Kako in zakaj analiziramo mikrobioto – hitri vodnik za zdravnike

How and why to analyze microbiota – a quick guide for clinicians

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Izvleček

Mikrobno združbo, ki naseljuje določen del človeškega telesa, imenujemo mikrobiota. Najbolj poznana je črevesna mikrobiota, vendar se zmeraj več raziskav izvaja tudi na področju mikrobiote ust, vagine in kože. Danes vemo, da imajo tudi številna področja, ki so nekoč veljala za sterilna, svojo specifično mikrobioto. Mikrobiote igrajo pomembno vlogo pri delovanju organov ter pri zdravju in razvoju bolezni in so zato predmet obsežnih raziskav, katerih namen je ugotavljanje možnih označevalcev zdravja oziroma bolezni ali možnosti uporabe mikrobiote v terapevtske namene. Skupaj z razvojem metod za analizo mikrobiote literatura na tem področju eksponentno narašča. Razumevanje osnovnih načel in izrazov, ki se uporabljajo v raziskavah mikrobiote, bo zato postalo pomemben del splošnega znanja zdravnikov.

Abstract

Microbiota is defined as a microbial population inhabiting a given body part. The best known example is the gut microbiota, but oral, vaginal, and skin microbiota have also been studied extensively. Many body parts previously thought to be sterile have been shown to possess their own specific microbiota. Each microbiota plays an important role in organ functioning, in health, or in disease development. Therefore, microbiotas are widely studied in an effort to establish possible health or disease markers, or to use microbiota as potential therapeutic targets. The literature on microbiota has increased exponentially along with the development of methods used for analysis. An understanding of the basic principles and terms used in microbiota studies are therefore an important part of a clinician's education.

MICROBIOTA – DEFINITION AND THE ROLE IN HEALTH AND DISEASE

Microbiota is a complex community of microorganisms inhabiting various parts of the human body. Although the gut microbiota has received the most attention, many other microbial communities have also been widely studied, such as the oral, skin, and vaginal microbiota (Table 1). Even body parts previously

Table 1. *General characteristics of different types of microbiota in healthy and disease states that are associated with dysbalance*

Microbiota	General characteristics in healthy state	References	Diseases	References
gut microbiota	- high microbial diversity	(11, 15, 16)	- colorectal adenomas and colorectal cancer	(17)
	- *Bacteroidetes* (*Bacteroides*), *Firmicutes* (*Anaerostipes*, *Blautia*, *Clostridium XIVa*, *Faecalibacterium*, *Lachnospiracea*, *Roseburia*), *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia*, *Ascomycota* (Saccharomycetaceae), Euryarchaeota (Methanobacteriaceae)		- infectious diseases (e.g. Clostridium difficile infection)	(18, 19)
			- inflammatory bowel diseases (i.e. Crohn's disease and ulcerative colitis)	(20)
			- metabolic diseases (e.g. obesity, diabetes)	(21)
			- cardiovascular diseases	(22)
			- liver diseases	(23)
			- respiratory diseases (e.g. asthma)	(24)
			- rheumatoid arthritis	(25)
			- neurological diseases (e.g. depression, autism)	(26)
oral microbiota	- high microbial diversity	(27, 28)	- dental caries	(29)
			- gingivitis	(30)
	- Streptococcus, Rothia, Neisseria, Candida, Veillonella, Actinomyces		- periodontitis	(30)
			- endocarditis	(31)
skin microbiota	- low microbial diversity	(32, 33)	- acne	(34)
			- atopic dermatitis	(35)
	- Propionibacterium, Staphylococcus, Corynebacterium, Moraxella, Malassezia		- chronic skin ulcers	(36)
			- psoriasis	(37)
vaginal microbiota	- low microbial diversity	(38, 39)	- aerobic vaginitis	(40)
	- Lactobacillus, Gardnerella, Mycoplasma/Ureaplasma		- bacterial vaginosis	(40)

thought to be sterile are now associated with microbial presence, such as the bladder, blood, eyes, and lungs (1-4). Although in utero colonization warrants further exploration (5), several recent studies have focused on microbial communities in the placenta and amniotic fluid from healthy pregnancies and/or meconium from newborns (6-8).

