ANALYSIS OF THE GUT MICROBIOTA COMPOSITION – POSSIBLITIES AND PERSPECTIVES FOR CLINICAL PRACTICE AND RESEARCH

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ABSTRACT

The digestive tract of each human is inhabited by gut microbiota – an environmentally and genetically shaped ecosystem that is composed of several hundreds of microbial species. Given the large inter-individual variation, several thousands of microbes can be part of the microbiota. Due to this fact, and the fact that microbiota has an extremely high density, the analysis of its composition are technically challenging. The majority of human gut microbes have never been cultured, and the presence of these organisms became apparent only after the introduction of molecular methods based on the sequence of phylogenetic marker – 16S/18S ribosomal RNA. Application of molecular methods enabled comprehensive and precise, qualitative and quantitative analysis of the microbiota and revealed its numerous important features, including the presence of dysbiosis – disturbed composition – in relation to a number of diseases. With growing awareness of the systematic impact of the gut microbiota on human health, analysis of the microbiota are increasingly being included in the design of clinical studies. Since gut microbiota composition determines health of an individual, but also the effect of various therapies, question of routine microbiota analysis in clinical practice becomes relevant. This paper reviews methods for microbiota composition assessment with specific attention on their applicability in routine analysis. The report of the relevant findings regarding the links between microbiota and health is complemented with recommendations for using this knowledge for improvement of diagnostics and therapeutics, as well as for future studies that would enable better understanding of gut microbiota role in human health.

Keywords: microbiota, dysbiosis, human health

INTRODUCTION

Gut microbiota is an extremely complex microbial ecosystem that inhabits the entire digestive tract. The density of the microbiota is the largest in the colon where it reaches the value of 10^{12} cell per gram of the luminal content. The gut microbiota is the densest ecosystem

on Earth (1) and it is so numerous that the microbial cells outnumber the human somatic cells. The total number of gut microbiota species has not yet been determined and estimates range from 5.000 (2) to 10.000 species (3). Currently more than 1.000 known microbial species have been detected in the human gut microbiota (4), while much larger number of completely unknown (uncultured) species occupy our digestive tract. Only a fraction of all these thousands of species inhabit digestive tract of an individual (5). Subject specific combination of few hundreds species form a stable ecosystem (6), a composition of which is determined by environmental factors and host genetics (7).

Although the first gut bacterium – *Escherichia coli* – was identified already in 1886 (8) gut microbiota has been excessively studied only in the XXI century (Fig. 1). The main reason for this is the fact that classical microbiological methods did not allow for the speed, precision nor resolution needed for the analysis of such a complex ecosystem (9). Microbiota contains a large number of species in different proportions of which the majority does not grow under laboratory conditions, while its composition varies dramatically between individuals. Only after the introduction of molecular methods in the field of microbiology it was possible to accurately analyze gut microbiota (10). Once the methods were in place, the field of microbiota research expanded and together with it our knowledge about the important role that our microbial symbiont has on establishment and maintaining of normal physiological functions as well as it's implications in pathological conditions.

This paper reports the most important methods for the analysis of the gut microbiota composition and discusses their suitableness for routine application in clinical practice. Furthermore, the most relevant findings that illustrate the importance of the microbiota for health and disease are reported. Finally, the paper discusses the need for the microbiota analysis in clinical practice and gives recommendations for using already existing knowledge for improvement of diagnostics and therapeutics, as well as for future studies that could contribute to better understanding of the host-microbe interactions and microbiota implications in human (patho)physiology.

