REVIEW

Mechanisms underlying gut microbiota–host interactions in insects

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ABSTRACT

Insects are the most diverse group of animals and colonize almost all environments on our planet. This diversity is reflected in the structure and function of the microbial communities inhabiting the insect digestive system. As in mammals, the gut microbiota of insects can have important symbiotic functions, complementing host nutrition, facilitating dietary breakdown or providing protection against pathogens. There is an increasing number of insect models that are experimentally tractable, facilitating mechanistic studies of gut microbiota–host interactions. In this Review, we will summarize recent findings that have advanced our understanding of the molecular mechanisms underlying the symbiosis between insects and their gut microbiota. We will open the article with a general introduction to the insect gut microbiota and then turn towards the discussion of particular mechanisms and molecular processes governing the colonization of the insect gut environment as well as the diverse beneficial roles mediated by the gut microbiota. The Review highlights that, although the gut microbiota of insects is an active field of research with implications for fundamental and applied science, we are still in an early stage of understanding molecular mechanisms. However, the expanding capability to culture microbiomes and to manipulate microbe–host interactions in insects promises new molecular insights from diverse symbioses.

KEY WORDS: Arthropod, Digestive tract, Fruit fly, Honey bee, Symbiosis, Termite

Introduction

The evolutionary success of many insects is closely tied to symbiotic associations with microbes (Douglas, 2015; Baumann, 2005). Multi-species microbial communities, or microbiota, inhabit the digestive system of many insects (Engel and Moran, 2013). They can offer a wide range of benefits to their host, ranging from increased oviposition (see Glossary) (Akami et al., 2019; Jose et al., 2019; Lee et al., 2017), fecundity (Pais et al., 2018) and longevity (Salem et al., 2014; Gould et al., 2018), to shorter larval development (Coon et al., 2014; Storelli et al., 2011; Shukla et al., 2018a,b; Vogel et al., 2017a; Shin et al., 2011), improved resilience to environmental disturbance (Almeida et al., 2017; Itoh et al., 2018a) or changes in host behavior (Liberti and Engel, 2020; Wong et al., 2017; Schretter et al., 2018). However, the nature of gut microbiota–host associations can be highly variable across insects, suggesting differences in the type and extent of benefits provided to the host (Engel and Moran, 2013). For example, termites (Brune and Dietrich, 2015), social bees (Kwong and Moran, 2016), cockroaches (Tinker and Ottesen, 2016) or certain ant (Hu et al., 2018; Sanders et al., 2017) and beetle species (Shukla et al., 2018a; Ceja-Navarro et al., 2019; Nardi et al., 2006) harbor specialized and dense

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gut microbial communities, representing longstanding microbiota–host associations. Conversely, fruit flies or mosquitoes are mostly colonized by transient and relatively sparse communities that are acquired from the environment (Wong et al., 2013; Bost et al., 2018; Coon et al., 2016b; Pais et al., 2018). Nevertheless, these bacteria can have strong positive effects on their host. Finally, there are insects which harbor very few or no bacteria in their guts, such as certain caterpillar species or aphids (Hammer et al., 2017), and if such bacteria are present, they have little or no detectable effect on the host (Phalnikar et al., 2019).

Given the large diversity of insects in physiology, morphology and ecology, it seems obvious why gut microbial communities are highly variable across species. Differences in gut anatomy and physicochemical characteristics require different microbial properties for gut colonization. Horizontal transmission (see Glossary) of specialized gut microbes is facilitated by insects living in social groups (Engel and Moran, 2013). Moreover, highly divergent dietary habits of insects create vastly different nutritional niches in the gut, favoring microbial partners with distinct metabolic capabilities and physiologies (Dietrich et al., 2014; Kešnerová et al., 2019). Which of these factors contribute to differences in the structure and function of gut microbiota may depend on the host species, as exemplified by the highly variable gut microbial associations found across ants, and is not yet fully understood (Hu et al., 2018; Łukasik et al., 2017; Jacoba et al., 2016; Sanders et al., 2017).

Diverse mechanisms must exist to mediate the crosstalk between gut microbes and their insect hosts. Until recently, molecular insights into these interactions have mostly been based on a few tractable model systems, such as *Drosophila* (Charroux and Royet, 2012). However, the establishment of experimental approaches to manipulate the gut microbiota of a wide range of host species (Leonard et al., 2018; Kikuchi et al., 2020) and the advances in 'omics' technologies have broadened our understanding of the molecular mechanisms of gut microbiota–insect interactions (Table 1). For example, the genetic basis of gut colonization has been elucidated for bacteria in several insect species (Kim et al., 2013; Jang et al., 2017; Powell et al., 2016; Vacheron et al., 2019). Moreover, we have learned that beneficial effects of the gut microbiota can include a wide range of functions, e.g. facilitation of nutrient assimilation (Hu et al., 2018; Zheng et al., 2019), colonization resistance against pathogens (Weiss et al., 2019; Raymann et al., 2017) or detoxification of xenobiotics or dietary components (Ceja-Navarro et al., 2015; Wang et al., 2020).

In this Review, we will summarize our recent understanding of host–gut microbiota symbiosis in insects, with a focus on the mechanisms involved in gut colonization and the provisioning of beneficial effects to the host (Table 1).

Mechanisms of insect gut colonization

Colonization of the digestive tract is the first step in the establishment of a symbiosis between gut microbes and their host. The digestive tract is exposed to the environment because dietary

Glossary

Click chemistry
A class of chemical reactions that allows to join two substrates, e.g. a biomolecule and a reporter, to track biomolecules *in vivo*.

Colonocytes
Epithelial cell type of the colon, i.e. the large intestine.

CRISPRi-Seq
Genome-wide sequencing of guide RNAs targeting an inactive (dead) dCas9 variant to inhibit gene expression, passaging a genome-wide library of guide RNAs (10,000–100,000 mutants) of a bacterium of interest through a given condition and identifying conditionally essential genes by sequencing the input and the output library of guide RNAs. Genome perturbation is mediated by repression of gene expression.

C-type lectins
A superfamily of proteins that recognize a broad repertoire of ligands, including carbohydrates. They play an important role in recognizing pathogens and activating the innate immune system by binding to carbohydrate motifs on microorganisms.

Detritivores
Organisms that obtain nutrients by consuming detritus (dead particulate organic material), thereby contributing to decomposition and the nutrient cycle.

Entomopathogens
Microbial pathogens of insects.

Hemolymph
Circulating fluid of insects, analogous to the blood in vertebrates; it is in direct contact with the tissues and contains hemocytes (immune cells) and various organic and inorganic compounds, such as amino acids, salts, proteins or sugars.

Horizontal transmission
Symbionts are taken up from the environment in each host generation, as opposed to vertical transmission where symbionts are transferred through the female (or male) germline.

IMD pathway
Immune pathway activated after recognition of Gram-negative bacteria leading to activation of NF-kB-like transcription factor Relish and subsequent induction of antimicrobial peptide production (e.g. Diptericin).

Melanization response
An innate immune response that, in insects, is initiated upon pathogen recognition and leads to the formation and deposition of melanin to engulf and isolate pathogens.

O-antigen
Also known as O-specific polysaccharides or O-side chains; major components of the surface lipopolysaccharide (LPS) of Gram-negative bacteria. They modulate the charge and integrity of the outer membrane.

Oviposition
The physical process of laying eggs.

Peritrophic matrix
An acellular semi-permeable chitinous layer that lines the midgut of most insects and protects the epithelium from damage and infection by pathogens.

TnSeq
Genome-wide transposon sequencing, passaging a genome-wide library of random transposon insertion mutants of a bacterium of interest through a given condition and identifying conditionally essential genes by sequencing the input and the output library. Genome perturbation is mediated by gene disruption.

Toll pathway
Immune pathway activated after recognition of Gram-positive bacteria, fungi and yeast leading to activation of NF-kB-like transcription factor dorsal and subsequent induction of antimicrobial peptide production (e.g. Drosomycin).

Trimeric autotransporters
Protein family with unique structural composition, found on the outer membrane of Gram-negative bacteria.

Trophallaxis
Food and fluid transfer among members of an insect species through mouth-to-mouth or anus-to-mouth feeding.

Type IV pili
Polymeric assemblies of pilin protein subunits. They build strong flexible filaments and among others are involved in host adhesion and biofilm formation.

content flows from the oral to the anal opening. Therefore, 'colonization' can refer to different types of associations ranging from the transient passage of environmental microbes to the replication and persistence of specific gut symbionts. Correspondingly, a wide range of mechanisms facilitating or constraining gut colonization have been described, reflecting different degrees of specificity of the symbiotic associations and including both host and microbial factors (Fig. 1).