Microbiota has an important role in protection, interaction with the immune system, and correct functioning in all anatomic sites (9, 10). Gut microbiota contributes to metabolism and functioning of all other organs. One of the best studied interactions of gut microbiota with other organ systems is the gut-brain axis (11, 12). Gut microbiota can also affect the metabolism of many drugs, thus impacting efficacy or toxicity (13).

A disbalance in microbiota, often referred to as dysbiosis, is associated with a broad spectrum of diseases (Table 1), but it is not known whether this association is a cause or effect (14).

Microbiota research is a rapidly developing field involving microbiology, clinical sciences, and molecular biology; however, it is beyond the scope of this manuscript to cover all possible aspects of microbiota. Thus, we have focused on explanations of the main principles and terms of microbiota analysis that should help clinicians when reading studies related to microbiota research. Also, because the literature is so vast, only a small fragment of selected publications is cited herein.

TYPES OF MICROORGANISMS THAT CONTRIBUTE TO MICROBIOTA

Human microbiota consist mostly of bacteria and viruses, while archaea, fungi, and other eukaryotes are found in lower proportions. The bacterial component of the microbiota has been the subject of intensive study in recent years, but less attention has been given to other microbial groups, in part because of the well-established tools that are easily accessible and enable insight into bacterial communities. Approaches to study other microbiota members (e.g., fungi and viruses) have not been established.

The composition of the bacterial community varies by body site (Table 1) and at each body site there is substantial variability between individuals (41, 42). It is because of this inter-individual variability that a large number of samples must be included in studies. Moreover, the composition of healthy microbiota in one geographic region can resemble the composition of disease-associated microbiota in other geographic regions. For example, there are the differences in vaginal microbiota among women from Western countries and Latin America (43).

Archaea have a similar cell morphology to bacteria but are actually distinct relatives. Until recently, archaea were thought to be associated with extreme environments, such as hot thermal springs, and were not considered to be human pathogens; however, it appears that archaea play a role in microbiota of several body regions (44). In fact, archaea are associated with skin and intestinal diseases (45, 46).

Viruses in any microbiota are diverse and all viruses in a given microbiota are referred to as a virome. A virome can also include pathogenic viruses, but the main component of a virome are bacteriophages, which are viruses that specifically target bacterial cells and are important for controlling the equilibrium of bacterial populations. A virome also contributes significantly to the structure and function of any given microbiota and health.

A mycobiome is the term describing fungi within a microbiota. A limited number of fungal species comprise a stable component of skin microbiota (33); however, in gut microbiota, fungi are generally present as the result of food intake or oral microbiota (47). A change in diet (e.g., a *Saccharomyces-free* diet) or improved oral hygiene drastically lower the amount of fungi detected in intestinal microbiota.

MAIN APPROACHES USED IN MICROBIOTA ANALYSIS

There are two main approaches used to study microbiota. One is based on the cultivation of microbes and the other involves molecular analysis without cultivation.

Early studies sought to identify the microbiota that colonize the human body, primarily in the

gut, by culture and characterization of physiologic properties of isolated bacteria. Due to specific growth requirements, however, only a minority of bacteria can be cultured. Recently, the introduction of culture-independent next generation sequencing methods has greatly expanded the repertoire of known microorganisms within the human body.

Molecular approaches are based on detecting the microorganisms with various molecular methods. Herein we describe only approaches based on next generation sequencing.

In a simple molecular approach we only search for one specific gene (a marker gene). This gene should be present in all microorganisms, but should also be specific for each individual species. Because microorganisms are diverse, there is no marker gene that is present in all microorganisms. All bacteria have the same gene that encodes a part of the ribosome (16S rDNA). Fungi do not have the 16S rDNA gene, thus, another genomic region (the ITS region) is used as a marker gene. Bacteriophages do not have a marker gene, therefore, the virome cannot be analysed using this simple molecular approach.

Based on the many sequences from one specific marker gene, we can generate a list of different microorganisms that are found in the studied microbiota. This will be described in more detail below using the example of bacterial microbiota analysis.

