Journal of Pharmaceutical and Biomedical Analysis 194 (2021) 113751

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Roadmap to functional characterization of the human intestinal microbiota in its interaction with the host

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ARTICLE INFO

Article history:
Received 22 May 2020
Received in revised form 28 October 2020
Accepted 29 October 2020
Available online 22 November 2020

Keywords:
Human
Intestine
Microbiome
Interaction
Function

ABSTRACT

It is known for more than 100 years that the intestinal microbes are important for the host's health and the last decade this is being intensely studied with a focus on the mechanistic aspects. Among the fundamental functions of the intestinal microbiome are the priming of the immune system, the production of essential vitamins and the energy harvest from foods. By now, several dozens of diseases, both intestinal and non-intestinal related, have been associated with the intestinal microbiome. Initially, this was based on the description of the composition between groups of different health status or treatment arms based on phylogenetic approaches based on the 16S rRNA gene sequences. This way of analysis has mostly moved to the analysis of all the genes or transcripts of the microbiome i.e. metagenomics and meta-transcriptomics. Differences are regularly found but these have to be taken with caution as we still do not know what the majority of genes of the intestinal microbiome are capable of doing. To circumvent this caveat researchers are studying the proteins and the metabolites of the microbiome and the host via metaproteomics and metabolomics approaches. However, also here the complexity is high and only a fraction of signals obtained with high throughput instruments can be identified and assigned to a known protein or molecule. Therefore, modern microbiome research needs advancement of existing and development of new analytical techniques. The usage of model systems like intestinal organoids where samples can be taken and processed rapidly as well as microfluidics systems may help. This review aims to elucidate what we know about the functionality of the human intestinal microbiome, what technologies are advancing this knowledge, and what innovations are still required to further evolve this actively developing field.

Contents

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https://doi.org/10.1016/j.jpba.2020.113751

1. Introduction

For more than hundred years it has been known that we are not sterile on our outer and inner surfaces but this fact was given little attention [1]. This especially applies to our intestinal tract where the number of microbial cells is exceeding the human cells in number. Following the isolation of *Escherichia coli* as the first species from the intestinal tract in 1885, over 1000 species have been cultured from the human intestine and validly described [2]. During the last dozen years the study of these microbes in the human context, here termed the microbiome, is receiving rapidly increasing attention and by now there is hardly any medical discipline that has not recognized the impact of the intestinal microbiome. While we are born virtually sterile, our intestine is colonized by the microbial communities that are involved in immune priming and therefore playing a critical role in us combating pathogens, are essential for metabolizing food substrates and therefore increasing the energy output, and produce a great variety of metabolites, including vitamins that contribute to the maintenance of general body functionalities [3]. Although it is clear that the importance of the intestinal microbiome relies on its functional interaction with its host, most of these interactions have not been characterized at the molecular level, simply because our knowledge and research tools are too limited. In fact, in many cases we do not even know what microbe is doing what in time and space. For example, the phylogenetic composition and richness of the microbiome is correlated with host health, such as insulin sensitivity, but it is not known what microbes are exactly involved or which are most important [4]. At the end, this can only be addressed in controlled human intervention studies and only few of these are now emerging. All microbiome research eventually aims to a better understanding of host-microbiome interactions. In this context the use of multi-omics approaches aiming at the host and its microbiome, including metagenomics, meta-transcriptomics, metaproteomics and metabolomics, can be mentioned as a holistic way to obtain a deep systems understanding of the function and host- interactions of the microbiome (see also [5]).

In this review we aim to discuss the insight in host-microbiome interactions and how we can reach a mechanistic understanding of these. Following an introduction of the general concepts which are required for conceptualizing host-microbiome interaction studies, we provide an overview of the state of the art analytic methodologies and what to consider when analysing and interpreting the data (Fig. 1). We focus on human studies for obvious reasons but where new technical advancements are discussed we also detail studies in model systems.

2. General Concepts for functional studying host-microbiota interactions

2.1. The host meets the microbiome

Deep metagenomic analysis has shown that the intestinal microbiome encodes over 10 million different bacterial genes [6]. Considering that the human genome encodes only for around 25,000 genes and their variation contributes to our individuality, the microbiome may have a substantial role in contributing to physiological differences across individuals. Genetic differences in the immune repertoire have been shown to affect the composition of the microbiome.

As the impact of millions of genes is hard to appreciate, the functional repertoire of the microbiome is summarized and visualized in comprehensive databases such as KEGG (Kyoto Encyclopedia of genes and genomes) [7,8] or COG (Clusters of Orthologous Groups) [9,10]. The use of these databases that predict the synthesis of proteins and other biomolecules ease the comprehension of the overall study results but are not enough in explaining phenotypic differences of not so evident conditions as illustrated by the following examples. Flagellins and LPS are important cell envelope located structures that signal to the host [11]. Different types of flagellins may be grouped to motility function but not all microbial flagellins may have the same immune or other host response [12]. It seems that flagellins of opportunistic pathogens differ structurally and functionally from that from commensals and hence may evoke a defence response while this is not the case with the commensal flagellins. Similarly, the lipopolysaccharides (LPS) from pathogens evoke an inflammatory response in contrast to that from some commensal bacteria [13]. Moreover, while enzymes like glycosyl hydrolases can be predicted quite well, their contribution throughout life may change dramatically. An example is formed by the hydrolytic enzymes from Bifidobacteria involved in the degradation of human milk oligosaccharides during lactation while later in life these may primarily degrade mucins [14].

