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Functional symbiosis and communication in microbial ecosystems. The case of wood-eating termites and cockroaches

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Summary. Animal hosts typically have strong specificity for microbial symbionts and their functions. The symbiotic relationships have enhanced the limited metabolic networks of most eukaryotes by contributing several prokaryotic metabolic capabilities, such as methanogenesis, chemolithoautotrophy, nitrogen assimilation, etc. This review will examine the characteristics that determine bacterial "fidelity" to certain groups of animals (e.g., xylophagous insects, such as termites and cockroaches) over generations and throughout evolution. The hindgut bacteria of wood-feeding termites and cockroaches belong to several phyla, including Proteobacteria, especially Deltaproteobacteria, Bacteroidetes, Firmicutes, Actinomycetes, Spirochetes, Verrucomicrobia, and Actinobacteria, as detected by 16S rRNA. Termites effectively feed on many types of lignocelluloses assisted by their gut microbial symbionts. Although the community structures differ between the hosts (termites and cockroaches), with changes in the relative abundances of particular bacterial taxa, the composition of the bacterial community could reflect at least in part the host evolution in that the microbiota may derive from the microbiota of a common ancestor. Therefore, factors other than host phylogeny, such as diet could have had strong influence in shaping the bacterial community structure. [**Int Microbiol** 18(3):159-169 (2015)]

Introduction

Bacteria in nature usually form complex multispecies communities. Living organisms constantly interact with their habitats, selectively taking up compounds from their surroundings to meet their particular needs but also excreting metabolic products and thus modifying their environment. It has been suggested that communication and cooperation, both within and among bacterial species, have produced emergent properties that give a selective advantage to such groups. Bacterial cells produce resources that benefit others in the same habitat. The recipients of such by-products will tend to lose their own costly pathways for those products, thus building dependency into the interactions. Such dependency can favor the spread of more obligate coevolved partnerships [58,59]. This paradigm suggests that bacteria might often form interdependent cooperative interactions in communities, and that

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bacterial cooperation should leave a clear genomic signature via complementary loss of shared functions. Adaptive genome rearrangement is known to be common in parasites and symbionts, both of which can benefit from losing costly functions that are provided by their hosts [39]. Examples of the drastically reduced genomes of obligate intracellular bacteria (endosymbionts) from several insects include the 450 to 653-kb genome of *Buchnera* (aphids), the 697-kb genome of *Wigglesworthia* (tsetse flies), the 792-kb genome of *Blochmannia* (ants) and the 686-kb genome of *Baumannia* (sharpshooter leafhopper). These arthropod-associated mutualists form distinct but related lineages in the Gammaproteobacteria. Gene losses in endosymbionts affect loci that perform functions that are unnecessary in an intracellular environment, or that can be provided by the host, but maintain others related to amino acids, vitamins biosynthesis or cofactors essential to their host.

The study of the complex relationship between host–microbe interactions and behavior requires an ecological perspective, involving several "stakeholders": the host, the microbiota and the biotope, that in combination constitute the holobiont. Microbes are part of animal/plant systems, and they must be included in the animal/plant life histories. The holobiont is an essential life-changing force that has resulted in a complex coordinated coevolution of life forms [28,77] (Fig. 1). Interactions between the host and its microbiota are not only nutritional, but also include tissue development, immunity, circadian regulation, etc. [74]. Those interactions involve multiple microbial species and their genotypes, so that functions depend on bacterial communities rather than on individual microbial taxa. Lifestyles of bacterial symbionts can vary in four important ways, all of which contribute to the long-term evolution of symbiotic microbial lineages as well as to the co-evolution of the holobiont: (i) host- symbiont specificity, (ii) the mechanisms of symbiont acquisition, development and maintenance, (iii) the functional mechanisms that the symbiont employs to benefit or injur the host, and (iv) the host response to the presence of bacteria [71].

Ecological interactions among members of the microbial communities may have different net impacts on host fitness based on the actual environmental circumstances [41,74]. Of all aspects of the environment, nutrition is the most important in shaping the responses of the microbiota and their host (the "holobiont system"). In the case of the gut microbiota, the nutritional resources are dependent on host feeding behavior. The composition and physical form of the food change 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 [62]. Insect gut symbionts play an essential role in the insect adaptation to various food types, especially in herbivorous insects. Herbivory can be a successful feeding mode, but only after key obstacles are overcome, such as low nutrient content, and indigestibility or toxicity of many plant tissues. The herbivorous microbiota has been shown to be important for lignocellulosic material degradation, nutrient production (amino acids, vitamins, etc.), and compound detoxification [12,19]. Disrupting insect gut symbionts can significantly reduce the fitness of insects and can even cause serious diseases such as Colony Collapse Disease (CCD) [16].