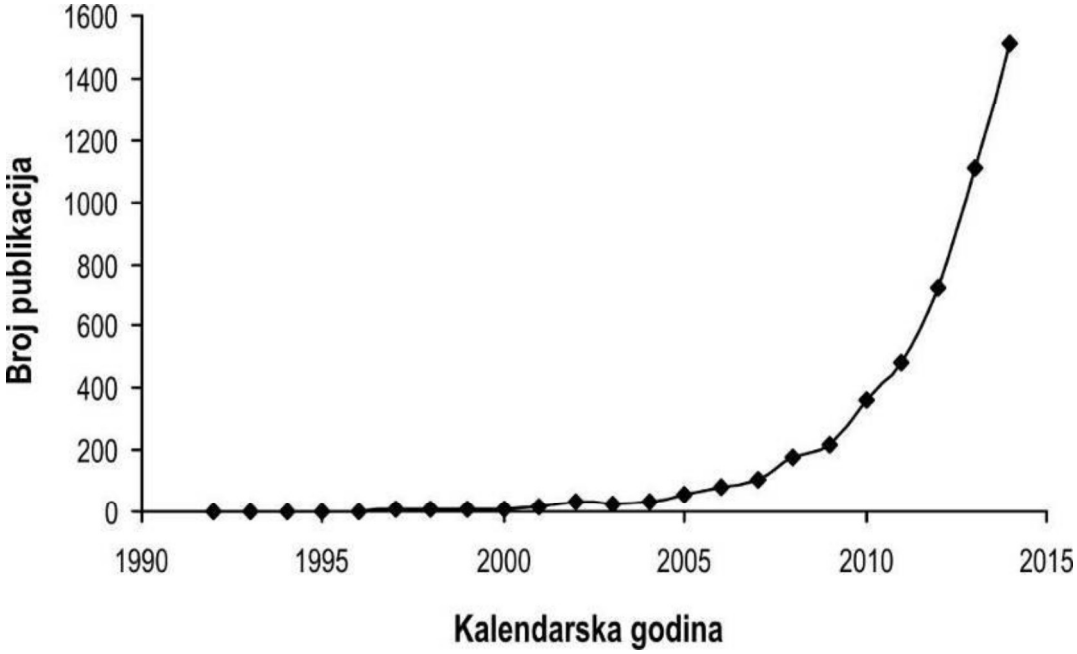

Figure 1. *Graphical representation of number of gut microbiota related publications. The graph was construced using the number of publications appearing on PubMed database using the search term "gut microbiota" (http://www.ncbi.nlm.nih.gov/pubmed/)*

METHODS FOR ASSESSMENT OF GUT MICROBIOTA COMPOSITION

Identification and quantification of microbes using classical microbiological methods is based on growth on specific media, colony counts, and its morphological and biochemical characterisation (11). To be able to grow a microbe in laboratory it is necessary to enable specific conditions, which for the majority of intestinal microbes includes the absolute absence of oxygen (12). In addition to strictly anaerobic conditions, gut microbes request for specific growth media, and as an example *Akkermansia mucniphila* grows only in media that contain mucus (13), while growth conditions for many of the gut microbes have not yet been defined. Another technical obstacle for the gut microbiota analysis using cultivation is the fact that some microbial groups are highly predominant (e.g. *Bacteroides* species reach density of 10^{11} cells per gram(14)), while the other groups are subdominant (e.g. *Actynomices* species reach density of 10^3 cells per gram (15)). In order to comprehensively analyse the microbiota composition, one would have to grow each sample on a large number of selective media, using wide range of dilution series and various growth conditions. Therefore, the analysis of the microbiota using cultivation are laborious, slow and costly (9). Still, the largest disadvantage of the classical cultivation is its inaccuracy. The natural system for species identification was achieved only after the introduction of the use of phylogenetic marker – sequence of 16S in prokaryotes and 18S ribosomal RNA in eukaryotes – for species identification (16). Using this phylogenetic marker it was determined that many microorganisms are misidentified, and the most striking example of it is the genus *Clostridium*. Application of 16S rRNA sequence analysis on the *Clostridium* members revealed that this genus comprises of highly unrelated species that should be reclassified into more than ten different genera (17). In line with this first observation, reclassification of several *Clostridium* species has been proposed including: *C. difficile* into genus *Peptoclostridium* (18), *C. coccoides* into genus *Blautia* (19), *C. glycolicum* into *Terrisporobacter* and *C. irregulare* into genus *Asaccharospora* (20). This reclassifications enable separation between those distantly related species and from the true clostridia such as *C. perfringens*, *C. tetani*, *C. botulinum* etc.