2.2. Ultimate goal of studying functionality of host-microbiota interaction

While for traditional medicine the best microbe would have been a dead microbe, we now move to an era where we know that our microbiome is an essential part of our body and its study needs to address the body as a whole. Hence, while the intestine is the major site where most of the microbiome is located, we know that its immune, metabolic and nervous system activities are connecting to many other organs, including brain, liver, lung and adipose and skin tissues. The developments in the microbiome research field has highlighted the importance of studies of the gut-brain-axis, gut-liver axis, gut-lung axis, gut-skin axis and gut adipose-tissue axis studies [15 –18].

Another layer of complexity is provided by the temporal development. The human-microbiome symbiosis cannot be considered to occur in separate life-time windows but has to be seen through a temporal continuum: while born virtually sterile, a newborn is colonized rapidly by the microbes in its environment, with a prominent role for its mother. New causal support for this maternal vertical transmission has recently been provided [19]. This colonization process is thought to continue in all life phases where in time previous events play a role in how the host will interact with its microbiome and vice versa. Basically, the genes we inherit from

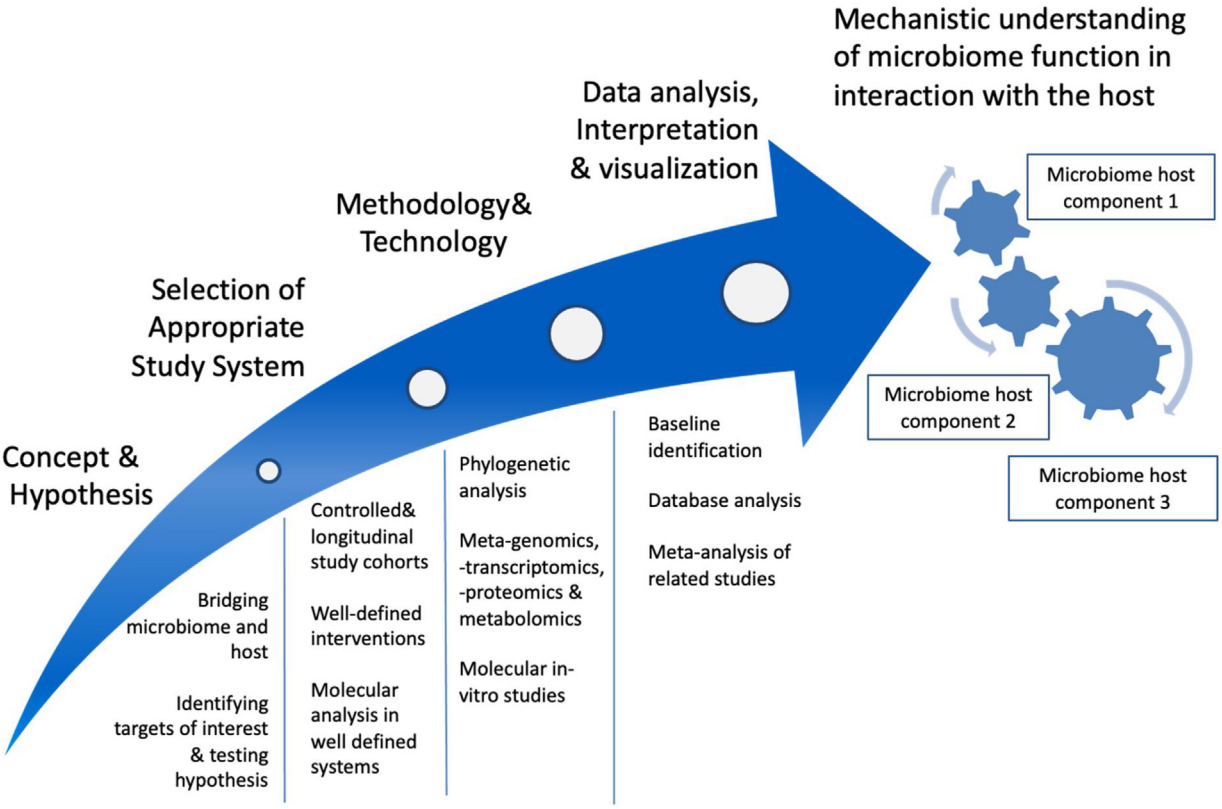

Fig. 1. Roadmap to functional characterization of the human intestinal microbiome in its interaction with the host.

our parents will later select for certain microbes although some of these signatures may disappear over time [20]. Moreover, the way how we enter the world and especially what we are fed [21], which antibiotics are prescribed [22] or other drugs we take [23], what we select as our standard diet [24], all impact our microbiome and health. Age, gender and physical activity are among others additional factors influencing the human microbiome (reviewed by Swann et al. [25]). Most studies focus on one life state or refer to different life states from different individuals. Hence, meta-data are increasingly collected for various life events potentially affecting the host-microbiome symbiosis. Longitudinal analyses of the microbiome have considerable advantages but life time studies do not exist and most studies have a limited duration. Hence, it is important to realize that there is always a certain time window that is selected and affects the functional interaction. Ultimately, by doing step-wise studies we may hope to reach to the final goal of understanding it all or at least being able to extrapolate with some confidence.

