Acta Biomed 2018; Vol. 89, Supplement 9: 52-59 DOI: 10.23750/abm.v89i9-S.7906

Review

The impact of intestinal microbiota on bio-medical research: definitions, techniques and physiology of a "new frontier"

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Summary. In recent years the metagenomics techniques have allowed to study composition and function of the intestinal microbiota. The microbiota is a new frontier of biomedical research to be explored and there is growing evidence of its fundamental health-promoting activity. The present review gives a synthetic overview on the characteristics and the role of the microbiota in the adult with particular reference to physiology, pathophysiology and relationships with the host and the environment. (www.actabiomedica.it)

Key words: microbiome, dysbiosis, metagenomics, aging, nutrition, exercise

The intestinal microbiota and the metagenomic approach

The human intestinal microbiome is the ensemble of microorganisms (mainly bacteria, but also viruses, fungi, protozoa and Archaea) that physiologically live in symbiosis with the host at the level of the digestive tract (1). The term microbiome is often used interchangeably with microbiota, which, instead, on a purely semantic level, identifies the set of proteins synthesized by such microorganisms (1).

Although there is a growing interest in the study of fungi and intestinal symbiotic viruses (so-called "mycoma" and "viroma"), actually most of the studies have analyzed the bacterial component of the intestinal microbiome. Therefore, in the common scientific language, when we speak of "microbiota" or "microbiome", we substantially refer to all intestinal bacteria, which in the past were designated with the improper terms of "microbial flora" or "resident bacterial flora" (2).

It is an extremely complex microbial community, with ecological characteristics not yet fully understood, including a large number of bacterial species (at least 1100, but some studies have hypothesized that this number exceeds 2000) (3-4). On overall, the intestinal microbiota of a healthy man can contain up to 10^{14} bacteria, with a genome that, in quantitative terms, is about 150 times larger than that of the host organism (3-4). The genome of bacteria hosted in the gastrointestinal tract is usually referred to as the "metagenome" of the host, and its study with sequencing techniques is called "metagenomics". It has been estimated that the entire human intestinal microbiota contributes to the body weight for a quota ranging from 175 g to 1.5 kg (5).

The concentration and the type of bacteria living in the intestinal lumen change according to the anatomical segment considered. In general, bacterial density increases from the proximal sections (duodenum, small intestine) to the distal ones (caecum, colon, sigma). The most represented bacteria are the obligate or optional anaerobes, especially at the colon level. The faecal microbiota is generally considered a reliable estimate of the microbiota present at the level of the lumen of the digestive tract. However, analy-

ses of stool samples are of course inaccurate to detect segment-specific alterations of the gut microbiome. Some techniques have been recently developed for the microbiota determination on intestinal biopsy samples (*mucosa- associated microbiota*) (6). These techniques have the great advantage of examining the microbiota present in a specific segment of the intestine and, therefore, of checking its possible interactions with the mucosa, but obviously they require invasive procedures (gastroscopy, operative colonoscopy) for sample collection. Thus, most of the studies on human intestinal microbiota have been conducted on faecal samples.

Most of the intestinal bacteria cannot be cultivated, even when using the most innovative and sensitive laboratory methods. It is estimated that around 60-80% of the bacterial species physiologically present in the gut microbiota share this characteristic. Thus, the complexity and diversity of the species contained in the human intestinal microbiota have been understood in only very recent years, thanks to the advent of laboratory methods of detection and identification of bacteria that are independent from culture media (*culture-independent*) and non-species-specific (7).

The "classical" microbiological techniques, currently applied until now in all clinical microbiological laboratories of the world, have in fact the great limitation of being partially or totally species-specific. So, in a biological sample with high microbial concentration such as a stool sample, they can only identify a single species or a limited range of species, for which a clinical question is posed, or a group of bacterial *taxa* that share certain biochemical and metabolic characteristics (7)

The culture-independent microbiological techniques, developed during the last decade, are based on the *high-throughput* sequencing of the bacterial DNA, and so fall within the definition of metagenomics. They allow to virtually identify all the bacterial species present in a complex ecosystem, basing on the genetic polymorphisms of some genes common to all prokaryotes and the subsequent comparison with genomic databases for taxonomic identification (8-9).