This review examines the relationships between two xylophagous insects (lower-termites and cockroach), their gut microbiota and the characteristics that determine bacterial fidelity over generations and throughout evolution with their host. Termites from the perspective of the "holobiont" are considered as a single functional unit in which host and symbionts are physiologically tightly connected.

Insect gut as a microbial habitat

The intestinal tracts of insects are small ecosystems comprising discrete and clearly delineated habitats that strongly differ in their abiotic and biotic environment. Many of those environmental features are intrinsic properties of the gut, whereas others result from physiological activities of the host or the microbial residents in the particular location. The basic structure of the digestive tract is similar across insects; it has three primary regions, foregut, midgut, and hindgut [23] (Fig. 2). The foregut transports food from the mouthparts into the crop, where it is incubated with secretions from the salivary glands. After further comminution by the gizzard, food passes into the midgut, where it is digested by enzymes secreted there. Part of the digestion products are resorbed by the midgut epithelium. The remaining material is transported into the hindgut. Many herbivorous insects have a tubular hindgut with several dilated compartments that harbor a dense gut microbiota. In these dilated compartments, or "fermentation chambers," the prolonged residence time of food allows its degradation by microbial symbionts, a situation analogous to that in the rumen or colon of mammals [13,23]. After the removal of water and ions, the residues of digestion are released as feces. The excretory system in insects consists of the Malpighian tubules that extend into the body cavity and absorb wastes, such as uric acid, which are sent to the anterior hindgut [23,51]. All insect guts are surrounded by tissues aerated by

Fig. 1. The "holobiont" as a single functional unit in which host and its symbionts are physiologically tightly connected. Relationship between host–microbe interactions involves the host, the microbiota and the environment. The holobiont a complex coordinated coevolution of different life forms. Interactions between the host and its microbiota are not only nutritional, but also include tissue development, immunity, etc. The human picture is based on the cover of *The Economist* magazine.

the insect's tracheal system. Oxygen penetrates the peripheral hindgut contents to a depth of up to 150–200 μm below the epithelium. The removal of oxygen by the respiratory activity of the gut microbiota creates a microoxic periphery around an anoxic center [11,33]. The diversity of the hindgut microbiota of termites depends on several factors, including the variety of specialized structures present in the gut, the effect of pH, the sharp redox gradient, the type of food ingested and coevolution with their host insect [3,12,15,64]. In insect guts, the midgut is endodermal but foregut and hindgut are of ectodermal origin, and are always lined with a cuticle, so during ecdysis, insects replace their entire cuticle, and the hindgut has to be recolonized after each molt.

In terms of host acquisition, symbionts can be acquired (i) horizontally from the environment, (ii) vertically from parental inheritance (e.g., endosymbionts such as *Buchnera*), or (iii) via a combination of these mechanisms. Horizontal symbiont transmission often leads to selection based on symbiont function rather than symbiont taxonomy [10]. Establishing horizontally acquired symbioses presents considerable challenges for both the host and the symbiont. The host may re-