3. State of the art and developing concepts on the functionality of host-microbiome interactions

3.1. Participation in host metabolism

The most obvious functional interaction between the microbiome and its host is in relation to the processing of food. The intestinal microbiome is located along the intestinal tract being responsible for digestion and therefore it inevitably plays a role in it. The most prominent microbial products are short chain fatty acids (SCFAs) derived from carbohydrates for which host enzymes are lacking and therefore providing the host with extra energy. The importance of these molecules is however beyond being a caloric addition but being involved in critical host events such as maintaining a normal turn-over of epithelial cells. Generally, the intestinal microbiome harbors many times more carbohydrate metabolising enzymes than the host. Hence, the human intestinal microbiome is in general a source rich in carbohydrate-active enzymes (CAZymes) and expands the human repertoire dramatically [26]. In spite of the extensive research on CAZymes, a recent systematic study indicated that not all functions are known yet [27].

The list of other microbiome-derived metabolites includes several dozens of molecules varying from processed body molecules such as secondary bile acids that affect host metabolism to *de novo* synthesized products such as LPS, trimethylamine (TMA) and specific amino acids or biogenic amines, that are in turn also processed by the body in various ways (for a recent review see Koh and Bäckhed [28]). Examples include the production of TMA-N-oxide (TMAO) that has been implicated in cardiovascular risks, while amino acids such as tryptophan and tyrosine have signalling effects beyond the intestine [29]. Histidine, polyphenols and lipids from the diet are metabolized as well and the derived metabolites play a role in maintaining host homeostasis. Moreover, products such as hydrogen sulphide, gamma-amino butyrate or serotonin have important neural signalling functions and contribute to understanding the gut-brain dialogue [30].

3.2. Intestinal integrity and relevant proteins

One of the central roles in human-microbiome interactions is the integrity of the intestinal cell-lining. This becomes obvious in circumstances where this important barrier is compromised resulting in health-impairing consequences. Often this is reflected in a reduced level of tight junction proteins that are important for closing the gaps between intestinal cells [31]. Their production is reduced in patients suffering from chronic inflammatory diseases, such as Ulcerative Colitis (UC), where a healthy intestinal barrier cannot be any longer maintained [32,33].

Another example is obesity, especially in context with metabolic syndrome. However, here it has not consistently been shown that the intestinal epithelial cells become less tightly closed. The reason for this is the limited accessibility of the intestine since it is basically impossible to measure cell-integrity in humans in-vivo as it would allow in situ determination of tight junction proteins or the recently discovered synaptopodin [34]. One has to rely on indirect markers such as the serum determination of administered non-digestible sugars such as lactulose/mannitol or measuring inflammation markers in faeces such as calprotectin, a protein for which different assays exist which limit cross-comparability. An alternative is to determine the serum LPS as a proxy for intestinal permeability and this has been used with some success in recent trials (see below; Depommier et al. [35]).

A recent review describes the components of the intestinal system from top to bottom and from the lumen to the intestinal epithelium and how intestinal homeostasis is maintained emphasising the interplay of diet, the host's immunity and the microbiome [36]. Both secretory immunoglobins (sIgA; see below) and mucins are the predominant structural proteins in the intestinal tract [37]. The structural mucins have important functions in the mucosal integrity [38]. Mucins represent an additional protection to the host side but also form a nutrient to the intestinal microbes which can in turn have an effect on other microbes. Intestinal enzymes include intestinal alkaline phosphatase which has been found to have very versatile functions in the intestine also in relation to the microbiome, since it regulates the absorption of fatty acids and calcium absorption and is involved in innate immunity [39,40].

A novel, potentially high throughput approach to determine both host and microbe functionalities in the intestine is the use of fecal metaproteomics that identified the presence of representative proteins for maintaining intestinal integrity [41,42]. In a study on healthy individuals which were sampled twice within one year the most abundant microbial protein group was glutamate dehydrogenase, which may play a role as an electron sink and therefore might be crucial for maintaining a low redox-potential [43]. Among the 20 most microbial functions was GroEL which takes part in iron-cluster formation and may be important for maintaining a constant mineral supply. In a study on 16 healthy individuals, the three most dominant host enzymes included alpha-amylase which might be involved in creating different ecological niches favoring certain microbes, alpha 1 antitrypsin, possibly regulating the proteinase activities and maintaining a balance of protein degradation in the intestine, and alkaline phosphatase which might be one way to maintain and restore equilibrium in the intestine [42]. Of interest, in subjects that were in majority morbidly obese, higher levels of host proteins than in the control group were observed and this may reflect a way the host reacts to compensate for the impact of the disease [44].

3.3. Gut-brain – a centralized memory

The enteric nervous system (ENS) is taking care of bowel movements and therefore affects the composition and activity of the intestinal microbes as they are experiencing a flow which may vary. While it is known that food components play a role in the signal transduction that affects bowel movements, various components produced by the gut microbes have a role in the ENS signaling, including hydrogen sulphide, gamma-amino butyric acid, and serotonin [30] (see above). It has been postulated that there is much more to the ENS than simply moving the chyme from top to bottom of the intestine and this process actually might be conditioned [45]. Due to its synaptic plasticity, alterations in neuronal sensitivity and possibility of structural changes the ENS might be capable of learning [45]. For example, post infectious irritable bowel syndrome (PI-IBS) might be caused by an ENS primed for the infectious condition which it experienced during a traveler's diarrhea. It is well known that subjects suffering from PI-IBS have compromised intestinal bacteria and host cross-talk as determined by intestinal cell expression [46].

