bacterial population levels

because the bacteria are enumerated directly in the environmental samples without selective amplification [41]. The relative abundances of the dominant members of the human gut microbiota as determined by FISH can be seen in Table **1**, which was adapted from [29, 42-45]. As part of two separate EU funded projects (Microbe-DIAGNOSTC, QLK1-2000-108 and CROWNALIFE, QLRT 2000-00067] FISH coupled with flow-cytometry for automated cell counting was employed to study the composition of the gut microbiota across the EU. Using a panel of 18 phylogenetic FISH probes Lay, *et al*. [44] were able to enumerate on average 75% of the bacteria present in faecal samples collected from 91 individuals from France, The Netherlands, Denmark and Germany. Although the authors did not find any effect of geography, age or gender on the relative abundance of different bacterial phylotypes, they did confirm that the gut microbiota of these Northern Europeans was dominated by two groups of Firmicutes, *Clostridium coccoides* group and *C. leptum* group, followed by the *Bacteroides*, comprising 28.0%, 25.2% and 8.5% of the total cells enumerated respectively. In a more recent study designed to examine age related differences within the gut microbiota of young adults 20-50 years, and seniors >60 years, Meuller *et al.* [45] found marked differences in the composition of the faecal microbi-

Table 1. Adapted from Flint [42], Stewart *et al.* [43], Louis *et al.* [29]. Relative Abundance of Dominant Human Gut Bacterial Groups and Corresponding Main Acidic Fermentation End-Products. The Abundance is Expressed as Mean Values of the Percentage of Total Bacteria Using FISH, Based on Data from Lay *et al.* [44] and Meuller *et al.* [45]. The Reported Fermentation End-Products are Indicative of Cultured Representatives

	Bacterial Group	Abundance (Typical % of Total Bacteria)	Fermentation End-Products
Firmicutes	Clostridial clusters XIV a+b		
	Eubacterium rectale - Clostridium coccoides	14.5-33.0	Butyrate, formate, lactate
	Eubacterium hallii	0.6-3.8	Butyrate, formate, acetate
	Ruminococcus obeum	2.5	Acetate
	Lachnospira spp.	3.6	Formate, acetate, lactate, succinate
	Clostridial cluster IV		
	Clostridium leptum	21.7 - 26.8	
	Faecalibacterium prausnitzii	4.9 - 20.4	Butyrate, formate, lactate
	Ruminococcus bromii, *Ruminococcus flavefaciens*	1.8 - 10.2 0.4 - 1.3	Acetate, formate, lactate, succinate
	Clostridium viride	0.5 - 2.6	Acetate, propionatc, butyrate, valerate, ammonia
	Eubacterium desmolans	0.1 - 0.4	Acetate, butyrate
	Clostridial cluster IX		
	Veillonella spp.	0.9 - 2.5	Propionate, various minor acids
	Clostridial cluster XVI		
	Eubacterium cylindroides	0.3 - 1.7	Butyrate, acetate, lactate, succinate, formate
	Lactobacillus/Enterococcus	0.2 - 2.7	Lactate, acetate
Actinobacteria	*Bifidobacterium* spp.	1.1 - 5.8	Lactate, acetate, formate
	Atopobium spp.	0.8 - 6.3	Acetate, formate, Lactate
Bacteroidetes	*Bacteroides-Prevotella* gp.	3.9 - 13.6	Acetate, propionate, succinate
	Bacteroides putredinis	0.1 - 0.8	Acetate, succinate
	Bacteroides fragilis	0.4 - 4.2	Acetate, propionate
Proteobacteria	*Escherichia coli* *Salmonella* *Klebsiella*	0.1-0.2	Lactate, acetate, succinate, formate
	Desufovibrio	< 5.5	SCFA cross-feeding, H_2S production
Archaea	*Methanobrevibacter smithii*	< 0.2	SCFA cross-feeding, CH_4 production

ota across ages and location in 230 individuals from 4 European countries, France, Germany, Italy and Sweden. Again using FISH coupled flow cytometry and a panel of 14 different group and species-specific 16S rRNA gene probes these authors noted age-related changes in the dominant gut bacteria (i.e. *Eu. rectale-C. coccoides*, *Bacteroides-Prevotella* or *Faecalibacterium prausnitzii*). Geographical location affected levels of bifidobacteria, with Italian citizens having 2-3 fold higher levels compared to other European countries, which is most likely due to their higher intake of fermentable fibre. Of the less dominant bacterial groups enumerated, the *Enterobacteriacaea* were much higher in the elderly, irrespective of country, while age-related differences in proportions of *Lactobacillus/Enterococcus* group were country-specific.

