Towards an integrated understanding of gut microbiota using insects as model systems

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ABSTRACT

Metazoans form symbioses with microorganisms that synthesize essential nutritional compounds and increase their efficiency to digest and absorb nutrients. Despite the growing awareness that microbes play key roles on metabolism, health and development of metazoans, symbiotic relationships within the gut are far from fully understood. Perhaps the most important obstacle to understanding these symbiotic relationships resides in the high diversity of bacterial communities living within the gut of most vertebrates. In this regard, insects, which generally harbor a lower microbial diversity within their gut, offer an interesting alternative to vertebrates and have recently emerged as potential model systems to study these interactions. In this review, we give a brief overview of the characteristics of the gut microbiota in insects in terms of low diversity but high variability at intra- and interspecific levels and we investigate some of the ecological and methodological factors that might explain such variability. We then emphasize how studies integrating a vast array of techniques and disciplines have the potential to provide a groundbreaking understanding of the biology of this micro eco-system.

INTRODUCTION

Extracting essential nutritious components from food can be challenging. Metazoans have partly faced this challenge by forming symbioses with microorganisms that both synthesize essential nutritional compounds and increase the efficiency of nutrient digestion and absorption (Fraune and Bosch, 2010; Moran, 2007). In insects, nutritional symbioses can be split into two main categories: (i) intracellular associations, that are generally found in arthropods with restricted diets such as blood and plant sap and involve only few types of symbionts, and (ii) extracellular associations, that are more common among metazoan and involve a complex community of symbionts that generally live within the gut lumen. Symbionts can serve a wide range of nutritional functions, from mobilizing stored nitrogen to contributing essential amino acids (Brune and Ohkuma, 2011; Douglas, 2009; Feldhaar, 2011; Kaufman and Klug, 1991), and hosts often rely on symbiotic microorganisms to supply nutrients required for viability and fertility (Dillon and Dillon, 2003; Douglas, 2010; Moran and Baumann, 2000).

In recent decades, numerous investigations have been devoted to understanding the metabolic roles of associated microorganisms (Douglas, 2009; Moran, 2007) with a particular emphasizes on the bacteria that compose the gut microbiota (Dillon and Dillon, 2003; Nicholson et al., 2012; Ryu et al., 2008; Storelli et al., 2011). In this context, it has been shown, for instance, that the gut microbiota can contribute up to 70% of a vertebrate's energy needs (Flint et al., 2008). However, despite the growing awareness that microbes play key roles on metabolism, health and development of metazoans (Fraune and Bosch, 2010; Lee and Brey, 2013; Maslowski and Mackay, 2011), symbiotic relationships within the gut are far from fully understood (Engel and Moran, 2013).

Perhaps the most important obstacle to understanding these symbiotic relationships resides in the high diversity of bacterial communities living within the gut of most vertebrates. In this regard, insects, which generally harbour a lower microbial diversity within their gut, offer an interesting alternative to vertebrates and have recently emerged as potential model systems to study these interactions. Insects are not only tractable and easy to manipulate, they also offer substantial genetic resources allowing investigations of conversed metabolic and immune pathways. However, capturing the properties of insect gut microbiota has been challenging so far due to a high variability in composition between individuals and closely related species. Here, we give a brief overview of the characteristics of the gut microbiota in insects in terms of low diversity but high variability at intra- and interspecific levels and we investigate some of the ecological and methodological factors that might explain such variability. We then emphasize how studies integrating the latest technological advances from molecular biology and stable isotopes based-technics can improve our understanding of host/symbiont interactions.

