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Review article

Microbiota and Human Health: Characterization techniques and transference☆

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ABSTRACT

The human microbiota comprises all the microorganisms of our body, which can also be categorised as commensals, mutualists and pathogens according to their behaviour. Our knowledge of the human microbiota has considerably increased since the introduction of 16S rRNA next generation sequencing (16S rDNA gene). This technological breakthrough has seen a revolution in the knowledge of the microbiota composition and its implications in human health. This article details the different human bacterial ecosystems and the scientific evidence of their involvement in different diseases. The faecal microbiota transplant procedure, particularly used to treat recurrent diarrhoea caused by *Clostridium difficile*, and the methodological bases of the new molecular techniques used to characterise microbiota are also described.

Microbiota en la Salud Humana: técnicas de caracterización y transferencia

Palabras clave:
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RESUMEN

La microbiota es el conjunto de microorganismos que reside en nuestro cuerpo, que a su vez pueden diferenciarse según su comportamiento en comensales, mutualistas y patógenos. El conocimiento de este ecosistema se ha visto considerablemente incrementado tras la introducción de las técnicas de secuenciación masiva del gen 16S ARNr (gen ADNr 16S). Este avance ha supuesto una verdadera revolución en el conocimiento de la composición de la microbiota y de su implicación en los estados de salud y enfermedad del ser humano. En este documento se detallan los diferentes ecosistemas bacterianos que podemos encontrar en el cuerpo humano y las evidencias científicas que existen en relación con diferentes enfermedades. También se describe el procedimiento de transferencia de materia faecal, particularmente utilizado para el tratamiento de las recidivas de la diarrea por *Clostridium difficile*, y las bases metodológicas de las nuevas técnicas moleculares utilizadas en la caracterización de la microbiota.

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Introduction

The microbiota is the set of microorganisms (bacteria, fungi, Archaea, viruses and parasites) residing in our bodies. These microorganisms may be classified as commensal, mutualistic or pathogenic. The term microbiome refers to the entire habitat, including the microorganisms, their genes and the environmental conditions. However, in practice, the two terms are used interchangeably as the suffix *-biome* (community) and the suffix *-oma* (set) are conflated.[1] In each of the different locations of our body we can find complex microbial ecosystems. The most complex, diverse and numerous is the one associated with the digestive tract, particularly in the caecum, where the density of micro-organisms is the highest in our body. These communities have a symbiotic and mutualistic behaviour with human eukaryotic cells, are essential for the proper functioning of our body, maintain an important dialogue with the immune system and have homeostatic functions that condition our health.[2] Numerous pieces of scientific evidence have implicated the intestinal microbiome and its metabolic potential in diverse pathological states over the last years, creating new therapeutic strategies to control and regulate this ecosystem.[3] Among these new approaches is the transfer of faecal microbiota, with increasing popularity given its success in the treatment of recurrent diarrhoea caused by *Clostridium difficile*.[4]

The knowledge of our microbiome has been considerably expanded after the use of molecular massive sequencing techniques, especially next-generation sequencing techniques. Microbiological cultures have always been used to determine the composition of the microbiota, but nowadays it is known that most of the micro-organisms of this ecosystem cannot be cultivated using traditional means, and their detection is only possible after DNA sequencing as a genetic fingerprint. The use of molecular techniques has enabled identifying and taxonomically assigning most microorganisms without the need to culture them.[5] This advance, as well as other microbiological techniques that will be detailed in this article, have led to a true revolution in the knowledge of the microbiota and its implication in the health and illness of human beings.

Clinical considerations

From birth, there is a symbiotic relationship between the microbiota and our cells that evolves over time, adapting to changes.[6] Due to its enormous metabolic capacity, the microbiota has been considered as an essential "organ" for life, with an influence on health and illness.[7] It is composed of unique features and characteristics of each individual, and may vary depending on genetics, diet and interaction with the environment.