One unique advantage of FISH coupled with flow cytometry is that it allows the physical separation or sorting of microorganisms present in mixed consortia into pylogenetically related groups of cells [46, 47]. The cell sorted bacterial groups can then be subjected to DNA extraction followed by PCR based metagonomic approaches to characterize the species make up of family glades enumerated by FISH. This allows generation information about which bacteria have hybridized with a particular group specific probe in mixed microbial consortia and has already been used to identify the species make up of viable, injured and dead fractions in human faeces [46]. Lay *et al*. [48] used this approach to determine which bacterial phylotypes were enumerated by FISH probes targeting the *C. leptum* group, which include important butyrate producing bacteria and constitute a dominant group within the gut microbiota. These authors used fluorescence-activated cell sorting (FACS) with probes for the *C. leptum* group (Clep866-CY5/cp or Fprau645-CY5) to isolate this target group of bacteria from faecal samples from 9 healthy volunteers (age 24-43 years). These cell sorts were then subjected to DNA extraction, PCR-TTGE to generate a profile of 16S rRNA species hybridized by the probes. The TTGE profiles were unique for each faecal sample analysed, they remained stable over time (2 months) and they comprised relatively low numbers of bacterial species (typically 4 to 6). The 16S rRNA gene fragments present in the cell sorts were then cloned, sequenced and found to be made up of 15 different phylotypes, two thirds of which belonged to previously uncultured bacteria. Considering that a sizable proportion of intestinal bacteria are new to science such an approach may be useful both in focusing probe resolution for a particular group of bacteria and in identification of novel phylotypes. Moreover, by employing sequence based metagenomics, the whole metagenome of previously unrecognised phylotypes may be characterized to derive functional information on specific phylogenetically related bacterial cell sorts directly in environmental samples in a culture-independent manner and without reference to previously cultured close relatives (see Fig. **1**).

CULTURE-INDEPENDENT FUNCTIONAL CHARACTERIZATION OF THE GUT MICROBIOTA METABOLIC POTENTIAL

The development of 16S rRNA based tools for measuring the species richness and relative abundances of bacteria in natural environments has provided unique and valuable insights into the make-up of the gut microbiota. These approaches have also highlighted the fact that in any given gut microbiota, many species present are novel, not represented in the 16S rRNA database and thus probably recalcitrant to laboratory cultivation [10, 21]. This highlights an important issue, how to assign ecological function to novel or unculturable bacteria? Recent advances in high through-put sequencing allow the rapid generation of large DNA sequence datasets many orders of magnitude more rapidly than traditional Sanger based sequencing and at a fraction of the cost [49]. The bioinformatics tools and access to high powered computing too has greatly improved over the last ten years enabling rapid and accurate annotation of gene function for large DNA sequence data sets. Taken together these advances have given rise to the metagenomics sequence based approach for functional characterization of microbial consortia. Sequence based metagenomics applies shot-gun sequencing of total DNA extracted from an environmental sample. Pioneered in marine and terrestrial microbial ecology, this approach has recently been applied to study the mammalian gut microbiota [10, 12, 50]. The functional or metabolic potential of the human gut microbiota can thus be accessed through large scale sequencing projects targeting not just community 16S rRNA genes, but potentially all bacterial genes present. Exploration of the genetic potential of metagenomic data bases may be carried out by comparative functional analysis using annotation schemes such as Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways or Clusters of Orthologous Groups (COGs) [51, 52]. KEGG analysis gives a comparison to enzymes already characterized in known metabolic pathways and COG analysis compares the degree of evolutionary relatedness between groups of functionally related genes. Sequence based metagenomics of the gut microbiota has already identified new levels of host:microbe and diet:microbe interactions [10, 12, 50].

Gill *et al*. [10] reported that the faecal metagenome of two unrelated American adults was enriched for genes involved in the metabolism of carbohydrates, amino acids and xenobiotics, metathogenesis and the biosynthesis of vitamins and isoprenoids, compared to the human genome and other bacterial databases. The authors pointed out that the faecal metagenome encoded many functions not represented in the human genome. In particular, few genes for the metabolism of major structural and storage polysaccharides present in dietary plants such as xylans, pectins, arabino-containing carbohydrates and fructans are encoded by the human genome, while in the metagenome of the gut microbiota such genes are common. At least 81 different glycosyl hydrolase families could be assigned to the gut microbiota as well as an enrichment of key genes involved in the production of SCFA (acetate, butyrate, propionate and succinate) highlighting the importance of carbohydrate fermentation in the energy economy of the colon. In a comparative study of the metagonomic make up of the gut microbiota of 13 healthy Japanese individuals of different age, Kurokawa *et al*. [12] found that genes involved in carbohydrate metabolism and transport were enriched in all 13 metagenomes, compared to a database constructed from COG assigned genome sequences of 243 different bacteria not known to be represented within the gut microbiota. Conversely, genes involved in lipid metabolism were under-represented in the gut metagenomes com-