Such descriptive analysis is simple and relatively cost effective, but only provides the composition of microbiota and gives no information about how this microbiota functions, i.e., which genes are present, which nutrients can be degraded, and what molecules can be produced. If we want to obtain a catalogue of all genes present in a given microbiota, then another sequencing method, such as a shotgun metagenomic approach, has to be used. In shotgun sequencing not only the marker genes but all DNA present in the sample is sequenced. From a shotgun sequence the information on bacterial, fungal, archaeal, and viral composition, as well as all functional genes, can be obtained. Shotgun metagenomic analyses are still not widely used because of the cost and the more difficult analysis of the obtained sequences.

Several other molecular and non-molecular "omic" approaches are also possible, such as metatranscriptomics to determine the current genes expressed by microbiota, metaproteomics to identify the enzymes being produced, and most of all, metabolomics, to identify microbial products (48, 49).

MOST OF THE CURRENT MICROBIOTA STUDIES ARE BASED ON 16S METAGENOME ANALYSIS, OTUs, AND RELATIVE ABUNDANCE

If we aim to determine the composition of bacterial microbiota, we have to sample the body site (e.g., swab, biopsy, or fecal sample), isolate the entire DNA from the sample, amplify the bacterial marker gene (16S rDNA gene), sequence the bacterial marker gene, and analyse the sequences to obtain the bacterial names, and determine the proportion of each bacterial group. To obtain sequenced DNA, the Myseq platform (Illumina) is widely used. Two analysis pipelines are commonly used for downstream analysis of 16S rDNA sequence data [mothur and QIIME (50, 51)].

Parts of the 16S rDNA gene are highly conserved and can be used for amplification of the gene. The other parts contain the so-called hypervariable regions. The sequence is specific for any given bacterial species. The definition of a bacterial species is arbitrary; two bacterial species have less than 97% identical base pairs in the 16S rDNA gene. Based on this definition, the various sequences obtained in a sequencing reaction from a single sample are first grouped according to the percent similarity.

In this way we obtain the main results of the 16S metagenome - a list of operational taxonomic units [OTUs]; Figure 1). An OTU is a group of sequences that are sufficiently similar (> 97%) to represent a bacterial species. The bacterial name is determined based on the OTU sequence compared to a large database. A number of different databases of 16S rDNA sequences are available to assign taxonomic identities to OTUs [i.e., The Ribosomal Database Project, Greengenes, and SILVA] (52-54). As will be described below, the sequencing reaction results in sequences that are too short to obtain bacterial species and identification is usually at the genus level.

The second main result of sequence analysis is the

Genus	OTU	Relative abundance [%] Individual level	Relative abundance [%] Population level
Faecalibacterium	Otu 00001	28.921	99.462
Bacteroides	Otu 00002	18.553	96.237
Bacteroides	Otu 00003	0.000	90.323
Prevotella	Otu 00004	5.919	22.581
Roseburia	Otu 00005	5.141	97.849
Blautia	Otu 00006	4.055	100.000
Bacteroides	Otu 00007	3.723	91.398
Bacteroides	Otu 00008	3.169	47.849
Ruminococcus	Otu 00009	2.780	78.495
Blautia	Otu 000010	2.049	99.462
Bacteroides	Otu 000011	1.861	37.634
Alistipes	Otu 000012	1.650	75.269
Lachnospiracea_ incertae_sedis	Otu 000013	1.629	99.462
Anaerostipes	Otu 000014	0.000	96.774
Barnesiella	Otu 000015	0.000	73.118
Bifidobacterium	Otu 000016	1.367	74.194
Gemmiger	Otu 000017	1.235	85.484
Bacteroides	Otu 000018	1.096	74.194
Parabacteroides	Otu 000019	1.011	84.409
Bacteroides	Otu 000020	0.947	18.817

Figure 1. *Presentation of microbiota analysis at the individual level with the list of OTUs and the relative abundances. One of the columns represents data for an individual sample and another column represents data for the entire population. The same bacterial genus is represented by different OTUs (species).*

relative abundance of each OTU. If the bacterial species is represented in the original sample in large numbers, there will be many copies of the specific marker gene in the isolated DNA, and therefore many copies will go into the sequencing reaction and will result in many sequences. The number of sequences in each OTU is represented in the percentage of all obtained sequences and is defined as the relative abundance (Figure 1). Relative abundance should be interpreted carefully. Relative abundance should be taken as an estimate of what OTU is more numerous than others but should not be taken as an exact quantitative measure.

