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The holobiont concept: The case of xylophagous termites and cockroaches[§]

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Abstract The rapid growth in microbiome research, particularly during the last 15 years, has revealed the crucial contributions of microbial communities to numerous physiological functions in animals, including digestion, immunity and reproduction. The permanent coexistence of these various bionts forms the holobiont (namely, the host and its microbiota). This review describes the relationships between xylophagous insects and their microbiota in an attempt to understand the characteristics that have determined bacterial fidelity over generations and throughout evolutionary history. Symbiotic interactions have probably played a central role in the evolutionary success of these insects, allowing their adaptation to unexploited ecological niches that are nutritionally deficient and/or unbalanced. Moreover, insect symbionts have provided the enzymatic capabilities that enable the synthesis of nutrients (carbon and nitrogen sources for the host) from recalcitrant plant polymers (cellulose, hemicellulose, pectin or lignin).

Keywords Holobiont · Endosymbionts · Gut microbiota · Termites · Xylophagus insects · *Cryptocercus* · Wood diet

[§]This paper is dedicated to Lynn Margulis (1938-2011), unforgettable teacher of one of us, unforgettable partner of the other.

1 Introduction

Evolution connects life through time. Prokaryotic microorganisms were the earliest cellular inhabitants of Earth, first appearing at least ~3850 million years ago (Guerrero et al. 2002; Zimmer 2009; Guerrero and Berlanga 2015). In fact, for 85 % of the history of life on Earth, prokaryotes were its only inhabitants. Symbiotic associations among prokaryotes gave rise to the ancestors of all the complex and varied biological forms that followed and that now exist on Earth. All of the currently known metabolic strategies developed in prokaryotic bacteria and archaea (Nealson and Conrad 1999; Guerrero and Berlanga 2006; Payne and Loomis, 2006). Yet many advances in evolution are due to changes that resulted in mechanisms and biochemical pathways very different from those extant until that time. Indeed, it was a metabolic change that led to the liberation of oxygen (as some cyanobacteria started to split water and not sulfide as a proton source) and thus to the evolution of aerobic life. Another change, in this case a strategic one, was endosymbiosis, which gave birth to the eukaryotic cell (with a differentiated nucleus, organelles, internal cytoplasmic membranes, etc.), as prokaryotic cells incorporated other thriving prokaryotes into their cytoplasm (by endosymbiosis), rather than digesting these invaders. Together, these changes allowed life to adopt very different forms and dimensions, from protists and microscopic plants and fungi, to redwoods, dinosaurs, whales, and humans. Today, it is common knowledge that the majority of microorganisms play essential roles in maintaining life on Earth. We, and our fellow “macrobes”, are ultimately reliant on the manifold activities of the “invisible” microbial world. The miniscule size of its members belies their tremendous importance (Guerrero and Berlanga 2009; McFall-Ngai et al. 2013).

The term “symbiosis” was coined by Heinrich Anton de Bary (1831–1888) to describe the living together of “differently named organisms” (De Bary 1879). Symbiosis is the long-term physical association of two or more partners, and both the establishment and maintenance of symbiotic relationships can occur only under certain environmental or metabolic conditions. In the case of endosymbiosis, the necessary condition is topological, as one partner lives inside the other. Symbiogenesis refers to the appearance of new morphologies, tissues, metabolic pathways, behaviors or other recognizable evolutionary novelties in holobionts. Joshua Lederberg (1925–2008) defined the holobiont as “the ecological community of commensal, symbiotic, and

pathogenic microorganisms that literally share our body space” (Lederberg and McGray 2001). As such, the holobiome (i.e., the assembly of genetic information contributed by the animal or plant and its associated microbiota) is an essential aspect of the evolving holobiont. The complex relationship between host–microbe interactions and behavior requires a new expanded ecological perspective, involving the host, the microbiota and the environment that in combination constitute the holobiont. The concept of holobiont is a life-changing force that has resulted in the complex, coordinated evolution of life forms. Microbes are part of the animal/plant systems and they must be included in the animal/plant life histories. Fundamental physiological processes including tissue development (Hooper 2004), nutrient absorption (Flint et al. 2012), immunity (Artis 2008), and circadian regulation (Leone et al. 2015) are emergent properties of the interactions between the host and its microbiota. Those emergent properties involve multiple microbial species and genotypes, reflecting the microbe composition at the level of community rather than individual microbial taxa. Ecological interactions among members of the microbial communities may have differing net impacts on host fitness according to the actual environmental circumstances (Mejía-León and Calderón de la Barca 2015; Wong et al. 2015). Of all aspects of the environment, nutrition is the most important in shaping the responses of the holobiont system. In the case of the gut microbiota, the nutritional resources are dependent on the host feeding behavior and on the host secretions. The composition and physical form of the food changes as it passes down the gastrointestinal tract, offering microbes at different locations a changing complement of nutrients. Finally, the host obtains multiple nutrients in appropriate quantities and balance to perform optimally (Simpson et al. 2015). Insect gut symbionts play an essential role in the insect adaptation to various food types, especially those herbivorous or wood-feeding insects, such as termites (Fig. 1). Herbivory is a successful feeding mode, but only after key hurdles are overcome, such as low nutrient content, indigestibility or toxicity of many plant tissues. The herbivorous microbiota has been shown to be important for lignocellulosic biomass degradation, nutrient production (amino acids, vitamins, etc.), or compound detoxification (Despres et al. 2007; Brune 2014). In termites, the disruption of host–symbiont interactions through the use of antibiotics can compromise and/or eradicate the gut microbiota (protists and/or bacteria) and significantly alters the termite's reproduction and colony establishment (Rosengaus et al. 2011).