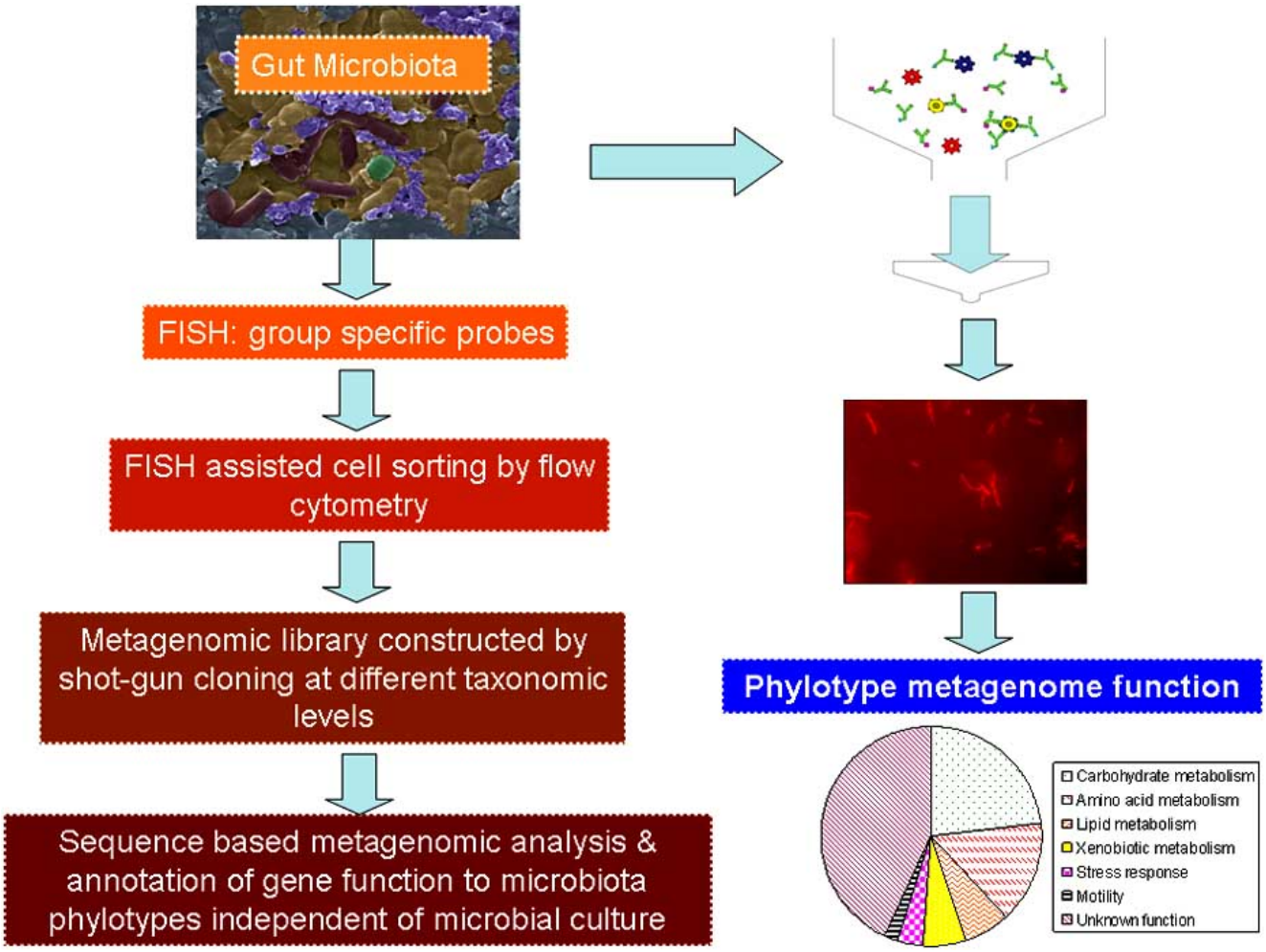

Fig. (1). Fluorescence activated cell-sorting (FACS) using FISH and 16S rRNA probes and sequence based metagenomics offers the potential to characterise the metabolic potential of phylogenetically related groups of bacteria irrespective of whether their representative species have ever been cultivated in the laboratory.