Molecular methods did not have impact only on the precision of the microbial identification and classification, but they have also enabled rapid and more informative analysis of complex ecosystems such as the gut microbiota. Assessment of the microbiota composition is based on the comparison of the 16S/18S rRNA sequence obtained from the real microbiota sample (typically stool or biopsy) with reference sequences from a known, cultured organisms (Fig. 2). Sequence similarity higher than 98.2% of intestinal sequence with the closest culture relative indicates that both sequences come from the same organism (21) and if this result is obtained it can be concluded that a particular species has been detected in a sample. However, if the sequence similarity is below this threshold, it is concluded that the sequence came from a currently unknown (uncultured) microbial species that can be positioned into the same genus as the closest relative (>95% similarity), family (>90% similarity) or higher taxonomic rank. The first molecular studies of the gut microbiota generated only a small number of sequences (few hundreds (22)), but today, in the era of the next generation sequencing it is possible to simultaneously sequence millions of DNA fragments (23) and process these automatically using pre-designed bioinformatical pipelines (24). Sequencing of the intestinal material had a major role in revealing many important facts about the gut microbiota including the presence of the dysbiosis in obese subjects (25) and inflammatory bowel diseases (IBD) patients (26), establishment of link between dietary pattern and microbiota composition (27), effect of antibiotics on this ecosystem (28) and many more.

Sequencing methods are widely used nowadays, despite the fact that they are technically demanding, and that their application calls for engagement of various experts. However, in case of inadequate data handling these methods can suffer from technical biases (evident through low reproducibility) or misinterpretation of the highly complex data. Having in mind these features of the sequencing methods, they will not be the first choice methods for application in clinical practice, although they have a undeniable and tremendously important place in research.

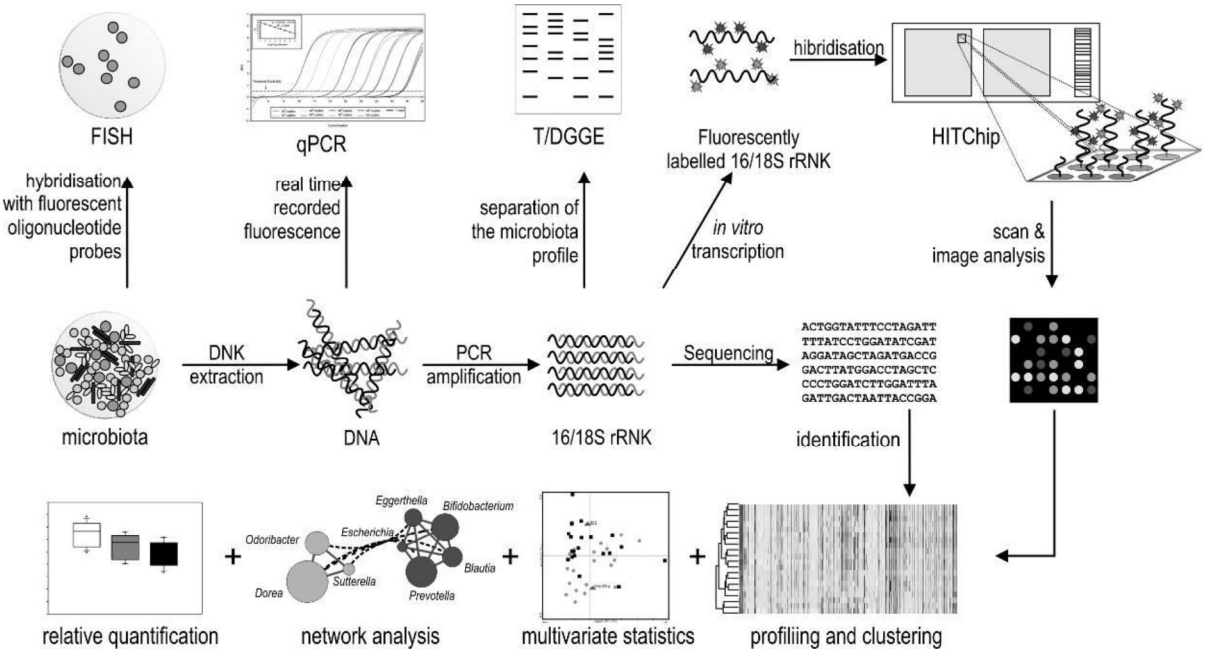

***Figure 2**. Schematic representation of experimental procedures and data analysis for microbiota compositon assessment using various molecular methods based on the sequence of the 16S/18S rRNK encoding gene.*