HOW TO DESCRIBE DIVERSITY, AN IMPORTANT FEATURE OF THE MICROBIOTA

In addition to taxonomic information (list of bacterial names), the diversity is also an important feature of each microbiota. Again, different rules apply for different microbiotas. For instance, healthy gut microbiota should have high diversity, while healthy vaginal microbiota should have low diversity.

Diversity can be described on two levels: the individual sample [one person] and the sample set [entire studied population].

Diversity within each sample is the alpha diversity and consists of two separate components, richness [the number of different OTUs present] and evenness [the number of individuals within a particular OTU]. High richness means that many different bacteria are present in the microbiota; however, a high number of different bacteria is not sufficient. We could have 100 bacterial species in the fecal sample, but if one of the species represents > 70% of the community, the microbiota is not healthy. Evenness is the measure that describes how even the OTUs are represented.

Alpha diversity is usually described by Shannon (H') and reciprocal Simpson (1/D) indices (55); the greater the value of the index, the greater is the diversity in the sample.

Beta diversity describes between samples diversity and can be assessed with several algorithms [e.g., UniFrac or AMOVA algorithms of mothur software] (56, 57). Beta diversity explains if the two sets of samples (e.i. microbiota of healthy individuals and microbiota of patients with a given disease) are statistically different with respect to diversity.

PRESENTATION OF MICROBIOTA ANALYSIS RESULTS

Information about microbial community composition (i.e., the relative abundances of OTUs) within a single sample can be presented in the form of tables (Figure 1) or diagrams (composition pie charts or bar plots; Figures 2 and 3, respectively). Within each microbiota, tens or hundreds of OTUs are detected, not all of which can be represented as a pie chart or bar plot. For individual presentation we usually show higher bacterial groups, such as families or phyla. The example of fecal microbiota composition at the phylum level is shown in Figure

