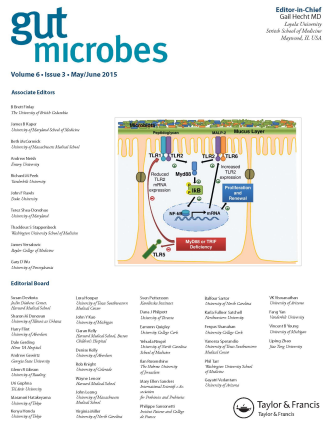

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Evolution of host specialization in gut microbes: the bee gut as a model

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Bacterial symbionts of eukaryotes often give up generalist lifestyles to specialize to particular hosts. The eusocial honey bees and bumble bees harbor two such specialized gut symbionts, *Snodgrassella alvi* and *Gilliamella apicola*. Not only are these microorganisms specific to bees, but different strains of these bacteria tend to assort according to host species. By using *in-vivo* microbial transplant experiments, we show that the observed specificity is, at least in part, due to evolved physiological barriers that limit compatibility between a host and a potential gut colonizer. How and why such specialization occurs is largely unstudied for gut microbes, despite strong evidence that it is a general feature in many gut communities. Here, we discuss the potential factors that favor the evolution of host specialization, and the parallels that can be drawn with parasites and other symbiont systems. We also address the potential of the bee gut as a model for exploring gut community evolution.

Keywords: coevolution, gut symbionts, host specificity, insects, *Snodgrassella*

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Bees serve critical ecological functions as plant pollinators. Some species, such as the Western honey bee (*Apis mellifera*), are indispensable for agriculture and have been prominent cultural icons in many human societies for thousands of years.[1] Only recently have the microbiomes of these ubiquitous insects been described,[2-4] and the bee gut has since emerged as an attractive model system for investigating gut community dynamics and host-microbe interactions. However, because of its novelty, the genomic and experimental data necessary for developing theoretical frameworks for this system have been lacking.[5,6] In our paper,[7] we sequenced the genomes of multiple strains of two common bee gut bacteria, *Snodgrassella alvi* and *Gilliamella apicola*, and showed that stringent host-symbiont compatibility is a characteristic property of this system.

The Specialized Gut Symbionts of Bees

Honey bees (*Apis* spp.) and bumble bees (*Bombus* spp) possess a distinctive gut microbiota dominated by about 8 bacterial phylotypes.[2-4,8] Three groups, *S. alvi, G. apicola*, and *Lactobacillus* spp., form the majority of the gut community.[9,10] Phylogenetic analyses indicate that they each comprise monophyletic clades of bee-associated bacteria, which is suggestive of an intimate symbiosis persisting over evolutionary time scales.[4,11-14] The simplicity of the bee gut community, and its analogy to more complex mammalian models, offer a unique opportunity to study gut microbiomes from the perspective of microbial evolution and ecology in an experimentally tractable system.

Specialized microbial symbionts often exhibit host range restriction and co-diversification with host lineages.[15-17] Indeed, various 16S rRNA surveys have consistently found patterns of correlation between bee gut symbiont strains and host species that cannot be explained by chance or geographic provenance alone.[4,18] This is striking, as, unlike endosymbionts,[19] gut associates possess greater avenues for dissemination, both through vertical transmission (e.g., from queen to daughter[18]), and through horizontal transmission (e.g., between workers[20]). Despite the capacity for transmission, different host species, including those living sympatrically, appear to harbor *specific* lineages of *G.*

apicola and *S. alvi* that can be resolved through phylogenetic reconstruction.[18]

However, specificity of host association, defined here as the restriction of a microorganism to a particular host species or set of host species, does not imply *specialization*, which we define as the adaptation of a microorganism to a particular set of hosts and adaptation of the host to the microorganism. One can imagine scenarios in which extrinsic barriers such as geographic separation or niche segregation prevent hosts of different species from interacting in ways that allow for sharing of gut symbionts. This would result in apparent specificity, but not necessarily specialization. A phylogenetic correlation between host and symbiont is yet another aspect that can reflect long-term evolutionary associations, but may or may not result in specificity or specialization. For specialization, a host-microbe pair should display a direct preferential relationship in addition to any phylogenetic correlation. Perhaps the most straightforward method of testing this is through transplantation experiments, whereby cultured strains (or entire gut communities[21]) are introduced into gnotobiotic animals, and the microbial colonization load recorded as a proxy for host-microbe compatibility.