3.4. Spatial organization of the intestinal microbiota: missing links to be discovered

To further characterize the functions of the intestinal microbiomes more knowledge is required on how the individual microbes are spatially distributed in the intestine. Mucus-degraders have been found in the vicinity of the mucus layer and a well-known example is *Akkermansia muciniphila* that exclusively grows on mucin and forms trophic chains with among others butyrate producers at the mucosal interface [47]. While the vertical chyme flow is the dominant force, vertical and horizontal movements by motile bacteria containing flagella or retractable pili may have specific functions and host interactions [48]. However, for most bacteria it is not clear where they are localized and which interactions might be linked to them. In some disease states, biofilms of anaerobic bacteria have been observed in the compromised intestine and these may be considered as a tipping point towards a further progression of disease and contributing to resistance to treatments [49].

The most abundant immunoglobulin in the intestine is sIgA that covers most if not all intestinal microbes but the specifics of its interaction with the intestinal microbiota are only starting to be understood. While sIgA may bind to the septation point in dividing pathogens and therefore prohibit the further spread and eases clearance by keeping them together in a process called enchainment, it may provide non-motile bacteria a docking site to attach to the host, enabling its replication and therefore contributing to the overall ecosystem [50].

Many ecological factors determine the functionality of the intestinal microbiome. Apart from the biotic factors illustrated above, also abiotic parameters are highly relevant, one is the luminal pH that is increasing throughout the intestine and is well described but the dynamics deriving from the interplay of nutrients, the host, and species being are still being learned [51]. Other factors such as oxygen and its distribution in the mucosal interface are still to be discovered and is of great importance as it affects the redox state of molecules and microbes. All these factors influence the functional composition such as that bacterial species appear not to fluctuate in their abundance continuously but rather appear in alternate states [52]. Into this also plays the fact that the growth rate in vivo is different than in vitro [53] and microbes may move different in different viscosities which may affect gene expression and interaction with the host but this needs to be confirmed in the host-human context.

4. Ways to study the function of the intestinal microbiome

4.1. System and sample choice

As we highlighted the concepts to take into account when studying microbiome function, what samples should now be taken to look at functions? Ideally, tissue is collected without any disturbance in the microbial ecosystem it contains and the analysis of its biomolecules may provide information on the host and microbiome at one time-point at one place (same spatial and temporal characteristics). A mouse study showed that it is possible to study the functions of the intestinal bacteria at the mucosal host interface [54]. Intestinal biopsies were taken from mice that had received a stable isotope-labelled amino acid and in combination with advanced spectroscopy and imaging it could be shown which

labelled bacteria contributed to which function in the mucosa. Only few studies are providing this type of information due to the obvious difficulty of accessing biopsy material. The inconvenient reality in human research lies in sampling the host non-invasively by collecting e.g. faeces or urine and minimal invasively by collecting blood. Microbial functions are usually either assessed by predictions from its metagenomic analysis or even only inferred from phylogenetic analysis or concluded upon changes in human physiology. However, the last decade of human intestinal microbiome research has produced hundreds of such studies and while contributing to reproducibility their impact is limited as many are observational and no cause effect relations can be deduced. Apart from specific intervention studies, what is required, is a systematic understanding of the microbiome functions in the interaction with its host and that might be acquired bottom up. Epithelial cell lines are being used to be either stimulated by microbial components or they are grown in co-culture, human cells growing on top of bacterial culture, the latter being carried out less often. While these systems are relatively easy to comprehend and functional outputs may be measured easily for instance by colorimetric assays, they lack the heterogeneity of cell-types in the intestine and the 3D orientation [55].

Different types of intestinal organoid models such as for example 3D cell structures consisting of only colon cells (colonoids) or consisting of several different intestinal cells (organoids) (see for a recent review, Verma et al. [56]). A mouse study measured the usage and reproducibility of such organoids and found a specific proteome (10 proteins) and transcriptome (14 messengers) signature to distinguish them from the parallel tested epithelial cell lines and fibroblasts. It was shown that components of the inflammasome were better represented in the organoid [57]. With few exceptions, intestinal organoids have been mostly used to study intestinal physiology or the effect of pathogenic agents [58]. Host-microbiome interactions have been studied by introducing for example supernatants of intestinal anaerobes on mouse organoids followed by analysis of its transcriptome [59]. A combination of *in vivo* (suckling rabbit model),*ex vivo* (Ussing chambers) and *in vitro* (epithelial cell lines and organoids) experiments found metabolites derived in the transition from breast-feeding to solid food to take part in the maturation of the intestine [60]. To take into account that differences in viscosity and flow impact the host-microbiome interactions, tissues on a chip in combination with microfluidics are being developed. Various systems have been developed to prototypes, including microfluidic gut on a chip [61] and also co-tissue chips, i.e. having tissue from multiple tissues such as intestine and liver on a chip system [62]. In a prototype of a hypoxic intestinal chip, it was found to be possible to maintain a stable microbial community of 200 unique operational taxonomic units from 11 different genera [63]. These studies are indicating that tools have been developed but also that they require further validation and applications to address relevant biological questions.

