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THE HUMAN INTESTINAL MICROBIOTA; FROM PHYLOGENETICS TO FUNCTIONAL METAGENOMICS

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SUMMARY

The human intestinal microbiota constitutes a complex ecosystem now well recognized for its impact on human health. It does contribute to prevention of colonization by pathogens and maturation of the immune system. Its possible implication in diseases of modern societies, currently increasing in prevalence, has been reported. These include allergies, inflammatory bowel diseases and possible obesity and cancer.

The analysis of the molecular composition of the human intestinal microbiota indicates marked inter-individual variations, which may seem paradoxical considering the high degree of conservation of major functions of the intestinal microbiota such as anaerobic digestion of alimentary fibres. We have characterized a phylogenetic core within the human intestinal microbiota, in terms of composition; i.e. a set of conserved species that could be responsible for major conserved functionalities. Based on culture independent molecular assessments, the current knowledge allows to define criteria qualifying the normal state of the human intestinal microbiota that we call "eubiosis". This further allows to identify specific distortion from eubiosis, i.e. dysbiosis in immune, metabolic or degenerative diseases. Noticeably, Crohn's disease, an inflammatory bowel disease of yet unknown aetiology, is associated with an intestinal dysbiosis with a lower representation of the *Clostridium leptum* group among the Firmicutes phylum. We further showed that the bacterial species *Faecalibacterium prausnitzii* is exerting anti-inflammatory properties *in vitro* and in animal models that could explain its ability, *in vivo* in patients, when it is detectable in the mucosa associated microbiota, to protect patients from post-operative recurrence of endoscopic signs of inflammation 6 months after surgical resection of the ileo-caecal region of the gut.

In confirming the major role of the microbiota in bowel related disorders, especially associated with a disruption of homeostasis, we are currently applying high throughput functional metagenomic screens in order to identify signal molecules and mechanisms of bacteria-host crosstalk. Together with the high resolution description of the human intestinal metagenome as well as explorations at the level of metaproteome and metabolome, these observations will further our understanding of the functional roles bacteria play in maintenance of health and well being in humans. It will open new perspectives for the monitoring and the design of strategies to modulate the microbiota for health.

INTRODUCTION

Microorganisms that colonize man from the very moment of birth on and establish with their host a mutualistic relationship that lasts for the whole life do play major roles that are well recognized today. Yet microorganisms that surround us are still most often perceived as dangerous potential invaders against which we should be ready to fight. This oddly negative perception contrasts with their acknowledged utility for environment, agriculture, industry and mankind.

The human organisms and before man, that of animals have long coevolved over millions of years with microorganisms, leading to the establishment of a mutually beneficial adaptation. Although fine mechanisms or temporal dynamics are not yet known, the host develops a tolerance towards non-pathogenic bacteria that colonize mucosal surfaces and skin and in return, the latter control the development and maintenance of numerous essential functions of their host. The intestinal microbiota contributes to bioconversion of food-born compounds unabsorbed in the upper parts of the digestive tract. Colonization by commensal microorganisms is key to immune development (*Duarte* et al., 2004; *Neumann* et al., 1998; *Souza* et al., 2004; *Oliveira* et al., 2005). The microbiota exerts in addition a direct role of colonization prevention keeping pathogens at levels of population preventing them from expressing virulence (*Freter*, 1983; *Wells* et al., 1988; *van der Waaij* et al., 1971). More recently, a potential role of the intestinal microbiota in regulation of fat storage has been described in mice (*Backhed* et al., 2005) and suggested to operate in man (*Ley* et al., 2006; *Duncan* et al., 2007).

Our vision of the human intestinal microbiota has recently been thoroughly revisited using molecular tools with a marker gene approach based on the use of ribosomal RNA as phylogenetic marker. For an overview of the main stages of the development of the complex microbiota associated to man the reader is invited to consult the appropriate chapter in this monograph. We herein will give an updated view of the intestinal microbiota composition and stability. We will thereafter outline its beneficial roles, and illustrate the association of microbiota dysbiosis and disease. We will finally give perspectives of application of metagenomic exploration of the functionalities of the dominant human intestinal microbiota.

