Human Gut Microbiome Analysis and Multi-omics Approach

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***Abstract*—Given the importance of human gut microbioma in human health, its role has been recently reviewed and even now is the subject of many investigations. It is known that traditional culture methods are limited for analyzing it and that high-throughput sequencing, omics technologies instead allow to deepen the behaviour of microbial ecology. With the application of omics technologies, as metagenomics, metascriptomics, metaproteomics and metabolomics, many progress has been made in this field. Their integration, referred to as multi-omics approach, provides more evidence for biological mechanisms. This review highlights that omics approaches can be powerful tools in analyzing the human gut microbiome. The current scenario and examples of recently published landmark work are discussed and some limitations are outlined. Their combination is analyzed and relevant studies allowing us to link the expression of disease-associated microbial functions to distinct taxa are treated. Furthermore, recent system level approaches for integrating different omics layers are discussed and some researches aimed to develop a framework for the reconstruction of a large microbial community are examined. However, even today, we are aware that the multi-layer datasets integration is a challenging issue and that a careful planning of a multi-omics application is thus required.**

***Index Terms*—metagenomics, metatranscriptomics, human gut microbiome, metaproteomics, metabolomics**

I. Introduction

The human gut microbiome has been considered another organ because of its products, its responsiveness to the environment and its integration with other systems [1]. It is a very elaborated system that may accommodate thousands of different species of bacteria. Particularly, the gastrointestinal tract consists of a complex and dynamic microbial community, including archaea, bacteria, viruses and eukaryota. Most of its microorganisms are bacteria, with a density of approximately 1013-1014 cells/g fecal matter, in which 70% of the total microbes colonize the colon. Furthermore, the gut microbiome is responsible for many metabolic relevant functions, including vitamin and short chain fatty acid production, amino acid synthesis, bile acid biotransformation, hydrolysis and fermentation of non-digestible substrates [2]. For all these reasons the human gut microbiome is defined as our second genome [3], [4].

Enumerating what organisms are present in it with standard microbiological techniques is not possible because many organisms have never been grown in culture and may require special and yet unknown growth conditions. In fact, traditional studies of the human gut microbiome, which are widely dependent on cultivation techniques, only cultivate 10%-30% of this microbial system [5].

Fortunately, the introduction of DNA-based methods that were culture-independent provided new opportunities for studying and analyzing our gut microbiome. Studies based on PCR using universal and group-specific 16S rRNA gene primers followed by electrophoreses were widely performed for analyzing microbiomes before the introduction of sequencing. Among PCR-based methods, real-time quantitative PCR has been one of the most commonly used techniques providing a quantitative estimation of the amount of the PCR products. However, it can only be used to detect known species. After, a rapid and high-resolution phylogenetic microarray-based method was developed, known as the human intestinal tract chip (HITChip), that allows the simultaneous comparison of the relative amount of over 1000 genus-like groups of gut bacteria. However using this method only known species can be observed [6].

Although DNA-based, culture-independent methods contributed to the identification of uncultured species, the characterization of the human gut microbiome basing on high-throughput approach has benefited considerably from the introduction of next-generation sequencing (NGS) techniques. These provide a good quantitative picture of the gut microbial communities allowing the determination of low abundance and previously unknown species [7], [8]. Many and effective DNA sequencing approaches, like 16S rDNA sequence analysis, are used to study uncultivated gut microbial [9].

Given the importance of human gut microbioma in human health, its role has been recently reviewed [10] [11] and even now is the subject of many investigations. Their fundamental goal is to define the intestinal microbiome structure and dynamics, the relationships between community members, what substances are produced and consumed, and the interaction with the host. A large-scale initiative known as the Human Microbiome Project in 2012 is considered as an important milestone in

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the characterization of human microbiome, within its scope a reference microbial genome database is defined [12] and the sequences of 178 bacterial species have been published. MetaHIT (Metagenomics of the Human Intestinal Tract) is another study aiming to provide a reference catalog of gut microbiome in association with obesity and inflammatory bowel disease (IBD) [13], [14].

II. Omic Approaches

A. Metagenomics

The first omics discipline to appear, genomics, focused on the study of entire genomes while genetics is based on individual variants or single genes. Technological progresses, that have made possible cost-efficient and high-throughput microbial analysis, have allowed significant improvements in this omics field. Current metagenomics studies are based on shotgun techniques, that can produce reads from DNA, aligned to reference genomes to identify variants and community population. Targeted sequencing such as 16S rRNA gene sequencing can be also used to take a microbial community census. These data are compared with databases to create matrix of taxa and abundance or analysed with appropriate software programs to cluster the reads into Operational Taxomonic Units (OTUs) [15].