>>Approximately here, Figure 1>>

Given their ecological ubiquity, it is not surprising to find many prokaryotic species in close relationships, including persistent associations, with members of many eukaryotic taxa (Guerrero and Berlanga 2013; Zilber-Rosenberg and Rosenberg 2008). According to the fitness effects of the symbiotic association on the partners, the relationship can be described as parasitism (involving two species, in which one benefits at the expense of the other), commensalism (in which the non-host species benefits from the interaction, while the host is neither positively nor negatively affected) or mutualism (a relationship that is beneficial to both species). Eukaryotic hosts can acquire their symbionts by maternal inheritance (transovarial, or acquisition in utero) or by environmental transmission (via the surrounding habitat, in which a new infection is established in each generation).

In animals, embryogenesis often occurs in the absence of direct contact with bacteria, but further development involves the formation of tissues that are destined to interact with coevolved microbial species. These interactions also ensure the formation of healthy stable microbial communities (McFall-Ngai 2002). Normal indigenous microbiota colonize those regions of the animal body that are exposed to the external environment, such as the skin, eyes, oral cavity and the respiratory, urinary, reproductive and gastrointestinal tracts. Despite the abundance of microbes in an animal's environment, only certain populations are able to permanently inhabit the available body sites, even though the composition of the specific microbial communities associated with a host may vary as a function of time and place. A principal challenge in the symbiotic relationship is the mutual elaboration of the features that will ensure the persistence of the complex interactions between the host and its specific bacterial partners. These features must be maintained with fidelity over the life history of a given organism, along generations of species and over evolutionary time.

No field of biology has been more affected by the recent progress in biotechnology, and no field had the potential to contribute more broadly to it than microbiology (Borre et al. 2014; Gilbert 2014; Moreno-Indias et al. 2014; McFall-Ngai 2015). Next-generation sequencing has led to an explosion in the descriptions of the microbial world and therefore to the identification and characterization of the uncultured microbial cells in complex communities, including those established in most animals and plants. Of all the animal–microbe interactions studied over the past 20 years, the human microbiota continues to receive the most attention. The unprecedented diversity and stability of the microbiota of the human body has been repeatedly

demonstrated, perhaps most notably by The Human Microbiome Project (McFall-Ngai 2015).

This review examines the characteristics that have ensured bacterial fidelity to certain groups of animals over generations and throughout evolution. In a case-study approach, it considers the relationships between two groups of xylophagous insects (termites and cockroaches) and their gut microbiota. Research on the wood-feeding *Dyctioptera* holobiont will contribute to the understanding of microbial metabolic integration and interdependency within hosts, and to the elucidating of mechanisms driving adaptations that lead to cooperation and coresidence of the microbial community.

2 Special bacterial symbiosis in insects

Invertebrates participate in an enormous diversity of symbiotic relationships. Indeed, symbiosis probably played a central role in the evolutionary success of these organisms, allowing their adaptation to ecological niches that are nutritionally deprived and/or unbalanced (e.g., wood, plant sap or blood).

Bacterial endosymbioses (in which the bacterial symbiont is located inside the host cell) are widespread among insects. The intensity of the relationship is such that the bacteria are trapped inside specialized host cells, the bacteriocytes, which generally aggregate and form an organ (bacteriome), and are vertically transmitted from the mother to her offspring over successive generations (Baumann 2005; Moya et al. 2008). Based on the degree of dependence between the partners, symbionts can be classified as primary (P-symbiont) or secondary (S-symbionts). P-symbionts are intracellular and have a codependent relationship with the host, one that is based on a long, shared evolutionary history. They are vertically transmitted to the host progeny. S-symbionts seem to have established more recent associations with their hosts (Moya et al. 2008; Moran et al. 2008).

The mechanisms used by endosymbionts (P- and S-symbionts) to establish and maintain their infection of host tissues indicate that invasion strategies are based on the same molecular mechanisms used by pathogenic bacteria (Dale et al. 2002; Wernegreen 2005; Moya et al. 2008). These include various secretion systems for the attachment and invasion of host cells and a quorum-sensing mechanisms for the regulation of virulence

or mutualistic traits, depending on the type of association (Sachs et al. 2011). Clearly, the host immune system must adapt so as to maintain rather than eliminate endosymbionts (McFall-Ngai 2007).

Endosymbionts have been described for a number of bacteria-insect systems, such as *Buchnera* and aphids (Shigenobu and Wilson 2011), the bacterium SOPE (*Sitophilus oryzae* principal endosymbiont) and the rice weevil (Heddi et al. 1999) (Fig. 2), *Wigglesworthia* and tsetse flies (Chen et al. 1999), *Blochmannia* and ants (Degnan et al., 2004), *Baumannia* and the sharpshooter leafhopper (Wu et al. 2006), *Carsonella* and psyllids (Nakabachi et al. 2010), *Blattabacterium* and cockroaches (González-Domenech et al. 2012), etc. Many of these arthropod-associated mutualists form distinct but related lineages in the Gammaproteobacteria. Generally, intracellular bacteria have smaller genomes than their free-living relatives, mostly due to a reduction in gene content (McCutcheon and Moran 2012; Bennett et al. 2014). Gene losses affect loci performing functions that are unnecessary in an intracellular environment or that can be provided by the host. Thus, highly reduced genomes (i.e., those from endosymbionts that have maintained a long relationship with their hosts) have typically lost most genes involved in DNA recombination and repair, have almost no gene duplications, lack transposable elements and probacteriophages and are characterized by high levels of structural stability (López-Madrugal et al. 2015). Examples of the drastically reduced genomes of obligate intracellular bacteria include the 450 to 653 kb of *Buchnera* (Shigenobu et al 2000; Lamelas et al 2011; Jiang et al. 2013), the 697 kb of *Wigglesworthia* (Akman et al. 2002), the 792 kb of *Blochmannia* (Degnan et al. 2005) and the 686 kb of *Baumannia* (Wu et al. 2006).