Host mechanisms involved in gut colonization
In many insects, the gut microbiota is acquired from the environment or ingested with the food; the host gut represents a selective habitat that determines bacterial colonization.

General gut properties
The strength of the host selection will depend on physicochemical features in the gut, such as the transit rate of food content, available nutrients, local pH, redox potential and immune effectors. These properties can differ along the gut, resulting in spatially distinctive colonization patterns (Vogel et al., 2017a; Ceja-Navarro et al., 2019; Powell et al., 2014). In many animals, the microbial density increases from the anterior to the posterior part of the digestive system (Kovatcheva-Datchary et al., 2013; Brune, 2014), thereby, the microbiota avoids competition with the host for nutrients, which are usually absorbed in the anterior part of the digestive system (Scott et al., 2013). One of the most distinctive features of insects compared with mammals and other vertebrates is that insects undergo metamorphosis, which has a huge impact on the assembly and persistence of the microbiota. Consequently, many holometabolous insects harbor different gut microbial communities at the larval stage and during adulthood, because the complete transformation of the gut environment at pupation poses constraints on gut microbial communities (recently reviewed by Hammer and Moran, 2019).

Table 1. Overview of insects discussed and their gut microbiota

Host species	Microbiota characteristics	Host colonization factors	Microbial colonization factors	Beneficial roles	Experimental tractability	References
African cotton stainer (*Dysdercus fasciatus*)	High specificity; low complexity	–	–	B vitamin supplementation	RNAi	Salem et al., 2014; Onchuru and Kaltenpoth, 2019; Onchuru et al., 2018b; Bauer et al., 2014
American cockroach (*Periplaneta americana*)	High specificity; high complexity	–	–	Nutrition from fermentation products	Partially culturable microbiota; RNAi	Tinker and Ottesen, 2016; Vera-Ponce de León et al., 2020
Bumble bee (*Bombus* spp.)	High specificity; low complexity	–	–	Colonization resistance	Culturable microbiota; gnotobiotic host; RNAi	Koch and Schmid-Hempel, 2011, 2012; Kwong et al., 2014,b; Praet et al., 2018; Zhang et al., 2020
Burying beetle (*Nicrophorus vespilloides*)	High specificity; high complexity	–	–	Colonization resistance; nutrient acquisition	–	Heise et al., 2019; Shukla et al., 2018a; Shukla et al., 2018b; Vogel et al., 2017a
Cabbage butterfly larva (*Pieris brassicae*)	Low specificity; low complexity; low biomass	–	*P. protegens*: T6SS		Culturable microbiota	Vacheron et al., 2019
Caterpillar	Low complexity; low biomass	Possible constraints: high transit rate of food content, high pH	*E. mundtii*: stress response, resistance to high pH; production and excretion of bacteriocins	Colonization resistance	Culturable microbiota; GFP-tagged *E. mundtii*; RNAi	Hammer et al., 2017; Phalnikar et al., 2019; Shao et al., 2017; Mazumdar et al., 2020 preprint
Coffee borer beetle (*Hypothenemus hampei*)	High specificity; high complexity	–	–	Detoxification via caffeine degradation	Partially culturable microbiota; RNAi	Ceja-Navarro et al., 2015
Fruit fly (*Drosophila melanogaster*)	Low specificity; low complexity	Uracil-dependent distinction of gut microbes via ROS-mediated immune response	*L. plantarum*: acetate metabolism	Nutrition and developmental improvement by co-factor and nitrogen assimilation and supplementation; immune system stimulation	RNAi, gene deletion, reporter genes in the host; gnotobiotic host; genetically amenable bacterial symbionts	Bost et al., 2018; Consuegra et al., 2020; Erkosar et al., 2015; Iatsenko et al., 2018, 2016; Kamareddine et al., 2018; Lee et al., 2018, 2015; Schretter et al., 2018; Storelli et al., 2011; Wong et al., 2017
Honey bee (*Apis mellifera*)	High specificity; low phylotype complexity, but high strain-level diversity	Ileum invaginations, slow transit rate in rectum	*S. alvi*: pili, adhesins, O-antigen, stress response and resistance, organic acid utilization, amino acid synthesis	Colonization resistance; nutrition from fermentation products	Culturable and genetically amenable microbiota; gnotobiotic host; RNAi	Ellegaard and Engel, 2019; Kešnerová et al., 2017; Kwong et al., 2017a; Leonard et al., 2018, 2020; Powell et al., 2016; Raymann et al., 2017; Zheng et al., 2019, 2017
Higher termites	High specificity; high complexity	–	–	Nutrition from fermentation products, organic nitrogen and co-factors	Partially culturable microbiota; RNAi	Hu et al., 2019 ; Tokuda et al., 2018 ; Liu et al., 2019
Lower termites	High specificity; high complexity dominated by protists	–	–	Nutrition from fermentation products, organic nitrogen, and co-factors; colonization resistance	Partially culturable microbiota; RNAi	Brune and Dietrich, 2015, Inagaki and Matsuura, 2018; Maurice and Erdei, 2018; Wertz and Béchade, 2020; Odelson and Breznak, 1983; Tholen and Brune, 2000

Table 1. Continued

Host species	Microbiota characteristics	Host colonization factors	Microbial colonization factors	Beneficial roles	Experimental tractability	References
Mosquito (*Aedes aegypti*; *Culex pipiens pallens*; *Anopheles gambiae*)	Low specificity; low biomass	C-type lectins	*C. neteri:* ompA, biofilm formation	Colonization resistance; hypoxia-induced nutrition and development improvement	Partially culturable and genetically amenable microbiota; gnotobiotic host; RNAi	Coon et al., 2016a, 2017; Correa et al., 2018; Hegde et al., 2019; Rodgers et al., 2017; Song et al., 2018; Valzania et al., 2018; Vogel et al., 2017b; Wei et al., 2017; Xiao et al., 2017
Mountain pine beetle (*Dendroctonus ponerosae*) and pine weevil (*Hylbius abietis*)	High specificity; high complexity	–	–	Detoxification and nutrition improvement via terpenoid degradation	Partially culturable microbiota	Adams et al., 2013, 2011; Berasategui et al., 2017; Boone et al., 2013
Parasitoid wasp (*Nasonia vitripennis*)	Medium complexity; two predominant phylotypes	–	–	Pesticide resistance	Partially culturable microbiota; gnotobiotic host; host gene deletion	Wang et al., 2020
Stinkbug (*Riptortus pedestris*)	High specificity; low complexity; single midgut symbiont *Burkholderia*	Symbiont sorting organ, midgut crypts	Stress resistance (PHA granules), O-antigen and LPS modifications, cork-screw flagella	Host development improvement; insecticide degradation	Culturable symbiont; fluorescence-labelled symbionts and gene deletions; RNAi	Itoh et al., 2018a; Jang et al., 2017; Kikuchi et al., 2020; Kim et al., 2017, 2016, 2013; Lee et al., 2017; Ohbayashi et al., 2015
Tick (*Ixodes scapularis*)	Low specificity; low biomass	–	–	Colonization resistance	RNAi	Narasimhan et al., 2014
Tortoise and leaf-mining beetle (Cassidinae)	Low specificity; low complexity; single foregut symbiont *Stammera*	Foregut symbiont organ	–	Nutrition by facilitating pectin degradation		Salem et al., 2017, 2020
Turtle ant (*Cephalotes rohweri*)	High specificity; low complexity	Proventriculus filter	–	Amino acid provision	Partially culturable microbiota	Hu et al., 2018, Lanan et al., 2016
Wood-feeding beetle (*Odonotaenius disjunctus*)	High specificity; high complexity	Physicochemical changes along the gut	–	Nutrition improvement via lignocellulose degradation		Ceja-Navarro et al., 2019, 2014; Nardi et al., 2006

RNAi, RNA interference of host genes.

The link between gut properties and microbiota is exemplified in bees and caterpillars. In honey bees, the bacterial density is relatively low in the fore- and midgut, but drastically increases, and changes in composition, in the ileum and the rectum (Powell et al., 2014; Martinson et al., 2012). The ileum seems to be selective for bacteria that can adhere to the ileal surface and colonize the epithelial folds, whereas the low transit rate in the rectum and the resulting accumulation of food content favors bacteria that reside in the rectal lumen and degrade non-digested dietary compounds (Powell et al., 2014; Martinson et al., 2012; Engel et al., 2015a). In contrast, the digestive system of caterpillars appears to be a relatively hostile environment for microbes given the relatively low microbial biomass detected (Hammer et al., 2017; Broderick et al., 2004). Microbial colonization is likely hampered by the tube-like morphology of the caterpillar gut (which lacks spatially structured niches for microbes), the extremely high pH in the midgut, the high concentration of immune effectors and the short transit time of luminal content (Dow, 1984; Johnson and Felton, 1996; Jiang et al., 2010; Brinkmann and Tebbe, 2007).