The study of this ecosystem is a field of rapid scientific progress, with it being universally accepted that to reach an adequate state of health it is also necessary to have a "healthy" microbiota. Our microbiota undergoes changes due to the influence of multiple factors, in a way similar to those experienced by any organ of our body from ontogeny to death. We are continually exposed to factors that may influence, although one of its characteristics is its great capacity for resilience[8] (ability to adapt to a disturbing agent or an adverse situation, with subsequent recovery of the initial state when the alteration ceases), immediately recovering its natural state, which is termed "eubiosis". The level of these changes is defined not only by the nature, strength and duration of the alteration, but also by the composition and stability of each microbiota, assuming that each is unique to each person. On some occasions, the nature of the alteration is so strong that it conditions alterations in its composition or functioning, reaching a state of dysbiosis. Dysbiosis can occur in a matter of days, particularly after the ingestion of antibiotics, but it can also be a consequence of other longer-term actions, fundamentally related to diet.

In an adult, the gastrointestinal tract can be home to between 500 and 1000 species of micro-organisms, with bacteria of the *Bacteroidetes* (≈25%) and *Firmicutes* (≈60%) phyla being the majority. In a lesser proportion, *Proteobacteria*, *Verrucomicrobia*, *Fusobacteria, Cyanobacteria, Actinobacteria* and *Spirochaetes*, Archaea, fungi, protozoa, viruses and other micro-organisms are detected. It is also important to keep the proportions balanced, and therefore the *Firmicutes*/*Bacteroides* ratio has been established as a parameter to evaluate the balance of the intestinal microbiota and its functionality. In obese people, this ratio is greatly altered by the increase in *Firmicutes.* The increase of *Firmicutes* has also been described physiologically in elderly people as a consequence of age.

The main functions of the intestinal microbiota are to prevent colonisation by other pathogenic microorganisms, help digest food, produce vitamins B and K that the human body is not able to synthesise and, finally, and by no means less important, to stimulate the immune system. After birth, the cells of the immune system lack stimuli, recognising all the antigens around them as part of the body and blocking the inflammatory response against them. That is why the first contacts of the microbiota with the undifferentiated immunological cell lines are very important, and they will help define what belongs to "the body" from what is "foreign". This system and the intestinal microbiota maintain a continuous mutualistic dialogue, but if this situation becomes unbalanced, a pathological process can begin. This seems to be the basis of certain autoimmune diseases where the antigens of the intestinal microbiota represent a stimulus large enough to trigger an inflammatory response. In other diseases, such as metabolic syndrome and obesity, the origin of the stimulus that causes a continuous basal inflammatory response is also attributed to the intestinal microbiota.

The existence of the gut-brain axis (GBA) has recently been described, which connects the central nervous system with the intestinal microbiota through the vagus nerve, the parasympathetic nervous system and bacterial metabolites, which can act as neurotransmitters, and the endocrine system associated with the digestive tract.[9,10] Thus, in addition to the diseases that have traditionally been related to alterations in the microbiota, such as obesity, type 2 diabetes, inflammatory bowel diseases and allergies, other diseases of the central nervous system have recently been associated with it, such as autism, anxiety, depression and alcohol dependence.

It is currently accepted that in order to reach a complete state of health it is necessary for our microbiota, particularly that associated with the gastrointestinal tract, to also be healthy. The main health indicators of the microbiota are its richness (number of microorganisms) and its biodiversity (number of species). Both parameters are evaluated with alpha-type biodiversity indices, such as the Shannon diversity index (reflects the heterogeneity of a community based on the number of species present and their relative abundance), and the Chao index (abundance and representation of each species in all samples).

Numerous associations between pathological states and alterations of the microbiota have been published, either due to the presence or increase of certain genera, or the complete opposite, i.e., the absence or reduction of their concentration. Computational methods are used to evaluate and compare the microbiota of a patient with that of a healthy subject, such as the clustering method, or reduction of the dimensions of the distance matrices that define the set of samples of each study. It is also possible to perform a principal component analysis, which allows the addition of other clinical variables.