>> Approximately here, Figure 2>>

Although many microorganism-insect associations are based on nutrient and metabolite exchange, such as P-symbionts *Buchnera*, *Blattabacterium*, etc., there is evidence of the involvement of symbionts in other functions, especially those related with S-symbionts such as protection from fungal pathogens [aphids with X-type enterobacteria (Heyworth and Ferrari 2015)] and from heat damage [aphids carrying “*Candidatus* *Hamiltonella defensa*” or “*Candidatus* *Regionella insecticola*” better tolerate high temperatures (Russell and Moran 2006)]. Other examples of host protection from predators, pathogens and parasites include the bacterial symbiont of *Paederus* beetles, which produces a polyketide toxin that protects the insect larvae against predators such as the wolf spider (Kellner and Dettner 1996). Nonetheless, not all

symbiotic relationships are beneficial to the host, as is the case for the arthropod S-symbiont *Wolbachia*. This bacterium is transmitted maternally with high efficiency, but it is also transferred horizontally, sometimes between distantly related hosts. It is present in many insect species and probably played an important role in their evolution. The genus *Wolbachia* (class Alphaproteobacteria) encompasses obligate intracellular bacteria that are transovarially transmitted in arthropods and filarial nematodes (Lo and Evans 2007; Li et al 2014). Based on the 16S rRNA gene phylogenies of *Wolbachia*, it has been grouped into eight major clades (A–H): Clades A and B include most of the parasitic *Wolbachia* found in arthropods; clades C and D contain the majority of the mutualistic *Wolbachia* present in filarial nematodes; and clades E–H are found in various arthropods. Clade F contains *Wolbachia* strains associated with termites, bedbugs, grasshoppers, etc. The clade E, G, and H are associated with springtails, spiders, and termites, respectively (Moriyama et al. 2015). *Wolbachia* clades F and H, while phylogenetically diverse, both infect Isoptera (termites). Members of clade F infect termites species that are phylogenetically “recent,” and those of clade H species considered to be phylogenetically “old” (Lo and Evans 2007). *Wolbachia* are associated with four distinct reproductive phenotypes that occur in a wide range of Arthropoda: feminization, parthenogenesis, male killing, and cytoplasmic incompatibility. Recently, it has been observed in bedbugs that *Wolbachia* clade F seems to contribute to the host fitness by synthesizing vitamins, such as biotin (Nikoh et al. 2014; Moriyama et al. 2015). Termites form colonies composed of different types of individuals (castes) that are phenotypically specialized to perform specific colony tasks: reproductives, soldiers, and workers. The caste composition of each colony can be altered depending on the circumstances. Although, is unknown about the possible phenotypes linked to *Wolbachia* in termites, soldiers, especially immature ones, have a significantly higher infection rate than workers and reproductives (Werren et al. 2008; Berlanga et al. 2011; Roy et al. 2015).

Cockroaches (including *Cryptocercus*) and the lower termite *Mastotermes darwiniensis* (which forms the basal branch in termite phylogenies) harbor endosymbiotic bacteria, such as *Blattabacterium* spp., within specialized cells—the bacteriocytes of the fat body—that are transferred vertically via the insects’ eggs (Lo et al. 2003). However, in other termites the bacterium has been completely eliminated. *Blattabacterium* belongs to the class Flavobacteria in the phylum Bacteroidetes (López-Sánchez et al. 2008). The genome of *Blattabacterium* strains isolated from the termite

Mastotermes or from the cockroach *Cryptocercus* lacks most of the genes encoding the enzymes of metabolic pathways required for the synthesis of essential amino acids; this is in contrast to the genomes of *Blattabacterium* harbored by other cockroaches (Sabree et al. 2012; Sabree and Moran 2014). Thus, additional members of the complex gut microbiota of the insects must compensate for this deficit and provide their hosts with the missing essential amino acids (Sabree and Moran 2014).

3 Gregarious and social Dictyoptera

Termites, cockroaches and mantids form a well-established lineage of insects, the Dictyoptera. In fact, termites are actually social cockroaches, with the family *Cryptocercidae*, a wood-feeding subsocial cockroach, as their closest relative and the Mantodea (mantids) as the sister group to the clade comprising cockroaches and termites. The order Blattodea is now made up of termites and all cockroach taxa (Beccaloni and Eggleton 2013). The most recent common ancestor of cockroaches and termites dates back to the Permian (~275 Mya), which contradicts the hypothesis of a Devonian (~375 Mya) origin of cockroaches. Stem-termites can be traced to the Triassic/Jurassic boundary, which refutes a Triassic origin (Legendre et al. 2015).

There are fundamental differences in the diets of termites and cockroaches. While termites feed almost exclusively on lignocellulose in various stages of decay, many cockroaches subsist on a highly variable diet. Examples of cockroaches that perform xylophagy are *Cryptocercus* spp. (family *Cryptocercidae*) from East Asia and North America, species belonging to the subfamily *Panesthiinae* (family *Blaberidae*) in Australia and Asia, and the cockroach *Parasphaeria boleiriana* (family *Blaberidae*) from Brazil.