Namely, the most used technique in current microbiological research is based on the identification of the polymorphisms of the bacterial gene encoding the 16S rRNA (*16S rRNA microbial profiling).* Each 16S rRNA gene sequence detected in a fecal sample is thus assigned to a specific operational taxonomic unit (OTU) basing on the degree of homology with other detected sequences. Then, each detected 16S rRNA gene sequence, corresponding to an OTU, is assigned to a given taxon (i.e. genus and species) or, in case of mismatch, a higher taxonomic level (phylum, class, order, family, genus) by means of bioinformatics analyses, by comparison with known sequences from taxonomic databases (8-9).

These techniques assure considerable advantages in the study of the human microbiota (9):

1. They allow the simultaneous identification of a large number of taxa that permit to understand the complexity and the diversity of the human intestinal microbiota better than any other currently known technique;
2. They allow to identify also bacterial species usually not cultivable or hardly cultivable, thus overcoming many limits of the classical microbiological techniques;
3. They allow the detection of previously unknown bacterial *taxa* in the human microbiota and assign them taxonomically to an order or family with a high degree of precision;
4. They allow us to estimate the relative abundance of the individual *taxa*, providing very important quantitative information to understand the structure of the microbiota.

Therefore, the metagenomics study of the human fecal microbiota have made it possible to clarify a long series of aspects, previously unknown, on its physiology, its interactions with the mucosa of the GI tract and with other organs, its alterations during acute or chronic diseases, its possible role in the pathogenesis of a long series of diseases, not only gastrointestinal (10-11). Furthermore, the scientific community has begun to develop "new generation" techniques for the manipulation of the human intestinal microbiota (12-13), with the aim of verifying the effects on the development and the progress of some diseases, reaching in some cases, as in *Clostridium difficile* enterocolitis, extremely significant results both from the biological and clinical point of view (14).

The study of the human intestinal microbiota represents therefore a "frontier" of translational biomedical

research and a topic of great relevance in medicine. At the date of 14th November 2018, there were 12897 scientific articles on "human gut microbiota" listed in the international database PubMed (https://www.ncbi.nlm.nih.gov/pubmed), including 5072 reviews, as a proof of the extreme relevance of the topic. Of these papers, only 266 (2.06%) were published before the year 2008 while 3199 (24.8%) went back to the year 2017 only.

Despite the huge amount of literature, there are still many areas of uncertainty and the understanding of the role of intestinal microbiota in human physiopathology is still far from being sufficient to significantly affect clinical practice, with probably the only exception of *Clostridium difficile* enterocolitis (13). In particular, there are still several uncertainties regarding the definition of the "normal" microbiota and the consequent clinical interpretation of a certain intestinal microbial profile. Moreover, for many chronic diseases, from IBD to kidney stones, the involvement of the microbiota in the pathophysiology and in the etiopathogenesis is not yet completely clear, although it is fully hypothesized in the light of existing evidence (15).

The physiology of the intestinal microbiota in adult

The first in-depth knowledge on the characteristics and composition of the normal human intestinal microbiota was acquired thanks to the Human Microbiome Project, a population study in which the composition of the faecal microbiota of 242 healthy adults between the ages of 18 and 40 was determined (14). This study has made it possible to clarify that in the human intestinal microbiota 10 bacterial *phyla* are generally represented, even though the large majority of the identified bacteria belong to two of them: *Bacteroidetes* and *Firmicutes*. There is generally an inverse relationship between the relative abundance of the bacteria belonging to one or the other *phylum*, so the subjects that have a microbiota rich in *Firmicutes* have a reduced representation of *Bacteroidetes* and vice versa (14). This project also allowed to clarify other very important concepts in the physiology of adult microbiota (14):

- even if, in the complexity of the microbial population, there is a high inter-individual variability, the most represented *taxa* in faecal samples of the healthy population are a relatively small number and constitute the so-called "*core microbiota*";
- there is a very high number of *taxa*, whose presence is inconstant across different individuals, that could play important metabolic and pathophysiological roles despite the low quantitative representation in absolute terms ("*minor players*");
- the microbiota composition of each individual remains stable over time during the adult life.