1. Gut microbiota in insects

1.1. A low diversity

In contrast to mammals, the bacterial diversity in insect digestive tracts is generally low and rarely exceeds a few tens of species (Colman et al., 2012). In *Drosophila*, the gut only contains 2 to 20 bacterial species in natural and/or field conditions (Apidianakis and Rahme, 2011; Bae et al., 2010; Blum et al., 2013; Chandler et al., 2011; Corby-Harris et al., 2007; Cox and Gilmore, 2007; Ren et al., 2007; Ryu et al., 2008; Storelli et al., 2011; Wong et al., 2011), while in humans the gut microbiota diversity generally exceed 1000 bacterial species (Ley et al., 2008b). Several immunological, physiological and morphological hypotheses have been proposed (see (Broderick and Lemaitre, 2012; Engel and Moran, 2013). The lack of adaptive immune function in invertebrates might partly explain this low diversity. Indeed, the innate immune system may only be capable of managing the simple communities of resident bacteria typically present in the invertebrate gut, while the adaptive immune system of higher metazoans might have facilitated association with a greater number of different microbes (McFall-Ngai, 2007). Invertebrates have developed physical barriers to separate the microbes from the host and this might explain why they rely primarily on innate immunity. This can be done either by the formation of a specific organ that will host the bacteria or by the existence of a specific tissue that effectively separates the microbes from the host tissues. For instance, the peritrophic membrane in insect digestive tract does not allow the passage of microorganisms and contains them in the gut lumen. This could also limit the diversity of microbes that would settle in the gut.

Profiling of the commensal community members in *Drosophila* has nevertheless revealed different bacterial constituents with high taxonomic diversity at the species level. *Lactobacillus* and *Acetobacter* are the most abundant and common genera in the gut of of *D. melanogaster*. Most of the commensal bacteria of the fruit fly are cultivable *in vitro*, which facilitates experimental manipulations of gut microbial communities and microbial genetic analysis. Also *Drosophila* is one of the major insect model systems in the study of innate immunity (Hultmark, 2003; Lemaitre and Hoffmann, 2007), aging (Bjedov et al., 2010), metabolism (Bharucha, 2009; Birse et al., 2010), intestinal stem cells homeostasis (Apidianakis and Rahme, 2011; Buchon et al., 2010; Casali and Batlle, 2009), large-scale dietary studies (e.g. Lee et al., 2008) and offers substantial molecular genetic resources. There is then strong expectations that laboratory experiments on *Drosophila* will define future research in biomedical systems (mammals and humans), which currently lacks a framework to better understand the relationships between nutrition, immunity and gut microbial ecology at different stages of life and in distinct environments. (see Bae et al., 2010; Broderick and Lemaitre, 2012; Charroux and Royet, 2012; Erkosar et al., 2013; Kau et al., 2011; Lee and Brey, 2013; Ponton et al., 2011, 2013; Ryu et al., 2010).

1.2. A versatile gut microbiota

One of the first steps to understanding the symbiotic relationship between gut microbes and their host is to characterize the baseline healthy microbiota and the differences that are associated with metabolic perturbations and disease. Once the healthy composition and functional states of gut microbiota are understood, the features that, when disrupted, are

111 associated with disease can be determined. Recent studies have however shown that defining
112 this “healthy” composition is challenging. Indeed, while it has been shown that in some insects
113 that have a restricted diet, some gut bacterial strains are specifically associated and can be
114 maternally inherited (see for instance), in lots of other insect species the composition of the
115 gut microbiota between individuals of a same species or closely related species varies not only
116 in total size but also in composition (Colman et al., 2012; Lozupone et al., 2012; Staubach et al.,
117 2013). Wong et al. (2013) explored the gut microbiota composition of drosophilid flies by first
118 investigating the prevalence of five strains of bacteria usually found in the gut of individual flies
119 for 21 strains in 10 *Drosophila* species; and, in a second analysis, they investigated the gut
120 microbiota of 11 species of *Drosophila*. Their results have shown that the five bacterial strains
121 were not systematically found in all individuals, without any evidence of a core gut microbiota
122 for the different species of *Drosophila*. In a recent review paper, Broderick and Lemaitre (2012)
123 summarized the results of different studies that also investigated the composition of gut
124 microbiota in *Drosophila* for laboratory stocks and wild-caught flies (see also Erkosar et al.,
125 2013). In laboratory reared flies, only two strains of bacteria seem to be consistently associated
126 with *Drosophila*: *Lactobacillus plantarum* and *Acetobacter pomorum*/*pasteurianus*. In wild-
127 caught flies, even if the diversity of bacteria present in the digestive tract was greater, the two
128 same bacterial genera, *Acetobacter*/*Gluconobacter* and *Lactobacillus,* were the only symbionts
129 to remain consistently present. In mosquitoes, Osei-Poku et al. (2012) catalogued the inter-
130 individual bacterial diversity in the guts of eight species collected from the coastal region of
131 Kenya. Extensive variation in gut microbiota has also been found between individuals of the
132 same species. Better understanding this variation in the composition of the gut microbiota is