There are few solitary cockroaches. The social structure of *Cryptocercus* is the equivalent of a newly founded termite colony. Adults excavate the nest, construct barriers, clean galleries and bury the inedible dead. After the eggs have hatched, adults feed the first few instars on hindgut fluids (proctodeal trophallaxis) (Nalepa 2015). The neonatal digestive tract is free of microbes, and establishment of the full complement of microbial symbionts is a sequential process that varies in length between species. Typically, it is not complete until the third instar, which is capable of nutritional independence but maintains close contact with adults (Nalepa 1990). Bacteria are established first, followed by the smaller flagellates and then the large flagellate genera

that not only phagocytose host-ingested wood particles but are themselves colonized by nitrogen-fixing bacteria, which cover their surfaces (Nalepa 2015). Once established, the gut symbionts are never lost under natural conditions, including during subsequent molting cycles. *Cryptocercus* retain their protists even through ecdysis, because the encystment cycles of the flagellates are finely regulated to the shifting hormonal titers of the host during this process (Nalepa 2015).

Termites display a distinctive form of eusociality that evolved independently from the forms found in Hymenoptera. Six families of termites (collectively grouped as “lower” termites) share with *Cryptocercus* sp. the unusual ability to degrade lignocellulosic plant material, which is carried out by the metabolic activities of the bacteria and protists of their gut microbiota (Ohkuma 2008; Berlanga et al. 2009; Guerrero et al. 2013). By contrast, “higher” termites, represented by a single highly diversified family, *Termitidae*, have lost their gut protists and harbor only bacteria. Among the lower termites, *Rhinotermitidae* is likely the sister group of the *Termitidae*. Lower and higher termites also differ in the developmental trajectories of their respective castes. Workers in lower termites are temporally arrested juveniles that retain developmental plasticity, whereas in higher termites most workers are terminally developed adults (Scharf 2015).

Termites are unique among social insects because they undergo incomplete metamorphosis and display a diversified caste polyphenism. Worker, soldier, reproductive and undifferentiated immature forms cooperate in an integrated manner in termite society. Developmental pathways are complex and vary between different termite families. In *Rhinotermitidae*, the larvae develop into nymphs or workers. Nymphs can then develop either into (i) alates, with wings and eyes (imagoes, first form) or (ii) brachypterous neotenic reproductives (second form), with rudimentary wings and eyes. Alates disperse and become primary colony founders, whereas brachypterous neotenic reproductives do not disperse but supplement or replace the reproductives within the colony. Workers can (i) become apterous neotenic reproductives (third form), with neither wings nor eyes, (ii) remain workers or (iii) become pre-soldiers that eventually molt into soldiers (Vargo and Husseneder 2009). Each caste plays a significant role within the colony: reproductive adults maintain the population, soldiers protect the colony from invaders and workers (the most numerous life stage in a colony) build and maintain the galleries, take care of the larvae and feed the other colony members.

Termites are strongly distinguished from other major eusocial insects by their food: their cellulose-based diet requires extensive, sequential processing that involves not only physical and chemical manipulations by the host but also the participation of multiple interacting symbionts (Nalepa 2015). Workers transfer food stomodeally (by regurgitation) and/or proctodeally (by excretion with the hindgut contents). Both oral trophallaxis (feeding) and coprophagy allow the direct or indirect transmission of microorganisms and thus promote the coevolution of specialized host-dependent symbionts (Dillon and Dillon 2004; Hongoh et al. 2005; Berlanga et al. 2007). Proctodeal trophallaxis is also the means by which microorganisms are transmitted vertically from workers to other individuals of the colony (Fig. 3). In many insects, the proctodeal part of the intestine, i.e., the hindgut, is shed during ecdysis. Consequently, in newly molted termite workers and soldiers, re-establishment of the gut microbiota depends on the contributions of fellow workers (Berlanga et al. 2011; Nalepa 2015). The termite colony is thus a physiological unit, and for an individual separated from the colony death is inevitable. Both oral trophallaxis (feeding) and coprophagy behaviour strategies of the host can promote a secure transmission of commensal microbiota between members of a colony (termites) or gregarious cockroaches and so, the maintenance of viability of the holobiont.

>> Approximately here, Figure 3>>

4 The role of hindgut microbes

Although plants represent the largest source of biomass in terrestrial systems, their tissues are largely composed of recalcitrant compounds, such as cellulose, hemicellulose, pectin and lignin, that for most animals are difficult or impossible to digest (Watanabe and Tokuda 2010). Furthermore, their levels of nitrogen relative to carbon are too low to meet the nutritional needs of animals. Major evolutionary transitions in animals (i.e., insects) that allowed them to use plant material as food required the aid of microbial symbionts (Moran 2007). Microbes offer new capabilities for synthesizing nutrients, digesting recalcitrant plant polymers and neutralizing plant toxins (Hansen and Moran 2014).

The basic structure of the digestive tract is similar across insects, although a broad range of modifications associated with adaptation to different feeding modes can be found. The insect gut has three primary regions, the foregut, midgut and hindgut

(Engel and Moran 2013); the foregut and hindgut originate from the ectoderm and the midgut from the endoderm. The Malpighian tubules comprising the excretory system in some insects and other animals extend into the body cavity and absorb wastes, such as uric acid, which are sent to the anterior hindgut, from which this system developmentally derives (Ohkuma 2008; Engel and Moran 2013). Many herbivorous insects have a tubular hindgut with several dilated compartments that harbor a dense gut microbiota. These compartments serve as “fermentation chambers,” in which the prolonged residence time of food allows its degradation by microbial symbionts, a situation analogous to that in the rumen or colon of mammals. All insect guts are surrounded by tissues aerated by the insect’s tracheal system. Oxygen penetrates the contents of the peripheral hindgut to a depth of 150–200 μm below the epithelium. The removal of oxygen by the respiratory activity of the gut microbiota creates a micro-oxic periphery around a highly anoxic center (Brune and Friedrich 2000; Köhler et al. 2012).