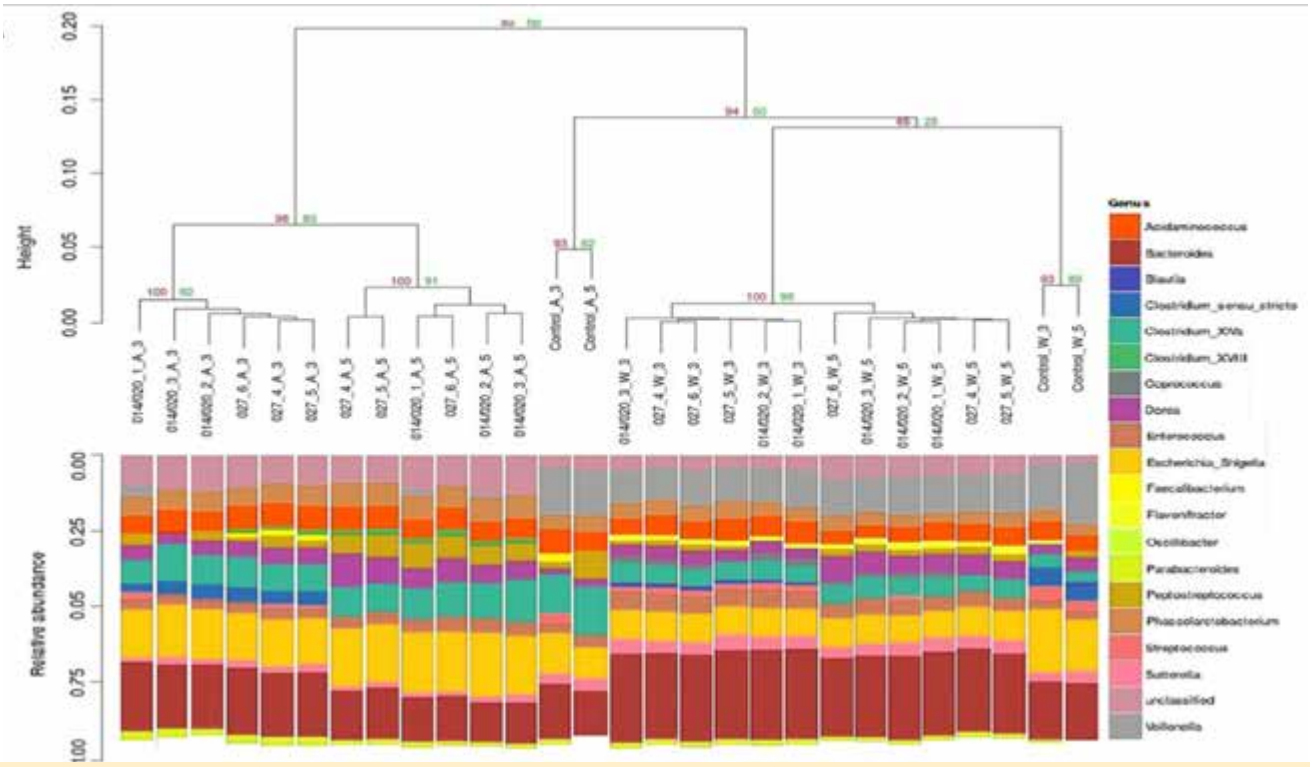

Figure 2. *Presentation of microbiota analysis results at the population level with hierarchical clustering of analysed samples (dendrogram) and corresponding relative abundances of OTUs within samples.*

3 and typically contains three-to-five phyla, of which Bacteroidetes and Firmicutes should be the majority.

When we transition from an individual sample to the sample set we have to decide how to present the comparison of tens or hundreds of samples each with hundreds of OTUs.

Many different algorithms are available that calculate sample similarities and can be used for grouping of samples into clusters. One method for such a presentation is a similarity dendrogram (Figure 2).

Another commonly used option is a non-metric multidimensional scaling (NMDS) analysis. This analysis transforms each microbiota (with several hundreds of OTUs) into a dot on a graph. The closer the two dots are positioned, the more similar are the two microbiotas (Figure 4).

Finally, we are also interested in which species (OTUs) are significantly different in one group of microbiotas compared to another group of microbiotas (as an example, which bacteria are different between healthy controls and patients with irritable bowel disease, IBD). Such significant differences between microbial communities of samples can be determined with several algorithms [e.g., the LefSe algorithm of mothur software] (58). The likeliness that a certain OTU (proxy for bacterial species) is present in one group and absent in the other group is described by the linear discriminant analysis (LDA) score. A list of such significantly different