133 important because (i) it will give new insights into the factors that modulate the gut microbiota
134 composition; (ii) it will allow to identify relevant bacterial diversity information and to target
135 species or functions that are key; and (iii) it will stimulate the development of functional
136 analyses that do not account for taxonomic diversity.

137 **1.3. Diet, a key driver of gut microbiota composition**

138 Several biological and ecological factors such as age, genetics and environment have been
139 proposed to explain gut microbiota composition. However, diet seems to be one of the main
140 factors driving variation in the composition of the gut microbiota in vertebrates and
141 invertebrates (Lozupone et al., 2012). In insects, effects of diet have been particularly
142 investigated and diet composition has been shown to influence the bacterial community in the
143 midgut of different species such as larval gypsy moths (Broderick et al., 2004) and cotton
144 bollworms (Xiang et al., 2006). Also, investigations in flies have shown that diet shapes the
145 microbiota composition regardless of taxonomy and geography (Chandler et al., 2011). Indeed,
146 whereas taxonomically- and geographically-distant fly populations collected from various food
147 sources have had very different microbiota compositions, when maintained on the same type
148 of food, they showed similar communities of bacteria in their gut (Chandler et al., 2011).
149 Similarly, Staubach et al (2013) have found no evidence for host species effects in lab-reared *D.*
150 *melanogaster* and *D. simulans*; instead the lab of origin has had pronounced effects reflecting
151 the importance of the culture conditions. The *Drosophila*–associated microbiota appears thus
152 to be predominantly shaped by food substrate with an additional but smaller effect of host
153 species identity. Also, one major difference between the human and insect microbiota is that all

154 insect bacteria seem to be aerobic and therefore capable of living on (and "digesting") the food
155 outside the fly. This may contribute to explaining why diet is such a key driver of microbiota
156 composition (erkosar??).

157 Food composition is then a major determinant of gut bacterial community (see also Ley
158 et al., 2008a; Ravussin et al., 2012; Wu et al., 2011). This might be explained by the fact that
159 diet composition promotes specific bacterial strains by providing them with appropriate
160 nutritional conditions. It has also been suggested that diet may influence the physical and
161 chemical milieu of the gut (Clissold et al., 2010; Flint et al., 2008; Ley et al., 2008b; Sorensen et
162 al., 2010) and will constrain the type of bacterial strains that can survive in the gut ecosystem.
163 In mosquitoes, for instance, blood meals have been associated with massive proliferation of
164 bacteria residing in the digestive tract (Kumar et al., 2010; Oliveira et al., 2011; Wang et al.,
165 2011). Blood meals decrease the levels of reactive oxygen species (ROS) in the digestive tract,
166 creating a favorable environment for bacterial growth (Oliveira et al., 2011). Although intestinal
167 microbes are more abundant after a blood meal, the diversity of the gut microbiota seems to
168 be affected with enteric bacteria being favored due to their capacity to cope with oxidative and
169 nitrosative stresses (Wang et al., 2011). When not ingesting blood, mosquitoes can feed on
170 nectars and these meals might also influence the composition of the bacterial communities
171 found in the gut (Lefèvre et al., 2013).