Therefore, metagenomics is an extremely powerful tool that can be used to describe the genetic potential of several kinds of microbiome. In fact, it has been used in different environments, including the soil and the sea. Environmental metagenomics was developed extensively before being applied to the human body, and these methods have had a significant effect on human-microbiome research [16]. Thereafter, a growing number of metagenomics studies have provided valuable results about the structure and diversity of the gut microbial community and their genetic composition. Many of them have had the goal to determine microbiome composition in various diseased patients using metagenomics analysis to identify potential interconnections between gut microbiome and diseases there should be [17], [18], and so forth. Other researches using synthetic metagenome generated from known gut microbiome provide very interesting results, as an iterative workflow having effective to enhance protein identifications [19]. Further studies have proved helpful to combine studies of metagenomics in the intestinal microbiome with other kinds of microbiome analyses, including cultivation methods. This approach has been adopted to ensure that the results are more accurate and convincing [20].

Anyway, despite numerous metagenomics studies of the human gut microbiome have been performed, according to some researchers it is important to investigate further in wider geographic areas, for longer periods [21].

Generally, there are some limitations in gut microbioma studies based on metagenomics approaches. Firstly, they only uncovered gene sequences that were present but do not provide information about their actual gene or protein expression levels. Besides, metagenomics analysis does not discriminate between microbiome that are active, dormant or dead. Consequently, it not provides enough information about the functions of the intestinal microbiome [22].

Furthermore, gut metagenomics require much higher sequence coverage than 16S rDNA sequence analysis and the costs and time involved in DNA sequencing projects are expensive.

Finally, in order to improve the research results in human gut microbioma metagenomics analysis, it is important to create a unified microbial DNA extraction method, improve computational algorithms, and complete the reference databases.

B. Metatranscriptomics

One of the major aims of the human microbiome studies is relating to the understanding of microbial ecology and of the biomolecular activities of the microbiome ecosystem [23].

Metagenomics has raised our awareness of gene content as well as of genetic variability in gut microbiome. However, it not provides enough information about the active bacteria and their functions in the human gastrointestinal tract.

Conversely, metatranscriptomics has focused on the genes activity and its approach is based on the retrieval and sequencing of environmental mRNAs from a microbial ecosystem to assess what genes may be expressed in that community.

Metatranscriptomics studies have been applied initially to samples from water and soil environments [24]. These efforts demonstrated the feasibility of RNA-based profiling of microbial consortium and also produced large amounts of novel sequence information (transcripts) [25]. Analyses based on this approach have subsequently been applied to the human gut microbiome, showing strong inter-subject in microbial gene expression [26], [27]. Other analyses of the intestinal microbiome performed during a diet [28] or a xenobiotic therapy have individuated significant alterations of the microbial gene-expression profile. Further metatranscriptomics researches using fecal samples aim to clarify the active members of the gut microbiome and their functionality under conditions of health [29]. In these studies the characterization of mRNAs revealed a uniform functional pattern in healthy individuals.

Generally, however, metatranscriptomics approaches have some limitations. In fact, it is very difficult to obtain high-quality and sufficient amounts of RNA from environmental samples. Furthermore, it is a challenge to separate the mRNA of interest from the more abundant types of RNA such as rRNA. Lastly, the metatranscriptomics classification is limited to not enough reference databases.

C. Metaproteomics

An analysis of proteins is important to understand microbial functions of human gut microbiome. According to this statement, over the past decade, metaproteomics has been applied to analyze human gut microbiome.

Particularly, metaproteomics is able to clarify functional aspects considerable to the underlying physiological states and to provide detailed insights about the link between microbial diversity and the impact on the host biology. Since metaproteomics could help to understand relevant pathologies it may be considered now an emerging research area in human gut microbiome field.

Concerning protein extraction approaches, efficient methods using microbial samples are critical to allow precise intracellular content representation. For human gut microbiome, several studies have showed that mechanical disruption by bead beating was an efficient protein extraction method, particularly for lysing Gram-positive bacteria [30].

Metaproteomics researches of human intestinal microbiome identified various proteins and revealed the presence of a common functional core that was mainly involved in sugar transport and degradation [31]. Other comparative metaproteomics analyses concerning both protein profiles of healthy individuals and protein profiles under altered physiological conditions have been performed. In the latter case, studies concerning the mucosal-luminal interface in Inflammatory Bowel Disease, changes in the bacterial phylotypes are associated with host immune response and inflammation [32]. At the same, several researches have also explored the functional profiles of dysbiosis in various diseases [33].