COMPOSITION OF THE HUMAN INTESTINAL MICROBIOTA

Microbial population densities in the human digestive tract reach their maximum values in the colon with 10^{11} bacteria per gram content. It is accepted today that this complex bacterial consortium known as the microbiota is made of hundreds of species in each individual. Yet a thorough description of all intestinal bacteria does not exist for two main reasons:

1. Traditional culture-based characterization (*Finegold* et al., 1974; *Holdeman* et al., 1976; *Moore* and *Holdeman*, 1974) does not allow to take into account more that 30% or so of the microorganisms that can be seen and enumerated by microscopic observation.
2. Species diversity of commensal intestinal bacteria at the level of the planet would be immense. In that respect, the use of molecular tools

indicated that the major part of dominant bacterial species observed in the faecal microbiota of an individual (approximately 80%) are specific of this individual (*Suau* et al., 1999; *Mangin* et al., 2004; *Eckburg* et al., 2005).

If species diversity of the dominant intestinal microbiota gives a faecal print essentially specific of the individual, composition at the level of taxa (genera and/or large phylogenetic groups) highlights consistent components, found in all individuals. Some of these taxa have been known for long and are represented by numerous collection strains; others have been evidenced only recently and via molecular approaches and still have no cultured representative. Culturable genera of the dominant faecal microbiota of adults are *Bacteroides*, *Eubacterium*, *Ruminococcus*, *Clostridium* and *Bifidobacterium* (*Moore* et al., 1974; *Finegold* et al., 1983). Accounting for non-culturable microorganisms allowed to refine this vision and to put it in a phylogenetic framework. The phylum Firmicutes is always highly represented. It comprises the *Eubacterium rectale – Clostridium coccoides*, often the most represented (14 to 31% of total bacteria depending on the studies) (*Franks* et al., 1998; *Jansen* et al., 1999; *Sghir* et al., 2000; *Rigottier-Gois* et al., 2003; *Seksik* et al., 2003). It is composed of species belonging to the genera *Eubacterium*, *Clostridium*, *Ruminococcus*, and *Butyrivibrio*. The phylum Firmicutes also comprises the *Clostridium leptum* group with the species *Faecalibacterium prausnitzii*, *Ruminococcus albus* and *R. flavefaciens*; a group that is also very often dominant (16 to 22% on average) (*Sghir* et al., 2000; *Lay* et al., 2005). Bacteroidetes are represented by genera related to *Bacteroides*. They are always present and share the dominance with the above groups (9 to 42% of total bacteria on average). The phylum Actinobacteria is less consistently detected in dominance, but represents a few percent of total bacteria. It comprises bifidobacteria (0.7 to 10%) and bacteria of the *Collinsella-Atopobium* group (0.3 to 3.7% on average) (*Harmsen* et al., 2000; *Rigottier-Gois* et al, 2003). Enterobacteria are more seldom observed in the top two logs of population in the faecal microbiota (0.4 to 1%), similarly to lactobacilli and streptococci (2%) (*Lay* et al., 2005). Also occasionally found are species related to *Clostridium ramosum*, *Eubacterium cylindroids*, *Phascolarctobacterium*, *Verrucomicrobium* or *Sporomusa-Selenomonas-Veillonella*.

Highly conserved composition traits at the level of phylogenetic groups and phyla on the one hand together with subject specificity at the level of species on the other hand suggest that there exists, on functional grounds, some degree of redundancy between species and the different levels of resolution bring complementary pieces of information.

Finally, bacterial species observed are strictly associated to the intestinal ecosystem. This derives from a long co-evolution with the host (*Ley* et al., 2006) that recent studies of cross-association of microbiota from/with different hosts do confirm (*Rawls* et al., 2006).

At present, phylogenetic reassessment of the human intestinal microbiota has been essentially restricted to the dominant fraction and our knowledge of subdominant bacteria (i.e. below 10^8 per gram stool) may be incomplete and remains restricted to culturable isolates. The ability to isolate and grow microorganisms *in vitro* remains a key step in knowledge building, especially con-

sidering that phylogeny does not inform on the *in situ* activity of microbes. On a more focussed standpoint, the assessment of the contribution of archaea or phages, that may be highly significant in terms of populations, has remained anecdotal up to now.