Differences in bacterial community structure may reflect different strategies to degrade food. Insects feeding on plant matter, especially wood (xylophagous), can harbor gut microbial communities involved in cellulose degradation such as Firmicutes, Bacteroidetes, Fibrobacteres, Spirochaetes, etc. (Anand et al. 2010; Brune 2014). In termites, it had been described also the participation of intrinsic enzymes in cellulose degradation (Tokuda and Watanabe 2007; Watanabe and Tokuda 2010; Ni and Tokuda 2013]. Enzymes glycoside hydrolases (GH)—necessary for cellulose and hemicellulose degradation—are classified into more than 100 families. All endogenous (provided by the termite) GH are affiliated with the glycoside hydrolase family (GHF) 9, and GHF1 (Ni and Tokuda 2013). The hindgut microbiota of insects is largely structured by exogenous (diet and local environment) and endogenous (gut environment) factors (Colman et al. 2012; Tai et al. 2014; Yun et al. 2014), but a dynamic core gut microbiota (commensal/symbiotic) were maintained even after environmental shifts (Schauer et al. 2014; Makonde et al. 2015). Figure 4 shows the gut microbial composition at phylum level of several wood-feeding insect, including two omnivores’ cockroaches. Each insect species presents a particular profile, but Bacteroidetes, Firmicutes and Proteobacteria are the most abundant bacterial phyla; and in termites also Spirochaetes. But, when the phylogenetic analyses of several particular phyla (such as Bacteroidetes and Spirochaetes) are performed, it can be observed that sequences from termites and cockroaches clustered together, but not with those from other animals, such as wood-feeding Orthoptera (e.g., *Acheta* sp.) and Coleoptera (e.g.,

Pachnoda sp.) or from other environments (Brune 2014; Dietrich et al. 2014; Makonde et al. 2015).

>> Approximately here, Figure 4>>

The gut of wood-feeding lower termites and *Cryptocercus* harbors a complex microbial community comprising protists, bacteria and archaea, but in higher termites lack symbiotic gut protist (Hongoh 2011; Brune 2014). The hindguts of termites and *Cryptocercus* accommodate bacteria belonging to more than 20 phyla and largely consisting of novel lineages that are unique to termites and without cultured representatives (Hongoh 2010; Berlanga et al. 2009; 2011; Dietrich et al. 2014; Tarayre et al. 2015). Among the lower termites examined, the phyla Spirochaetes, Proteobacteria, Firmicutes and Bacteroidetes predominate, but other phyla are also represented, including Actinobacteria, Synergistetes, Verrucomicrobia and Elusimicrobia [formerly ‘*Candidatus* phylum Termite Group 1’ (TG1) and ‘*Candidatus* phylum Termite Group 2’ (TG2)]. In higher termites, Spirochaetes, ‘*Candidatus* phylum TG3’ and Fibrobacteres are the dominant groups. Distinct termite species harbor different bacterial species with community structures specific to the host species, but most of those bacteria belong to phylogenetic clades that are unique to termites and are shared among diverse termite species (Hongoh 2010; Mikaelyan et al. 2015). While Firmicutes and Bacteroidetes are generally more abundant in cockroaches than in termites, Spirochaetes are absent or have yet to be described in omnivorous cockroaches.

The gut protists of lower termites and of the related cockroach genus *Cryptocercus* belong to either the phylum Parabasalia or the order Oxymonadida (phylum Preaxostyla). Most of these organisms are unique to termites and to the cockroach. Parabasalia was traditionally divided into two orders, Hypermastigida and Trichomonadida. Hypermastigida were subsequently reclassified into three different orders, Trichonymphida, Spirotrichonymphida and Cristamonadida (Adl et al. 2005; Noda et al. 2009). Recently, it has been proposed dividing the parabasalids into six classes: *Trichonympha*, *Spirotrichonympha*, *Cristamonadea*, *Tririchomonadea*, *Hypotriconomadea*, and *Trichomonadea* (Cepicka et al. 2010; Noda et al. 2012). It is remarkable that the diversity of flagellate species in *Cryptocercus* cockroaches is greater than that of any extant termite species (Cleveland et al. 1934; Dolan 2001). Representative protist genera found in the gut of *Cryptocercus* are: *Trichonympha*, *Eucomonympha*, *Urinympha*, *Barbulanympha*, *Idionympha*, *Leptosironympha*,

Macrospironympha (order *Trichonymphida*); *Prolophomonas* (order *Cristamonadida*) and *Saccinobaculus* (order *Oxymonadida*) (Ohkuma et al. 2009). As noted above, symbiotic flagellates were established in an ancestor common to *Cryptocercus* and lower termites, vertically transmitted to their offspring through proctodeal interaction and subsequently became highly diversified depending on the host and the symbiont lineages (Ohkuma et al. 2009; Tai et al. 2014). While lower termites and *Cryptocercus* support a characteristic community of gut protists, many protist species are not necessarily restricted to one termite species. Moreover, they may be simultaneously associated with different bacterial ectosymbionts, such as Spirochaetes, Bacteroidetes and Synergistetes, and endosymbionts, such as Bacteroidetes, Elusimicrobia, methanogens (genus *Metanobrevibacter*) (Noda et al. 2007; Ohkuma 2008; Ikeda-Ohtsubo and Brune 2009) and, as recently described, spirochetes (Ohkuma et al. 2015).