Transfer of faecal matter

At the moment the only indication for faecal transfer is the recurrence of diarrhoea caused by *C. difficile*, where the aim is to ecologically restore the bacterial diversity and the dysbiosis caused by diarrhoea and the pathogen. The first guidelines recommended carrying out the transfer in the third recurrence; however, in a recent review, it is recommended that it be carried out after the second episode of diarrhoea. In cases in which the transfer does not obtain immediate results, a second transfer can be done under the same conditions, and in the event that it fails again it can be re-performed, but using another donor.[11]

There are other diseases where the transfer of faecal matter has great therapeutic potential: inflammatory bowel disease, obesity, metabolic syndrome, autoimmune diseases, allergies, chronic fatigue syndrome and some neuropsychiatric diseases. For all these diseases, studies have been published with transfer of faecal matter, although the results have not been as successful as in diarrhoea due to *C. difficile*. Of all of these, ulcerative colitis has the best clinical results, although it seems that success is dependent on the donor for each patient.

The side effects of this procedure are usually scarce and not very relevant. Recently, a review was done of 50 publications related to faecal transfer, which shows that a large number of patients presented adverse effects.[12] Many of these side effects are associated with the route of administration of the infusion, with colonoscopy being the safest route. Cases of death due to aspiration pneumonia have been described, almost always linked to administration by nasogastric tube. However, all studies indicate that, for the time being, the long-term adverse effects of faecal transfer are not known. The absence of communicable diseases in the donor is an essential requirement, and international guidelines include the disorders that must be ruled out before accepting a donor. Basically, the study that is done on the donor is similar to the one done in any organ transplant. Another possible application of the transfer of faecal matter is the intestinal decontamination of patients colonised by multidrug resistant bacteria. For the time being, the eradication of vancomycin-resistant enterococci, methicillin-resistant staphylococci and carbapenemase-producing *Klebsiella* spp.[13,14]

Choice of the sample

As previously described, each zone of our body hosts its particular microbiota and, therefore, the sample will depend on the area to be studied. The vast majority of studies focus on the intestinal microbiota, since it is the largest and most involved in our state of health. For the study of this community, the most used sample is faeces, because of its simple, non-invasive collection procedure. The disadvantages of faeces are that they do not represent the totality of the microbiota adhered to the intestinal epithelium and that the bacteria of the uppermost intestinal tracts can be completely degraded, preventing their correct detection. In some disorders, such as inflammatory bowel disease, biopsies obtained during routine endoscopies can be used. The biopsy has the advantage of being a more real sample, whose collection and storage are more standardised, although it also has the drawbacks of its invasive nature, which only collects the microbiota from a specific point, and that the preparation enemas of the patient for colonoscopy can modify its composition. For all these reasons, faeces samples are used more commonly than biopsy samples. Another option is to use rectal exudates, which reproduce microbiota profiles similar to those found in faeces. This sample is easily obtained and can be stored immediately when taken in the centre itself. This sample is also currently used in all hospitals for the study of patients who are carriers of multidrug resistant bacteria with good results, although the total representation of the microbiota is not assured.

Collection, storage and transport

In the case that the sample is faeces, the recommendations are to collect it as for a stool culture and freeze it immediately at −80 °C, although higher temperatures are also acceptable (up to −20 °C). Freezing prevents possible changes in microbial communities until nucleic acid extraction can be performed. Freezing speed is especially important in the case of RNA extraction, because it degrades easily at room temperature. Rectal swabs can be stored for up to 2 h at room temperature in a stabilising buffer without impact on the composition of the microbiota.