pared to this reference database. These observations suggest that the human gut microbiota has evolved along-side its human host to complement human encoded functions and allow the host access to dietary nutrients not digested or absorbed in the upper gut. SCFA produced during colonic fermentation are thought to contribute about 10% of our daily energy intake [53]. Similarly, 90% of plant derived polyphenolic compounds reach the colon and are there transformed into biologically active and available intermediates by the colonic microbiota [54]. This has important implications for the design of healthy human diets and nutrigenomics. For much of our evolutionary history, humans have consumed diets rich in whole plant foods particularly high in dietary fibre and polyphenols (see Box **1**). The maintenance of a stable, fermentative gut microbiota would have been essential to maximize energy recovery from such diets, maintain vitamin production by the gut microbiota [55] and to take advantage of the biologically active polyphenolic compounds present in many whole plant foods [56]. Conversely, modern diets which are often low in dietary fibre, low in whole plant foods and in general, richer in fat and refined sugars, are out of step with our "pre-historic" hunter-gatherer gut microbiota. There is a growing body of evidence suggesting that modulating our modern gut microbiota through dietary supplementation with prebiotic fibres (defined below) in particular can mediate positive health effects on a number of important physiological functions including *de novo* lipogenesis [57], mineral absorption [58, 59], regulation of satiety and body fat deposition [60, 61] and importantly, providing butyrate as an energy supply to the colonic mucosa a key process in controlling mucosal proliferation, differentiation and maintenance of mucosal integrity [62].

One of the more surprising insights provided by recent metagonomic studies has been the observation that the gut microbiota of obese people appears to differ from that of the lean individuals. Over the past 15 years there has been a sharp increase in the incidence of obesity in the developed world, and obesity is set to reach epidemic proportions with 60% of the UK male population projected to become obese by 2050 at current prevalence rates [63]. There is a genetic component to obesity and there is a sub-population geneti-

Box 1: Reconstructing ancient Paleolithic (50,000-10,000 years ago) or pre-agricultural diets is extremely difficult and prone to some error but certain facts can be derived from diverse sources. Human skeletal remains may tell the nutritional status of our ancestors surviving on particular diets, while the study of human coproliths (fossilized faeces) from archaeological finds may reveal specific dietary details such as types and quantities of animal bones, fish shells and botanic remains which can be identified in order to calculate the macronutrient content (e.g. animal protein content, carbohydrates or fibre). Data from extant hunter-gatherer (or forager) populations can give unique insight into the gross composition of the types of diets our pre-agricultural ancestors may have adhered to. For the vast majority of human evolution our ancestors followed a hunter gather diet made up of wild fruits, vegetables, fish and game. Agriculture, a fairly recent occurrence in evolutionary time, emerging some 10,000 years ago, resulted in dietary change which continues to this day. The table below compares "typical" hunter gather/late paleolithic type diets with modern counterparts in different regions of the world and in populations following very different diet and life-styles and only serves as a very rough comparison on average fibre intake between the different populations [99, 100]. The percentage of carbohydrates intake in the late Paleolithic diet was similar to that in modern western diet, 45-50% of daily energy. However, there was a remarkable qualitative difference. Under most circumstances during the late Paleolithic, the vast majority of carbohydrates were derived from fruits and vegetables, very little from cereal grains and none from refined flours [99]. Western diets contain carbohydrate foods with relatively high glycaemic indices (e.g. potatoes, bread) whereas the wild plant foods (fruits and vegetables) consumed by hunter-gatherers are high in fibre content and slowly or lowly digestible carbohydrates which produce low glycaemic and insulin responses. The late Paleolithic diet was 3-5 folds higher in fibre content compared to modern western-type diet.