The coevolution of ectosymbiotic spirochetes that attach to the cell surfaces of protists in the termite gut reflects a complex process. A single protist cell usually harbors multiple spirochete species, and different protist genera share the same spirochete species (Iida et al. 2000; Noda et al. 2003). As demonstrated by 16S rRNA analysis, the majority of spirochetes from termites belong to *Treponema* clusters I, II and III. Cluster I termite spirochetes have been found in all termites examined so far and include both ectosymbionts attached to protists and free-swimming gut spirochetes, whereas those of cluster II have been identified as the ectosymbionts of oxymonad protists and those of cluster III are related to *Spirochaeta* (Iida et al. 2000; Noda et al. 2003; Berlanga et al. 2007).

The main metabolic compounds produced by spirochetes are acetate, H₂ and CO₂, all of which are consumed by sulfate-reducing bacteria and methanogens (with both groups present in the termite gut). Spirochetes from termite hindguts possess homologues of a nitrogenase gene (*nifH*) and exhibit nitrogenase activity (Lilburn et al. 2001). The identification of key genes from the gut community of the wood-feeding termite *Hodotermopsis sjoestedti* revealed that the endosymbiotic bacteria of the cellulolytic protist belonging to the genus *Eucomonympha* are uniquely responsible for substantial gut activities—nearly 60 % of reductive acetogenesis and nitrogen fixation. The endosymbionts were identified as a spirochete species of the genus *Treponema* (Ohkuma et al. 2015). The dual functions of reductive acetogenesis and nitrogen fixation attributed to the endosymbiotic treponeme of the *Eucomonympha* protist,

together with the cellulolytic ability of the protist, clearly benefit the host termite in terms of efficient carbon, nitrogen and energy metabolism (Ohkuma et al. 2015).

Functional genome analyses from two uncultured endosymbionts, '*Candidatus Endomicrobium trichonymphae*' (phylum Elusimicrobia) (Hongoh et al. 2008a) and '*Candidatus Azobacteroides pseudotrichonymphae*' (Bacteroidetes) (Hongoh et al. 2008b), suggested that both supply essential nitrogenous nutrients, such as amino acids and cofactors, to their host protists; in turn, they receive monosaccharides derived from the lignocellulose digested by the latter. *Candidatus Azobacteroides pseudotrichonymphae* has the additional ability to fix atmospheric nitrogen and to recycle the nitrogen wastes of its host protist. Bacteroidales ectosymbionts of gut flagellates shape the nitrogen-fixing community in dry-wood termites (Desai and Brune 2012). The ectosymbiont of the protist *Devescovina* spp., '*Candidatus Armantifilum devescovinae*' (Bacteroidetes), contains functional genes for nitrogen fixation. These are missing in the ectosymbiont of the protist *Dinenympha* spp., '*Candidatus Symbiothrix dinenymphae*' (Bacteroidetes), which instead imports ammonium and uses it to synthesize more complex nitrogenous compounds. In addition, the genome of this endosymbiotic bacterium contains genes encoding various glycoside hydrolases, such as endoglucanases and hemicellulases. Therefore, it has been proposed that *Candidatus Symbiothrix dinenymphae* contributes to termite gut symbiosis in different ways, namely by hydrolyzing and fermenting (hemi)celluloses and by providing complex nitrogenous compounds (Yuki et al. 2015).

5 Final remarks

Apart from the human microbiome, the best studied holobionts are those that emerged from symbioses involving insects, especially those with plant material diets. The presence of symbiotic associations throughout most of the evolutionary history of insects suggests that they were a driving force in the diversification of this group. Termites and *Cryptocercus* cockroaches are strongly distinguished from other insects by their food: a cellulose-based diet that requires extensive, sequential processing that involves not only physical and chemical manipulations by the host but also the participation of multiple interacting symbionts.

Thus, eukaryotic organisms can be considered as coevolved, tightly integrated communities in which natural selection acts on the holobiont as if it were a single unit

(Guerrero et al. 2013). Symbioses combine the development potential of two or more genomes. Selection pressures on the holobionts compel their intimate interactions. The reward is their ability to exploit niches where the presence of other life forms is ruled out, including extreme environmental conditions or where important nutrients are lacking.