In our study, we inoculated *S. alvi* strains isolated from the honey bee *A. mellifera* and two bumble bees (*Bombus bimaculatis* and *B. vagans*) into lab-reared, germ-free adult workers of *A. mellifera* and *B. impatiens*.[7] Consistent with the hypothesis of strain-level host-specialization, we observed higher levels of colonization in bees inoculated with their native *S. alvi* strains (**Fig. 1**). Although the *Bombus*-derived *S. alvi* strains were not isolated from *B. impatiens*, their hosts of origin were from the same subgenus (*Pyrobombus*), suggesting some flexibility within a general trend of decreasing compatibility with increasing host genetic distance. Cross-inoculation experiments with isolates from more distantly related *Bombus* will be needed to prove this.

We also conducted co-inoculations and found that the native *S. alvi* strains were able to become dominant in the gut despite an initial numerical disadvantage (**Fig. 1**). These kinds of competition assays are another simple way to indirectly test for host specialization. Because non-resident microorganisms may colonize opportunistically in the absence of the normal flora, the mere observation of colonization is insufficient to determine specialization. In a competition between a specialized community and an artificially introduced one, however, the one that has evolved to thrive in the gut of that particular host species will almost invariably win out.[21]

There are myriad reasons why we should care about host specialization. From a practical standpoint, specialized gut communities are indicative of intimate, evolved interactions between host and microbe, and hence are key to mediating symbiotic benefits that affect host biology. Gut microbial incompatibilities may lead to detrimental outcomes for host immunity and development.[22,23] From an evolutionary perspective, specialized gut bacteria represent a unique but ubiquitous form of symbiosis that has thus far escaped close scientific scrutiny. The forces and mechanisms that shape symbioses in the gut remain largely unknown.

Evolution of Specialization

Host ecology, neutral genetic drift, and selective forces all likely contribute to host specialization in gut microorganisms. Disentangling these factors is challenging, but we suggest that the propensity for a gut bacterium to be specialized can be ascribed to 3 general characteristics: transmission mode, cost/benefit to host, and cost/benefit to the microbe. Systems in which vertical transmission dominates will enforce allopatry of microbial lineages in closely related hosts, enhancing divergence due to both drift and divergent selection reflecting distinct ecological niches of different host species. On the other hand, horizontal transmission between host species would lead to homogenization and fewer opportunities for specialization.

Beneficial microbes are expected to be preferentially retained by hosts due to selection, and thus will also be favored to become specialists. Microorganisms that harm their hosts, and thus threaten the persistence of their own microenvironment, would be unlikely to form the long-term associations needed for evolution of specialization, unless this is offset by a tremendous fitness advantage to the microbe. This would be the case for pathogens, for which the benefit of residing in a particular hostile host is greater than that of any other host or abiotic environment.

Ecological factors, chance, and selection are obviously not constant for a system, but shift through time. A host-microbe interaction that initially provides small benefits to the host or to the microbe may lead down the road to greater specialization, and would be aided by the establishment of a stable mode of transmission. Absent horizontal gene transfers, this would tend to be an irreversible process: genomic erosion, co-evolution with host immune function, and development of genetic incompatibilities (a Bateson–Dobzhansky–Muller model,[24] but with incompatible loci between host and microbe genomes) would discourage promiscuity and host switching.

For the eusocial corbiculate bees, there appear to be at least 4 lineages of gut bacteria exhibiting host specificity: *S. alvi, G. apicola, Lactobacillus* spp, and *Bifidobacterium* spp.[11,12,18] However, specialization to particular host lineages remains mostly untested by transplantation experiments, and 16S rRNA lacks sufficient resolution to reconstruct detailed phylogenetic histories of these bacteria at the strain level. New approaches leveraging the power of high-throughput genomics may help unravel the processes behind the evolution of specialization: shotgun metagenomics and metatranscriptomics enable functional profiling of whole communities,[5,25] 16S rRNA gene surveys allows broad assessment of community composition at the genus level,[10] and an increasing number of sequenced strains and single cells[7,26-31] permit analysis of diversity at the individual bacterium level.

These studies are beginning to reveal the intricate tapestry that is the history of the corbiculate bee gut microbiota, and point to a complex web of gene flow and recombination,[9,30] as well as strong signals of specificity reflecting millions of years of host-microbe codivergence (**Fig. 2**).[7,18] Within an individual host, deeply branching symbiont lineages also appear to coexist – cryptic species of gut bacteria that are all but invisible by 16S rRNA analysis.[30]

Such parallel lineages could reflect specialization of function to distinct ecological niches within the gut,[5] a process likely common in gut microbes.[32,33] The existence of reliable transmission routes, possible benefits to host, and an enriched habitat for gut symbionts may ultimately facilitate evolution of both sympatric diversification within hosts and specialization between hosts.