However, given the great deal of species and diversity of human gut microbiome, robust approaches for quantitative metaproteomics studies are even now lacking. Furthermore, technical limitations are present and standardized procedures are still to be established. Studies involving a larger set of subjects are also necessary to catch a careful functional input of the human gut microbial system. Finally, similar to metatranscriptomics, the ability to assign functional classifications is limited to not enough reference databases.

D. Metabolomics

mRNA gene expression data and proteomic analyses reveal the set of gene products being produced in the cell, data that represents one aspect of cellular function. Conversely, metabolic profiling can give an instantaneous snapshot of the physiology of the cells.

Therefore, metabolomics allow to define the metabolic gut microbioma profile, to individuate and quantify classes and compounds of interest. Biological systems based on this omics approach have opened a new scenario in the comprehension of the gut microbiome by supporting the understanding of its state, modulation and interaction with microorganisms and the knowledge of the role of nutrients in health [34].

Mass spectrometry and nuclear magnetic resonance spectroscopy are the fundamental technologies applied to metabolomics. Biostatistics and mathematical approaches coupled with metabolomics are essential tools in the extraction of biologically information from large datasets.

Some human intestinal disorders have been studied using metabolomics [35]. For instance, a study on patients with ulcerative colitis shows a growth of quantities of taurine and cadaverine. At the same time, a higher bile acid concentration and lower levels of branched-chain fatty acids characterise the inflammatory bowel disease. Moreover, metabolomics studies in human gut microbiome have focused on the generation of new biomarkers, which could lead to the development of hypotheses potentially applicable for effective therapies [36].

Currently, metabolomics is increasingly used to study the gut microbiome [37]. However, some limitations restrict the development of metabolomics in health. In fact, the obtained metabolites are mixed, and it is difficult to identify the information coming from the host and that coming from the gut microbiome. In addition, the metabolomics databases are incomplete and there are metabolites not included.

III. MULTI-OMIC APPROACH

With the development and application of metagenomics, metatranscriptomics, metaproteomics and metabolomics, it is possible to identify significant microbial data able to support diagnosis and treatments in human health. However, as discussed in the previous sections, each single approach has in itself some limitations. In other words, single omics technologies provide a comprehensive gut microbial community view, but it is generally limited only to single layers, like genomic, transcriptomics, proteomic, metabolomics layers. On the contrary, their integration generates a global view which provides more evidence for biological mechanisms. “Fig. 1” shows this combination, which is referred to as multi-omics approach.

In this context, different multi-omics studies have been carried out achieving very interesting results. A multi-omics study revealed drastic changes in the protein profiles of the human gut microbiome following β-lactam antibiotic therapy [38]. This analysis reflects functional adaptation of the microbiome in response to the drug. However, more studies are needed to understand how different antibiotics can shape the human intestinal microbiome.

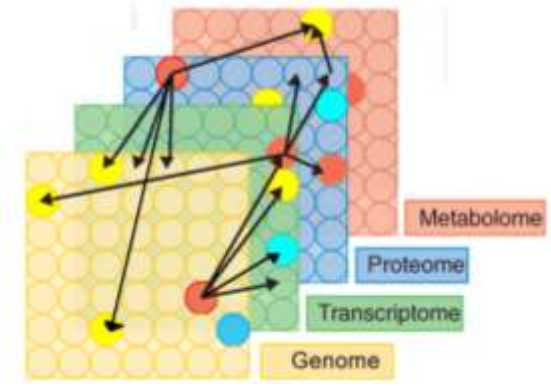

Figure 1. Multi-omics approach.

Recent insights in the combination of metagenomics, metatranscriptomics and viromics can provide more detailed description on the interplays between

microorganisms and viruses in gut microbiomes, because of the potential involvement of viruses in microbial dysbiosis. Generally, studying the molecular interactions using the combination of these omic approaches can considerably improve our understanding of human microbiome [39].

Other studies highlight that multi-omics is an effective approach in medical field and, as compared to studies of a single omics type, it offers the opportunity to understand the flow of information that underlies disease. Among these studies, a research activity, in which different omics layers are linked to expand from genomics to multi-omics, allowing to meet the goals of personalized medical therapies [40]. Further studies integrate metagenomic and metabolomic approaches and underline the importance of gut bacteria in reducing and eliminating cholesterol from the gastro intestinal tract [41]. According to these results the gut microbiome may play an important role in host lipid metabolism.

IV. Conclusions and Discussion

Given the relevance of the multi-omic applications in the analysis of the human gut microbiome it is important to make some considerations that may be useful for researchers aiming to design a study based on this approach. Multi-omics studies, by their nature, rely on large numbers of comparisons, statistical analyses, and a considerable investment of time and skilled human resources. Therefore, careful planning is required and experimental parameters should be considered [42].