quire mechanisms for the selection and retention of specific microbes from the environment whereas at the same time, it needs to retain a functioning immune system to destroy opportunistic or potentially pathogenic microorganisms [9]. The basic insect life cycle also presents potential challenges for transmission of microorganisms between generations. In most insects, females abandon eggs after depositing them. In this case, opportunities for direct transfer of gut symbionts between adults and juveniles are more limited compared to mammals and birds, which have extended parent–offspring contact. However, some insect species, including cockroaches, termites, ants, and some wasps and bees, show gregarious or social behavior, which can enable direct or indirect social transmission of their microbiota. In cockroaches, the neonatal digestive tract is free of microbes, and the 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 [14,44,45]. Worker caste termites transfer food stomodeally (by regurgitation) and/or proctodeally (by excretion of the
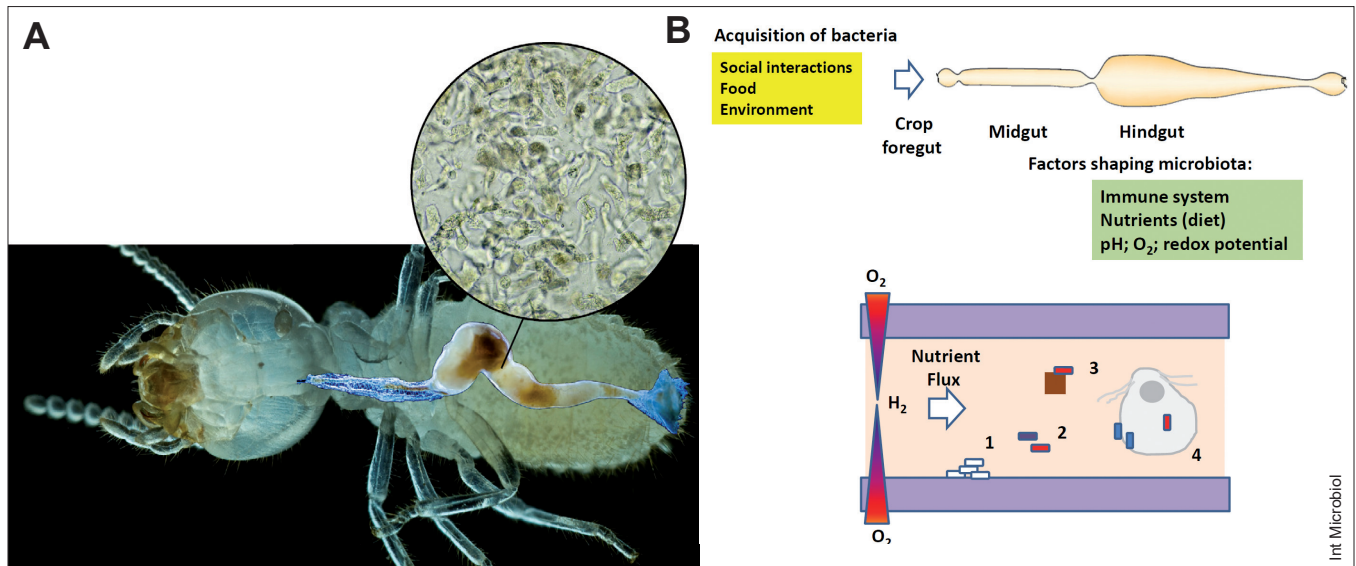
Fig. 2. (A) Macroscopical aspect of the worker caste from the lower termite *Reticulitermes grassei* showing the intestinal tract and a detail of their complex microbiota (Photo by R. Duro). **(B)** Generalized gut structure of insects, focusing on the structure of the gut of the lower termite *R. grassei* and factors that may influence the microbiota composition and distribution in the gut. Bacteria may colonize the intestinal wall (1); may be free-swimming in the lumen (2); they can be attached to food particles (3); or existing as ecto- or endosymbionts of protists (4).

hindgut contents). Both oral trophallaxis (feeding by mouths contact) and coprophagy (feeding feces to mouths) can promote a secure transmission of commensal microbiota between members of a colony of termites or of gregarious cockroaches [21,55].

Prokaryotic community in wood-feeding Dictyoptera

Termites (Isoptera), cockroaches, and mantids form a well-established lineage of insects, the Dictyoptera. In fact, termites are actually social cockroaches, with the family Cryptocercidae as their closest relative and the Mantodea (mantids) as the sister group to the clade comprising cockroaches and termites [69]. The order Blattodea is now made up of termites and all cockroach taxa [2]. 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 [36]. There are fundamental differences in the diets of termites and cockroaches. While termites feed almost exclusively on lignocellulose in various stages (i.e., wood, leaves, humus, detritus, and herbivore dung), cockroaches subsist on a highly variable diet (omnivorous or xylophagous).

The gut of wood-feeding "lower" termites (e.g., *Reticulitermes grassei*) and that of, the cockroach *Cryptocercus* harbors a complex microbial community comprising protists and bacteria [31]. "Higher" termites lack the symbiotic gut protists, instead having a gut microbiota composed of prokaryotic microorganisms [12]. Transient bacteria acquired from food and/or from the environment could modify the composition of gut microbial communities, but a dynamic core gut microbiota (commensal) is maintained even after environmental shifts [8,38,61,63].