Systematic studies such as the introduction of individual bacteria or a consortium to the organoid to exploit the host-microbiome interactions are still to come. Such a study would for example label a host or a microbial protein and follow its interaction by microscopical analysis and determine the transcriptome and proteome of individual cells by single cell transcriptomics and proteomics [64,65]. This could lead to determining the interaction partners of a specifically labelled protein. In addition, organoid research could shift from mouse-organoids pre-dominated to human-dominated organoid research. Target molecules can be identified from cohort studies such as dietary intervention studies. The human body is the only system where all components crucial for host-microbiome interaction are in place at the same time: i.e. the consortium of epithelia and immunological cells from the host, host mucins, the right growing conditions in regard to pH, oxygen and nutrient availability and the community of intestinal microbes taking part in this relation with their enzymes, metabolites and surface structures [66]. As human intervention studies are complex studies due to the huge amount of confounding factors, dietary and pharmaceutical intervention studies require a careful study design. A recent guidance on study cohort considerations as well as possible readouts is given in [25]. It highlights for example the use of cross-over studies where each individual serves as its own control and emphasises the importance of taking age, lifestyle and diet into account as confounding factors and to control for them. Short chain fatty acids are given as one example readout as evidence for microbial activity as not all observed changes may be microbiome related. Guidelines as the one discussed here will eventually assure quality and ease comparativeness between studies when carrying out intervention studies to study the effect of food on gut microbiota interactions. The usage of stable-isotope labelled food components may assist in following the metabolic path a certain food ingredient may take [67,68]. To take one step ahead of the complexity of human studies, intestinal simulators can be used to focus on the processes regarding a specific substrate e.g. certain types of fibres. Here the use of synthetic communities are needed and various efforts have been reported (for recent reviews see Elzinga et al. [69]; Shetty et al. 70]). Such defined consortia in proof of principle studies have been proposed with specific attention for the effect of different pH settings [71]. An advantage of these in vitro systems is their miniaturization that will allow high throughput studies with expensive substrates such as stable isotopes in combination with advanced mass spectroscopy or nuclear magnetic resonance analysis.

4.2. Analysis methods

Recently, several studies have applied and combined different meta-omics technologies to advance the understanding of the human intestinal tract in health and disease. The most recent ones are summarized here and include phylogenetic analysis of bacteria, archaea and fungi, some combined with metagenomics, metatranscriptomics or metaproteomics (see Table 1 for an overview). The studies varied from early life to healthy adults or patients with specific attention for Inflammatory Bowel Disease (IBD) and Type 1 Diabetes (T1D) and insulin sensitivity. It is evident that most studies are proof of concept studies that address a limited number of adults or infants. Only few large-scale studies target over 100 subjects but as indicated above this is a trend that will further develop.

Moreover, these summarized human meta-omic studies do not show much cause-effect data as they were rather observational. Hence, we also summarized some of the recent human intervention studies that allowed to provide a causal relation (Table 2).

4.3. (Meta-)Genomics

It is important to identify the pool of genes and species present in the intestine as references for functional studies and hints for potential targets of functional assays. Metagenomic information has been collected for the human microbiome for the whole life span from immature babies [72], via infants, toddlers and children [85,86], adolescents [87] and adults [6] to centenarians [88]. Most studies have focused on Westernized populations but a variety of indigenous groups has also been studied (Turroni et al. [89; Pasolli et al. 90], review by Wilson et al. [91]). Especially the metagenomic data of non-Western populations provide a rich source for identifying novel, or forgotten, functions of the microbiome. Many different diseases and the effect of various diets on the genetic potential of the microbiome have been studied. Therefore, read archives such as in the NCBI, ENA and other databases, are rich in all kind of genetic information and provide a profound basis to mine specific func-

Table 1
Landmark studies in humans assessing global functions of the intestinal microbiome*.