HOMEOSTASIS OF THE INTESTINAL MICROBIOTA

By definition, a microorganism that colonizes a given niche will persist and multiply without requiring re-inoculation. Dynamics and homeostasis of the intestinal microbiota may be considered in time (for any given individual) and space (i.e. between individuals or intestinal compartments). The global composition of the dominant intestinal microbial community appears conserved between individuals and with time. The same major phyla are present with proportions that vary between individuals but most likely remaining within the same log-unit equivalent in terms of population. Dominant species diversity appears remarkably stable with time for a given individual from day-to-day and even across years (*Zoetendal* et al., 98; *VanHoutte* et al., 2004; *Seksik* et al., 2003) while a large fraction of the dominant species appear specific of the subject. At the level of strains stability is more or less evident depending on the subject (*McCartney* et al., 1996; *Kimura* et al., 1997). Hence the stability observed at the level of groups and species would hide an important rate of renewal at the level of strains. Genomic plasticity may in fact come into play in that respect. It has also been shown that species diversity for subdominant groups (ex. Lactobacilus) is far less stable with time than that of dominant ones (VanHoutte et al., 2004) and that stability of communities is greater in the colon than in the ileum. It must also be considered that lactic acid bacteria brought by the ingestion of fermented food may occasionally have a survival rate during transit that leads to their transient passage in dominance in the small intestine and colon.

For a given individual, modifications of the intestinal microbiota may derive either from colonization by exogenous microorganisms or by modulation of population levels of commensal bacteria. In most cases it will essentially be the consequence of relays in dominance, in response to factors modulating ecological niches. Numerous factors may affect stability of microbial communities - among others transit time, pH, quality and quantity of exogenous substrates and endogenous mucins. Although microbial communities appear ready to deal with changes in ecological settings, it seems difficult to induce durable alterations of established dominant populations, at least in terms of composition. Numerous observations hence illustrate the ability of the dominant intestinal microbiota to resist modification. The administration of an allochtonous strain such as a probiotic or an exogenous non-absorbable substrate such as a prebiotic often lead to transient modifications of microbial equilibrium. Even a major stress such as an antibiotic administration can be followed by a return of the community to its initial dominant species profile within a month or so (*de la Cochetiere* et al., 2005; *Dethlefsen* et al., 2008). This ability to recover its original make-up following a stress, known as resilience, suggests a fine-tuned adaptation of the microbiota to the gut and even to the host that harbours it. This can be linked to the observation that

monozygotic twins have faecal microbial communities that have significantly more closely related patterns than these of unrelated individuals, suggesting that genotype may play a role in the development and structuration of the intestinal bacterial populations.

Analysing the spatial distribution of intestinal microbes as a function of digestive sites is difficult to study; it requires collection of samples within and along the intestine, hence via invasive methods. The preservation of topological relations between bacteria and epithelium is also a challenge. This explains some remaining controversy on this topic.

The luminal microbiota (within the intestinal cavity) has been explored in several ways. The proximal colon luminal microbiota differs from the faecal microbiota of which the composition only represents the distal parts of the colon (*Marteau* et al., 2001). Between the proximal and the distal colon, microbial populations increase overall by a factor of 100 and the increase is essentially due to an increase in strictly anaerobic bacteria. The layer of mucus that covers the intestinal wall constitutes a specific ecological niche. Several studies have shown that the microbial community that colonizes this niche is stable with time and remarkably comparable from the ileum to the rectum for a given individual (*Lepage* et al., 2005; *Wang* et al., 2005). Conversely, species that dominate in the mucus layer differ from dominant luminal species found in faeces (*Eckburg* et al., 2005; *Lepage* et al., 2005).

The ability of commensal gut bacteria to adhere *in situ* to intestinal epithelial cells has so far not been documented in an unequivocal manner. Indirect evidence does exist, derived from the presence of genes encoding adhesions in the genome of strains of *E. coli* able to durably colonize their host. Adhesins could nonetheless contribute to the recognition of mucosal sites and structures or sloughed cells. In ecological terms, a given strain must divide at least as quickly as its offspring's are eliminated in order to maintain itself at a stable level of population in the ecosystem (*Lee* et al., 2004). Hence adhesion to the epithelium does not appear as an absolute necessity, but recognition of sites within the mucus or in the contents would provide a selective advantage for slow growing strains (*Freter* et al., 1983). If adhesion to the epithelium does not seem to be a relevant criterium for commensal bacteria, this property has been associated with intestinal bacteria in patients with inflammatory bowel diseases, and of course many intestinal pathogens.