Using the data obtained by sequencing of the 16S/18S rRNK (or its encoding gene) from known organisms or DNA from intestinal samples, a basis for development of other molecular methods has been generated (29, 30). These molecular methods include profiling techniques that enable simple profiling of the total microbiota or its subgroups (methods DGGE and TGGE – Denaturant or Temperature Gradient Gel Electrophoresis). Profiling techniques are based on separation of DNA fragments from different members of an ecosystem during electrophoresis. Each specific sequence stops its migration on specific position, which results in a development of an ecosystem profile. Profiling techniques have also played an important role in discovering features of the microbiota, including the development of the newborn microbiota (31) and the discovery that the gut microbiota is individual-specific ecosystem that is stable in time (32). The biggest disadvantage of the profiling techniques is that they do not allow for identification of organisms in the developed profile, which seriously limits their wider application. Quantification molecular methods (qPCR quantitative PCR and FISH – Fluorescent *In Situ* Hybridisation) enable rapid and precise quantification of groups of organisms (typically genus or species members) in complex samples. qPCR can generate results similar to those obtained by cultivation, which is in the format that most microbiologists are familiar with, and therefore it can be anticipated that this method is going to be the most appealing for application by clinical microbiologists. Given that qPCR produces results with higher sensitivity, better precision and that it is less laborious, one could anticipate that this method will replace cultivation based detection in the near future. Quantification using molecular methods also had an important role in revealing the truths about the gut microbiota and among the other findings these methods were used for determination of the low *Faecalibacterium prausnitzii* counts in relation of

Crohn's disease (33), increased abundance of *Fusobacterium nucleatum* in inflamed appendicitis tissue (34), as well as for quantification of several uncultured species that are markers of the irritable bowel syndrome (IBS) dysbiosis. (35). The FISH technique enables *in situ* visualization of cells in the tissue which is a valuable feature. However, this is also the most technically demanding method, and therefore one could expect only its limited application for specific questions in clinical practice.

Phylogenetic microarrays (DNA arrays) represent another important group of molecular methods. This methodology is based on the simultaneous hybridization of thousands of molecular probes with nucleic acids material from a complex microbiota sample. Several phylogenetic microarrays have been developed for the analysis of the human gut microbiota (36-38) and among them the HITChip (Human Intestinal Tract Chip) found the widest application. The HITChip generates matrices of data suitable for further bioinformatics processing similar to that applied in sequencing technologies (Fig. 2). In contrast to sequencing, microarrays have a very high reproducibility, and they enable automatic identification and quantification, which implies that this technology does not call for specific experts. The most important clinical application of the HITChip was the discovery that stabilization of the microbiota diversity is an important phenomenon that occurs during successful faecal transplantation on recurrent *C. difficile* associated diarrhoea (CDAD, 39). In addition, the use of the HITChip enabled defining the IBS associated dysbiosis, but also identification of specific microbial markers associated with IBS patients symptoms (40, 41). Furthermore, the HITChip was used for determining the effects of probiotics and prebiotics on the microbiota as mediator of the health effects of these functional foods (42, 43). Among others, difference between microbiota of infants with and without colics were determined (44), but also the specific microbiota signatures of children with atopic diseases (45). The main disadvantage of the microarrays is the fact that they can target only known microbial species. The coverage of the microarrays includes also uncultured species, but only after they have been identified as members of the gut microbiota. Having in mind that the total number of gut inhabitants is still unknown and that the number of gut species increases dramatically over years (Fig. 3) testifies for the importance of regular update of the microarrays probe design. With regular update, it is feasible that phylogenetic microarrays can become widely applied in clinical practice at least at the level of application of human genomic expression microarrays.

Classical cultivation methods still have an important place in the studies of the gut microbiota composition since learning about the ecosystem comes through learning about its the members. Currently the majority of gut microbes are uncultured species that were reported only on the basis of their 16S/18S rRNA sequence (Fig. 3). The majority of these uncultured species clusters within the *Firmicutes* phylum, which is by far the most predominant group of gut microbes. Function, metabolism and interaction with host of these predominant microbes is completely unknown, and only after isolation and description of these species there will be a basis for studies that will elucidate the gut microbiota function in health and disease. An example that testifies for the importance of having a cultured representative of gut microbes is *Akkermanisa muciniphila*– mucolytic species isolated in 2004 (13) that has a major impact on glucose level regulation, metabolism and obesity (46). The fact that *A. muciniphila* was available as pure culture enabled studying of the host-microbe interactions using various *in vitro* and *in vivo* systems (47). The scientific society more and more acknowledges the importance of cultivation, and it is expected that classical microbiology will still play an important role in future research.