Filtering organs

Certain insects have evolved specific anatomical features that selectively filter bacteria from the environment. For example, stinkbugs house a single gut symbiont of the genus *Burkholderia* in a specialized symbiotic tissue in the midgut. This bacterium is critical for insect growth and development (Kim et al., 2017; Jang et al., 2017), but needs to be selected from the soil environment, which contains thousands of other microbial taxa. In the stinkbug *Riptortus pedestris*, this is achieved by the 'sorting organ', a constricted region between the anterior and posterior midgut. Bacteria of the genus *Burkholderia*, but not *Escherichia coli*, can pass this region and reach the posterior midgut (Ohbayashi et al., 2015). Strikingly, the constricted region degenerates after bacterial colonization, decoupling the anterior from the posterior midgut, and thereby selectively controlling the colonization of the symbiotic tissue (Kikuchi et al., 2020). Turtle ants (*Cephalotes rohweri*) possess a similar mechanism to control gut colonization. Upon larval eclosure and symbiont inoculation via anal–oral trophallaxis (see Glossary), the proventriculus of adult ants (a valve-like structure between fore- and midgut) is coated with a thick,
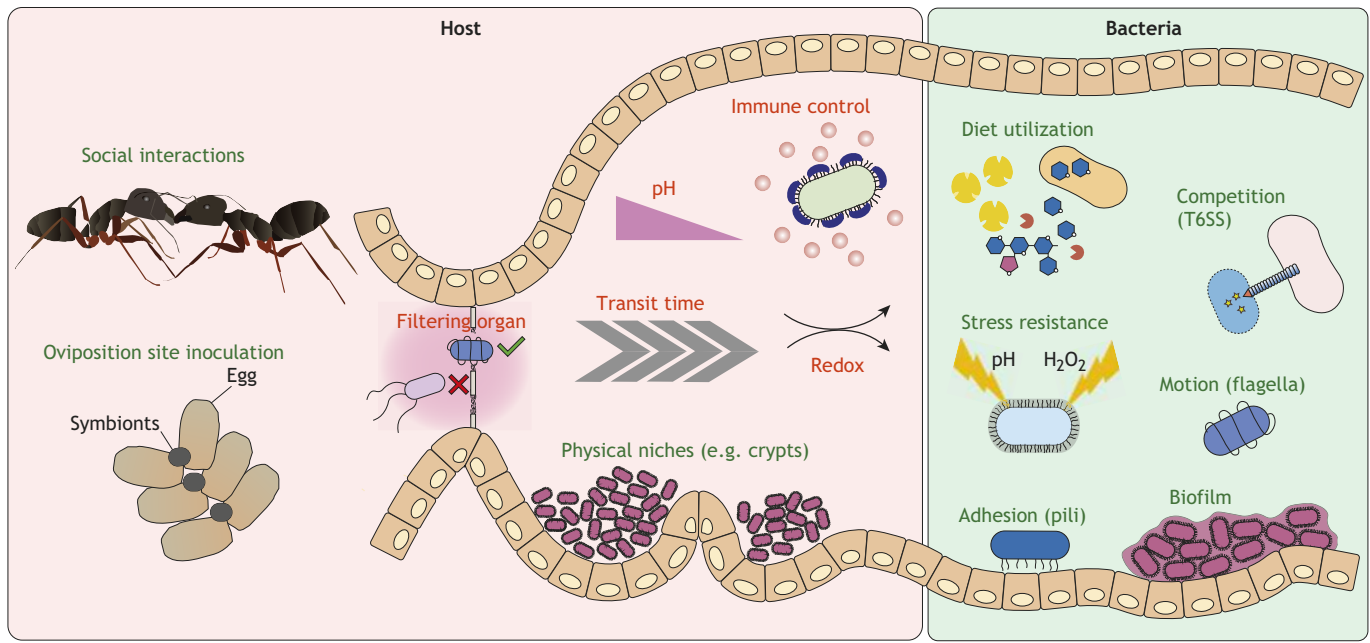
Fig. 1. Mechanisms facilitating or constraining insect gut colonization by bacteria. Overview of mechanisms facilitating (green) or constraining (red) gut colonization. The different factors are separated into host factors (pink background) and bacterial factors (green background). Social interactions and inoculation of eggs with microbes facilitate microbiota transfer. Physical niches provide a spatially structured, and hence protected, microenvironment for microbes. Physical filters, redox potential, pH, short transit time or immune effectors can hinder gut colonization. Bacterial properties that facilitate gut colonization are dietary utilization, stress resistance, interbacterial competition, host adhesion and biofilm formation.

non-cellular mucilaginous layer (Lanan et al., 2016). This acts as a filter that blocks the passage of small particles and hence prevents bacteria from entering the posterior gut after the microbiota has been seeded. In both cases, however, the detailed molecular determinants of these sorting systems have not yet been elucidated.

Immune control

Insects can also mount specific immune responses and secrete effector molecules that regulate gut colonization. In the mosquitoes *Aedis aegyptii* and *Culex pipiens pallens*, host-derived C-type lectins (see Glossary) protect gut bacteria from the bactericidal activity of antimicrobial peptides (AMPs) through binding to carbohydrates on the bacterial cell surface (Pang et al., 2016). Interestingly, C-type lectin and AMP expression are both induced by the gut microbiota via the immune deficiency (IMD) pathway (see Glossary) suggesting that the protective effect is mediated via an immune response. It is possible that specialized gut bacteria are adapted to this immune response by producing cell surface carbohydrates that bind C-type lectin, whereas opportunistic pathogens are not and, hence, can be selectively eradicated by the host (Pang et al., 2016). However, the specificity of this mechanism is not yet fully resolved (Pang et al., 2016).

Further evidence for the existence of mechanisms that allow insects to discriminate between beneficial symbionts and potentially harmful pathogens comes from work on *Drosophila*. In fruit flies, bacteria-derived uracil is perceived as a pathogen signature, as it is produced by opportunistic pathogens, but not by beneficial gut symbionts, triggering the host to produce reactive oxygen species (ROS) and leading to pathogen elimination (Lee et al., 2013a). This highlights that bacterial uridine catabolism is recognized by the host and helps to mount selective immune responses against pathogens (Kim et al., 2020).

Microbiota transplantation

Another strategy of insects to control gut colonization is to ensure microbiota transfer from one generation to the next (reviewed in

Salem et al., 2015). In social and sub-social insects, this is achieved by grooming or trophallaxis (Currie et al., 2006; Marsh et al., 2014; Hongoh, 2010; Koch and Schmid-Hempel, 2011; Powell et al., 2014). However, non-social insects have also evolved specific behaviors and reproductive strategies that ensure the transfer of their microbiota (see Box 1).

In summary, the above studies show that general differences in gut properties or social behavior are important host factors that influence gut microbiota colonization. In addition, insects that rely heavily on the beneficial functions of their gut microbiota have established specific mechanisms that increase partner fidelity between bacteria and host. Elucidating the molecular mechanisms of specialized structures, such as the symbiont sorting organ, will hopefully provide a better understanding of the evolutionary developmental biology of gut symbiont–host relationships.

Microbial mechanisms involved in gut colonization

Bacteria need to be equipped with particular features to allow successful colonization of the selective gut environment. Comparative genomics (Bradley et al., 2018; Kwong et al., 2014; Engel et al., 2012; Warnecke et al., 2007), transcriptome analyses (Tokuda et al., 2018; McNulty et al., 2013; Ryan et al., 2020) or targeted gene disruption strategies (Lee et al., 2013b; Kim et al., 2013) have helped to identify genes underlying gut colonization across animal models. Particularly powerful methods are experimental screens to determine the genome-wide set of host colonization factors, such transposon or CRISPR interference sequencing (TnSeq or CRISPRi; see Glossary) (Liu et al., 2020; Cain et al., 2020). TnSeq approaches have been successfully applied to several mammalian gut symbionts (Cain et al., 2020) and recently also to the bee gut symbiont *Snodgrassella alvi* (Powell et al., 2016). Bacterial colonization factors identified across insect hosts by the above methods can be categorized into the following five