172 Extracellular symbionts in insects are not usually inherited from the mother or
173 transmitted from host-to-host, but they mainly come opportunistically from the environment
174 (see for instance Storelli et al, 2011 and Blum et al, 2013; but see also Kikuchi et al, 2009) . Food

can be itself a vector of commensals, and different diets will provide microbial inoculates of different community compositions (Broderick and Lemaitre, 2012; Gendrin and Christophides, 2013). In a recent study, Blum et al. (2013) investigated the role of food in supplying bacteria to flies and allowing insect to maintain a gut microbiota. They have shown that flies establish and maintain their gut microbiota by frequently consuming food containing bacteria. Hence, food can be considered as a bacterial reservoir for flies and the establishment of *Drosophila* microbiome is only possible if flies consume exogenous bacteria. Food sources such as living animals can also be vectors of bacteria for blood feeding insects that become infected when feeding on the host (Gendrin and Christophides, 2013). Unravelling the relationships between nutrition, food composition and, the composition and function of symbiont populations is fundamental to predicting the outcome of parasitic infections. This is particularly significant when the application of microbial symbionts is considered to reduce vector competence and to control the spread of arthropod-transmitted pathogens (Weiss and Aksoy, 2011).

2. Towards an integrated understanding of gut microbiota: from genes to function

Molecular techniques that have been employed to analyse gut microbiota might also be sources of variability, particularly for taxonomic identification. The integration of studies combining different methods might allow a better understanding of individual and communal roles of bacteria in the physiology and ecology of the host

2.1. History and limitation of 16S rRNA as a universal marker

For decades, the study of microbial diversity has been hampered by the resistance of the vast majority of bacteria to cultivation under artificial conditions. In the late 1970s, the work of Carl Woese brought a new perspective on microbial diversity by providing the first bacterial phylogeny based on ribosomal RNA sequence (16S rRNA) (Woese and Fox, 1977). The 16S rRNA is a molecular marker that is universally present in bacteria and has highly conserved domains flanking hypervariable sequences, which can easily be used for gene amplification using PCR and further sequencing and analysis, enabling phylogenetic identification of bacteria without any cultivating steps (Dave et al., 2012). The seminal work of Woese and Fox led to a breakthrough in the classification of the "uncultivated majority of microbes", which are estimated to account for over 80% of human gut microbes (Eckburg et al., 2005). The emergence of next-generation sequencing technologies, which became commercially available in 2005, has further increased the speed of phylogenetic coverage and decreased the cost through massively parallel sequencing methods. As a result, over 3.8 million 16S rRNA sequences are now available in databases such as Silva (QUAST et al. 2013) (http://www.arb-silva.de/). Despite these enormous advances, the microbial community of the gut remains unclear, hiding behind its diversity and unexpected variability between individuals (Lozupone et al., 2012).

There have been an increasing number of studies targeting gut microbial communities in insects based on the sequencing and analysis of 16S rRNA (see Broderick and Lemaitre, 2012; Colman et al., 2012 for review). However, determining and comparing the taxonomic composition of the bacterial communities based on sequencing and analysis of highly conserved genes should be considered with caution particularly when the identifications

were done in separate studies. Not only are protocols usually different in terms of tissues and methods used for extraction, but also taxonomic analyses can be different and use different taxonomic levels to distinguish the bacterial groups. Modern sequencing technologies define microbial taxonomic groups by 16S rRNA sequence similarity (OTUs, or operational taxonomic units), the species-level OTU being generally defined by a percentage of similarity greater than 97% between two sequences (Gevers et al., 2005). The intriguing question arises as to whether the level of diversity and variability found in the gut microbiota communities of different individuals may reflect the level of taxonomic classification (i.e. percentage of similarity) chosen (also discussed in Wong et al., 2013). It is very likely that at higher-order taxonomic levels (e.g., phylum), the gut microbiota communities between individuals begin to resemble one another more closely. It is still unclear whether studies should consider strain-, species-, genus-, or higher order-level to assess the diversity of gut microbiota and search for differences among individuals or conditions. Future work evaluating critically the appropriateness of taxonomic level used for assessing the diversity of microbial communities in the gut of insects, and more generally in metazoans, is therefore required. It is important to note that the differences in gut microbiota communities can also be measured as changes in the proportional representation of OTUs or Phyla. For example, the relative abundance of the bacterial phyla Firmicutes, Actinobacteria, and Bacteroidetes in the gut has been shown to be consistently associated with obesity in both humans and mice (Turnbaugh et al., 2006; Turnbaugh et al., 2009). Also, De Filippo et al. (2010) demonstrated a significant increase in the abundance of Bacteroidetes relative to Firmicutes in the gut microbial communities of children characterized by a rural diet compared to a modern western diet. Despite the considerable progresses realized in this field,

240 the general consensus is that microbial studies based on 16S rRNA enable little by way of
241 functional conclusions and give little information on the metabolic capabilities of the different
242 bacterial groups.