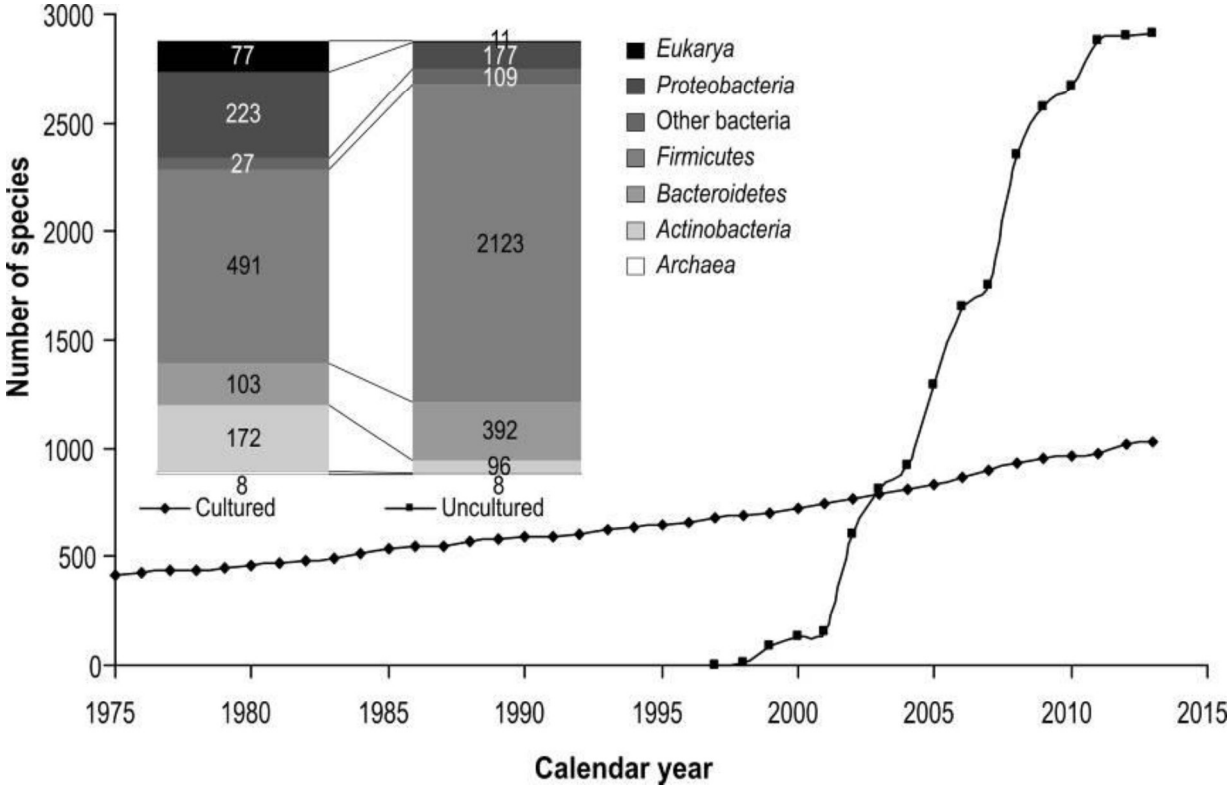

Figure 3. *Graphical representation of the cummulative number of cultured and uncultured gut microbital species as a function of time. Phylogenetic composition on phylum level of known cultured and ucultured gut microbiota species in 2015 is graphically presented.*

Examples of high throughput cultivation where cultivation is coupled with rapid molecular-based identification of isolates, showed that process of retrieving currently uncultured gut species can be speeded up (48). Despite undeniable place of cultivation in the field of microbiota research, these methods cannot be applied in routine analysis due to the numerous incompatibilities of classical microbiological methods with requirements for studying complex ecosystems.