In the event that the sample chosen is faeces, the most common procedure is for patients to collect it at home, keep it refrigerated (4 °C) or frozen (−20 °C) and deliver it as soon as possible to the centre where it will be processed. There may be factors that modify the final results, such as contamination during collection, the time until they are frozen, freezing at not-very-low temperatures or thawing during transport to the laboratory. When the sample remains at room temperature, the composition of its microbiota can be altered, so immediate freezing is recommended. Another possibility that has been studied with good results is to freeze the sample in the 15 min following defecation and store it for up to 3 days in the freezer at home.

Depending on each centre, based on the type of sample and the time that it will be stored until processing, it will be necessary to choose the most appropriate method according to different criteria, which include cost, availability, difficulty of use, time required for handling and if it is compatible with other diagnostic methods that will be used in that same sample.[15]

Sample processing: extraction of nucleic acids

Within one sample there may be different microenvironments with variations in the composition of their microbiota, and it is therefore very important to perform a good mechanical homogenisation before beginning the extraction process. It is also very important to fully dissolve the aliquot to be processed from the sample, usually 0.5 g of faeces in 5 ml of water, by vortexing or with the use of glass pellets.

Undoubtedly, the most critical step of the whole process is the extraction and purification of nucleic acids, usually DNA, since it is necessary to achieve a good quantity and quality without bearing substances that can inhibit the subsequent PCR reactions. For the extraction there are different commercial protocols and manuals with which good results are generally obtained.

Sequencing method

With the technological progress in massive sequencing, new methods have been developed that can directly sequence fragmented or amplified DNA without the need for cloning. These sequencing methods are known as next-generation sequencing methods. The next-generation methods have many advantages, including lower cost, reduced preparation time of the libraries and the sequencing process, an acceptable quality of the data and, above all, the generation of a large number of sequences.

Mass sequencing with next-generation methods requires a previous step of PCR amplification that can lead to errors, especially by overestimating the majority populations. Even so, these techniques represent, at present, the standard for the study of microbial communities, particularly the human microbiota. Among the next-generation sequencing methods are those based on Roche's 454

technology: a previously pioneering method which is no longer found on the market. The most common technologies currently available are Solexa, marketed by Illumina, and Ion Torrent, marketed by Thermo Fisher.

In order to compare the results of different works, it is important to know the different methodologies and their biases. The PCR process has limitations, since the most abundant is always amplified, and minority populations are disregarded. It is also important to choose correctly the primers that will be used. The most widely used amplify the V3–V4 region of the 16S rDNA gene, and were chosen based on their "universality" for most bacterial species, although recently it has been described that some species of bifidobacteria and lactic acid bacteria are not amplified with these primers due to failures in hybridisation.

In the study of the microbiota based on the taxonomy of the 16S rRNA gene, the relative abundance of each phylum, family or genus is quantified. However, this method has a significant drawback, since this gene has a different number of copies in each genus or species. For example, *Mycobacterium tuberculosis* has one single copy of the gene, *Helicobacter pylori* has 2 copies, *Staphylococcus* has 7 copies and, finally, *Clostridium beijerinckii* has 14 copies.

In spite of the possible biases and technical limitations that exist in massive sequencing, these molecular approaches are very powerful tools which are usually reproducible and allow for a determination of structural changes in microbial communities. For all this, the next-generation massive sequencing is considered as the best option to study the human microbiome.

The third generation of sequencers is already on the market with several pieces of equipment under development. The great improvements they offer include the direct sequencing without the previous step of PCR amplification, and the length of the obtained sequences, which can reach up to several kilobases. However, one of its disadvantages is that they have to improve the quality of the sequences that are obtained. Some of these third-generation methods include Pacific Biosciences (PacBio), with the new Sequel System, and MinION, from Oxford Nanopore.