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Figure legends

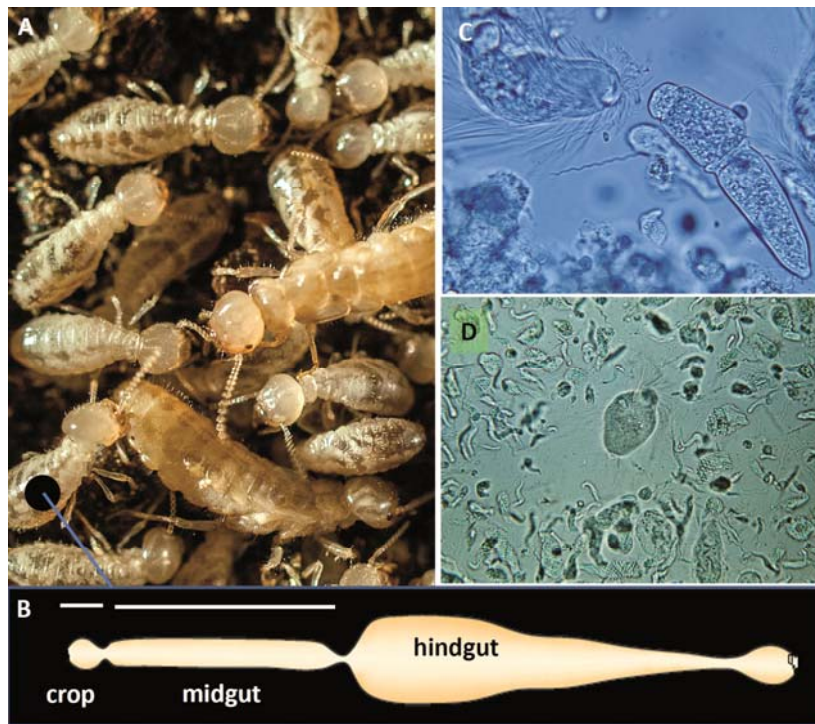


Fig. 1 The holobiont is an essential life-changing force that has resulted in a complex coordinated coevolution of life forms. Insect gut symbionts play an essential role in the insect adaptation to various food types, especially those herbivorous or wood feeding insects, such as termites; disrupting insect gut symbionts can significantly reduce the fitness of the insect (A) Macroscopical aspect of worker caste from the lower termite *Reticulitermes grassei* (Photo by R. Duro). (B) Generalized picture of the gut structure of the lower termite wood-feeding *Reticulitermes grassei*. (C) Detail of the complex microbiota from midgut of *R. grassei* (Photo by R. Duro). (D) Detail of the complex microbiota from the hindgut of *R. grassei* (Photo by R. Duro).

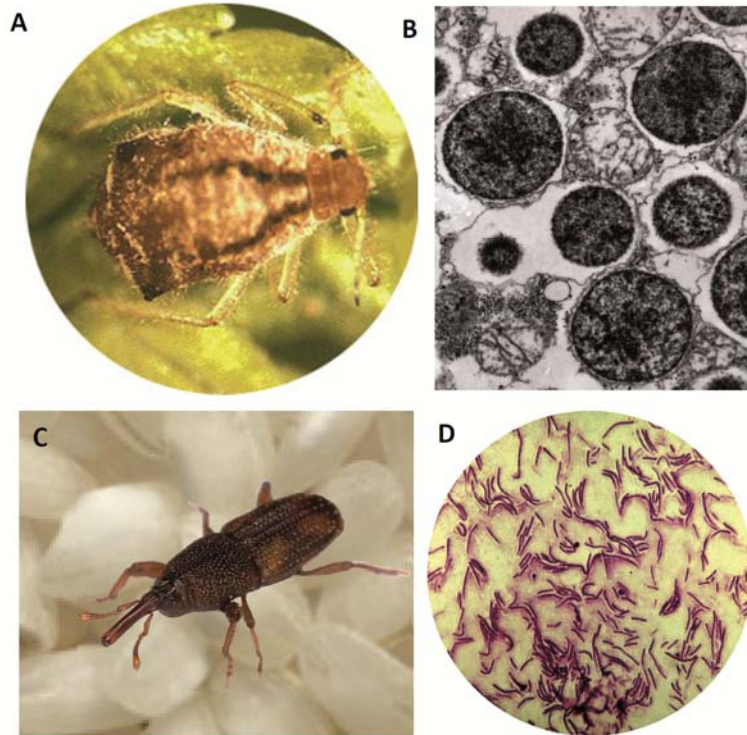


Fig. 2 Insects and their endosymbiont bacteria. (A) The aphid *Cinara tujafilina* (Insecta: Hemiptera: Aphididae). (Photo by A. Latorre and J.M. Michelena. From International Microbiology 5(3) 2002, cover, with permission). (B) Transmission electron micrograph of several cells of *Buchnera*, a bacterial symbiont of aphids (Insecta: Hemiptera: Aphidids), in a cell of their host. (Photo by D. McLean, M. Kinsey and P. Baumann. From International Microbiology 5(3) 2002, p.150, with permission). (C) *Sitophilus oryzae*. (D) Squash preparation of a larval bacteriome of SOPE (*Sitophilus oryzae* principal endosymbiont), a Gammaproteobacterium. (Photo by P. Nardon and A. Heddi. From International Microbiology 11(1) 2008, cover, with permission).