Multi-omics approaches generate data to provide biological insight based on statistical analysis coming from datasets that are usually large. For this reason, the power to individuate associations and to detect accurately the flow of information strongly depends on sample size. The ability of omics approaches to produce meaningful insight into human gut micromione study is very much dependent on available samples.

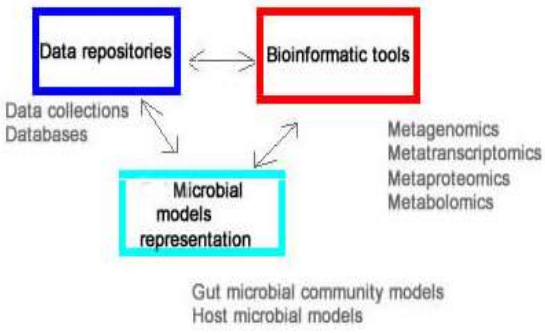

Figure 2. Resources and software tools supporting multi-omics approach.

Therefore, an initial power calculation to ensure sufficient sample size is necessary in large-scale studies.

Another aspect very important when a multi-omics approach is designed, it is the analysis of data requirements, that involves with multi-omics data integration. Generally different omics data were generated by using different technical platforms. Therefore, combining several omics approaches requires the development of a pipeline that integrates these data and that often requires a large storage space. Furthermore, it is important to ensure that the differences observed in the samples before integration are due to biological variability and not a technical issues.

It is generally accepted that a good design of a multi-omics research activity includes the definition of a system level approach to adopt in order to integrate the different omics layers. In this context, to have available an integrated software system supporting a combined omics approach it can be crucial. "Fig. 2" shows an example of such system, constituted by resources (data repositories), knowledge representation model (gut and host microbial models), omics tools (bioinformatic tools) relating respectively to data resources, to data model and integration, to data processing.

Recently, methods based on an integrated multi-omic analysis of microbial consortia have been developed. Below, we discuss some studies that adopt a system level approach in multi-omics applications. A study concerns a research on the biological wastewater treatment [43], but the adopted approach may be adopted also in other areas. Its aim is to develop a computational framework for the reconstruction of a large microbial community from multi-omic data. These provide an integrated overview of the functional capabilities by incorporating gene copy, transcript and protein abundances. A comparative integrated omics analysis including metagenomics, metatranscriptomics and metaproteomics was carried out. Particularly, the considered framework allows integration of systematically generated multi-omic data within reconstructed community-level metabolic networks. Here, genes encoding key functionalities are identified and these are traced back to the community members which encode them. Keystone species are also identified.

The linking of key functionalities to the microbial consortium through integrated omics opens up exciting possibilities for supporting prediction and control strategies for this community in the future.

The reconstruction of metabolic networks based on genomic data presents a good alternative to metabolomics for obtaining the metabolic features of organisms. Traditional approaches, which used to progress from single to multi-species metabolic network reconstructions, fail to explain how variations in gene or species composition affect the overall metabolic state of a given microbial community. Such bottom-up approaches may be limited by the impossibility to extrapolate community-wide networks and behaviour from isolate omics data sets.

The potential of the approach for the reconstruction of metabolic networks starting from genomic data, has been highlighted in a study on human gut microbioma [44], which identified enzyme-coding genes in samples of human individuals with obesity or inflammatory bowel disease and identify key metabolic traits within microbial consortia. Here, a metagenomics system biology computational framework integrates metagenomics data with an in silico systems-level analysis of metabolic networks. In our opinion, the system-level approach

presented on this research lays the foundation for a framework for studying the human microbiome, its organization, and its impact on health.

Ideally, top-down and bottom-up approaches should be combined to identify links between microbial community structure and function, trying to eliminate the gap between the metabolic networks and the larger microbial community networks to build a systems-level model of interactions between species [45].

Another relevant study allowed us to link the expression of disease-associated microbial functions to distinct taxa, which demonstrates the necessity for integrated multi-omics analyses of human microbiome [46]. It expands the system-level method developed in the previously described study [43] for integrated multi-omics analyses of microbial consortia and adopts an integrative approach to resolve the taxonomic and functional attributes of gastrointestinal microbiota at the metagenomic, metatranscriptomic and metaproteomic levels. In this study it is also demonstrated that the patterns of gut microbiome individuality are discernible on all omics levels. The adopted approach and the achieved results may be generalized and may constitute a reference point for future large-scale integrated multi-omics analyses of the gastrointestinal microbiome in the context of host–microbe interactions in human health and disease.

Considering the studies successfully carried out, we are convinced that despite the technical difficulties outlined before multi-omics approaches will allow to study in depth the modulation of the intestinal microbiome and that will be sufficiently powerful to elucidate the ecologic roles of the human gut microbiome.

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