It remains clear that not all mechanisms involved in maintenance of homeostasis of the human intestinal microbiota are understood to date; noticeably determinants of resistance to change and resilience. It hence is still fully relevant to question the right level of phylogenetic depth and time period for which stability of the ecosystem should be defined. As far as resilience is concerned, it is also reasonable to speculate that a certain level of stress will disturb the equilibrium of the gut ecosystem such that it will be irreversibly perturbed. The threshold above which the human intestinal microbiota loses its ability to return to its original balance is still unknown today.

Finally, parameters of homeostasis will apply to functionalities as much as composition; yet the relevance of these parameters on a functional standpoint is totally unexplored. Functional resistance and resilience of the intestinal microbiota have yet to be determined, as well as the link between phylogeny

and functions. There are only speculations at present on a potential link between "quantity of diversity" and functional resistance and resilience.

EUBIOSIS AND DYSBIOSIS OF THE HUMAN INTESTINAL MICROBIOTA

Based on culture independent molecular assessments, the current knowledge allows to define criteria qualifying the normal state of the human intestinal microbiota that we call "eubiosis". Obviously, emphasis has been put for technical reasons on phylogenetic evaluation of composition, diversity, core species and the dynamics of these over time and space and it is obvious that defining eubiosis will benefit from the addition of functional parameters. This context further allows to identify specific distortions from eubiosis, i.e. dysbiosis, which can be specifically investigated in immune, metabolic or degenerative diseases. We have recently validated the concept in the case of Crohn's disease. Crohn is an inflammatory bowel disease of yet unknown aetiology, that has a prevalence of one per 2000 in European countries. We have demonstrated that Crohn's disease is associated with an intestinal dysbiosis with a lower representation of the *Clostridium leptum* group among the Firmicutes phylum (*Seksik* et al., 2003; *Sokol* et al., 2006, 2008, 2009). We further showed that the bacterial species *Faecalibacterium prausnitzii*, when detectable in the mucosa associated microbiota of the ileum of patients, is protective against post-operative recurrence of endoscopic signs of inflammation 6 months after surgical resection of the ileo-caecal region of the intestine (*Sokol* et al., 2008). We finally demonstrated that *Faecalibacterium prausnitzii* could exert anti-inflammatory properties *in vitro* and in animal models with chemically induced inflammation.

The exploration of dysbiosis may be viewed as a primary step providing key information for the design of strategies aiming at restoring or maintaining homeostasis and eubiosis. Although so far restricted to microbiota composition and/or diversity, dysbiosis has been proposed and in a few cases well documented in irritable bowel syndrome (*Kassinen* et al., 2007), ulcerative colitis (*Sokol* et al., 2008; *Martinez* et al., 2008), obesity (*Ley* et al., 2007; *Kalliomäki* et al., 2008), Type-1 diabetes (*Dessein* et al., 2009; *Wen* et al., 2008), type-2 diabetes (*Cani* and *Delzenne*, 2009), celiac disease (*Nadal* et al., 2007; *Collado* et al. 2009), allergy (*Kirjavainen* et al., 2002; *Björkstén*, 2009), and in cases of infections with *Clostridium difficile* (*Hickson* et al., 2007) or HIV (*Gori* et al., 2008). These observations, just as that of dysbiosis in Crohn's disease above, are not indicative of a causal relationship between microbiota imbalance and onset of the disease. Indeed it is quite reasonable to argue that, once such diseases are declared, owing to the disruption they cause in the immune system and in physico-chemical properties of the intestinal milieu, dysbiosis could in fact be a consequence rather than a cause. In the case of *Faecalibacterium prausnitzii* in Crohn's disease, we nevertheless have a situation in which a deprivation in populations of a normal commensal bacterium, belonging to the most dominant core species of the healthy gut microbiota and potentially anti-inflammatory *in vivo*, will be associated with a reduced ability of the ecosystem to promote a return to immune

homeostasis. It can even be anticipated that a vicious circle is into place combining the detrimental effects of higher bacterial densities close to the mucosa (*Swidsinski* et al., 2005), increased populations of Gram-negative, pro-inflammatory, endotoxin producing bacteria usually subdominant in healthy subjects (*Baumgart* et al., 2007; *Darfeuille-Michaud* et al., 2004), reduced proportions of anti-inflammatory commensals (*Sokol* et al., 2008) and even increased occurrence of protein biomarkers, potentially promoting auto-immune reactivity (*Juste* and *Doré*, personal communication).