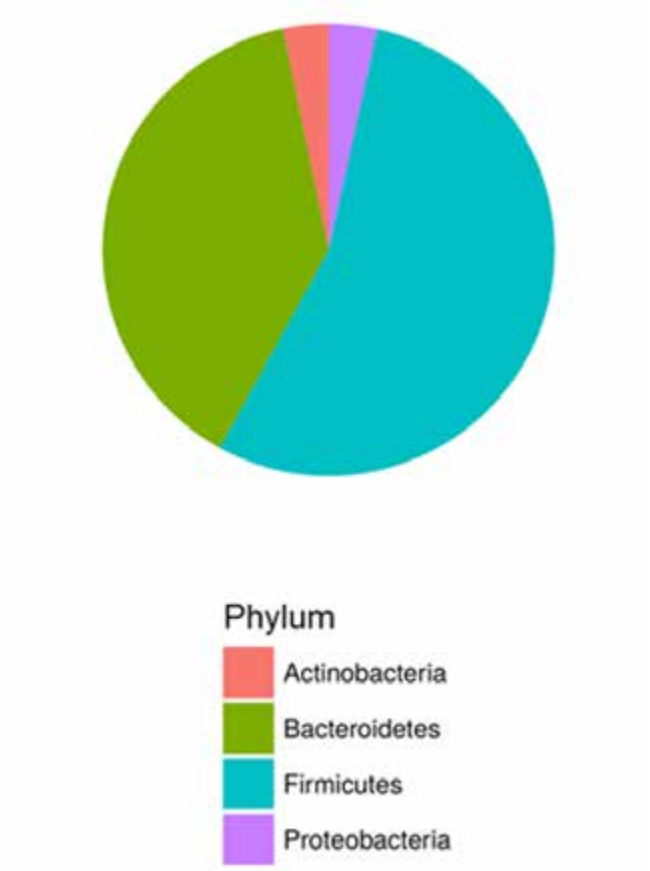

Figure 3. *The composition pie charts at the phylum level*

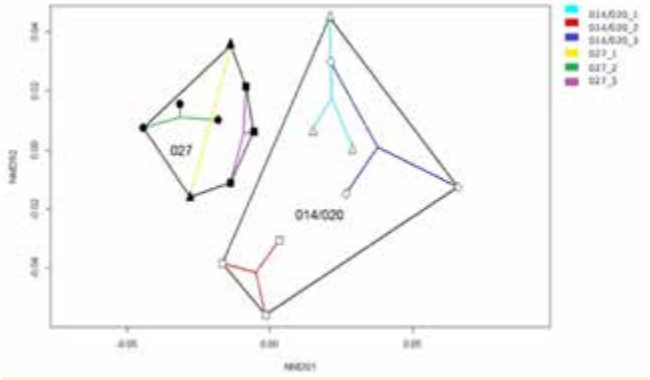

Figure 4. *Relationships between samples according to NMDS analysis*

bacteria together with the LDA values is usually shown in publications as presented in Figure 5.

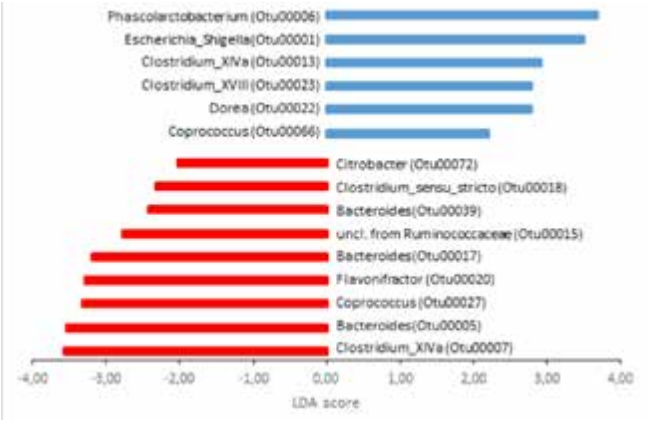

Figure 5. *Presentation of differentially represented OTUs in different samples identified by he linear discriminant analysis (LDA; calculated with LEfSe test in mothur software).*

HOW INFORMATIVE IS AN INDIVIDUAL MICROBIOTA ANALYSIS?

The analysis of a specific microbiota in a single person has a very limited informative value at the time. Although some commercial companies currently offer this analysis and claim that health-related information can be deduced, this is very unlikely.

The reason is simple. We know only broadly what are the features of a healthy microbiota. For gut microbiota, for instance, we do know that diversity must be high and that Firmicutes and Bacteroidetes should be very numerous. But what ratio of Firmicutes-to-Bacteriodetes is a health marker, if at all, remains to be determined. In addition, we do not know how important each bacterial species is and what are the normal relative abundances for the most common bacterial species that are found in gut microbiota.

If we compare the situation with blood analysis, we determine different markers (such as the white blood cell count, CRP, and albumin), knowing what the expected normal values would be for each of them and we also know what elevated or decreased values indicates clinically.

With microbiota analysis, we are now at the stage when we have the methods to determine the features, but we still do not know the normal reference values by which to interpret the results.

MICROBIOTA ANALYSIS IS BASED ON HUMAN STUDIES, ANIMAL MODELS, AND IN VITRO SYSTEMS

Microbiota studies are comparative and use human populations, animal models, or *in vitro* systems. Studies on humans and animal models provide important insight into the disease being studied. While findings from human studies have greater translational potential than animal models, the ability to perform invasive tests, the control of environmental factors, and the availability of exact biological replicates are some of the important advantages of animal models. Humanized germ-free animals are attractive tools for human microbiota research (59).

The main drawback of animal models, however, is the ethical concerns. A number of *in vitro* models have therefore been developed recently to study microorganisms that are difficult to culture, such as the *in vitro* gut model (60). Properly designed *in vitro* models combined with next generation sequencing methods can explain microbial interactions and the associated impact on disease under investigation (61).