Mechanisms for Maintaining Specificity

Specialization, defined as adaptation through natural selection for the ability to use a host or to accept a symbiont, produces specific mechanisms that help establish and maintain the association. The molecular bases for symbioses are still poorly understood, particularly for gut microbes. Genome sequencing is now typically the first step in elucidating specificity determinants, and our genomic analysis of *Snodgrassella* and *Gilliamella* uncovered a large repertoire of cell-cell interaction genes which may perform such roles.[7] These include RTX toxins, type VI secretion systems, type IV pili, capsular polysaccharides, and trimeric autotransporter adhesins.[7] While these are the most promising candidates, the suite of host-specificity determinants undoubtedly extend beyond direct interaction genes and will require additional experimental evidence to be identified and validated.

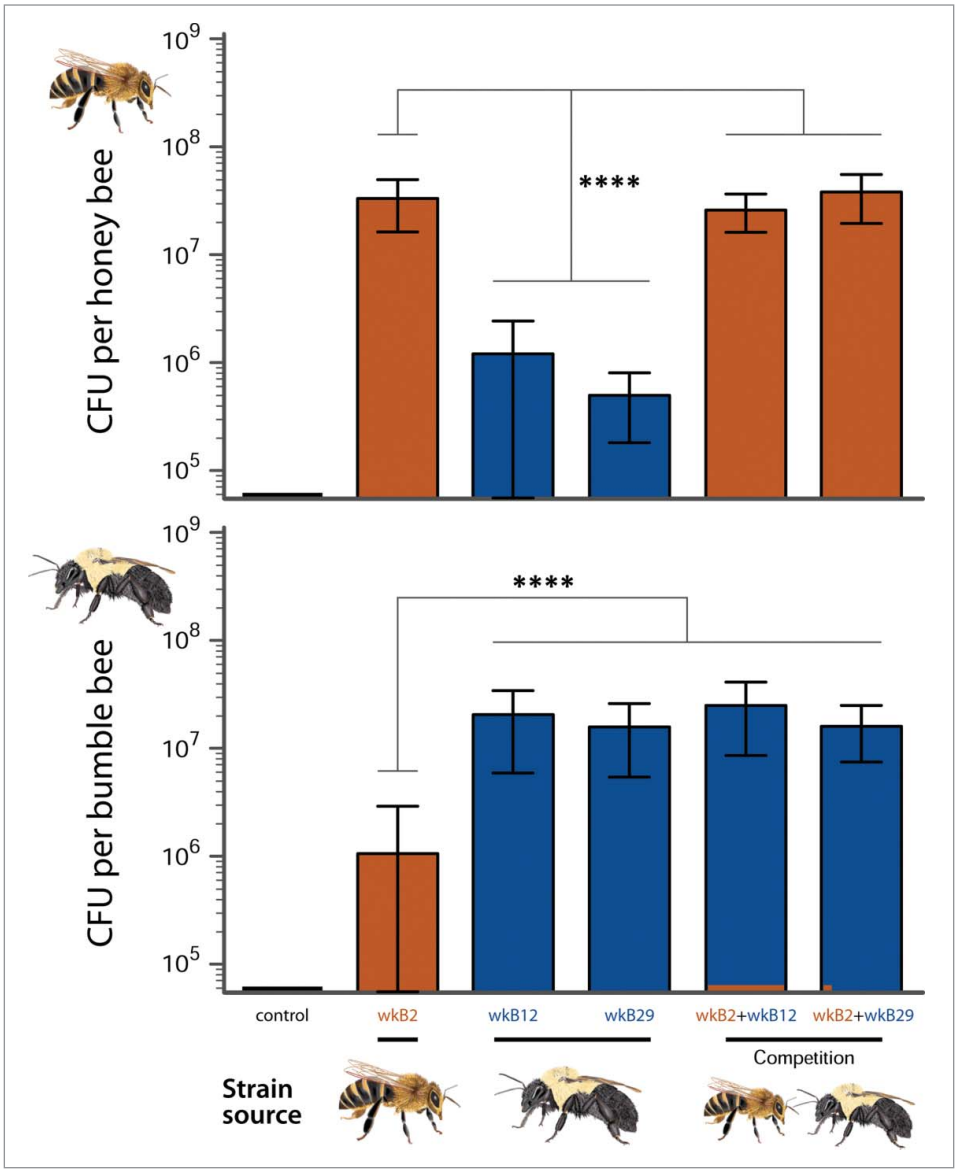

Figure 1. Host-specialized *S. alvi* strains, as demonstrated by transplantation and competition assays. *In-vitro* cultured strains were fed to sterile, newly-emerged adults, and total CFU counted from guts after 5 days. In competition assays, the inoculum consisted of a 1:10 ratio of native to non-native strain. Recovered proportions of each strain type are represented as bar colors: Orange, *Apis*-derived strain (wkB2); blue, *Bombus*-derived strains (wkB12, wkB29). ****$P < 0.0001$, bars denote 95% CI of means. Figure adapted from Kwong et al.[7]