243 **2.2. Molecular approaches to investigate potential functions of bacteria**

244 Recently, some studies based on 16S rRNA gene have suggested that it may be possible to
245 predict the functional potential of microbes from phylogeny (Langille et al., 2013). PICRUSt, an
246 automated method based on evolutionary modeling uses phylogenetic information contained
247 in 16S marker gene sequences in relation to existing reference genomes to predict the function
248 of microbial communities (Langille et al., 2013). Although PICRUSt does not infer function for
249 viruses or eukaryotes and its accuracy is affected by phylogenetic dissimilarity among
250 environmental organisms and sequenced genomes, it can predict and compare probable
251 functions for bacteria across many samples from a wide range of habitats at a limited financial
252 cost. This approach has the potential to provide the first functional glimpses into the vast
253 amount of existing samples for which only 16S data are available and can be seen as an
254 important step for future cost-effective studies including two steps: (i) integration of completed
255 genome sequences and 16S rRNA gene studies to approximate functional information and then
256 (ii) design of more costly metagenomics or functional studies to assess precisely the metabolic
257 role of gut microbiota communities.

258 Metagenomics allows direct sequencing of genomes contained within a community
259 providing access to the functional gene composition of microbes and therefore to a much
260 broader description than phylogenetic surveys based on the diversity of 16S rRNA. Despite the

fact that deep, and therefore expensive, metagenomic sequencing is generally required to access rare organisms and genes, more than 100 metagenomics sequencing projects have been completed so far (Liolios et al., 2010). Some of these projects have been responsible for substantial advances in the study of microbiomes over the past 10 years, enabling the characterization of microbial assemblages at a functional level. Suen et al. (2010) did the first functional characterization of the fungus garden microbiome of leaf-cutter ants using metagenomics and whole-genome sequencing. They have shown that the microbial community within the fungus gardens of leaf-cutter ants contains not only the fungal cultivar, but also a diverse assembly of bacteria. Using metagenomics analysis of a carbohydrate-active enzyme, they further showed that these bacteria are likely to participate in the symbiotic degradation of plant biomass in the fungus garden while previous studies suggested that the fungal cultivar was solely responsible for this process. Metagenomics therefore provides important opportunities for the emerging field of eco-systems biology (i.e. considering molecular systems biology at the ecosystem level) which unites molecular microbiology and ecology to develop an understanding of community function (Raes and Bork, 2008). Also, looking at the functional capabilities of communities reveals conservation of metabolism even when species composition varies. One intriguing result of this emerging field is that completely different microbial communities found in different individuals or habitats appear to converge on the same functional gene repertoire (Dinsdale et al., 2008). For instance, using the same set of samples from lean and obese twins, different species assemblages appear to lead to very similar functional profiles, demonstrating the presence of a shared core of metabolic capabilities in the microbiome (Turnbaugh et al., 2009). Discovering the relationships between these consistent

283 functional signatures and the sum of all metabolic processes (i.e. the nutrients and energy
284 cycling) occurring within the gut will be an especially important step to better understand the
285 biology and functioning of this micro eco-system (Figure 1). Further down the track,
286 transcriptomic approaches, which allow investigating change in gene expression in both the
287 insect host and the microbial symbionts, are powerful to understand the interplay between the
288 different actors within this micro-ecosystem. Although the vast progress of transcriptomics
289 approaches are well described elsewhere (REF) and beyond the scope of this review, it is
290 important to note that combined metagenomic and metatranscriptomic datasets further allow
291 examining the link between gene expression level and sequence conservation, revealing broad
292 evolutionary patterns across taxonomically and functionally diverse communities (Stewart et al,
293 2011 http://genomebiology.com/content/12/3/R26).