Estimated Daily Fibre Intake in Palaeolithic Diet and Modern Diet

Dietary Pattern	Fibre Content	Reference
Palaeolithic diet reported in 1985 (35% meat, 65% vegetables)	45.7g	[99]
Palaeolithic diet modified in 1997 (50% meat, 50% vegetables)	104g	[100]
American coprolite data I	>100g	[101]
American coprolite data II	150-250g	[102]
Rural Chinese diet	77g	[103]
Rural African diet	60-120g	[104]
Current US diet	12-18g	[105]
Recommended fibre content in US	20-35g	[105]
Current UK diet	12g	[106]
Recommended fibre content in UK	18g (minimum)	[106]

cally predisposed to obesity [64]. However, the sudden increase in obesity over the past 15 years has occurred at a rate which far outstrips that of human genomic evolution, showing that the obesogenic environment has a major role to play. This obesogenic environment impacts on the quantity of food we eat, the types of foods we eat, satiety, energy recovery from the diet, epigenetic programming, mental state and exercise, all of which play important roles in determining the risk of obesity. There are strong epidemiological data linking diets high in fat and refined carbohydrates with obesity [65]. Conversely, diets rich in fibre and whole plant foods are inversely associated with obesity [66]. Similarly, there is a growing body of evidence from animal studies that certain fibres particularly prebiotics, may reduce the risk of obesity itself and its associated pathologies [67]. Interestingly, prebiotics, "non-digestible (by the host) food ingredients that have a beneficial effect through their selective metabolism in the intestinal tract", act through modulation of the gut microbiota, which has been identified as an important environmental determinant in mammalian energy metabolism [68, 69]. Recent data from the metagonomic analysis of the gut microbiota of animal models of obesity and obese humans suggests that the gut microbiota differs between obese and lean subjects at the phylum level [71-73]. It appears that obesity is associated with a reduced abundance of intestinal Bacteroidetes and a higher abundance of Firmicutes in the genetic *ob*/*ob* mouse model of obesity and in humans. This altered microbiota is associated with increased fermentation end products in caecal and faecal samples, and differences in metagenomic composition [72, 73]. Interestingly, when germ-free animals are associated with caecal contents of conventional, but obese counterparts carrying this obese-type microbiota, they too become obese, despite being maintained on the same diet [73, 74], indicating that the obese type microbiota may be an important contributor towards the onset of obesity itself. However, it also appears that diet can strongly influence the composition of the obese-associated microbiota. When obese humans are put on weight reduction diets (either low carbohydrate or low fat) a gut microbiota composition resembling a lean profile is observed upon weight loss [72]. Gut microbiota differences may go some way towards explaining why the obese are more effective at extracting energy from food, and less effective at regulating energy storage than lean individuals. In fact, a change in our eating habits may foresee a forced "directed microbiota evolution", in the case of high-fat Western-style diets, towards a

more detrimental gut microbiota composition. However, further studies are necessary to delineate any aetiological role of the obese-type microbiota in body weight gain and conversely, in the ability of different diets to induce an obese-type microbiota or reduce the risk of becoming obese by modulating gut microbiota composition and activity.

The metagonomics approach provides a powerful tool for measuring the metabolic potential of the gut microbiota. Identifying genetically encoded functions which are enriched or under-represented within a microbial community can shed light on the ecological role of that community as a whole [50]. However, as observed by Kurokawa *et al.* [12] not all open reading frames (ORFs) can be annotated to known function by comparisons with reference databases. These authors found that between 45 and 80% of protein-coding genes observed in the 13 Japanese [12] and 2 American [10] faecal metagenomes could not be assigned a metabolic function at the 90% threshold identity upon BLASTP analysis against their reference database of 243 non-gut bacterial genomes. Another limitation of the metagonomic approach is that it generates data on the metabolic potential of a microbial ecosystem, the potential encoded by the genetic material of the organisms present which is only translated into metabolic kinetic in response to particular environmental stimuli. Another post-genomics approach has recently emerged with the capability to measure this metabolic kinetic or flux in microbial metabolites which occur in functioning ecosystems.