Gut protists of termites belong to either the phylum Parabasalia or to phylum Preaxostyla (order *Oxymonadida*) (Fig. 3). Most of those protists are unique to termites and the related cockroach genus *Cryptocercus*. Parabasalia was traditionally divided into two orders, Hypermastigida and Trichomonadida. Hypermastigida were subsequently reclassified into three different orders, Trichonymphida, Spirotrichonymphida, and Cristamonadida [48]. Representative protist genera found in the gut of *Cryptocercus* are: *Trichonympha, Eucomonympha, Urinympha, Barbulanympha, Idionympha, Leptospironympha, Macrospironympha* (order Trichonymphida); *Prolophomonas* (order Cristamonadida) and *Saccinobaculus* (order Oxymonadida) [52]. Protists observed in *Reticulitermes* sp. (lower termite)
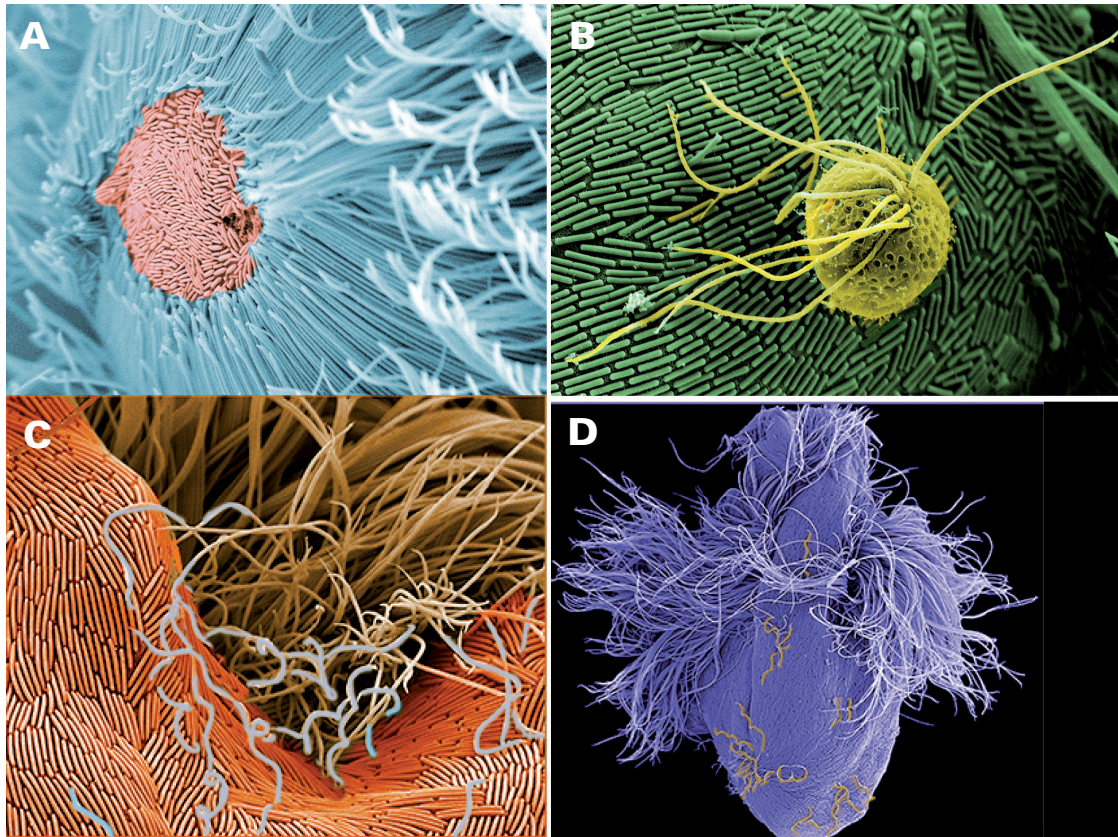
Fig. 3. Scanning electron micrographs showing several protists from the gut of lower termites and the cockroach *Cryptocercus*. Note the presence of ectosymbiotic bacteria. (Scanning micrographs by Kevin J. Carpenter; shown at the exhibition at the Exploratorium museum, San Francisco, CA, USA. Micrographs were artificially colored by M. Berlanga to contrast the ectosymbionts from the protists.) (**A**) *Trichonympha* showing their rod shaped symbiotic bacteria (red) and flagella (blue). (**B**) The protist *Saccinobaculus* in front of the larger protist *Barbulanympha*, which is completely covered by rod-shaped bacteria (green). (**C**) The protist *Staurojoenia* showing spirochaetes (blue), rod-shaped symbiotic bacteria (orange) and flagella (brown). (**D**) The protist *Staurojoenia* (whole cell) showing spirochaetes in their surface (orange).

were: *Holomastigotes*, *Microjoenia*, *Trichonympha*, *Spironympha*, *Monocercomonas* sp., *Dinenympha* sp., *Spirotrichonympha* sp., and *Pyrsonympha* sp. [49]. 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*) [32,47,51] and, as recently described, spirochetes [53].

The hindgut of termites and *Cryptocercus* accommodate diverse bacteria from more than 20 phyla, with the majority constituting novel lineages with uncultured representatives that are unique to termites [4,5,20,30;38,65]. Among the lower termites examined, the phyla Spirochaete, Proteobacteria, Firmicutes, Bacteroidetes are predominant. Other phyla are also represented, such as Actinobacteria, Synergistetes, Ver-

rucomicrobia, Elusimicrobia (formerly candidate phylum Termite Group 1-TG1), and candidate phylum Termite Group 2 (TG2) (Fig. 4). In higher termites, Spirochaete, candidate phylum TG3 and Fibrobacteres were the most dominant groups. Firmicutes and Bacteroidetes were generally more abundant in cockroaches than in termites. Spirochaetes were absent or not described in omnivorous cockroaches. Distinct termite species harbor different bacterial species with community structures specific to their host, but several of those bacteria are unique to termites/*Cryptocercus*, and were shared among diverse termite species (e.g., Spirochaetes, Bacteroidetes). Those bacteria may derive from the microbiota of a common ancestor before the diversification of cockroaches, and then diversified and adapted in each host [3,4,30,42,64].