Studies of bacterial symbionts (pathogens as well as mutualists) suggest that host specificity is mediated through at least 3 types of processes: host recognition and colonization, compatibility with host immune systems, and acquisition of nutrients specific to the host environment.[34] In *Vibrio fischeri*, a bioluminescent symbiont of marine animals, specificity to the squid *Euprymna scolopes* critically depends on RscS, a sensor kinase that detects an as-yet unknown host factor and induces expression of exopolysaccharide that enables colonization.[35] *V. fisheri* strains that colonize fish, in contrast, lack RscS.[35] The mouse gut symbiont *Lactobacillus reuteri* also relies on biofilms for colonization, and the inability of *L. reuteri* strains from humans, pigs or chickens to establish in mice likely stems, in part, from the absence of particular genes for biofilm production.[36]

Human-specific bacterial pathogens can evade host defenses by utilizing proteases to break down antibodies or by binding down-regulators of complement-mediated immunity.[34] Conversely, the host may develop specialized immune responses to encourage colonization of a beneficial microbiota, such as has been proposed for antimicrobial-peptide-mediated host specificity in *Hydra*.[37] Nutritionally, each host presents a unique selective environment

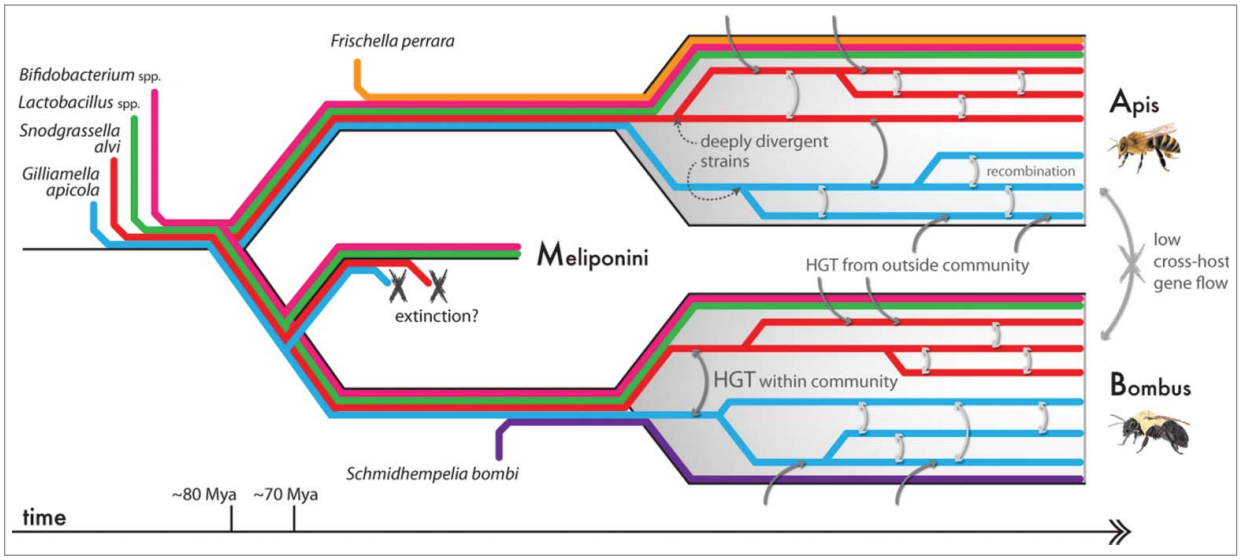

Figure 2. Evolution of the corbiculate bee gut microbiota. The eusocial origins of the coribiculate bees[59] may have facilitated the development of a specialized gut microbiota by enlarging host reservoirs and providing a reliable transmission route. The symbiont genera *Gilliamella, Snodgrassella, Lactobacillus*, and *Bifidobacterium* may be ancestral to corbiculates (originating ca. 80 Mya[60]), and are presently all found in *Apis* and *Bombus* bees.[4,12,14,18] Stingless bees (*Meliponini*) appear to have lost *Gilliamella* and *Snodgrassella*,[18,61] but retain *Lactobacillus* and *Bifidobacterium*.[14] Two bacteria related to *Gilliamella* (order: *Orbales*) were likely acquired sometime later: *Frischella* by the *Apis* lineage,[31] and *Schmidhempelia* by *Bombus*.[29] Within *Apis* and *Bombus, Gilliamella* and *Snodgrassella* strains have substantially diverged at many genomic loci, suggesting the existence of deeply-branching lineages co-existing within the same host.[7,30] This may be due to niche differentiation in the gut.[5] Recombination between some lineages still occurs, however, and likely explains the high 16S rRNA identity between strains.[7,9,30] Horizontal gene transfer (HGT) between gut symbionts[7] and from other/environmental sources[62] may allow for dynamic gene repertoires in the bee gut microbiota. Nonetheless, it appears that gene flow between strains native to different bee hosts is generally limited.[7] It is possible these evolutionary characteristics also extend to the other bee gut species, but, unlike *Gilliamella* and *Snodgrassella*, they have not yet been closely examined. Events other than that of known host splits (timings marked) are speculative and are for illustrative purposes only.

for a microbe. For example, cattle-specific *Campylobacter* strains tend to possess a vitamin B_5 synthesis locus lacking in chicken-specific strains, presumably due to vitamin B_5 scarcity in grasses compared to chicken feed.[38] Microbes and their hosts may also have to compete for the same scarce resources. Opportunistic pathogens such as *Neisseria* and *Haemophilus* have evolved receptors to pick up iron in host-bound molecules of transferrin, leading to a co-evolutionary arms race and accelerated adaptive evolution at the responsible loci in both host and microbe.[39]