MICROBIOTA IN HEALTH AND DISEASE

Gut microbiota composition is individual-specific and stable thorough long periods of time in adulthood (6, 49). The ecosystem develops very dynamically in the first year of life (50), but also later through childhood and adolescence (45, 51). Gut microbiota composition is determined by genetic and environmental factors (7, 52). It was determined that gut microbiota composition of both adults and children from developed western societies differs significantly from the microbiota of populations form rural areas of Africa and Central America (3, 53, 54). In one study the microbiota of African Americans and native Africans was compared (55). Using this approach the influence of genetics was to a large extent eliminated, and the study showed that the majority of the difference in the microbiota of subjects that live in developed and developing countries is a result of differences in the lifestyle, and particularly dietary pattern change (55). The microbiota of subjects living in developed countries is characterized by low proportion of specific groups, including those that are capable of resistant carbohydrates degradation (3, 53-55). In addition to dietary changes other factors such as application of oral antibiotics (28), daily consumption of food additives (56) and high hygiene standards (57) have had a major impact on the gut microbiota composition. With the societal development, many diseases were diminished, yet other, non-infectious diseases are in expansion. Many of the non-

infectious diseases are characterized by specific microbiota composition – dysbiosis (Fig. 4). Although the presence of dysbiosis does not provide evidence for microbiota's aetiological role, it can be anticipated that the microbiota is a mediator which composition and function is affected by various environmental factors linked to societal changes, that eventually induces an effect on human health.

Disturbed microbiota composition is associated to a number of digestive diseases including IBS (40, 58), IBD (26, 33, 59, 60), CDAD (61)and colon cancer (CRC, 62). While dysbiosis in digestive diseases could be, to some extent, anticipated, it is intriguing that gut microbiota dysbiosis is associated to other, non-digestive diseases such as type 1 and type 2 diabetes (63-65), atopic diseases (66, 67) and even autism (68-70). The data indicates that microbiota dysbiosis is a wide spread phenomenon among non-infectious diseases and that it is affecting, basically, all members of the microbiota (Fig. 4). Although this data cannot be directly applied for treating patients, it provides basis for selection of therapies that take into account the microbiota, its interaction with human host and its response to various therapeutics.