Spirochetes in the guts of termites and *Cryptocercus* fall into three clusters named *Treponema* cluster I, II and III. *Treponema*-termite cluster I comprises both ectosymbionts at-
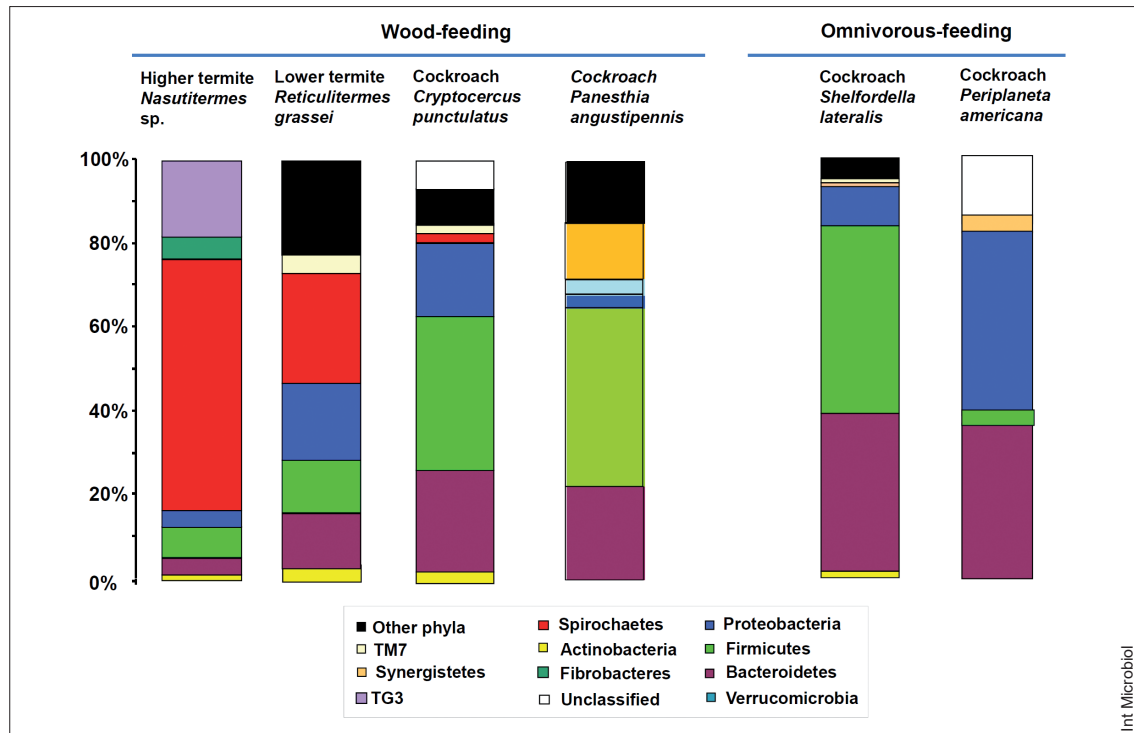
Fig. 4. Phyletic composition (%) of the gut microbiota in several xylophagous insects. Data from *Nasutitermes* spp. (Termitidae) [33]. Data from *Reticulitermes grassei* (Rhinotermitidae) [5]. Data from the xylophagous cockroach *Cryptocercus punctulatus* [4]. Data from the cockroach *Panesthia angustipermis* [20]. Data from the cockroach *Shelfordella laterals* (omnivorous) [20]. Data from *Periplaneta americana* [7].

tached to protists and free-swimming gut spirochetes from lower and higher termites. Treponema-I includes the only three isolates of the entire cluster, *Treponema primitia* [25], *Treponema azotonutricium* [26], and *Treponema isopterico-lens* [22]. Although none of its cultivated members seem to be cellulolytic, *Treponema primitia* is able to catabolize catechol under microoxic conditions, which suggests a possible role of *Treponema* bacteria in the breakdown of aromatic compounds released from the lignin fraction of lignocellulose [37]. Members of Cluster II are ectosymbiotic spirochetes of oxymonad protists. However, not all ectosymbiotic spirochetes are in Cluster II. *Treponema*-termite cluster III contained *Treponema* sequences from other cockroaches and from higher termites, such as in fungus-cultivating termites [3,42].

Ohkuma et al. [50] classified the *Bacteroidetes* in five clusters (I–V). Group V consists of sequences from uncultured strains isolated from termites and cockroaches, such as *Shelfordella* (omnivorous) and *Cryptocercus* (wood-feeding). Many of these *Bacteroidetes* represent symbionts of gut flagellates from the protist *Pseudotrichonympha*. Group V also contains the diazotroph *Azobacteroides pseudotrichonymphae* [18] and

ectosymbionts described previously in Devescovinid flagellates from the *Kalotermitidae* (lower-termite) [17].

