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GUEST EDITORIAL

Beyond sequencing: fast and easy microbiome profiling by flow cytometry

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Analyses of the microbiome are increasingly important to understand the impact of the microbiota on physiological functions, diseases, and toxicity in the host organism. The microbiome is the sum of all bacteria, viruses, fungi, and phages forming communities present in any habitat, including human mucosal tissues, the gut, the lung, or the skin. The scientific literature dealing with microbiomes is a simple, yet impressive, indicator for the exponentially growing interest. Using "microbiome" as a key word, a PubMed search pulls up only 524 papers for the year 2008, and 10 years later, 10,884 citations are available. For instance, toxicologists addressed questions, how chemical compounds change the bacterial gut microbiome with adverse effects on health, as was done for glyphosate and bees. Other toxicological topics, including papers in Archives of Toxicology, comprised effects of food ingredients, drugs, air pollution on the microbiome, or the role of various genes in the host–microbiome relationship. Indeed, there is no limit to the kind of questions asked; even curious relationships between social behavior in baboons and microbiome shifts were explored. Of course, knowledge of dysbiosis as such does not inform about cause–effect relationships, i.e., does an environmental insult cause dysbiosis, or do changing microbiomes affect the host response/host health, for instance, via the metabolites they secret. Besides the phylogenetic characterization, bacterial-derived metabolites enable a detailed mechanistic understanding. Changes in the microbial composition and consequently changes in metabolites can have profound effects on the host immune system. These include, for example, the amino acid metabolite tryptamine, a ligand of the aryl hydrocarbon receptor, a critical regulator of immunity, and inflammation in the gut.

The explosion of microbial profiling became possible by the development of deep-sequencing methods, often called next generation sequencing (NGS), which happened around the year 2008. There are several sequencing methods available to date. First, 16S ribosomal RNA or DNA sequencing makes use of the fact that bacteria have taxon-specific variable regions within highly conserved sequences in their ribosomal RNA. The frequency of specific sequences found within the data estimates the relative frequency of the bacteria in the original mixture. In other words, the method detects both the diversity and the abundance of bacteria in a microbiome. Methodological pitfalls exist, such as the need for a balanced DNA isolation—harsh enough to include difficult to break up bacteria, yet soft enough not to lose bacterial DNA from easily lysed species. Read length and quality, and sequencing depth are important for reliable identification of species and quantitative detection of rare species in a mixture. Contamination from the environment is an issue as well. Taxonomic identification of the organisms is possible, but requires pre-existing sequence data for the annotation. However, 16S rDNA sequencing only allows taxonomic classification to varying degrees depending on the variability of the sequenced 16S variable regions. Similar to the approach of 16S rDNA/rRNA sequencing for bacteria, 18S rDNA/rRNA sequencing can identify fungi. Compared to 16S or 18S rDNA/rRNA-based sequencing, whole genome sequencing allows assessing the entire microbial community in a sample, with taxonomic classification potentially down to species and strain level and with the additional information of the genetic repertoire in the sample. Whole genome sequencing is much more expensive and requires the assembly of the genetic fragments sequenced. Importantly, both 16S rDNA and whole genome sequencing require bioinformatics expertise and sophisticated tools to decipher, analyze, sort, and present the data. Recently, computational methods reconstructed prokaryotic genomes

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in human gut content from such operational taxonomic units (OTUs) (Davey 2002).

NGS is time-consuming and can become expensive with many samples. Thus, despite its power, extensive microbiome analyses are primarily performed in research settings, and only slowly catch on in medicine. It is worth mentioning though that companies jumped the bandwagon and offer microbiome analysis from stool samples for a substantial fee, sometimes combined with dubious "personalized" recommendations for nutrition or behavioral interventions and promises of health improvements. Despite such budding commercialization, NGS is not yet commonly done as routine analysis, in personalized medicine, or numerous applications in industrial, agricultural, or community settings such as wastewater management.

Very often, a shift in the microbial community as such is of primary interest or even sufficient, with knowledge of the taxa of the changing composition being only of secondary importance. This might be the case in large screening experiments, time kinetics with many sampling points, or epidemiological projects. In addition, speed or in-time analysis might be an essential parameter for the analysis and choice of method. An exciting novel alternative to NGS is single-cell-based analysis by flow cytometry, also called cytometric microbiome profiling. This high-throughput method provides a fast and inexpensive tool for the analysis of the microbiota and offers the unique opportunity to monitor dynamic changes over time. Importantly, direct isolation by cell sorting of defined bacterial populations is possible for further molecular and functional analyses. Needless to say, flow cytometry is well established in many laboratories. How does cytometric microbiome profiling work? In flow cytometry scatter light and fluorescent emission characteristics of single cells are measured, at rates of several thousand cells per second (Davey 2002). Originally, characterization of bacteria by flow cytometry was restricted to enumeration of life bacteria. Coating with IgA antibodies in intestinal microbiota samples permits discrimination of certain functional subsets. Such an approach restricts analysis, of course, as anti-bacterial immune responses resulting in IgA antibodies are not comprehensive. Flow cytometric microbiome profiling uses light scatter as an approximation of sizes and fluorescent staining for quantitative DNA-content assessment of bacteria. For instance, in 2016, one of us showed the feasibility of microbiome profiling—in this case, the analysis of gut dysbiosis during colitis—using such flow cytometric microbiome profiling (Zimmermann et al. 2016). The principle is simple: the microbes are cleaned from debris (such as present in a fecal sample) and fixed with formalin. Then, the microbes are stained with a DNA dye, and analyzed on a flow cytometer for scatter and fluorescence parameters. For each event, i.e., a DNA-containing organism in the sample, both parameters are noted and stored. Data are analyzed by gating. A grid/gate template is defined and the frequency of events in each section of the grid used for down-stream statistics in assessing the microbial patterns and possible shifts. Gating or laying a grid needs supervision and software tools are emerging to make the process as objective as possible. For instance, tools with different operator expertises have been described, which automate analysis (Koch and Muller 2018; Zimmermann et al. 2016). One of these tools, the Cytometric Histogram Image Comparison (CHIC), is a fast image-based analysis procedure, which is operator independent, and results in a dissimilarity matrix. Our labs currently work on another operator independent approach, using the freely available R-software to create gating templates over the samples and for further multivariate analysis of variance. Efforts are ongoing to make those methods more user-friendly and amenable for routine analysis.

In conclusion, microbiome analysis informs many fields and the recognition of the interaction and manipulation of hosts by "their" microbiome triggered new and far-reaching questions. Next generation sequencing and the accompanying statistical tools have brought huge progress in understanding the microbiome and its symbiotic relationship with higher eukaryotes, including humans. Flow cytometric microbiome profiling as a novel, cost-effective, and low-threshold method adds to the toolbox and has the potential to push microbiome research towards routine analysis and applications. This tool to analyze microbial shifts and resolve kinetic changes is useful and has come just in time. Still, there is a need for even simpler down-stream analysis of the flow cytometry data to make the technique accessible and bring it forward for routine research, toxicological assessments, or even for use in clinical diagnosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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