Analyzing the composition of the faecal microbiota of 39 healthy adults, Arumugam and colleagues later confirmed the presence of a high inter-individual variability, identifying however the presence of some "enterotypes", that is, groups of individuals characterized by the presence of a very similar *core microbiota*. In particular, enterotype 1 is rich in *Bacteroides spp*. and *Parabacteroides spp.*, enterotype 2 is characterized by a high relative abundance of *Prevotella spp*. and *Desulfovibrio spp*., and enterotype 3 is rich in bacteria with mucin degrading capacity, such as *Ruminococcus spp*. and *Akkermansia spp*. (16). The factors that affect the presence of one or the other enterotype remain largely unknown, as dietary patterns do not seem to have a predominant role except for enterotype 2, where the abundance of *Prevotella spp*. can be positively related to fiber consumption and negatively to the consumption of animal proteins (16).

However, the diet and the place of residence remain two important factors in determining the composition of the intestinal microbiota (17-18). Yatsunenko and colleagues (17) showed significant differences in the composition of the *core microbiota* between two groups of subjects, one resident in Malawi and the other in the United States of America, hypothesizing the presence of environmental factors (diet, food preservation methods, exposure to animals, domestic hygiene) as the reason of these differences.

A diet rich in animal protein can increase the relative abundance of bacteria tolerating the exposure to high concentrations of bile acids (*Bacteroides, Alistipes, Bilophila*), and decrease the relative abundance of bacteria of the *phylum Firmicutes* metabolizing vegetal polysaccharides (*Roseburia, Eubacterium, Ruminococcus*) (18). A mainly vegetarian or vegan diet is instead associated with a greater abundance of the genus *Prevotella*, which is significantly correlated with the dietary fiber

intake (18). Furthermore, a diet with a high content of animal proteins is generally associated with a reduced complexity of the intestinal microbiota, and therefore with reduced microbial diversity (19). Finally, Wu and colleagues have shown that enterotypes, or at least the presence of a *Bacteroides* enterotype compared to a *Prevotella* enterotype, are significantly related to long-term eating habits and not to nutrient intake in the days or weeks preceding the analysis of the microbiota (20). These results partly discard the initial conclusion of Arumugam and colleagues that diet only marginally influences the *core microbiota* and enterotypes (16).

Other studies have analyzed the relationship between diet and human intestinal microbiota focusing only on specific nutrients, without reaching conclusive evidence (21). For example, a diet rich in non-digestible waxes is associated with an increase in the relative abundance of bacteria capable of degrading such compounds, such as *Eubacterium rectale* and *Oscillobacter spp*. (22). A high dietary intake of inulin, a fiber present in some vegetables, and fruit-oligosaccharides is associated with the increase of *Bifidobacterium* bacteria (23-24), while a diet rich in polyunsaturated fatty acids is associated with an increase in relative abundance of *Eubacterium rectale* and *Clostridium coccoides* (25). These changes are generally of little importance in absolute quantitative terms, mainly regarding "*minor players*" in the microbiota. However, they could assume great importance from a metabolic and functional point of view (21).

In fact, an intestinal microbiota characterized by a high diversity ("species richness") is generally considered a marker of good health and it is associated with a lower body adiposity, a greater tendency to maintain body weight over time and a better metabolic profile with reduced insulin resistance (26). Compared to the normal-weight subjects, a reduced microbial diversity is often found in overweight individuals, probably due to different eating habits and lifestyle (26).

Among other factors related to lifestyle, physical exercise also seems to be a determinant of the composition of the intestinal microbiota in healthy adults. Clarke and colleagues have shown that agonistic sport practice is associated with a greater intestinal microbial diversity compared to a sedentary lifestyle, independently of dietary caloric intake and body mass index (19). Furthermore, the peak of oxygen consumption under stress, i.e., cardiorespiratory fitness index, is correlated with the microbial diversity of the intestinal microbiota according to a study performed in a group of Canadian young adults (27). Similar results have also been obtained in studies conducted on animal models (28-30), allowing to hypothesize that at least part of the health benefits of physical exercise are mediated by exercise-related improvement of microbial diversity in the GI tract (31).