Functional symbiosis as a driving force of cooperation

Identifying microbes responsible for particular environmental functions is challenging. Termites may harbor different microbial symbiont populations with specialized functionalities geared towards different feeding regiments that performed similar functions such as lignocellulose degradation and homoacetogenesis and nitrogen fixation [13].

Carbon source of the host. The major component of plant material is cellulose (20–40%), a linear polysaccharide consisting of glucose units. It represents the most abundant biomass on earth. Hemicellulose is a general term for major noncellulosic polysaccharides in plant cell walls. Main chains of hemicellulose are composed of xylose, or glucose and mannose, which are often acetylated or shortly branched with

arabinose, galactose, or other acidic sugars. Chemical compositions of hemicelluloses vary across plant species. Lignocellulose consists of cellulose (20–50%), hemicellulose (15–35%), and lignin (18–35%) [68].

Insects feeding on plant matter, especially wood (xylophagous), can harbor gut microbial communities involved in cellulose degradation [1], at least from the Miocene [72]. Cellulose exists as crystalline or amorphous microfibrils in plant cell walls and thus is not readily accessible to the host [70]. In the gut, the cellulose fibers are broken down into simpler sugar residues, a process which microbiota are typically involved in [12,76]. Termites have also been found to have their own cellulases [46,70]. The degradative process of cellulose/lignocellulose differs in higher and lower termites. In lower termites and *Cryptocercus*, cellulose digestion is mostly accomplished by protists and to a lesser extent by bacteria. The cellulolytic activity of bacteria in higher termites might replace the functions provided by protists in lower termites (Fig. 5). Glycoside hydrolases (GH), necessary for cellulose and hemicellulose degradation, are highly represented and expressed in termite hindguts. Based on peptide sequence similarities, glycoside

hydrolases 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 [46]. The endogenous cellulolytic system of wood-feeding higher termites is thought to contribute to cellulose digestion more significantly than that of lower termites. Compared to host cellulases, symbiotic protistan communities in lower termites produce more complex cellulolytic enzymes, such as GHF3, GHF5, GHF7, etc. In higher termites, hindgut bacteria, principally Spirochaetes, Fibrobacteres and TG3, apparently took over the role of flagellates in cellulose degradation [70]. Also, members of Bacteroidetes and Clostridia are thought to be specialized in the degradation of complex organic matter, including lignocellulosic compounds [43,76].

Ingested cellulosic particles are fragmented in the foregut. There, the wood particles that are produced by the mandibles mix with enzymes secreted by the salivary glands. Any glucose that is released in the midgut is resorbed via the epithelium, whereas the partially digested wood particles pass through the enteric valve into the hindgut. In lower termites, the wood particles are immediately phagocytized by
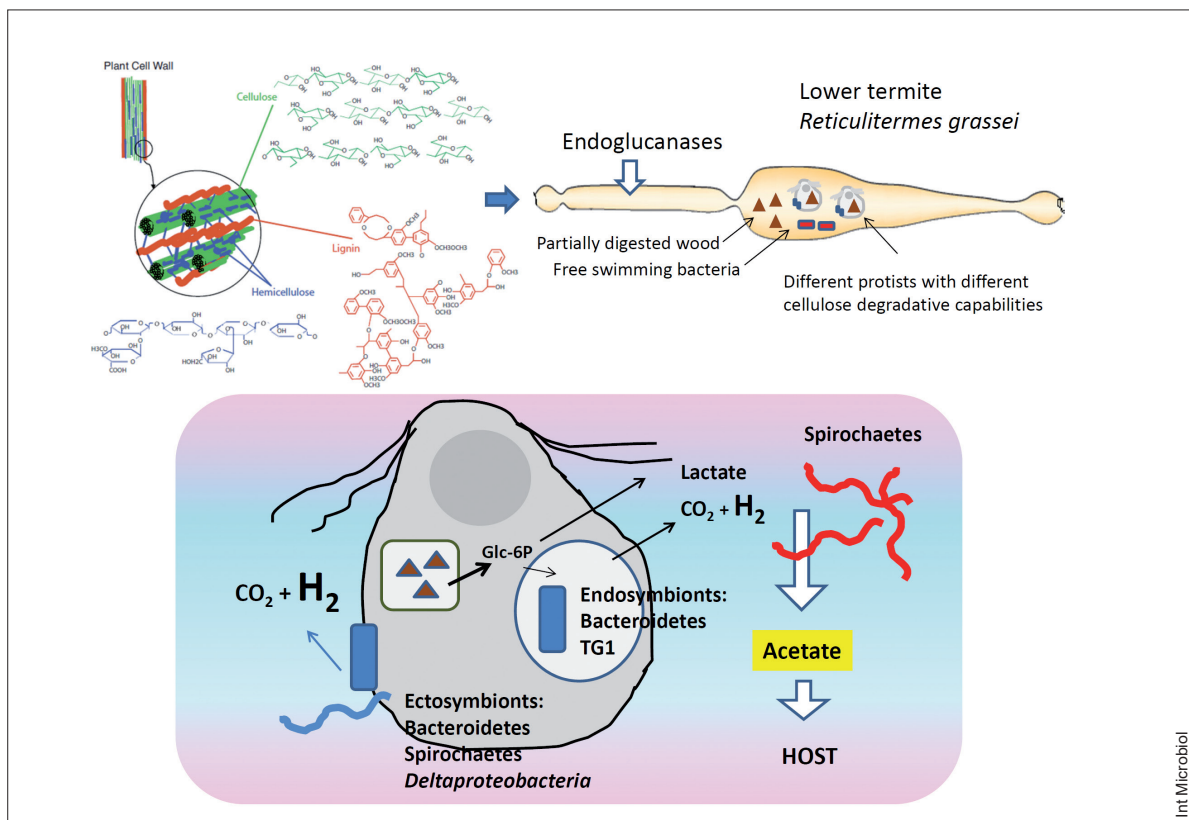