Box 1. Host behaviors ensuring bacterial colonization

In termites and cockroaches, newly-emerged larvae or nymphs acquire their specialized hindgut microbiota by eating the feces of their nestmates (Brune and Dietrich, 2015; Ohkuma and Brune, 2011). This type of fecal transplantation is also referred to as coprophagy and may be widespread among insects (Weiss, 2006) (Fig. 1). It has been reported to be the mode of symbiont transfer in the blood-sucking kissing bug *Rhodnius* spp. (Beard et al., 2002) and was also suggested to play a role in gut microbiota acquisition in honey bees and ants (Koch and Schmid-Hempel, 2011; Lanan et al., 2016; Powell et al., 2014; Onchuru et al., 2018a). Whereas in social insects the transfer of the microbiota is facilitated by group living, a number of solitary insects have also evolved specific mechanisms to transplant the microbiota from one generation to the next. A prevalent transfer mechanism includes egg-smearing or oviposition site inoculation (Fig. 1), in which adults contaminate the egg surface or the intimate environment of oviposition with the gut microbiota, ensuring that the hatchlings acquire the beneficial symbionts upon emergence (Hosokawa et al., 2013; Hayashi et al., 2015; Crotti et al., 2009; Kaltenpoth et al., 2009). Two remarkable forms of oviposition site inoculation have been described for plataspid and urostylidid stinkbugs. In the Plataspidae, the females produce gut symbiont-containing 'capsules', which are deposited between the laid eggs (Fukatsu and Hosokawa, 2002). The newly hatched nymphs actively feed on the symbiont capsules, resulting in gut symbiont colonization. In the Urostylididae, the females secrete and distribute jelly-like material onto the egg mass (Kaiwa et al., 2014). Intriguingly, the nymphs not only acquire the gut symbiont from the jelly-like matrix but also obtain essential nutrients stored in the jelly. This allows them to hatch in midwinter and grow in the absence of their natural food source, i.e. plant sap. These two examples show how gut symbiont–host co-evolution can drive the development of unique reproductive and symbiont transfer mechanisms in some insects.

categories: stress response, immune resistance, extracellular structure, metabolic capabilities and interbacterial competition.

Stress response

A recurrent pattern across several insect species is that genes involved in the stress response are critical for gut colonization, corroborating that gut microbes are exposed to physiological challenges and need to develop mechanisms that allow them to withstand harsh conditions. In the genome-wide screen for colonization factors of *S. alvi*, genes involved in protein quality control, glutathione synthesis (antioxidant) and SOS DNA-damage response were beneficial during bee gut colonization (Powell et al., 2016). Transcriptome analysis of the caterpillar gut colonizer *Enterococcus mundtii* showed that genes involved in the response to alkaline stress are differentially regulated during gut passage, likely facilitating resistance to the highly alkaline conditions (pH 10–12) present in the digestive tract of these insects (Mazumdar et al., 2020 preprint). The *Burkholderia* gut symbiont of *R. pedestris* accumulates intracellular granules of polyhydroxyalkanoate (PHA) during gut colonization, and deletion of genes essential for PHA granule production decreases colonization levels (Kim et al., 2013; Jang et al., 2017). Although these granules have been linked to bacterial tolerance to various stresses (Kadouri et al., 2003), their precise role in gut colonization is not yet understood.

Immune resistance

Gut bacteria also need to be capable of withstanding host defense mechanisms. In both the bee gut symbiont *S. alvi* and the stinkbug symbiont *Burkholderia*, pathways for the assembly and modification of O-antigen (see Glossary) biosynthesis are beneficial for gut colonization (Powell et al., 2016; Kim et al., 2017, 2016). In pathogens, these LPS modifications change the surface charge and integrity of the outer membrane, promoting resistance to antimicrobials and evasion of the host's innate immune response (Lindell et al., 2012; Post et al., 2012; Ilg et al., 2009). As immune effectors are known to be released into the gut tissue, it seems conceivable that beneficial gut symbionts also need to withstand such host defenses. Indeed, several bee gut bacterial symbionts were shown to exhibit elevated resistance towards honey bee antimicrobial peptides (Kwong et al., 2017a).

Extracellular structures

Microbes may need to attach to the epithelial cell envelope of the host or form biofilms to persist in the gut and to engage in interactions among each other and with the host. In the bee gut, *S. alvi* forms biofilm-like structures on the host epithelium together with other symbionts (Martinson et al., 2012; Emery et al., 2017; Engel et al., 2015a). Accordingly, genes for adhesion, such as Type IV pili (see Glossary) and trimeric autotransporters (see Glossary), were shown to be important gut colonization factors, and mutants of the pili genes show impaired biofilm formation *in vitro* (Powell et al., 2016), suggesting they have reduced colonization capabilities. Likewise, a mutant of the outer membrane protein OmpA in the Enterobacteriaceae *Cedecea neteri* shows impairment in biofilm formation and colonization of adult mosquitoes, corroborating the importance of adhesion for gut colonization (Hegde et al., 2019).

Evidence for the importance of extracellular bacterial structures in gut colonization comes from the *Burkholderia* gut symbiont of *R. pedestris*. Passage of the sorting organ and colonization of the midgut crypts is dependent on a functional flagellum (Ohbayashi et al., 2015). Motile *E. coli* bacteria are not able to pass through the organ, suggesting that specific properties of the flagellum of *Burkholderia* enable colonization (Kikuchi et al., 2020). Indeed, using advanced microscopy, it was observed that the flagellum of the symbiont is wrapped around the cell body like a cork screw (Kinosita et al., 2018). This specific utilization of the flagellum may propel the bacterium forward through the mucus-filled constricted area of the sorting organ, facilitating host colonization.

Metabolic capabilities

As microbes need to obtain energy and nutrients to replicate in the gut, their metabolic capabilities to utilize the available resources, and synthesize those that are missing, constitute important colonization factors. The honey bee gut symbiont *S. alvi* obtains energy from the respiration of organic acids (Kwong and Moran, 2016) and, accordingly, genes for lactate and acetate utilization, as well as the TCA cycle, have strong fitness benefits *in vivo* (Powell et al., 2016). Moreover, amino acid and co-factor biosynthesis genes were found to be important for host colonization, highlighting that despite the nutrient-rich diet of the host, some metabolites are not readily available to bacteria in the distal hindgut, possibly because of prior host absorption or competition with other microbes (see below).

The importance of metabolic genes for gut colonization was also demonstrated in an evolution experiment conducted with the fruit fly gut symbiont *Lactobacillus plantarum*. Passaging this bacterium through the gut, or its addition to the insect's diet, led to the emergence of variants with improved colonization capabilities and host fitness effects (Martino et al., 2018). Adaptive mutations were found in *ackA*, an acetate kinase gene involved in the conversion of acetate to acetyl-phosphate (Martino et al., 2018).

The importance of dietary adaptation for host colonization is particularly evident in the gut microbiota of many herbivorous insects. These communities harbor large arsenals of metabolic

genes, including glycoside hydrolase and polysaccharide lyase, for the extraction of energy and nutrients from non-digestible plant-derived molecules such as lignin, cellulose, hemicellulose or pectin (Tokuda et al., 2018; Vera-Ponce de León et al., 2020; Engel et al., 2012; Zheng et al., 2019; Liu et al., 2019; Wertz and Béchade, 2020). However, as plant polysaccharide-degrading gut symbionts are often not culturable or amenable to genetic manipulation, it remains unclear which of their carbohydrate breakdown genes are most important for host colonization, and under which conditions.

Interbacterial competition

Nutrients and space are limited in the animal gut. Therefore, many gut symbionts are in competition for host colonization (Coyte et al., 2015) and need to be equipped with functions that allow them to either evade niche competition or engage in inter-microbial warfare. Bumble bees and honey bees are colonized by distinct lineages of the same gut bacteria (Kwong and Moran, 2016; Bonilla-Rosso and Engel, 2018). For two of these gut bacterial lineages, *S. alvi* and *Lactobacillus* Firm-5, it was shown that microbiota-depleted honey bees can be colonized with strains isolated from bumble bees, although at lower levels. However, colonization is completely abolished in the presence of competitor strains isolated from honey bees (Ellegaard et al., 2019; Kwong et al., 2014, 2017b). A similar observation was made for gut colonization of the stinkbug *R. pedestris*: although non-symbiotic *Burkholderia* are able to pass the restricted region and provide beneficial effects to the host, they are outcompeted when co-inoculated with native competitor strains (Itoh et al., 2019). These examples highlight the importance of interbacterial competition for niche colonization and host-symbiont specificity. Accordingly, co-existing symbionts often evade competition for nutrients by metabolic diversification as reflected by the high variation in gene content for polysaccharide utilization in gut symbionts of mammals and certain insects (Zheng et al., 2019; Tokuda et al., 2018; Sonnenburg et al., 2010; Flint et al., 2012) (Ellegaard et al., 2019; Ellegaard and Engel, 2019).