Study description	Main methods/ Methodological achievement	Main/significant finding	Reference
Functions early in life			
4 premature infants, 2 of whom developed necrotizing enterocolitis (NEC) 4–6 times fecal samples at different time points per infant	Establishment of diametric ratio as quantitative metric for metatranscriptomics; sample-specific meta-genomic assembled genomes	Significantly higher diametric ratios of genes associated with low oxygen levels in samples of infants later diagnosed with NEC than in samples without NEC	[72]
428 infants; Caesarean-born and vaginal-delivered; age: 3 months Probiotic intervention Faecal samples	Phylogenetic 16S rRNA analysis with subset of samples analysed with metagenomics and metaproteomics	Probiotic-supplemented groups with high levels of beta-galactosidase (lactose degradation) and beta-galactosyl N-acetyl hexosaminephosphorylase (HMO degradation)	[73]
Studies to establish a functional baseline in healthy individuals			
3 individuals, 3 time points over one year Faecal samples	Correlation of phylogenetic 16S rRNA and metaproteome analysis	Personalization at metaproteome level stronger than temporal development; good correlation of metaproteome and phylogenetic results	[43]
16 individuals, 3 time points over six weeks Probiotic intervention with *Lactobacillus rhamnosus* Faecal samples Serum measurements	Metaproteomics analysis with phylogenetic 16S rRNA analysis Taxonomic associated functions	Individual effect stronger than time effect from peptide to COG level; no direct effect of probiotic intervention catalogue of human and host functions as a history of the digestive process	[42]
Studies to establish functional insight into ecological mechanism			
180 healthy, 53 IBD: 14 Crohn's Disease (CD), and 39 Ulcerative Colitis (UC) patients Faecal samples (subjects from earlier studies of Nielsen et al. [74] & Qin et al. [75])	Combination of 16S rRNA based phylogenetic analysis and metatranscriptomics to identify coexistence-associated gene-expression changes; co-cultivation to verify findings	Identification of transcriptionally modulated genes with species having an effect on another one; central metabolism such as anaerobic respiratory pathway, environmental sensing and uptake of substrates community context specific	[76]
(Food)Intervention studies			
39 participants with reduced insulin sensitivity Two intervention arms: diet high in resistant starch vs. low Faecal samples	Integration of 16S rRNA data, metatranscriptomics and metaproteomics by likelihood of relatedness with network visualization	More discriminating features for the diet high in resistant starch than the one low in resistant starch	[77]
27 subjects, healthy but impaired insulin-sensitivity and obese Cross-over 3 day perturbation of either caloric under- or underfeeding and either placebo or vancomycin (exact same foods for each 3-day period)	Measuring of ingested calories, 16S rRNA and for a selection metagenomics	*A. muciniphila* increased upon underfeeding and vancomycin treatment; less butyrate as proxy for decrease in the capacity of the gut microbiome to harvest nutrients microbial metabolism in blood in underfeeding and vancomycin treatment	[78]
Disturbance of functions in disease			
132 subjects, controls, IBD patients with UC or CD, up to 24 time points within one year Faeces, blood and biopsies	Integration of metagenomics, metatranscriptomics, metaproteomics and host parameters	*F. prausnitzii* in network hubs with numerous EC downregulated in dysbiosis, *E. coli* in hubs with upregulated ECs	[79]
Familial type 1 diabetes mellitus (T1DM) 36 samples from 18 individuals	Reference genome-independent workflow to integrate metagenomics, metatranscriptomics and metaproteomics	Differences in the availability or microbial use of cellulose in T1DM subjects; protein difference in immune functions	[80]
13 non-overweight, 16 overweight individuals	Phylogenetic 16S rRNA data Metaproteomics Serum biomarkers	Separation of protein profiles of obese and non-obese individuals; correlation of serum marker and proteins	[44]
Cystic fibrosis; 15 children and unaffected siblings (age 1.6–15.6)	Phylogenetic 16S rRNA data Fecal and serum markers Metaproteomics	Potential human and bacterial faecal biomarkers for cystic fibrosis	[81]
16 subjects undergoing allogeneic hematopoietic stem cell transplantation	16S & 18S rRNA phylogenetic data Metatranscriptomics	Higher overall number and higher expression levels of antibiotic resistance genes in posttreatment microbiome	[82]

tions and identify novel functional groups. However, connecting the reads to the specific metadata is not always trivial and therefore impairs the harvesting of information while read coverages are at times too shallow to allow the identification of certain functions.

Few studies have performed genome wide association studies (GWAS) linking human genetic variation to the metagenome [92,93]. In principle, this can deliver genetic evidence for co-evolution of host-microbiome interactions. However, only limited

Table 2
Landmark studies on specific functions of the intestinal microbiome.

Study description	Main methods	Main finding	Reference
32 subjects completing study Three treatment arms: placebo, live *A.muciniphila*, or pasteurized *A. muciniphila* as supplement for 3 months	Supplementation with *Akkermansia muciniphila* in overweight and obese human volunteers: a proof-of-concept exploratory study.	Pasteurized *A. muciniphila* improved insulin sensitivity	[35]
20 subjects Cross-over study with white and sourdough bread	Metagenomics and advanced continuous glucose monitor based glycemic response	Prediction of type of bread that inducing lower glycemic response in each person based on microbiome only	[83]
24 subjects with metabolic syndrome *Four week Anaerobutyricum soehngenii* intervention	Effect on dose response insulin clamp Phylogenetic 16S rRNA analysis, qPCR, metagenomics and replication analysis based on metagenomics	*A. soehngenii* was replicating and this is related to insulin sensitivity improvement.	[84]

correlations have been described, the most important being the observation that adults lacking sufficient levels of lactase and therefore not being capable of digesting lactose, appear to contain higher levels of *Bifidobacterium*. The hypothesis is that these individuals harbour *Bifidobacterium* species to compensate for the loss of lactase activity but this has still to be confirmed [94]. Another example with some controversy is the impact on the intestinal microbiome of the host fucosyl transferase (FUT2) gene that is not functional in approximately 20 % of the general population. It has been established that the FUT2 gene determines the fucosylation degree of human milk oligosaccharides and this was found to affect the early life microbiota in newborns, notably delivered by Caesarean section [65]. However, since the FUT2 gene also affects the fucosylation of the intestinal mucin, an effect was to be expected on adult microbiome. This was indeed observed in a study with Finnish adults [95] but not in a well-studied UK cohort [96]. However, the impact of antibiotics or other confounders may have affected the outcome of the latter study.

Functional metagenomics present a way to look into specific functionalities of the intestinal microbiome. A study on metagenomes of elderly identified *flagellin* sequences to contribute to an inflammation risk [97]. The screening of several hundred metagenomes identified microbe-mediated vitamin metabolism networks [98]. And the identification of genes involved in the degradation of human O- and N-glycans identified in the metagenome of one subject led to a lectin-binding assay which identified mucin-modifications [99].