Even the events of the infantile age, and in particular the type of childbirth, breastfeeding and the age of weaning, may have significant repercussions on the composition of the adult microbiota. In fact, at the moment of birth, the intestinal microbiota is substantially absent or characterized by extreme simplicity with low bacterial load. The intestine is therefore contaminated with the microbial flora present in the birth canal (for those born by eutocic delivery) or with that present on the maternal skin (for those born by cesarean delivery). Therefore, in the newborn the intestinal microbiota is dominated by *Lactobacillus spp*. if the birth was eutocic or from *Staphylococcus spp*. if the birth was cesarean (32). With breastfeeding, part of the microbiota present on the skin of the mother's breast and part of milk microbiota are transmitted to the infant, contributing to increase the intestinal microbial complexity (33). After weaning, there is a noticeable increase in microbial diversity, with progressive reduction of *taxa* such as *Lactobacillus* and *Staphylococcus* and increase of those *taxa* representing the adult *core microbiota*, such as *Bacteroides* and *Prevotella* (34). At the age of 3 years, the microbiota then reaches a composition that, from a quantitative and qualitative point of view, is very similar to that of an adult (17).

However, special events that occur in childhood, such as diseases and/or exposure to drugs including antibiotics, can induce significant changes in the intestinal microbiota under development, which are maintained over time even in adolescence and adult age (35). Likewise, prematurity can also lead to alterations in the development of the microbiota that are maintained in later adult life (35).

The type of living environment in childhood also plays an important role in shaping the intestinal microbiota. The presence of siblings (36) and domestic

animals (37) is in fact capable of influencing the microbial populations present in the children's microbiota. It has also been shown that people living in the same domestic environment, regardless of age, and even their animals, share some common characteristics in their microbiota (38).

It has also been postulated that genetic factors of the host may influence the type of intestinal microbiota during the development phases. The study by Yatsunenko and colleagues (17) seems to disprove this hypothesis, since it found significant differences in the composition of the intestinal microbiota of mono- and dizygotic twins of different geographic origin. Bonder and colleagues (39) have instead recently demonstrated through genome-wide analysis, conducted on 1514 healthy adults, that some host *loci* are related to the relative abundance of some intestinal *taxa* such as *Bifidobacterium*. These *loci* are related to the function of the immune system and to some receptors or adhesion molecules expressed by the intestinal epithelium.

Two fundamental characteristics of the intestinal microbiota of healthy adults are stability over time and resilience. It is in fact known that, if no perturbative factors are involved, the composition of the microbiota can be estimated as constant from adolescent age up to the age of 60-65 years (40). Indeed, very complex balances are established in the relative abundance of the individual components of the microbiota, which depend on the availability of the metabolic substrates, dietary habits, function of the intestinal mucosa and the activity of the local immune system (11). In these balances, some *taxa* grow to form the *core microbiota*, while others remain confined to some ecological niches, for which they present a relative lower abundance (minor players). These balances are to a certain extent predictable through complex mathematical models that refer to the law of the equilibrium of Nash (41).

When a perturbative event, such as an acute illness, infection, antibiotic therapy, or a sudden change in dietary habits, occurs, the equilibrium changes due to the new factor (41-42). For example, in a study conducted on 10 healthy volunteers, the rapid change of diet (from high protein to vegetarian and vice-versa) caused significant changes in the relative abundance of some *taxa*, which however rapidly disappeared with resoration of steady state when the restrictive diet was suddenly suspended (18). This phenomenon, whereby the global composition of the microbiota tends to return spontaneously to the pre-existing equilibrium, is called *resilience* and is a fundamental characteristic of the intestinal microbiota of healthy adults (41-43).

Because of this resilience, age is generally not a factor influencing the composition of the intestinal microbiota in the range between 10/15 and 60/65 years old (40). In the elderly, however, some physiological changes occur that may have biological and clinical relevance.

Much of the current knowledge on the intestinal microbiota of the elderly comes from the study by Claesson and colleagues, published in Nature in 2012 (44), which analyzed the intestinal microbiota of a group of 178 Irish older subjects, either institutionalized or community-dwelling, followed-up for one year. Briefly, these authors have shown that, over 65 years of age, inter-individual variability increases, while microbial diversity, i.e., the number of species detectable with metagenomics techniques, is reduced. These changes, which may in part derive from changes in dietary habits, are more pronounced in those who exhibit a lower degree of functional autonomy, in those who live in nursing homes (44,45) and in patients with polypharmacy (46). The most interesting finding of these studies is the circumstance that the reduction of microbial diversity and therefore the alterations in the overall composition of the microbiota do not depend so much on the "chronological" age, but rather on the "biological" age or on the functional performance (47). Thus, frailty, the age-related reduction of homeostasis and functional reserve preceding disability, may be significantly associated with the microbiota composition (48).