The production of molecules that harm other microbes can also provide competitive advantages in multi-species communities and hence facilitate gut colonization (Garcia-Bayona and Comstock, 2018). Type 6 secretion systems are prime examples. These nanomachineries allow bacteria to deliver toxic effectors and kill neighboring cells, and they have been shown to be important colonization factors in the mammalian gut in the presence of competition (Wexler et al., 2016). Evidence for a role of T6SS in insects comes from the pathogen *Pseudomonas protegens*, which uses its T6SS to invade the gut microbiota of cabbage butterfly (*Pieris brassicae*) larvae (Vacheron et al., 2019). T6SS and corresponding toxin genes are also highly abundant in several gut symbionts of bees (Steele et al., 2017; Engel et al., 2015b; Kwong et al., 2014). Although experimental evidence for the role of T6SS in these species is currently lacking, the conservation of the corresponding genes across strains isolated from different host species, their expression *in vivo* and the large and highly diverse set of effector proteins suggests an important role in the bee gut (Steele et al., 2017; Powell et al., 2016). We can expect that these nanomachineries are also important in other insect gut symbioses, considering the ubiquity of T6SS among bacteria (Bingle et al., 2008; Coulthurst, 2019).

Despite recent progress, we are still in an early stage of understanding the importance of bacterial colonization factors of the insect gut. For many insect gut symbionts that exhibit intriguing host colonization phenotypes, the underlying genetic determinants of colonization have not yet been identified. For example, the Gammaproteobacterium *Frischella perrrara* adheres to a small region in the honey bee gut, where it causes a host melanization response (see Glossary) that appears as a brown band around the inner gut circumference (Engel et al., 2015a; Emery et al., 2017). Other gut bacteria of bees do not induce this phenotype, suggesting a symbiont-specific interaction with the host. Moreover, in lab-reared fruit flies, gut bacteria of the family Acetobacteraceae are typically transient colonizers (Blum et al., 2013; Pais et al., 2018). However, several Acetobacteraceae isolates that persist and proliferate in the *D. melanogaster* foregut were recently identified (Pais et al., 2018), but it remains unclear what promotes their more permanent colonization. The comparison and genetic manipulation of related gut bacteria with different colonization properties provides a great opportunity to unravel the molecular basis of persistence in insect models (Ma and Leulier, 2018).

Mechanisms underlying beneficial roles of the insect gut microbiota

Despite a high variation in community structure, the gut microbiota of many insects can have beneficial effects on the host. The best-studied functions are polysaccharide breakdown, detoxification, nutrient provisioning and colonization resistance, which will be discussed in the following section. However, in some cases the underlying mechanism has not yet been elucidated or the beneficial effect is mediated by yet another function, such as in the case of the gut microbiota of mosquitoes (see Box 2).

Box 2. The role of the gut microbiota in the larval development of mosquitoes

In contrast to many other insects, mosquitoes do not harbor specialized microbial communities in their gut, which rather seems to be colonized by bacteria present in their immediate environment (Coon et al., 2016b). Coon et al. (2014) found that when mosquito larvae (*Aedis aegypti* and *Anopheles gambiae*) were reared under sterile conditions, they could not develop to adulthood (Coon et al., 2014). Supplementing germ-free larvae with bacteria rescued development, independent of the bacterial species used (Coon et al., 2014). Intriguingly, not only bacteria, but also eukaryotes, including yeasts, insect cells and algae, were able to rescue larval development (Valzania et al., 2018). Moreover, the beneficial effect of the microbiota was not only found for detritivore (see Glossary) mosquitoes, but also for predaceous species (Coon et al., 2020). These findings highlight that the gut microbiota of mosquitoes, or more broadly the ingestion of living organisms, plays a conserved role for host development, and that this beneficial effect must be mediated by a relatively unspecific interaction with the host. In a follow-up study, it was described that the presence of bacteria (and other organisms) induces hypoxia in the gut, which serves as a signal for mosquito development via activation of the host transcription factor hypoxia-induced factor (HIF) (Coon et al., 2017; Valzania et al., 2018). Moreover, sterile mosquitoes exhibit alterations in gene expression related to the assimilation of nutrients, suggesting a role for the microbiota-induced hypoxia in nutrient acquisition (Vogel et al., 2017b). This mechanism was challenged by another study, which highlighted that sterile larvae could develop to adulthood when provided with a nutrient-rich diet and in the presence of high amounts of heat-inactivated bacteria (Correa et al., 2018). However, sterile larvae had much longer development times and showed stunted growth as opposed to conventional larvae, suggesting that nutrition alone cannot be the sole role of the gut microbiota. Together, these studies exemplify that relatively unspecific microbial associations can mediate critical host phenotypes in insects, and that the underlying mechanism can be multifaceted, including the activation of host pathways in response to microbial signals.

Symbiotic digestion of plant-derived polysaccharides

Non-digestible dietary compounds typically end up in the animal hindgut, where gut bacteria can utilize them to cover their own energy and nutrient requirements, but also to convert them into metabolites that are of nutritional value for the host.

The most prominent example is the degradation and fermentation of polysaccharides, which leads to the production of short-chain fatty acids (SCFAs), such as butyrate, acetate or propionate (Cummings et al., 1987). In the mammalian gut, these metabolites are absorbed by

the host and serve as energy sources for colonocytes (see Glossary) or as building blocks for gluconeogenesis and *de novo* lipogenesis (Scott et al., 2013; Donohoe et al., 2011; den Besten et al., 2013). Moreover, SCFAs can have profound effects on neural and immune pathways in mammals (Cryan and Dinan, 2012).

Polysaccharide breakdown is also the predominant metabolic activity of the gut microbiota in many herbivorous insects (Fig. 2) (Wertz and Béchade, 2020). This degradation is facilitated by microbiota-derived glycoside hydrolases or polysaccharide lyases,
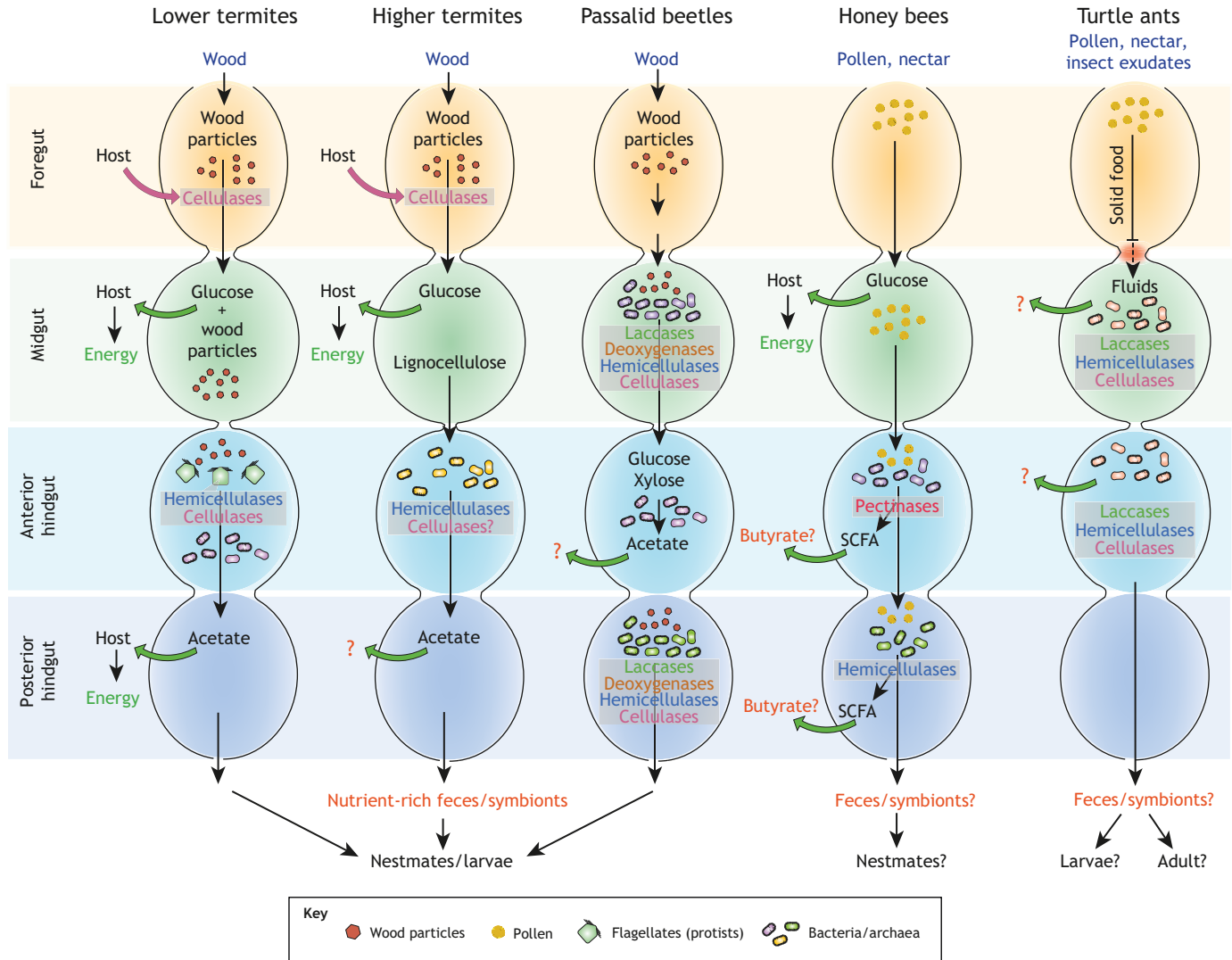