MEASURING THE METABOLIC KINETIC OF THE HUMAN MULTI-ORGANISMAL SYSTEM - METABONOMICS

From the above metagenomic studies it is becoming clear that there is a high degree of co-operation between the human genome and the gut microbiota and that the gut microbiota has co-evolved alongside its human host over time [13]. The concept of gut microbiota:human genome co-evolution, resulting in a symbiotic relationship, has previously been put forward by Nicholson *et al.* [18]. These authors suggested that gut microbiota structure and composition reflects this symbiotic relationship with only the bacterial populations beneficial to the host predominating in the human microbiota in health and contributing to co-metabolism of a range of dietary components and xenobiotic compounds. Recognizing the contribution of microbiota derived compounds observable in metabolite profiles of human biofluids (e.g. blood, urine, faecal water) to metabolic processes at the whole organism level, Nicholson *et al.* [18] combined high resolution analytical techniques with image analysis and multi-variate statistics to establish another "post-genomics" approach. Metabonomics has been defined as "a systems approach to examining the changes in hundreds or thousands of low-molecular-weight metabolites in an intact tissue or biofluid" [18]. The human metabonome thus comprises; the metabolites derived from human encoded genetic determinants, metabolites of microbiotal origin, and the flux in these combined metabolite profiles under different perturbations e.g. consumption of different foods or drugs, carriage of parasites, and chronic disorders like cardiovascular disease. Metabonomics employs ^{1}H-nuclear magnetic resonance (NMR) spectrometry and mass spectrometry-based techniques to generate profiles of metabolites in biofluids including urine, plasma and faecal water, which characterize the metabolic kinetic of an organism at a particular point in time. By applying image analysis followed by multivariate statistics, metabonomics can follow changes in these metabolite profiles in response to dietary (e.g. probiotic) or pharmaceutical interventions and to generate distinctive metabolite profiles in disease states such as inflammatory bowel disease and cardiovascular disease, offering the possibility to develop new diagnostic tools and identify novel therapeutic targets [75-78]. Recent metabonomics studies have highlighted the potential contribution of microbiotal derived metabolites or co-metabolites in the aetiology of chronic diseases. Dumas *et al.* [79] found that in mice genetically predisposed to impaired glucose homeostasis and non-alcoholic fatty liver disease (NAFLD) maintained on a high fat diet, microbial activities within the gut lead to reduced choline bioavailability, mimicking choline-deficient diets already known to induce NAFLD and insulin resistance (IR), key initial steps in the development of the metabolic syndrome and obesity. Specifically, ^{1}H-NMR spectral profiling was able to identify low circulating plasma phosphatidylcholine and high urinary excretion levels of methylamines (dimethylamine, trimethylamine and trimethylamine-N-oxide), co-metabolic products of the host animal and gut microbiota, as differentiative of high-fat fed animals with NAFLD and impaired glucose metabolism. Holmes *et al.* [78] recently illustrated the power of this approach in grouping individuals according to their urine metabolite profiles at the population level, and relating these profiles to geography, diet and disease risk, in this case showing an inverse association between urinary formate (and, to a lesser extent, hippurate) with blood pressure (BP) and coronary vascular disease (CVD) risk. Upon ^{1}H-NMR metabolite profiling of two 24h urine samples taken from 4,630 human volunteers in China, Japan, the UK and the USA, these authors were able to distinguish the East Asian from Western populations, Japanese living in Japan and Japanese living in the USA, and populations of northern (Guangxi) and southern (Beijing and Shanxi) China. Urinary profiles of people in the UK and USA were similar. The main differentiating metabolites were of dietary origin, including amino acids, creatine and trimethylamine-*N*-oxide; acetylcarnitine, tricarboxylic acid cycle intermediates involved in energy metabolism and dicarboxylic acids like suberate. They also identified a group of compounds of microbial origin or which derive from the co-metabolic processing by host and gut microbiota including hippurate, phenylacetylglutamine, methylamines and formate. Formate can either derive from endogenous one-carbon metabolism or through fermentative metabolism of non-digestible carbohydrates by intestinal bacteria including certain clostridia (mainly belonging to clusters XVI, IV, XIV), the Actinobacteria including the bifidobacteria, and to a lesser extent, the Proteobacteria, see Table **1**. Hippurate, an end product of polyphenol co-metabolism by the host and gut microbiota, was also inversely related to BP and positively correlated with fibre intake, while high BP was associated with diets high in animal protein and the urinary metabolite, analine. The same group have also recently shown that the close relationship between mammals and their intestinal microbiota extends through-out life from infancy to old age. Distinct metabolite profiles as determined by ^{1}H-NMR

urine profiling were found in dogs at different stages of their lives and in response to dietary change (a calorie restricted diet compared to normal chow). The trajectory demonstrated initial rapid shifting of urine metabolite profiles before age 1 year (early life) after which the metabolic signature stabilized between 1 and 2 years of age. A second metabolic shift was observed in middle-age (years 5-9) before profiles again underwent a metabolic transformation in old age (>10 years old). Many of the differentiative metabolites had their origins in the microbiota:host co-metabolism highlighting the role of the gut microbiota in the ageing process [80]. Although these studies identified microbiota associated metabolites as playing important roles in metabolic disease, CVD risk and the ageing process respectively, they did not attempt to link these metabolite profiles with specific bacteria or groups of bacteria within the gut microbiota.