The advantage of metagenomics is that it is a rather mature method among the meta-omic approaches. One of the critical steps is the extraction of DNA from faecal samples and efficient methods with limited bias based on mechanical lysis have been reported following comparisons of a dozen different methods [100]. Advancements in sequencing technologies both in speed and price have contributed to the maturation of metagenomics that resulted in miniaturization of sequencing as well as developing single molecule sequencing as an alternative for massive parallel sequencing of short DNA molecules. Moreover, a suite of processing tools for metagenomic sequence reads are available further advancing this field together with an ever increasing database of human microbiome sequences [6,101].

4.4. Metatranscriptomics

Shortly after the establishing of metagenomics as major analytical technique metatranscriptomics evolved. Early work highlighted the prominent role of the intestinal microbiome in carbohydrate metabolism [102]. However, one of the issues with the application of metatranscriptomics in intestinal systems is the short half time of bacterial messengers that are in the order of minutes. Hence, the fecal transcriptome only reflects the events that have happened the last few minutes. A way to circumvent this, is the use of somewhat evasive methods to obtain intestinal samples such as by using endoscopes. An alternative solution has been the study of ileostoma patients where immediate sampling is possible and RNAseq analysis provided insight in the metabolic processes in the small intestine [103]. Metatranscriptomics is generally co-applied with metagenomics and the major findings are highlighted in Table 1. As especially the data-analysis of metatranscriptomics requires special attention metatranscriptomics analysis pipelines are reviewed in Niu et al. [104].

4.5. Metaproteomics

Metaproteomics targets the global protein content of the intestinal ecosystem but to date has identified only a small fraction of the predicted proteins. By interrelating host and microbial proteins and looking for the contribution of functions by certain species metaproteomics has been show cased to contribute in unravelling the host-microbiome interrelationship [42].

Recent years have mostly seen activity on developing computational tools [105,106]. There have been efforts on sample preparation attempts but there is no state of the art sample preparation. However, the field still needs to focus on explaining biology with the technique [107]. The by now largest set of intestinal samples in one study consists of 450 metaproteomic profiles [79]. As the profiles have been mostly integrated with several other layers of the study such as metagenomics and metatranscriptomics this dataset provides a good basis for further data harvesting and learning about the identifiable proteins. During the last five years analytical improvements on general LC–MS approaches e.g. faster mass analyzers and up-scaling of absolute quantifications were introduced [108,109]. These technical advancements are expected to also affect the throughput and sensitivity in metaproteomics. In addition, high-throughput analysis of target proteins has become possible. While major investigators in metaproteomics used to be microbiome researchers also core-proteomics institutes have been joining the field [110,111]. This highlights the acceptance of metaproteomics as a discipline, although the output on the intestinal microbiota is so far limited.

Only a very few human intestinal microbiome studies actually integrate several layers of data like the recent one [79]. Collecting multi-level data is becoming a more common practice but often it stays with showing the different data in parallel and using one dataset to confirm the other but without truly integrating the different data-sets i.e. by correlating them or constructing networks. A recent review is addressing the considerations as to when performing data integration of microbiome data and what methods one can choose from [112].

4.6. Metabolomics

As introduced above, the intestinal microbiota contributes substantially to overall metabolites found throughout the human body. The rationale of performing metabolomics is that the metabolites serve as readout of the pathways which have been activated both on host and microbiome side and therefore are being used as reporters of the host-microbiome interactions. Due to their prominence as described above SCFAs have been in the focus of many studies and several protocols for their analysis in faecal samples exist [113]. In the context of the human microbiome metabolomics has been performed from faecal, urine and blood samples. As with the other omics approaches standardization of processing methods and analytic methods has not been realized and is more complex than isolating DNA from fecal samples that already has been a major technical hurdle (Costea et al. [100]; see above). When the faecal and urine metabolomes were compared in a high fibre intervention, it appeared that the urine metabolome was most diagnostic of the rapid changes due to the diet [114]. Another group of metabolites gaining much attention are lipids that because of their complexity and interest developed into lipidomics, almost a discipline in itself. At present, many *m/z* features in lipidomics get assigned a compound class without being able to assign the individual compound. Global metabolic maps are the harder to interpret and there is a vast amount of unknown metabolites, especially in faeces. However, the increase in reference spectral libraries will improve this issue. For now, the interpretable part of data is still small and therefore hard to draw overall conclusions, notably since only few studies address temporal development [115]. However, few labs perform metabolomics analysis by themselves because of the technical complexity of identifying thousands of relevant compounds and more commonly this analysis is outsourced to companies that use proprietary technologies and databases.

4.7. Physiological tests

To build a basis for understanding the host-microbiome interaction it is useful also to focus on the microbial part by performing microbial physiological tests. A large scale study investigating the effect of many different drugs on a variety of intestinal microbes identified that by far not only antibiotics effect the growth of intestinal commensals [23]. The isolation of microbes upon an intervention in the host allows studying their physiological properties. A single cell genomics approach was performed on bacterial responders on dietary fibers from bacterial strains isolated from mice [116]. This type of analysis in humans is a good addition to the meta-omics approaches. It might also be interesting to study the physiological characteristics of a species isolated from different locations or donors to acknowledge isolate differences [117].