Fig. 2. Degradation of dietary carbohydrates compared across insects. Lower and higher termites produce endogenous cellulases for lignocellulose degradation in the fore- and midgut. Released glucose is absorbed in the midgut and used as energy/carbon source. In lower termites, cellulose- and hemicellulose-degrading flagellates continue to degrade lignocellulose in the anterior hindgut, although the bacterial contribution to lignocellulose degradation is debated. This is in contrast to higher termites, where hindgut flagellates are absent and bacteria are thought to facilitate further lignocellulose degradation using hemicellulases and possibly also cellulases. In wood-feeding passalid beetles (*Odontotaenius disjunctus*), distinctive microbial communities across gut compartments enable a spatially separated, step-wise lignocellulose deconstruction: lignocellulose depolymerization starts in the midgut and continues in the posterior hindgut, facilitated by the availability of oxygen and the presence of diverse microbe-encoded enzymes. In contrast, fermentation of sugars occurs in the anterior hindgut under anoxic conditions (Ceja-Navarro et al., 2019; Nardi et al., 2006; Ceja-Navarro et al., 2014). In both termites and wood-feeding beetles, symbionts and digested nutrients are transferred to adult and larval nestmates via anal–oral trophallaxis. In honey bees, the degradation of the primary plant cell wall of pollen is supported by pectinases encoded by *Gilliamella apicola* in the anterior hindgut and by hemicellulases encoded by *Bifidobacterium* and *Lactobacillus* spp. in the posterior hindgut (Zheng et al., 2019). Turtle ants harbor microbial communities that encode carbohydrate-degrading enzymes in their mid- and hindgut. However, solid food such as plant fibers are unlikely to pass to the midgut owing to the thick, non-cellular layer between the fore- and midgut (shown in red). It is speculated that the mid-/hindgut symbionts are transmitted to either larvae or the infrabuccal pocket of adults (a cavity located beneath the tongue of worker ants), where food digestion may occur. To what extent anal–oral trophallaxis plays a role in nutrition and symbiont transfer in honey bees and turtle ants is not completely understood. Question marks indicate processes that are not yet fully understood. Grey background shading indicates key enzymes involved in carbohydrate degradation in a given gut compartment.

which cleave terminal sugar residues or internal glycosidic bonds in homo- or heteropolymers of polysaccharides. The released sugar residues can, in theory, be absorbed by the host and used as an energy source (Caccia et al., 2007; Treherne, 1957; Crailsheim, 1988). In tortoise leaf beetles, for example, an extracellular gammaproteobacterial symbiont, located in a specific organ in the foregut, releases pectinases to help the host degrade plant-derived polysaccharides and absorb the released sugars (Salem et al., 2017).

In most other herbivorous insects, however, it is believed that the gut microbiota itself utilizes the liberated sugars and ferments them into SCFAs (Fig. 2). These metabolic end-products are then released into the gut lumen, where they can be absorbed by the host to support nutrition, as reported for mammals (Scott et al., 2013; Koropatkin et al., 2012). In this regard, one of the arguably best-studied insects is the lower termite, which feeds on lignocellulose (Brune, 2014). The lower termite hindgut microbiota contains highly specialized protists, which are key for lignocellulose degradation and whose depletion has a huge effect on host survival (Cleveland, 1925, 1924). The current understanding of this symbiosis is that a complex mix of host- and microbiota-derived enzymes digests the cellulose and hemicellulose components of lignocellulose (Brune, 2014). The major fermentation product is acetate, which is transported into the hemolymph (see Glossary) and respired by the host (Tholen and Brune, 2000). It has been suggested that 77–100% of the energy requirements of a termite could be covered by the oxidation of the acetate produced in the hindgut, highlighting the crucial role of microbiota-mediated polysaccharide degradation for host nutrition (Odelson and Breznak, 1983).

Similarly, wood-feeding passalid beetles (*Odonotaenius disjunctus*) harbor highly complex gut microbiota, which are composed of fungi, archaea and bacteria and aid in the degradation of lignocellulose (Ceja-Navarro et al., 2019). As in termites, large amounts of SCFAs accumulate in the hindgut of these beetles, likely serving as a major energy source for the adults and their offspring (Fig. 2). Genomic analyses have revealed that the microbial communities of cockroaches, honey bees and cephalotes turtle ants are also capable of degrading plant polysaccharides (Tokuda et al., 2018; Vera-Ponce de León et al., 2020; Engel et al., 2012; Zheng et al., 2019; Liu et al., 2019). In cockroaches and honey bees, SCFAs accumulate in the gut, highlighting that polysaccharide breakdown and fermentation are the dominant microbial processes in these insects (Wada-Katsumata et al., 2015; Zheng et al., 2017). In honey bees, the presence of the gut microbiota results in increased amounts of butyrate in the hemolymph (Zheng et al., 2017). However, the transfer of this SCFA from the gut into the hemolymph and its contribution to host nutrition and metabolism still needs to be experimentally demonstrated. In contrast to beetles and termites, honey bees survive without their microbiota under laboratory conditions (Kešnerová et al., 2017; Raymann et al., 2017), suggesting differences in the contribution of microbiota-mediated polysaccharide degradation to host nutrition and energy extraction.

Insects can substantially differ in how polysaccharide degradation is partitioned among the different symbiotic partners. In contrast to mammals, many insects encode glycoside hydrolases in their genome and, hence, do not necessarily rely on microbial associations for polysaccharide breakdown (Fig. 2) (Fischer et al., 2013). In lower termites, lignocellulose degradation involves both host-derived glucanases in the fore- and midgut and protist-derived cellulases in the hindgut. In wood-feeding higher termites, protists are absent. Instead, bacterial hindgut communities seem to support lignocellulose degradation (Hu et al., 2019; Tokuda et al., 2018; Liu et al., 2019; Warnecke et al., 2007). In particular, a divergent group of Spirochaetes has adopted the function of hemicellulose degradation via horizontal

acquisition of the necessary genes (Tokuda et al., 2018). The American cockroach (*Periplaneta americana*) does not encode any of the enzymes required for the hydrolysis of complex dietary polysaccharides (e.g. pectin) and therefore seems to rely on its microbial symbionts for their degradation (Bignell, 1977). Isolation, cultivation and genomic sequencing of *Bacteroidetes* from *P. americana* revealed several species which possess the ability to degrade pectin, cellulose and starch (Vera-Ponce de León et al., 2020).

The case of the termites also illustrates that the digestion of plant-derived polysaccharides occurs step-wise, and is not only divided between different symbiotic partners but also across gut compartments (Tokuda et al., 2018; Tokuda et al., 2012). This is confirmed by studies on wood-feeding passalid beetles, honey bees and also turtle ants (see Fig. 2) (Ceja-Navarro et al., 2019; Nardi et al., 2006; Ceja-Navarro et al., 2014; Zheng et al., 2019; Lanan et al., 2016; Hu et al., 2018).

In addition to polysaccharide degradation, other digestive functions of gut microbes are certainly also contributing to the release of dietary-derived metabolites with possibly beneficial effects on the host. In honey bees, the gut microbiota facilitates the digestion of flavonoids, ω-hydroxy acids and phenolamides (Kešnerová et al., 2017). Although not necessarily supporting host nutrition, the degradation of such compounds could influence other aspects of host health. Flavonoids, for example, have been shown to regulate weight gain in mammals by influencing host signaling or increase antioxidant activity in the gut (Thaiss et al., 2016).

Degradation of toxic compounds
The digestive capabilities of gut microbes can also aid in removing or inactivating toxic compounds from the diet. Although some insects encode such functions in their genome, detoxification symbioses have been described across a wide range of hosts (reviewed in Itoh et al., 2018b). They are of particular relevance for herbivorous insects, given that plants produce a wide range of phytotoxins (Itoh et al., 2018b).