294

295 **2.3. Stable isotopes based technics to quantify metabolic activity of bacteria in situ**

296 By allowing the researchers to simultaneously investigate genes, their functions and the
297 bacteria that exert them, molecular approaches such as metagenomics have greatly improved
298 our understanding of microbial communities and their metabolic potential. However, genetic
299 information is not always well correlated to the metabolic activity of specific bacteria *in situ*. In
300 this context, the development of single cell approaches using radioactive or stable isotope-
301 based techniques such as microautoradiography, Raman microspectroscopy and Secondary Ion
302 Mass Spectrometry (SIMS) has been a revolutionary step in modern microbial ecology,
303 revealing individual microorganisms that are metabolically active in their natural habitat and

within complex communities (for review on these techniques and their application in microbiology (see Musat et al., 2012; Orphan and House, 2009; Wagner, 2009). Among these single-cell approaches, the latest version of high spatial resolution SIMS instrument (NanoSIMS) can provide direct imaging and precise quantification of up to 7 different isotopes at a micrometer or submicrometer scale (up to ~50 nm) (Hoppe et al., 2013). Recently, Carpenter and coworkers (Carpenter et al., 2013) applied, for the first time, this technique to insect gut microbiota studies, combining stable isotope labeling using 13C-cellulose with NanoSIMS analysis to investigate nutrients flow within the gut microbiota of the desert dampwood termite *Paraneotermes simplicicornis*. Their results suggested an unexpected tripartite nutritional interaction, the protist *Oxymonas dimorpha* degrading wood fragments ingested by *P. simplicicornis* and subsequently transferring carbon derived products to bacterial symbionts. Given its spatial resolution and detection limit (parts per million), NanoSIMS allows the quantification of the relative metabolic contribution of different partners (i.e. metabolic rates of individual host and symbiont cells) within an intact symbiosis (Pernice et al., 2012). This technique can also be used in concert with *in situ* hybridizations to simultaneously identify individual microbial cells and quantify their substrate uptake (Behrens et al., 2008; Musat et al., 2008). Using this combination, Berry et al (2013) elegantly demonstrated that within the complex communities of the mouse gut microbiota, two bacterial species, *Bacteroides acidifaciens* and *Akkermansia muciniphila*, are important host protein foragers. In this context, the use of stable isotope approaches in aposymbiotic animals that can be infected selectively has a great potential to experimentally test hypotheses about nutritional function of a specific bacterial species (Salem et al, 2012). Despite the fact that the application of NanoSIMS to study

insect gut microbiota is still in its infancy, there is no doubt that future studies integrating this powerful analytical technique can dramatically improve our understanding of the nutritional interactions that lie at the very heart of insect gut microbiota. Together, these investigations are revolutionizing our understanding of the gut microbiome.

CONCLUSION

There is a growing interest in using model systems to provide a comprehensive and integrated understanding of the functioning of the gut microbiota and its interactions with the host metabolism and immunity. Insects are relevant systems in many ways (Apidianakis and Rahme, 2011; Charroux and Royet, 2012; Erkosar et al., 2013) and numerous studies already provide an abundance of data on the taxonomic diversity of gut microbiota, but also the ecological factors that might influence this diversity and the *in situ* roles of bacteria residing in the digestive tract. However, readability of data and harmonization of the data collection and analyses could be the largest obstacle to providing reliable and repeatable results that might be translated to mammalian models, thereby achieving the full potential of this field. There is therefore a need for a consortium where researcher working on gut microbiota share technical details (see also Broderick and Lemaitre, 2012) and produce some standard guidelines that can be followed by the researcher community when publishing gut microbiota data. These guidelines will allow an adequate, more transparent and comprehensive reporting of experimental details. This has already been successfully applied for studies based on quantitative real-time PCR for instance (see Bustin et al., 2009). Also, the successful development of research on gut microbiota will result only with the integration of a vast array of techniques and disciplines allowing a deeper

understanding of the functioning of this micro eco-system. Technological advances in combination with ecological and evolutionary approaches will soon provide groundbreaking understanding of the function of microbiomes and comprehensive knowledge of the effects of gut microbiomes on the host biology, physiology and immunity; and might provide new efficient treatments against infection and metabolic diseases.