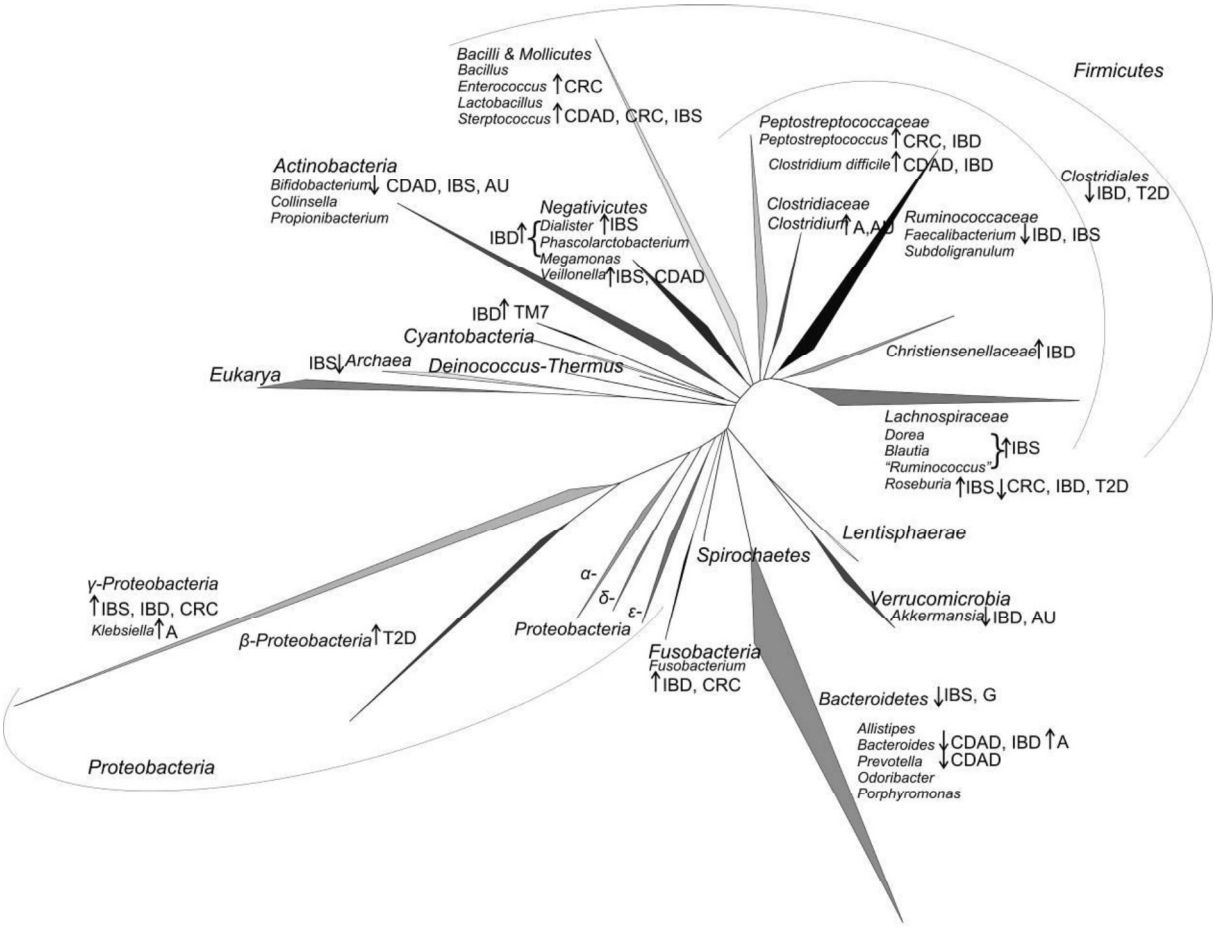

Figure 4. *Phylogenetic tree representing the diversity of the human gut microbiota, with indicated microbial markers of dysbiosis associated with colorectal cancer (CRC), C. difficile associated diarrhoea (CDAD), inflammatory bowel diseases (IBD), irritable bowel syndrome (IBS), type 2 diabetes (T2D). atopic diseases (A) and autism (AU). Figure is adapted from previously published work (71) and arrows pointing up and down indicate phylogenetic groups that in the microbiota of patients are increased and decreased, respectively.*

MICROBIOTA IN DIAGNOSIS AND THERAPY – STATE OF THE ART AND PERSPECTIVES

The microbiota dysbiosis is associated with many diseases, which brought up a question if resolving dysbiosis could lead to the health improvement. An example of successful therapy based o normalisation of the gut microbiota is treatment of recurrent CDAD with faecal transplantation. Patients suffering from CDAD have disturbed microbiota composition and drastically decreased microbiota diversity due to the domination of the pathogen – *C. difficile*. As an alternative to the typical antibiotic therapy, and in case of failure of the antibiotics, as the last line of therapy, CDAD patients are treated with faecal transplantation. This method appeared to be successful both in cases of transplantation using upper (39) and lower gastrointestinal route (72). Subsequent to faecal transplantation, the microbiota composition changed to resemble that of the donor (39). After stabilisation of the ecosystem, it had ability to resist pathogen invasion and lead to the improvement of health.

Decreased microbiota diversity is also linked to IBD (59, 73), and these patients were also treated with faecal transplantation. In contrast to CDAD patients, the faecal transplantation was not successful treatment method for the majority of IBD patients, although clinical picture of some ulcerative patients did improve upon this therapy (74). An important concern with regard to faecal transplantation is that it can lead to serious adverse effects due to the incompatibility of the transplanted microbiota and immunology of the recipient. It should be noted that ulcerative colitis patients that have experienced improvement of the symptoms upon faecal transplantation had a different microbiota composition than the patients that did not respond positively on the therapy (75). This illustrates that this potentially dangerous therapy should be applied only after determining compatibility of the patient and donor, in line with procedures applied for blood (or organ) transplantation.