The mountain pine beetle (*Dendroctonus ponerosae*) and the pine weevil (*Hylbius abietis*) are two major pests of conifer forests. Both seem to have adapted to the highly toxic terpenoids present in the bark of pine trees with the help of their gut microbiota. In the mountain pine beetle, the gut microbiota is enriched in bacterial genes for diterpene degradation (Adams et al., 2011, 2013), and several Gammaproteobacteria isolates are capable of degrading diterpenes *in vitro* (Boone et al., 2013). In pine weevils, microbiota depletion results in an increase of terpenoids in the gut, and gene clusters for terpenoid degradation can be identified among the microbiota (Berasategui et al., 2017). However, insects with intact microbiota exposed to diterpenes do not show increased survival when compared with microbiota-depleted ones, but they do lay more eggs (Berasategui et al., 2017), suggesting that the microbiota increases the nutritional value of these compounds rather than playing a role for detoxification.

The coffee borer beetle (*Hypothenemus hampei*) engages in a detoxification symbiosis to facilitate dietary adaptation to coffee beans, which contain high concentrations of the toxic alkaloid caffeine. Metagenomics revealed that the beetle harbors a distinctive gut microbiota, dominated by *Pseudomonas* species, which are shared across beetles from different coffee-producing countries (Ceja-Navarro et al., 2015). Pseudomonads isolated from these beetles could grow on caffeine as a sole carbon and nitrogen source, and reinstated caffeine degradation in beetles that were pretreated with antibiotics (Ceja-Navarro et al., 2015).

In addition to facilitating dietary adaptation, multiple pest species have been reported to harbor gut symbionts that are able to degrade insecticides (Almeida et al., 2017; Itoh et al., 2018b). When

exposed to soils contaminated with the insecticide fenitrothion, *R. pedestris* was shown to acquire specific strains of its *Burkholderia* gut symbiont, capable of degrading the insecticide and increasing host survival (Itoh et al., 2018a). As another example, the wasp *Nasonia vitripennis*, was found to possess higher survival after treatment with the pesticide atrazine in the presence of its microbiota as compared to germ-free wasps (Wang et al., 2020). Atrazine treatment over several wasp generations led to an increase in atrazine resistance, which was accompanied by a change in gut microbiota composition. Strikingly, the atrazine resistance was carried over to subsequent wasp generations in the absence of selective atrazine treatment (Wang et al., 2020). These studies highlight the potential of the gut microbiota to increase the adaptive potential of its insect host, which has important implications for the management of important pest and pollinator insects.

Provisioning of organic nitrogen and production of co-factors and vitamins

Organic nitrogen and vitamins can be limiting nutritional factors. The gut microbiota can complement these dietary needs of the host. For example in wood-feeding termites, which feed on an extremely nitrogen-poor diet, hindgut bacteria fix atmospheric nitrogen or recycle uric acid into ammonia, which is subsequently assimilated by the bacteria in the form of amino acids. To make these compounds accessible to the host, termites feed their feces to nestmates (anal–oral trophallaxis, see Fig. 2). In the midgut of the nestmates, proteolytic digestion occurs and the released amino acids are absorbed (Brune, 2014).

Provisioning of nitrogen is also an important microbiota function in turtle ants. In an elegant stable isotope tracer experiment, Hu et al. (2018) showed that the gut microbiota recycles urea into essential amino acids, which are subsequently transferred into the hemolymph of the host. How these organic compounds are reabsorbed by the host is unclear. However, antibiotic treatment of workers being sustained on urea as the only nitrogen source leads to decreased survival (Hu et al., 2018).

A potential role in vitamin provisioning was described for a gut symbiont of the African cotton stainer, *Dysdercus fasciatus*: symbiont loss negatively impacts host development and survival, but this can be reverted by the supplementation of additional vitamin sources

within the diet (Salem et al., 2014). Multiple studies have also demonstrated an important role of the gut microbiota for nitrogen and co-factor assimilation in *Drosophila* (Storelli et al., 2011; Sannino et al., 2018). In a large-scale experimental screen based on chemically defined fruit fly media, Consuegra et al. (2020) comprehensively analyzed the extent to which two major gut symbionts of *Drosophila* (*L. plantarum* and *A. pomorum*) complement the nutritional requirements of the host during larval development. The study revealed that the two gut symbionts differentially provide the host with all essential amino acids, many vitamins and trace metals. Previous work has shown that *L. plantarum* can also support larval development by enhancing host peptidase gene expression, leading to increased intestinal proteolytic activity and amino acid availability (Erkosar et al., 2015). Combined, these findings exemplify that gut bacteria can support host nutrition by several different mechanisms. Nutritional screens with chemically defined media, such as those conducted by Consuegra et al. (2020), could reveal important insights into the integrated nutritional network of the gut microbiota and its host in other tractable insects.

Colonization resistance against pathogens

The digestive system is constantly exposed to the environment, making it vulnerable to pathogens that either establish in the gut and cause inflammation or use the gut as an entry point to cause systemic infections. The gut microbiota can protect the host against such pathogenic attacks, a beneficial effect that has been referred to as colonization resistance (Bohnhoff and Miller, 1962). Colonization resistance can be mediated by different mechanisms as indicated in Fig. 3. It has been extensively studied in insect vectors (Saldaña et al., 2017; Huang et al., 2020; Dennison et al., 2014) because of the potential to harness their gut microbiota to interfere with pathogen transmission and hence reduce the spread of human diseases (Wang et al., 2012).

One of the major mechanisms by which the mammalian gut microbiota protects the host against pathogens is competition for nutrients and space (Mullineaux-Sanders et al., 2018). In insects, this is likely also the case, but probably only for species that harbor dense gut microbial communities. Several studies have reported that the gut microbiota can induce physicochemical changes in the insect gut, which hinder colonization by potential pathogens. For example, perturbation of the gut microbiota alters the peritrophic matrix (see
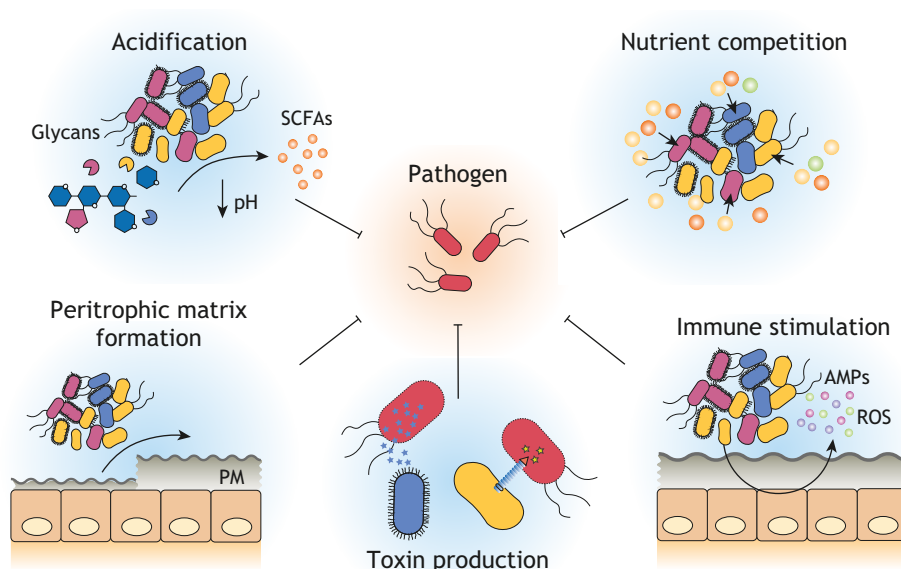

Fig. 3. Five different mechanisms of colonization resistance. Glycan degradation and sugar fermentation lead to the production of short chain fatty acids (SCFAs) and subsequently to the acidification of the gut environment, which can hinder pathogen colonization. By utilizing the same nutrients as pathogens, gut microbiota members can outcompete pathogens. Microbiota members trigger immune responses such as production of reactive oxygen species (ROS) or antimicrobial peptides (AMPs), which incoming pathogens are susceptible to. Similarly, microbiota members can modulate the gut surface, such as the peritrophic matrix (PM), hindering pathogen colonization. Finally, gut microbes may secrete toxins that kill incoming pathogens.

Glossary) of the deer tick (*Ixodes scapularis*), which leads to a reduction of the pathogen *Borrelia burgdorferi* (Narasimhan et al., 2014). Likewise, in the malaria vector *Anopheles coluzzii*, the synthesis and integrity of the peritrophic matrix is dependent on the microbiota, thus limiting the growth of and systemic infection by certain gut bacterial taxa and reducing vector competence (Rodgers et al., 2017; Song et al., 2018).