A CULTURE INDEPENDENT "TRANS-OMICS" APPROACH TOWARDS FUNCTIONAL CHARACTERISATION OF THE GUT MICROBIOTA

A key recent development has been the application of a "trans-omics" approach combining ^{1}H-HMR based metabolite profiling of human biofluids and metagenomic fingerprinting of the resident intestinal microbiota. Li *et al*. [81] examined the urinary metabonomic profile of a single Chinese family, spanning four generations, infants to great grandparents, and correlated it with the metagenomic profile of their faecal microbiota generated by PCR-DGGE of community 16S rRNA. Unique urinary metabolites were identified which differentiated these individuals and these metabolites correlated with unique bacterial species in faecal PCR-DGGE profiles. Specifically, the authors found that the prevalent and dominant member of the gut microbiota, *Faecalibacterium prasnutzii*, correlated strongly with eight urinary metabolites of diverse structure, involved in numerous systemic metabolic pathways. This functional metagenomics approach, briefly outlined in Fig. (**2**), provides a unique tool for dissecting the metabolic contribution of the human gut microbiota and more over, since it employs culture independent methodologies, has the potential to generate testable scientific hypotheses concerning the functional and ecological role of bacteria thus far recognisable only as entries in 16S rRNA gene sequence databases or completely new to science.