Less aggressive method for treating microbiota dysbiosis is (oral) antibiotic therapy. This treatment appeared to be relatively successful in improvement Crohn's disease patients symptoms (reviewed in 76). Various clinical studies tested affect of four different antibiotics in different dosages on Crohn's disease patients . None of the studies followed the effect of the therapy on the gut microbiota. Crohn's disease associated dysbiosis its characterized by decreased diversity (73), and substantial depletion of *Faecalibacterium prausnitzii* and related species (33). *F. prausnitzii* has anti-inflammatory properties (77) and health beneficial effect of this bacterium is a result of positive effect of its major metabolite butyrate (78). Butyrate is a substance that can be directly utilized by colonocytes and its production by gut microbes leads to the decrease of inflammation and improvement of gut barrier function (79). An independent study in which urinary infection of paediatric patients were treated with two different antibiotics, showed that ciprofloxacin induces dramatic and non-desirable changes of the microbiota in contrast to rapidly absorbed antibiotic – nitrofurantoin (80). Since nitrofurantoin treatment decreased the level of *Clostridium* species and increased the abundance of *F. prausnitzii* and related species, authors of the study have suggested that this antibiotic could potentially be used for treating the dybiosis of Crohn's disease patients (80). In contrast to ciprofloxacin, nitrofurantoin has never been used for treatment of IBD patients, given that for this application antibiotics with low bioavailability were typically selected, since the goal of the antibiotic therapy was to have a significant effect on gut microbes (81). In addition to ciprofloxacin, rifaximin, antibiotic with very low bioavailability (82), was also widely used for treatment of IBD patients. Selection of such antibiotics is not optimal from the microbiota perspective, given that highly diverse and abundant microbiota is necessary for normal human physiology, and clinical studies with other antibiotics will show if this therapy when taking into account the microbiota can improve symptoms of IBD patients.

The information of the gut microbiota composition can be used not only for design of therapy, but also for diagnostics. The fact that the symptoms of IBD patients are to a large extent overlapping with symptoms of IBS patients, call for invasive endoscopic checks of IBS patients, in order to make a reliable diagnostics. Such tests are often burden for patients and medical system. Non-invasive diagnostics of IBS based on presence of specific microbiota markers in stool samples has recently been patented (83). In addition to presence of specific microbiota markers, the decrease of microbiota diversity in IBD patients, in contrast to IBS patients (40, 59, 73) could be another feature suitable for microbiota-based diagnostics. Finally, it is of high relevance that molecular methods enable more precise detection of pathogens than the currently applied methodologies. In a study that have assessed the gut microbiota composition of ulcerative colitis patients during remission, it was determined that the majority of patients had *C. difficile* in their stool samples. However, the study design was developed to exclude *C. difficle* harbouring patients, and all recruited subjects tested negative for presence of *C. difficile* exotoxins A and B (59). Patients with exotoxin negative *C. difficile* colonisation represent particularly vulnerable group, prone to risk of complications pending the antibiotics therapy.

Gut microbiota composition of healthy subjects and patients is highly relevant for its physiology and it to a large extent defines the response on therapy with oral drugs and supplements with low digestibility and bioavailability. Inclusion of the gut microbiota analysis before and after therapy has a potential to increase our understanding of inter-individual variation to various therapies including faecal transplantation, antibiotics but also functional foods such as probiotics and prebiotics. The data about microbiota changes through therapy could lead to a design of better therapeutic approaches for treatment of pathologies associated with gut microbiota dysbiosis. Recent research has shown that gut microbiota composition influences the response on vaccination of healthy children (84), but also immunosuppressive drugs when treating paediatric IBD patients (85). These results indicate the systemic impact of the gut microbiota and illustrate that assessment of the gut microbiota composition could be beneficial in various medical fields.

Currently a wide range of methods for rapid and precise microbiota composition analysis are available and allow for more and more frequent inclusion of microbiota composition assessment in clinical studies. As a result number of publications that tackle the subject of the gut microbiota increases rapidly (Fig.1). With increasing number of publication, our knowledge about the importance of the systemic impact of the gut microbiota rises. In addition to the inclusion in clinical studies in gastroenterology, endocrinology, immunology, and other medical fields, it can be expected that the gut microbiota composition assessment will, in some extent, become part of the clinical practice in the near future. This step will be first taken in procedures that directly aim the gut microbiota manipulation, such as faecal transplantation. In line with steps taken towards personalised medication, it is reasonable to expect inclusion of gut microbiota assessment since it defines response to various therapies. In order to enable gut microbiota composition analysis either novel methods and procedures should enter clinical centres, or collaboration with centres able to perform high throughput and comprehensive microbiota analysis should be established. While it is uncertain which of these scenarios will occur in the future, it is certain that gut microbiota assessment is a part of modern and future clinical practice.

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