Changes in gut pH may also be a major mechanism by which the microbiota mediates resistance. Colonization of tsetse flies (*Glossina morsitans morsitans*) with the bacterium *Kosakonia cowanii* leads to the acidification of the midgut, inhibits proliferation of trypanosomes (the causative agents of human sleeping sickness) and increases host survival after infection with *Serratia marcescens* (Weiss et al., 2019). Likewise, acetate production by the gut microbiota of the dampwood termite *Zootermopsis nevadensis* also provides resistance to *S. marcescens* (Inagaki and Matsuura, 2018), and in honey bees, the same pathogen is suppressed in the presence of a normal microbiota (acidifying the gut environment) (Raymann et al., 2017; Raymann et al., 2018). Colonization resistance against the trypanosomatid pathogen *Critidia bombi* in bumble bees may also be mediated by the capability of the microbiota to modulate the pH (Koch and Schmid-Hempel, 2011; Koch and Schmid-Hempel, 2012): two recent studies have identified bacteria that inhibit the growth of *Crithidia in vitro* (Praet et al., 2018; Palmer-Young et al., 2019), and in one of them it was shown that the acidification of the culture medium is sufficient to mediate the inhibitory effect (Palmer-Young et al., 2019).

In vector insects, multiple reports have shown that experimental depletion of the microbiota dampens the immune response in the gut. Moreover, it was observed that such microbiota-depleted insects often show elevated pathogen susceptibility. This led to the idea that the microbiota can control vector competence via its modulatory effect on the host immune system (Dong et al., 2009; Dennison et al., 2014). Several bacteria isolated from the gut of *Anopheles* spp. can induce immune pathways/effectors that inhibit *Plasmodium* colonization (Bahia et al., 2014; Eappen et al., 2013). Likewise, certain gut bacteria were able to control Dengue virus infections in mosquitoes via the induction of the Toll pathway (see Glossary) and the production of antimicrobial peptides (Xi et al., 2008; Ramirez et al., 2012). It is worth noting that the immuno-modulatory effect of gut microbes can also decrease colonization resistance. A fungus (*Talaromyces*) associated with the midgut of *Anopheles aegypti* was shown to increase vector competence of mosquitoes for Dengue viruses by down-regulating genes encoding blood-digesting enzymes (Angleró-Rodríguez et al., 2017). Similarly, infection of mosquitoes with the fungus *Beauveria bassiana* results in increased mortality in the presence of the microbiota by dampening antimicrobial peptide production and dual oxidase expression, resulting in the systemic spread of the pathogen *S. marcescens* (Wei et al., 2017).

Finally, the gut microbiota can also employ direct mechanisms to inhibit or kill pathogenic invaders (see 'Interbacterial competition'). The dominant gut symbiont of the caterpillar *Spodoptera littoralis*, *Enterococcus mundtii*, secretes a bacteriocin, a peptide-based toxin that acts against closely-related entomopathogenic (see Glossary) *Enterococcus* strains. The purified peptide, named mundticin, was sufficient to suppress bacterial invasion of entomopathogenic *Enterococcus* in the caterpillar gut (Shao et al., 2017). Contact-dependent killing mechanisms, such as the delivery of toxins via T6SSs can also present a potential mechanism of colonization resistance (Anderson et al., 2017). However, the requirement of physical proximity and the high specificity of some of the effectors may limit the effectiveness of T6SSs to act against pathogens, and it yet needs to be demonstrated in insects. There are many other

examples of insect gut microbes that confer colonization resistance; however, the underlying mechanisms are often not fully understood. A particularly curious case is the burying beetle *Nicrophorus vespilloides*, which appears to harness its gut microbiota to regulate the microbiome of the feeding environment of its progeny (Box 3).

Outlook

This Review highlights recent advances in understanding molecular processes involved in insect-gut microbiota associations. Although the structure and function of the gut microbiota substantially differ between insects, we are starting to see common patterns emerging regarding the mechanisms involved: e.g. similar bacterial genes are needed for gut colonization, the breakdown and utilization of complex carbohydrates is a step-wise, compartmentalized process and there is evidence for common mechanisms of colonization resistance. Yet, we are only just starting to understand these processes and, despite the implications for fundamental and applied science, considerable challenges are faced when studying insect–gut microbiota interactions.

Genome-wide screens, such as TnSeq have the potential to identify bacterial factors needed for the colonization of diverse insect gut environments. However, such approaches depend on the genetic manipulation of bacteria and the experimental tractability of the host. Yet, recent advances in the culturing of gut bacteria from diverse host species combined with the availability of versatile and broadly applicable genetic tools hold promise for the future (Leonard et al., 2018; Cain et al., 2020; Dantas et al., 2013; Wexler and Goodman, 2017).

The inability to genetically manipulate many insects presents another important limitation for molecular studies of specialized insect–gut microbiota associations (Emery et al., 2017; Engel et al., 2015a; Kikuchi et al., 2020; Ohbayashi et al., 2015; Lanan et al., 2016). As outlined above, insects can control bacterial colonization,

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Box 3. The curious role of the burying beetle microbiota in animal cadaver conservation

Animal cadavers are nutrient-rich resources. However, they are highly susceptible to microbial overgrowth and decomposition. Therefore, necrophagous animals need to consume the cadaver shortly after death or preserve it from decay. The burying beetle *Nicrophorus vespilloides* uses small animal cadavers (e.g. mice) as the major nutrient source for its developing larvae. The beetle buries the cadaver underground, prepares a feeding cavity for the larvae and smears anal and oral secretions on top. The resulting matrix-like structure controls the amount and type of microbes that can grow on the carcass, thereby preventing its decomposition (Shukla et al., 2018a). Nutritive enzymes, lysozymes and antimicrobial peptides are transmitted to the carcass via the secretions (Vogel et al., 2017a; Shukla et al., 2018b). Moreover, the matrix structure also contains a large variety of bacteria and yeasts present in the gut microbiota of adult and larval beetles (Vogel et al., 2017a; Shukla et al., 2018b). It is believed that the combined action of the released enzymes and immune effectors, together with the beetles' gut microbiota, shape the carcass environment and hence aid in the extra-intestinal digestion, detoxification and defense of the host's nutritional resource (Vogel et al., 2017a; Shukla et al., 2016; Shukla et al., 2018a,b). This illustrates that insect gut microbial communities can have additional functions that go beyond their host-associated niche. Yet, the relative contribution of host-derived versus microbiota-derived functions to the preparation of the feeding cavity needs to be elucidated. However, a few microbiota members, including *Yarrowia* yeasts and *Serratia*, which are characteristic for the burying beetle, were shown to produce antimicrobials against microorganisms present in beetle-tended carcasses (Vogel et al., 2017a; Heise et al., 2019).

and may even distinguish between pathogenic and beneficial gut symbionts (Kim et al., 2020). However, how widespread such host selection mechanisms are and how they have evolved is currently unclear. To follow up on these questions, there is a need to map out the molecular processes at play between the microbiota and the immune system and to go beyond what is currently known from fruit flies and mosquitoes. Future studies will hopefully profit from the recent advances in CRISPR-based genome editing tools and their applicability to non-model insect species. An alternative, but highly promising approach is to employ gut bacteria to interfere with host gene functions. Two studies have shown that exogenous dsRNA expression by gut bacterial symbionts results in highly efficient gene knockdowns, not only in the gut, but also in distal host tissues (Leonard et al., 2020; Whitten et al., 2016).

One of the most important beneficial functions of the insect gut microbiota is the support of the host's nutrition. However, which metabolites are taken up by the host, where they are absorbed in the gut and how they mediate beneficial effects is far from clear. Metabolomics approaches combined with advanced imaging technologies and isotope- or click chemistry-based labeling methods (see Glossary) are likely to provide important new insights in the future (Alonso-Pernas et al., 2017; Hu et al., 2018).

Furthermore, most studies have investigated the effects of the gut microbiota on host survival or development, which are easily measurable fitness effects. However, benefits provided by the gut microbiota may be subtle or context-dependent, or linked to complex behavioral traits of the host. For example, there is evidence that the gut microbiota can influence the olfactory behavior, dietary intake or cognition of the host, but only some of the underlying mechanisms have been identified (Wong et al., 2017; Schretter et al., 2018; Liberti and Engel, 2020). Moreover, it is unclear if the effects of microbiota on host behavior are evolved traits with beneficial roles for the host or simply by-products of natural selection (Johnson and Foster, 2018).

Finally, studying mechanisms underlying insect–gut microbiota interactions will not only address fundamental questions of symbiosis, but also holds promise for applied research such as the control and maintenance of economically important insect species. In honey bees, the gut symbiont *S. alvi* was used to deliver dsRNA constructs into the host to target deformed wing virus and *Varroa* mites, which resulted in a strong protective effect against these two major bee parasites (Leonard et al., 2020). Similar engineering approaches have been applied to gut symbionts of insect vectors to either reduce the fitness of the host or specifically antagonize the transmitted human disease agents (Huang et al., 2020; Whitten et al., 2016). Such genetic approaches are obviously faced with formidable challenges related to the release of genetically modified organisms. However, recent proof-of-concept studies exemplify their potential and can serve as starting points to new approaches for managing insect populations.

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