5. Data analysis and interpretation of results

Recent years have seen accumulation of genetic information from the microbiome in public resources. While the EU-funded MetaHIT project provided millions of metagenome reads [75,6], the NIH-funded human microbiome project (HMP) produced hundreds of reference genomes and additional metagenomic reads. These can all be used to search for specific functions (www.hmpdacc.org) whereas the jgi img/metagenome (JGI IMG Integrated Microbial Genomes & Microbiomes img.jgi.doe.gov) is a good resource to get an overview of available metagenomic data and their characteristics, such as how many COGs have been identified. In addition, the availability of raw data and scripting code increased, also due to the technical requirements by many publishing houses, but it nevertheless eases re-analysis and meta-analysis. The development of software packages including visualization options, such as the protein centered software tools MetaproteomAnalzyer [118] and the unipept tool [119] or the widely applicable ggplot package for the statistical software R to gain a comprehensive overview of one's data have lowered the threshold of performing functional microbiome studies.

The common approach in meta-omics studies is to map the identified sequences to an extensive omics database. In this way, sequences are identified which have been characterized before.

As it is still useful to gain an overall impression of the represented functions of a microbiome study, a variety of computational tools is available and these have been recently described with a dataset derived from the salivary metaproteomes [120]. The tools differ in requirements for pre-computation, which indicates that there is still room for the development of a homogenous workflow. Moreover, the outputs of these computational tools differ quite dramatically and their comparability is limited. This highlights the difficulty of cross-study comparison when already the same dataset produces different outcomes depending on the computational tool used.

The high and unique complexity of the human microbiome has led to the development of many new statistical analysis methods [121–123]. However, the practise of comparing data with existing datasets remains and can also lead to confirming earlier findings. However, in many cases additional sets of statistically significant differences are found. An example is the thousands of correlations between unknown proteins and phylogenetic taxa of the human microbiome [42]. Recent work addressed this and provided an approach to identify partners in networks as a way to discover the function of any not yet annotated protein by grouping it in the pathway where it could be related to [124]. This approach may facilitate the time-consuming process of manually going back to all relevant published studies.

6. New directions for functional research into the microbiome: from function to artificial intelligence

It requires some humility to acknowledge the fact that human microbiome science is a young science that with the big data it is producing has to further mature and develop. An important point is the individuality of the human microbiota that has been discovered decades ago [125]. The personalized microbiome is the main driver of microbiome differences and its impact cannot be underestimated and needs to be appreciated at all levels similarly as stem cell research is well aware of the individuality of a cell. Moreover, with the possible exception of the phylogenetic analysis, most microbiome techniques are not yet fully matured and many technological challenges remain. This also holds for the computational challenges and even in the phylogenetic analysis a large variety of settings and variables as well as confounders may affect many interpretations. The public deposition of the phylogenetic, metagenomic and other sequences, however, allows meta-analysis studies to confirm results and, if needed, identify errors and retroactively correct these.

What is further missing are stable baseline measurements. The first baseline studies addressing a healthy or normal microbiome function have emerged but there are no established parameters to define what is the healthy state. While blood-based measurements provide well-established proxies for health, such values do not yet exist for the microbiome. An important reason is that the blood components reflect homeostasis while the fecal microbiome is basically an end of the pipeline product. While the richness and diversity of the microbiome has been used as a proxy for health [4] this is only useful in certain instances. It has been well doc-

umented that disturbances such as antibiotic intake or a major shift in diet results in an initially more diverse community and these confounders should be addressed. Hence, a functional objective measure for a healthy microbiome has not been established. To reach to that gold standard it is required to systematically combine large longitudinal cohort studies, which are still rare, and link these with large sets of in-vitro molecular approaches. Computational improvements such as the application of artificial intelligence methods will ease the interpretation of cohort studies [126].

In the context of metagenomics and other sequence derived methods, it is important to realize that it is not enough to know the sequence of a gene but it is essential to understand what that gene is doing. This function may differ substantially in different microbial and environmental contexts. Moreover, it is also dependent on the gene integrity as minor sequence differences may have a large impact on the functionality. Hence, further work on the improvement of gene annotations is required and for now one has to be aware that the assigned function might not represent the real function but an approximation often based on automatic annotation. High throughput crystallographic approaches and advancements in 3D modelling for structure predictions will ease a structured harvesting of the millions of identified sequences of metagenomic data resulting in the discovery of new functions.

Finally, more studies need to drill down to the causal and molecular level. Some of the causality studies have been summarized here and provide ways to address the mechanisms in humans by the integration of omics approaches (Table 2). It is expected that more of these well-controlled cause effect studies are to follow and provide new molecular insight. Moreover, recent studies have addressed effects beyond the molecular dogma of gene, transcript and protein. It has been shown that the innate immune host response to a pathogen may lead to epigenetic changes enabling a rapid further reaction with cross-reactivity between gram-negative bacteria and a virus [127]. It is also evident that epigenetic effects are introduced by commensals – an obvious mechanism is the production of butyrate or propionate that are known to affect histone modifications and hence affect host gene expression [128].

In conclusion, the microbiome field is rapidly developing and we live in exciting times. Blessed are those who now start to research the microbiome field as there is an untapped potential for new discoveries.

Declaration of Competing Interest

The authors report no declarations of competing interest.

Acknowledgements

Part of this research was funded by the SIAM Gravitation Grant 024.002.002 and Spinoza Award of the Netherlands Organization for Scientific Research (WMdV).

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