Fig. 5. Lignocellulose digestion in termites (showing in this case a lower termite) involves activities of both the host and its gut microbiota. The illustration shows the fermentative breakdown of wood polysaccharides.
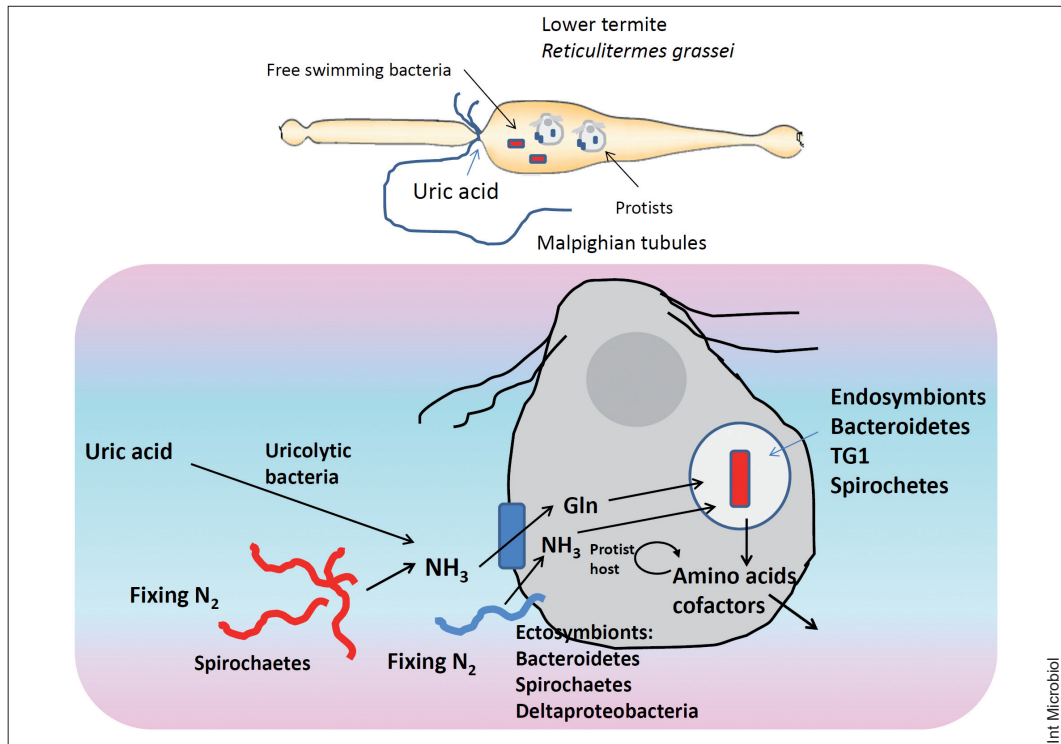
Fig. 6. Nitrogen supplied to the host by their bacterial symbionts.

cellulolytic flagellates, which hydrolyze the remaining polysaccharides using glycoside hydrolases that are secreted into their digestive vacuoles. Protists convert cellulose to acetate, H_2 and CO_2 [12]. The flagellates are probably also responsible for the production of lactate [54]. Lactate may be rapidly converted to acetate by bacteria that are located in the gut periphery in an oxygen-dependent process. Furthermore, formate is produced in the hindgut of many termite species. Depending on the termite species, hindgut formate is either accumulated, or oxidized to CO_2, or reduced to acetate, presumably by homoacetogenic bacteria [54]. The microbial fermentation products (which are mainly short-chain fatty acids, e.g., acetate) are principally resorbed by the host, and the lignin-rich residues are voided as feces (Fig. 5).