Further studies have shown that, in healthy older individuals, the *core microbiota* tends to be maintained both qualitatively and quantitatively. Conversely, in frail or institutionalized elderly subjects, a quantitative reduction of the *core microbiota* can be detected, with a simultaneous increase of taxa such as *Anerotruncus*, *Desulfovibrio* and *Coprobacillus*, that can be considered as biomarkers of reduced health status (46).

These changes, which occur slowly over time, are accompanied by a labile equilibrium. Thus, the intestinal microbiota becomes more sensitive to possible per-

turbators and, ultimately, shows a lower resilience (50). In a study performed on a group of 728 elderly women, the Frailty Index, a global measure of fitness, was positively correlated with the relative abundance of species such as *Eggerthella lenta* and *Eubacterium dolicum* and inversely related to *Faecalibacterium prausnitzii* in the intestinal microbiota (50). Moreover, specific alterations in the intestinal microbiota of older individuals seem to be associated with reduced cognitive performances, and even be involved in the pathophysiology of Alzheimer's disease (51).

Aging then results in a reduced relative abundance of a series of bacteria, including bifidobacteria (52-53), whose metabolic activities have been defined as health-promoting (52-53). These alterations can be reflected in a reduced cross-talk between the microbiota and the intestinal mucosa, with greater activation of the local and systemic inflammatory response and less functionality of the cells of the innate immune system, with negative effects not only on the GI tract but also on the whole body (54).

Some studies on the intestinal microbiota of centenarians and supercentenarians have shown that extreme longevity, albeit accompanied by a reduction in intestinal microbial diversity, is associated with the expansion of the representation of bacterial *taxa* with health-promoting activity, such as *Eggerthella, Anaerotruncus, Bilophila* and *Akkermansia*, and *taxa* with still unclear

Table 1. Overview of physiological and pathological factors influencing the composition of the intestinal microbiota in adult subjects

Involved factors	Comment
Physiological factors	
Dietary habits	- Influence on the enterotype – Influence on microbial diversity – Influence on the relative abundance of some taxa by particular metabolic substrates (eg waxes, fibers) or sensitive to different concentrations of bile acids
Geographic origin	- Influence mediated by dietary habits, methods of food storage, exposure to animals, domestic hygiene
Physical activity	- Increase in microbial diversity and in the concentration of health-promoting bacteria
Type of childbirth, breastfeeding/lactation, age of weaning	- They can influence the overall composition of the microbiota in childhood, leaving a fingerprint even in adulthood
Presence of cohabitants and pets	- Over the time the microbiota of people and pets that live in close contact tends to resemble each other in the global composition
Genetic factors	- The presence of some taxa depends on the types of receptors expressed by epithelial cells of the mucosa
Living environment (home vs. institution)	- Reduction of microbial complexity with high inter-individual variability in institutionalized subjects
Age	- The microbiota is stable in adulthood up to 65-70 years – Then there is an increase in inter-individual variability with a reduced number of species and a tendency to dysbiosis
Pathological factors	
Direct exposure (therapy) or indirect (environmental contamination) to antibiotics	- It causes dysbiosis with profound changes in the composition of the microbiota that are not necessarily associated with a decrease in the number of bacteria – Dysbiosis depends on the type of antibiotic taken, the dose and duration of therapy
Chronic pharmacological therapies	- The main evidence is for antiblastic chemotherapy. On overall, polypharmacy is related to dysbiosis
Immunological alterations	- Immunosuppression promotes the growth of pathogenic strains

activity, such as *Oscillospira*, *Odoribacter* and *Butyricimonas*, at the expense of other bacteria with beneficial metabolic activities such as *Faecalibacterium prausnitzii* (55-57). These results allow at least to hypothesize an active role of the intestinal microbiota in the phenomena of aging and in the promotion of longevity, also through the modulation of inflammation (55).

A summary of the main factors involved in modulating the composition of the intestinal microbiota in adult is shown in Table 1.

Conclusions

The study of the intestinal microbiota with metagenomics techniques offers a new point of view for the understanding of human physiology and physiopathology. Growing evidence suggests a significant role of the microbiota in the maintenance of the homeostasis of the body and in helping to determine the state of health or illness. Biomedical research in the near future will have to focus on clarify microbiota-host relationships and on planning microbiota manipulation to prevent and possibly modify the natural history of many diseases.

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