LEGEND TO FIGURES

Figure 1: Gut eco-system biology: from genes to function.

An overview of the different methods that can be used for understanding the function of the gut eco-system by integrating different levels (Diversity, Gene function and Metabolic activity). Recently developed methods (listed below a figure of representative output) can be used to assess changes in the different levels of response to different factors (such as change in microbial diversity and host diet). Arrows between the different levels indicate interdependencies (for example, metabolic activity depends on gene function and ultimately on microbial diversity). The representative output for isotope-based technics and 16S rRNA analysis are modified from (Pernice et al., 2007; Pernice et al., 2012).

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575 **Figure 1**: Gut eco-system biology: from genes to function

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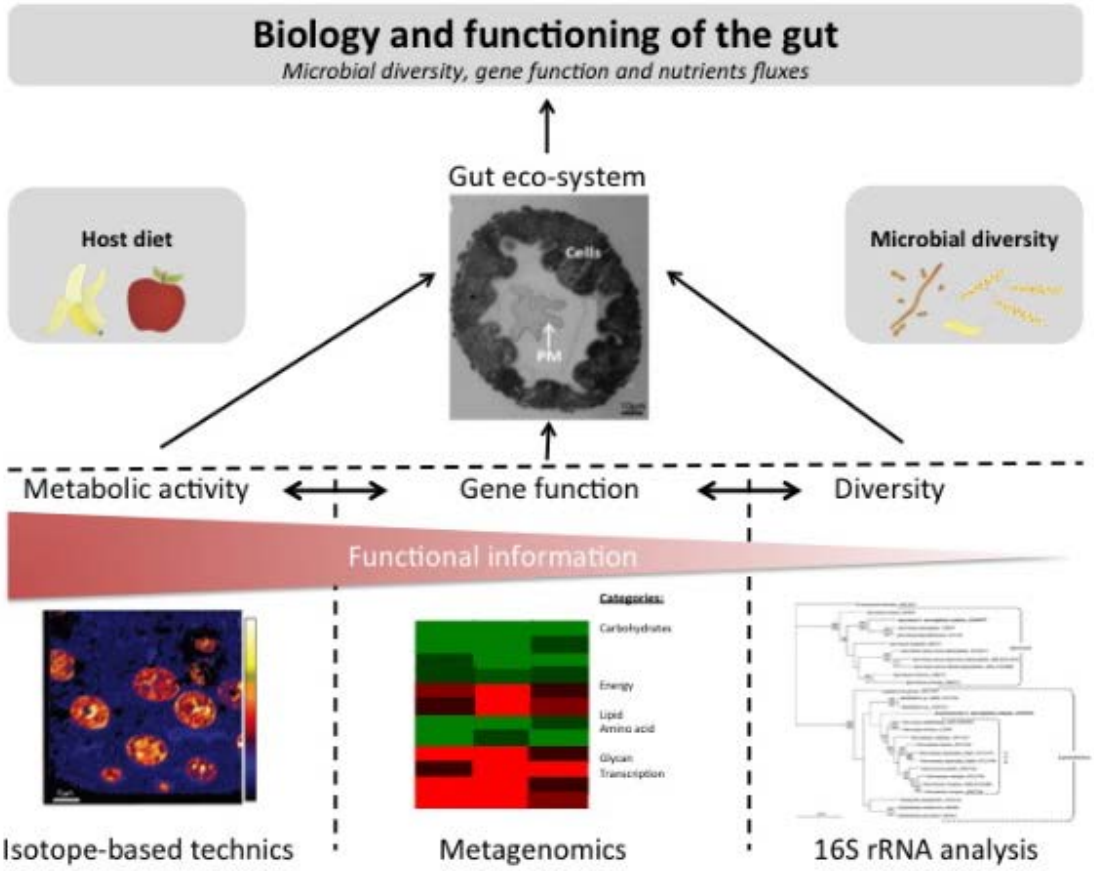

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