Hydrogen source of the host. Hydrogen is a key fermentation product that fuels many bacteria in the gut [54], and can be generated and consumed through nickel-iron (NiFe)-hydrogenase or iron-only (FeFe)-hydrogenase activities. (NiFe)-hydrogenases were present in members of the Synergistetes and Deltaproteobacteria. (FeFe)-hydrogenases, which are widely distributed in termites, have been assigned only to Spirochaetes. Homoacetogenesis (i.e., CO_2 reduction to form acetate) is the major H_2 sink in wood-feeding termites

[34]. Formyl tetrahydrofolate synthase (FTHFS), a key enzyme in the homoacetogenic pathway, has been assigned principally to Spirochaetes. It was suggested that termite symbiotic Spirochaetes may have acquired their CO_2 reductive acetogenesis capability through lateral gene transfer from Firmicutes. However, whether the gene transfer event occurred before or after Spirochaetes having initially become termite symbionts has not been determined [60].

Nitrogen source of the host. Wood is poor in nitrogen content, which it is an important constraint to the growth of wood-feeding termites and *Cryptocercus*. The microbiota plays an important role in the fixation, recycling, and upgrading of nitrogen [75]. Dinitrogen fixation by hindgut diazotrophic bacteria can represent 30–60% of the new acquisition of nitrogen. The *nifH* genes in termite guts are present among Spirochaetes, Clostridia and Bacteroidetes [18,48]. The bacterial symbionts (ecto-, or endosymbionts) of the protists seem to play important roles in nitrogen fixation, the assimilation of ammonia, and the synthesis of amino acids and vitamins [17,53]. The major waste product of nitrogen metabolism is uric acid. It is formed in the fat body and secreted into the hindgut via the Malpighian tubules, where uricolytic hindgut bacteria convert uric acid nitrogen to ammonia. The recy-

cling of uric acid nitrogen is an important and significant aspect of the symbiosis with gut microbiota. The assimilation of ammonia into new microbial biomass completes the nitrogen cycle [67]. Uric acid recycling by gut bacteria seems to be a general mechanism for the conservation of nitrogen in terrestrial insects, particularly those whose natural diets are low in combined nitrogen [12] (Fig. 6).

The endosymbiotic bacterium *Blattabacterium* is present in cockroaches (including *Cryptocercus*) and the lower termite *Mastotermes darwiniensis* but is absent in the rest of termites (lower and higher). The members of *Blattabacterium* that inhabit the fat bodies of cockroaches are thought to participate in uric acid degradation, nitrogen assimilation, and nutrient provisioning. Genomic analysis and metabolic reconstruction indicate that *Blattabacterium* sp., despite lacking recognizable uricolytic enzymes, is able to recycle nitrogen from urea and ammonia (both of which are uric acid degradation products) into glutamate by using the enzymes urease and glutamate dehydrogenase [56]. The genome of *Blattabacterium cuenoti*, whether from the termite *Mastotermes darwiniensis* or the social wood-feeding cockroach *Cryptocercus punctulatus*, lacks most of the pathways for the synthesis of essential amino acids found in the genomes of relatives of this bacterium isolated from non-wood-feeding hosts. This deficit may be filled by the other members of the complex gut microbiota, which provide their host with all essential amino acids [57].

Final conversations

In 2004, Carl Woese wrote: "The time has come to replace the purely reductionist 'eyes-down' molecular perspective with a new and genuinely holistic, eyes-up, view of the living world, one whose primary focus is on evolution, emergence, and biology's innate complexity." [73].

Today, it is common knowledge that the majority of microorganisms play essential roles in maintaining life on Earth. We, and our related "macrobes", are ultimately dependent on the assorted activities of the "invisible" microbial world. The miniscule size of its members belies their tremendous importance [6,27,29,40].

Interactions between animals and microbes are not specialized occurrences but rather are fundamental aspects of animal biology [40]. Symbiotic microbes are fundamental to nearly every aspect of host form, function, and fitness, including the traits that once seemed intangible to microbiology: behavior [24], and sociality [66]. Symbionts recognize one another and communicate. The gut is likely the most dynamic

organ, with intense and constant cross-talk between the huge diversity of microbes it hosts and the epithelium and the rest of the host. It is intriguing to consider that these kinds of communication evolved to conserve an association's balance with its hundreds of beneficial bacterial species, and that pathogens have "taken control of" these conversations to enhance their fitness through disease.

As Lederberg wrote [35], reminding us of August Krogh's principle, [f] "for any given scientific challenge there is a critter fittest towards its solution."

Conflict of interests. None declared.

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