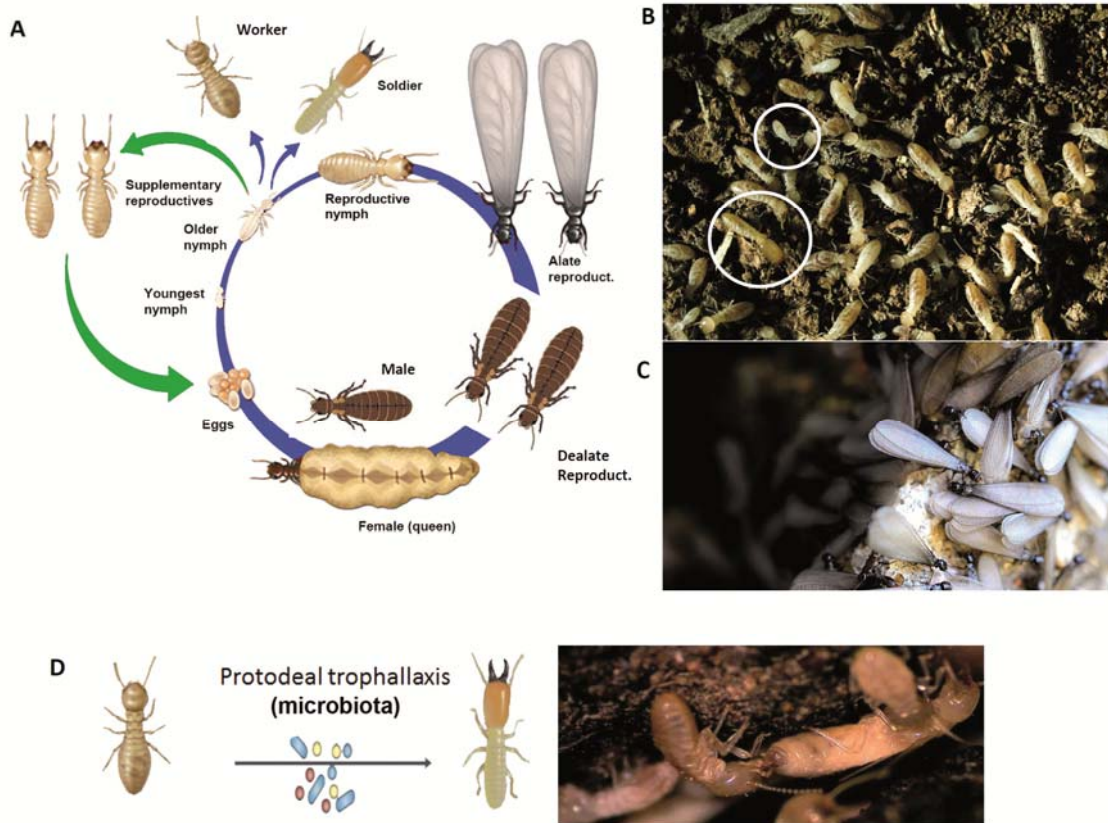


Fig. 3 Eusocial termites. (A) The termite life cycle. (B) Photo showing a colony of the lower termite *Reticulitermes grassei*. A nymph and a soldier castes were inside circles, the rest of termites correspond on workers caste (photo by R. Duro). (C) Alates from *R. grassei* (photo by R. Duro). (D) Trophallaxis and coprophagy allow the direct or indirect transmission of microorganisms among workers and other castes (photo by R. Duro).

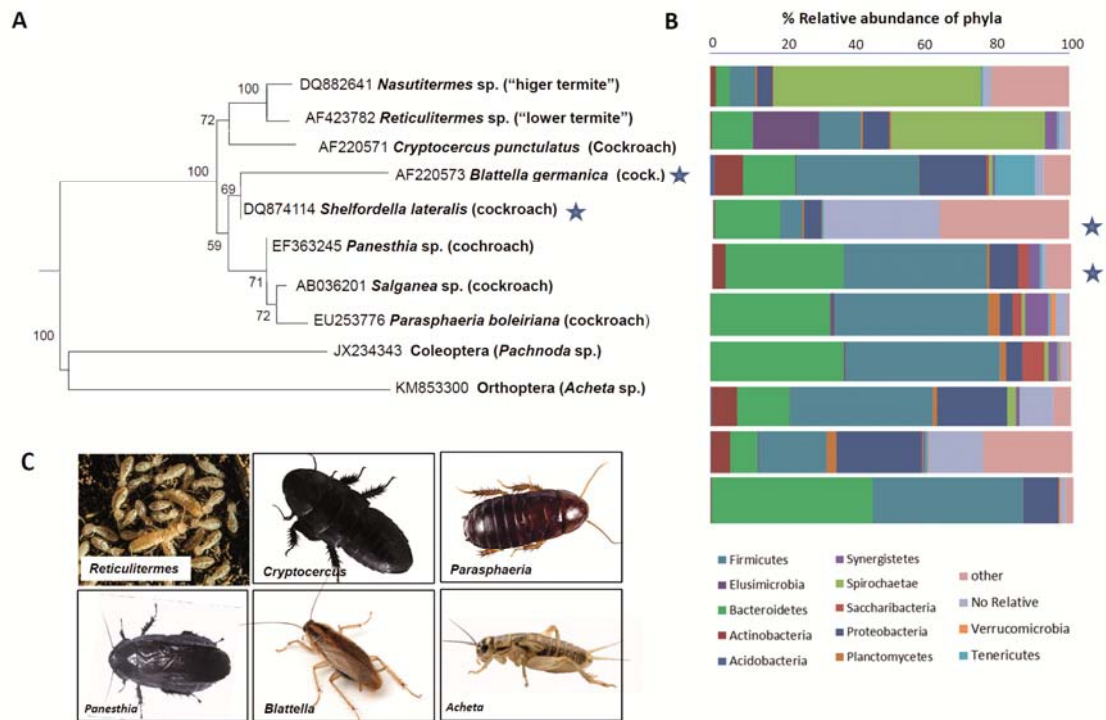


Fig. 4 (A) Phylogenetic tree of several insects belonging to the Orthoptera, Coleoptera and Dictyoptera. All insects present wood-feeding diet except the *Blattella* and *Shelfordella* that are omnivore's cockroaches (blue star). The tree was estimated by Maximum-parsimony (MP) and maximum likelihood (ML) and bootstrap support values are depicted at the nodes. Accession number sequence for the NCBI data base for insect species is indicated in the tree. (B) Hindgut relative abundance of bacterial phyla for each insect is shown in the order of: *Reticulitermes grassei* (data from Berlanga et al. 2011); *Nasutitermes* sp. (data from Köhler et al. 2012); *Cryptocercus* and *Parasphaeria* (data from Bioproject PRJNA284583); *Blattella germanica* (data from Bioproject PRJEB3414); *Shelfordella* sp., *Panesthia* sp., *Salganea* sp., *Pachnoda* sp., *Acheta* sp. (data from Bioproject PRJNA217467). (C) Photographs of insects in the phylogenetic tree are shown."