Strategies for the characterisation of the microbiota

1) Metataxonomy is the most widely used strategy for characterising the composition and the relative quantity of microbial communities, as well as their evolution with the passing of time and other clinical variables. From a stool sample from which the total DNA is extracted, the 16S rDNA gene is amplified with universal primers and then the amplifications are sequenced massively. Each sequence is assigned the taxonomic group by searching public databases such as the *Ribosomal Database Project*, and then the results are analysed with bioinformatic tools, first to validate the quality (identity $\geq$ 98%) and length ($\geq$200 pb) of the sequences. The usual order of bioinformatic analysis includes: (a) quality control of the sequences; (b) elimination of chimeric sequences; (c) clustering of the sequences by characteristics of similarity and overlap (clustering); (d) taxonomic allocation, and (e) statistical analysis to determine significant differences.[16]

In general, the complete process of massive sequencing is usually left to the sequencing services, due to the high technical specialisation required and the limitation of the availability of the devices required for this methodology. These units offer the knowledge and the skills necessary to carry out the construction of the libraries and the sequencing according to the standards of each chosen technology. Although the final processing of the data is usually carried out by a bioinformatics expert, it is always necessary to attribute a biological and microbiological meaning to the results.[17]

2) Metataxonomy of the active fraction. Metataxonomy studies allow us to learn the composition of the microbiota without differentiating between live and dead, latent or inactive bacteria. The functional information of bacteria is also important, as it can directly or indirectly affect our health. In order to identify the active bacteria, it is necessary to extract the RNA from the sample, transform it into cDNA by retrotranscription and, finally, sequence it. In this way, only the bacteria that are dividing are identified. The techniques and technologies for the study are the same as in the case of metataxonomy, except that in this case the starting material is the 16S rRNA molecule.
3) Metagenomic. This approach is based on the massive sequencing of DNA or RNA (or cDNA) to study how alterations in the microbial composition influence the content of genes and their expression. This method, developed in the 1980s and 1990s, is known as shotgun and enables the sequences of DNA or RNA fragments to be read without prior amplification. It is a method that requires a greater computation of the data and that usually makes the process more expensive. The set of all these fragments is considered representative of the set of bacterial genomes present in the original microbiota. Metagenome sequencing makes it possible to avoid the significant bias introduced by the PCR process, since the fragments obtained are randomly selected from the all the genomes present in the original sample.[18]
4) Metabolomic. This strategy addresses the identification and characterisation of metabolites from a functional point of view.[19] The qualitative and quantitative determination of the metabolites is considered one of the best markers of microbial activity, since they are the final product of a metabolic reaction, regardless of which microorganisms or what number of enzymes participate in it. The metabolic analysis of a sample such as faeces is very complicated, since it not only contains the metabolic products of microorganisms and epithelial cells, but it also receives a constant flow of substances with the consumption of food. From the perspective of analytical chemistry, 2 types of tools are required for the analysis of metabolomes: nuclear magnetic resonance and mass spectrometry. In addition, regardless of the techniques, there are 2 types of metabolomic approaches: directed and non-directed, depending on whether or not the nature of the metabolite to be identified and/or quantified is known.

The most studied bacterial metabolites in faeces are short chain fatty acids (SCFA), originated in the bacterial fermentation of the complex carbohydrates of the diet (fibre and starch). The main SCFA detected in faeces are the acetic, propionic and butyric acids (>90% of all SCFA), but there are also other branched acids—isobutyric and isovaleric acid—which are less prominent (representing around 5% of the total) and which derive mainly from the metabolism of proteins and amino acids, having been less studied in general than the majority.

Most of the SCFA produced in the colon are absorbed into the colonic mucosa by diffusion and specific transporters. While the epithelium of the colon almost completely consumes the butyric acid, which is the main source of energy for the colonocytes, the acetic and propionic acids pass into the portal circulation and are used as precursors in the liver or peripheral tissues for hepatic gluconeogenesis and lipogenesis. The SCFA are of great importance in the physiology and nutrition of the gastrointestinal tract, presenting anti-inflammatory and anti-cancer properties. Thus, butyric has been related to the reversion of neoplastic cells, being able to participate in the prevention of carcinogenic processes.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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