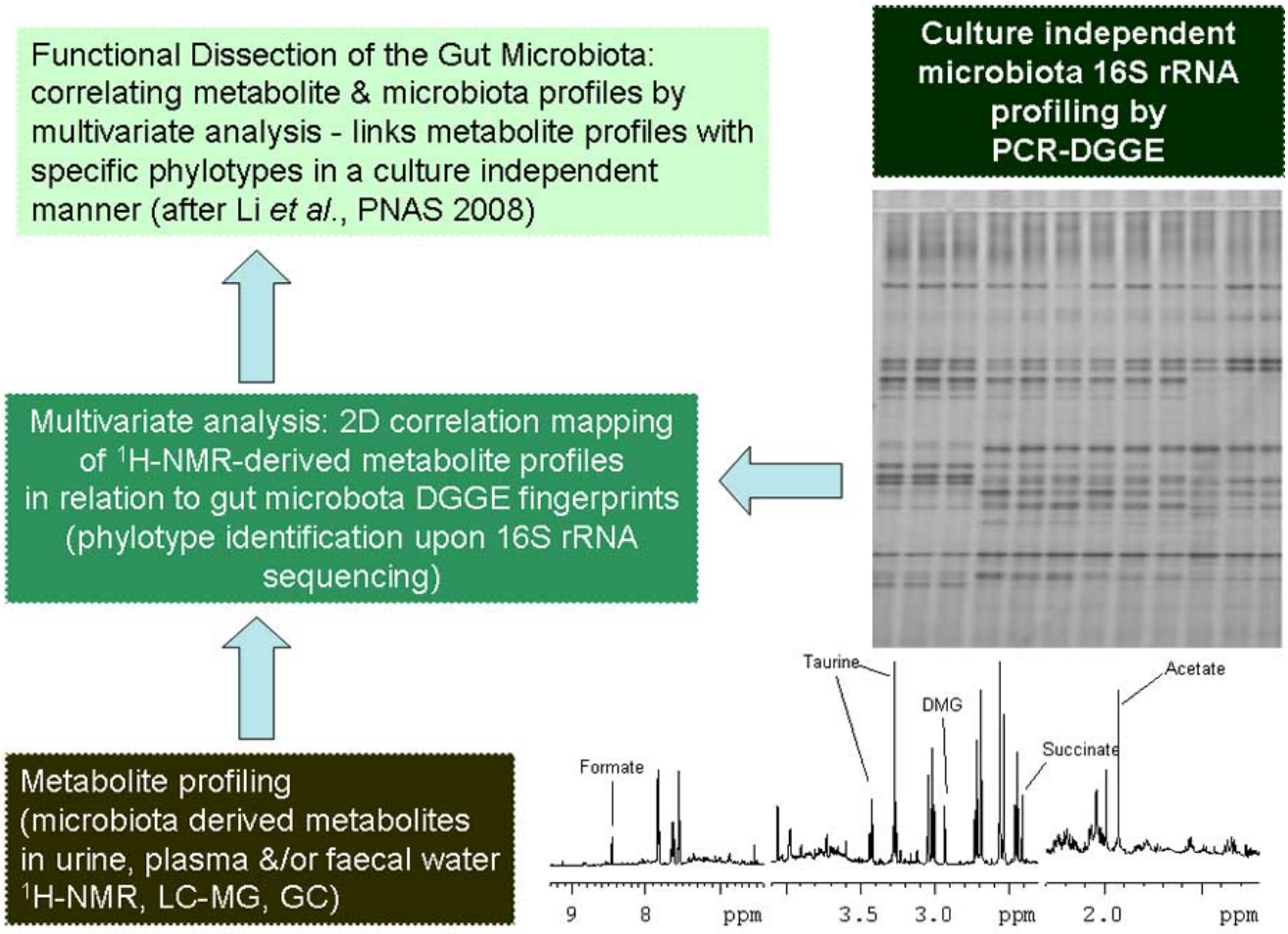

Fig. (2). Schematic representation of the "trans-omics" approach as described by Li *et al.* [81]. By combining ^{1}H-NMR based metabonomics and culture-independent metagenomics fingerprinting of the human gut microbiota Li *et al.* [81] presented a powerful tool for measuring the metabolic contribution of specific members of the gut microbiota, or phylotypes, with no prior requirement for species identity. This approach may provide key insights into the ecological role of novel and unculturable bacterial species which account for a sizable proportion of the human gut microbiota.

FUNCTIONAL METAGENOMICS- THE SEARCH FOR NOVEL BIOLOGICAL AGENTS (ENZYMES AND ANTIBIOTICS)

As described above, the human intestinal microbiota is a diverse ecosystem with high microbial species richness and a high density of bacteria, up to 10^{12} cells/g intestinal contents. These bacteria compete for growth substrates, ecological niches and attachment sties on the gut wall and on food particles within the luminal contents. This competitive environment may therefore be a rich source of biological agents, both enzymes, useful in breaking down carbohydrates, proteins, lipids, xenobiotics or polyphenolic compounds, and clinically important molecules like antibiotics and bacteriocins. Sequence based metagenomics approaches combined with the function-driven-enrichment or screening strategies have greatly accelerated the rate of discovery of novel genes for antibiotics like tubromycin [82, 83], biocatalysts especially lipases [84-87], xylanases [88], amidases [89]. To date soil environments have been the most common subjects of these metagenomic studies due to the vast diversity and history as sources of commercially valuable biomolecules [90]. However, the industrial biotechnological demand for novel enzymes and other biomolecules has driven metagenomic expansion into other complex microbial ecosystems including marine environments (e.g. sponges) and the gastrointestinal tract [12, 91, 92]. Metagenomic expression libraries of the bovine rumen have been useful in unearthing many novel enzymes including, esterases [93], hydrolases [94], amylases [95, 96] and phenol oxidase [97]. BAC libraries of mouse gut metagenome have revealed glucanases, genes involved in environmental sensing, nutrient acquisition and microbial co aggregation [91]. The recently commissioned Human Microbiome Project will pave the way for identification of novel biocatalysts, restriction enzymes, antimicrobials, antimicrobial resistance genes and other potential biomolecules encoded within the gut microbial metagenome [50]. In addition, metagenomic data of the enzymatic mechanisms of host:microbe interactions will facilitate improved understanding of the role of gut microbiota in human diseases like colon cancer and inflammatory bowel disease (IBD) [98].

CONCLUSIONS

Traditionally studies on the composition and function of the human gut microbiota were greatly limited by our inability to cultivate the majority of gut microorganisms, the fact that many of these bacteria are novel without any previously cultured close relatives, and that there was a paucity of useful biomarkers of microbiota functioning in the body. Epidemiological studies have highlighted the importance of dietary fibre (comprising the main substrates for microbial growth in the colon) and dietary intervention studies (with foods like prebiotics or antibiotics) have shown that our microbial partners contribute greatly toward human health and disease. The recent application of molecular microbial ecological tools like FISH, DGGE, FACS, and the post-genomics technologies, metagenomics and metabonomics in particular, for studying the gut microbiota is generating a completely new perspective on the contribution of our gut microbiota to human systems biology. A picture of close co-evolution between the human genome and the resident gut microbiota is evolving, with both genetic and environmental, especially dietary, factors contributing towards the establishment and optimal functioning of an individuals gut microbiota. Wider application of these approaches in human clinical and nutritional studies holds great promise for elucidating further the mechanistic principles underpinning host:microbe interactions in disease states like colon cancer, ulcerative colitis and more systemically, obesity and coronary vascular disease, autism, schizophrenia, senile dementia, and osteoporosis. These tools too hold great potential for the rational design and *in vivo* validation of functional foods, neutraceuticals and pharmaceuticals which can intervene in these host:microbe interactions to improve host health.

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