PROBLEMS WITH MICROBIOTA ANALYSIS

Microbiota analysis has several problems that must be taken into account when designing studies, interpreting results, or reading the published research. One of the important problems is sampling. For gut microbiota analysis, fecal samples are usually analysed because fecal samples are easily obtained; however, these samples lack microorganisms attached to the intestinal mucosa, and therefore, do not properly represent the gut microbiota. In addition the gut microbiota profile can be altered by temperature and duration of storage of the fecal sample. For skin microbiota, swabs are obtained, but a great portion of skin microbiota resides within hair follicles.

There are also several drawbacks in approaches that are currently being used for microbiota analysis. Here we will describe the approaches using the bacterial part of microbiota as the focus in most studies.

If the same sample will be studied by cultivation and a molecular approach, the overlap would be surprisingly small; some species will be demonstrated by cultivation and others with molecular methods.

The list of bacterial names obtained in the molecular analysis is only an estimation, as are the obtained relative abundances. The 16S rRNA region is present in several copies in bacterial genomes, which might influence the apparent relative abundance of microorganisms in 16S metagenome studies (57). If the bacterium was not detected, this does not mean it is not present. The bacterium could be there, but below the level of detection. Biases could also be introduced due to a primer design for marker genes, which may select for or against particular groups of microorganisms or through the contamination of reagents (58-59).

Current methods of next generation sequencing can only produce sequences that are up to 600 base pairs long. The entire 16S rRNA gene has 1500 base pairs and cannot be sequenced in the entire length. Researchers must determine which part of the 16S rRNA gene will be sequenced and this is described in papers as the V3V4 or V1V3 region. The selected region can influence results and if two laboratories work with the identical DNA samples, but use a different 16S rRNA gene region each, a different list of bacterial groups would result.

Another problem of 16S rRNA gene sequencing is that 600 base pairs are not sufficient to determine bacterial species. Therefore, the current method enables us to study microbiota at a very low resolution. As shown on Figure 1, when OTUs in a group of fecal samples are determined, it is clear that several different species of Bacteroides (Otu00002, Otu00003, Otu00007, Otu00008, Otu00011, Otu00018, and Otu00020) are present. Much higher resolution in sequencing and in post-sequencing analysis is needed to develop personalized microbiota-associated markers.

Finally, when examining the samples with a low expected microbial load, such as blood or prosthetic joint infections, it is difficult to differentiate between contamination and clinically-relevant OTUs.

MICROBIOTA STUDIES BY THE MEDICAL FACULTY AT THE UNIVERSITY OF MARIBOR

Our research group is focused on gut microbiota studies, with a particular interest in the gut communities (bacterial, archaeal, and fungal) of healthy individuals and *C. difficile* patients (16, 62). Our unpublished work on different microbiotas and complex microbial populations was expanded to IBD, hospitalized patients, chronic wounds, and prosthetic joint infections.

Disturbances in gut microbiota are typically associated with *C. difficile* infections (CDIs) and can be successfully restored by transplanting fecal material of healthy donors (63). Colonization resistance mechanisms through which the gut microbiota influences *C. difficile* colonization are well-described and include regulation of *C. difficile* growth and germination by bile salt metabolism or *C. difficile* inhibition via inhibitory substances, competition for nutrients, and immune response activation (63, 64). Our studies using a simple *in vitro* batch model, however, showed that the interaction between *C. difficile* and gut microbiota goes in both directions. *C. difficile* can also affect the gut microbiota balance and these changes are likely associated with nutrient availability (65). We have also shown that healthy microbiota interacts differently with *C. difficile* than dysbiotic microbiota (66). These findings are crucial for proper understanding of *C. difficile* pathogenesis and should be considered when planning therapeutic approaches for CDIs.

CONCLUSIONS

Microbiota can be found in almost any part of the human body and has profound importance for health maintenance. Chronic diseases are especially associated with disturbances in microbiota.

Microbiota composition with OTUs and relative abundances as the main result is easy to determine but is less informative. More important but also more difficult is to determine microbiota function.

Despite the methodological difficulties, microbiota research provided important insight into microbial species that contribute to given diseases, influence our mental well-being, or protect us from pathogens.

Current research is devoted to microbiota-associated disease marker establishment, personalized microbiota-associated markers for medication or diet, and to establish ways in which microbiota modulation sustains health.

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