The current strengthening of the concept of eubiosis/dysbiosis confirms the major role of the microbiota in bowel related disorders, especially associated with a disruption of homeostasis. It stresses the need to apply the emerging tools of microbiomics to provide diagnostic models and also to identify signal molecules and describe bacteria-host crosstalk mechanisms at play.

HUMAN INTESTINAL MICROBIOMICS

Beyond the phylogenetic level extensively explored so far lie the combined genomes, transcriptomes, proteomes and even metabolomes of the members of the intestinal microbial community. These are known today as the metagenome, metatranscriptome, metaproteome, .. and all-together the human intestinal microbiome. Since the vast majority of dominant intestinal bacteria are not yet cultured to date, the genomic content of this microbial community and its derived components at the various levels of omic integration are essentially unknown. Metagenomics is emerging today as this most powerful approach to characterize the repertoire of genes of any complex microbial setting, independent of the culturability of its components. The development of very high throughput sequencing technologies is further contributing to this development. In practical terms, the microbial community may be extracted from its environment and its DNA purified and/or cloned in order to determine its sequence. The complete gene repertoire of culturable and non-culturable dominant microbes can hence be obtained. This further offers the possibility to design high throughput profiling tools informative at the level of functional potentials of the complete community. In addition, cloning of genome fragments of intestinal bacteria in large insert metagenomic libraries allows to use functional screens in order to seek functions of non-culturable bacteria after heterologous expression in *E. coli*, giving access to yet totally unexplored biological resources. Beyond its innovative character, the potentials of metagenomics have already largely been documented (*Riesenfeld* et al., 2004; The new science of metagenomics: revealing the secrets of our microbial planet, National Research Council of the National Academies, The National Academies Press, Washington DC, USA, 2007).

The first developments of the metagenomics approach applied to the human intestinal microbiota have focussed on diversity of the microbial community (*Manichanh* et al., 2006, 2008) and the gene repertoire for a few subjects (*Gill* et al., 2006; *Kurokawa* et al., 2007). Functional metagenomics applications have remained confined to the soil ecosystem (*Williamson* et al., 2005) and animal guts (*Beloqui* et al.,

2006). We initiated its application to the human intestinal context (*Gloux* et al., 2007). Although still in its infancy, this approach brings major promises for an improved understanding of microbe-food, microbe-host and microbe-microbe interactions.

The microbiomic exploration of the human intestinal microbiota is hence ongoing thanks to several programs such as the European Commission-funded program MetaHIT (http://locus.jouy.inra.fr/metahit/), and the French Agency for Research-funded program GMGE Micro-Obes (http://www.inra.fr/micro_obes), as well as the NIH Roadmap programs (http://nihroadmap.nih.gov/hmp/) and many others worldwide. At the international level these programs are structured within the International Human Microbiome Consortium (IHMC) co-chaired by the NIH and the European Commission. These programs will deliver a huge mass of information that will in turn allow to identify conserved and variable genomic and functional traits of the ecosystem, to describe those specific to the gut environment and bearing the best diagnostic and/or prognostic potential, to reconstruct the metabolic food-chain of the microbial community, and to start describe ecotypes and model their relationships in a systems' ecology endeavour.

CONCLUSION

Application of molecular ecology tools to the intestinal microbiota has allowed very significant improvements in our understanding of this ecosystem in terms of composition and dynamics of species diversity. The single gene approach based on ribosomal RNA as a universal phylogenetic marker had nevertheless left aside the functions microorganisms exert in their environment.

It has become possible to sequence combined genomes of complex microbial communities giving access to their potential activities. The following steps towards environmental transcriptomics, expressed proteins, activities and metabolites are being taken. The global functional exploration of the human intestinal microbiota is hence underway, with perspectives as large and fascinating as these of the former decade.

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