Like *V. fischeri*, host specificity for the nematode symbiont *Xenorhabdus* can be mediated by a single locus. Here, the genes *nilABC* are unique to strains infecting the nematode *Steinernema carpocapsae* but are absent in other *Xenorhabdus*; heterologous expression of *nilABC* in the other *Xenorhabdus* enable their colonization of *S. carpocapsae*.[40] These findings beg the question as to whether single-locus dependent specificity, such as *rscS* and *nilABC*, are extreme outlier cases or, rather, represent a more general basis for host specialization. In *Salmonella enterica*, a widespread pathogen of mammals and birds, adaptation to hosts is thought to be multifactorial, with both gene gain and loss playing a part.[41,42] However, it is unclear whether these events are the cause or consequence of specialization. A recent gene-swapping study of *V. fischeri* strains hosted by Australian or Hawaiian *Euprymna* squids suggested that multifactor-mediated host specificity is not incompatible with single loci of large effect: there may in fact be multiple genes in a genome capable of greatly altering host affinity.[43]

These studies demonstrate that horizontal gene transfer, whether by an experimenter or by natural processes (as proposed for *rscS*[35] and *nilABC*[40]), can greatly alter a microbe's host range. In the plant pathogens *Xanthomonas* and *Pseudomonas*, type III secretion system effectors are likely important determinants of host specificity.[44,45] The horizontal acquisition of the permissive effector genes can lead to effective colonization of the same host plant by distantly related pathogen strains, thus breaking apart the phylogenetic host-microbe correlations typically associated with co-evolved symbioses.[44,45]

Both horizontal gene transfer and genomic degradation probably play prominent roles in the evolution of specialization,[42,46] but to what extent remains an unresolved question. There is also the host perspective to consider, as interplay between host immunity and the microbiota constitutes an ongoing dialog between partners that often have competing evolutionary interests.[47] Behavioral mechanisms by the host (e.g. coprophagy, egg-smearing) may also evolve to facilitate symbiont maintenance. Delineating the diversity of mechanisms behind host specialization and the dominant forces influencing their evolution will be critical steps going forward, as will be the description of any general rules governing differences in these properties among mutualists, pathogens, and commensals, and between

Table 1. Examples of host specificity in extracellular bacterial symbionts. The degree of specialization and the mechanisms involved remain areas of active investigation in these systems.

	Host ranges	Reported mechanisms of specificity	References
Gut microbes			
Campylobacter jejuni	mammals, birds	vitamin B_5 biosynthesis	38
Lactobacillus reuteri	mammals, birds	biofilm production	36
Salmonella enterica	mammals, birds, reptiles	pathogenicity factors, loss of metabolic pathways	42
Snodgrassella alvi	honey bees and bumble bees	unknown	7
Other symbionts			
Pseudomonas syringae, Xanthomonas spp.	plants	virulence factors, type 3 secretion effectors	44,45
Rhizobia	legume plants	Nod factors	51,52
Streptomyces philanthi	beewolf wasps	host response or behavior	53
Vibrio fischeri	squid, fish, environmental	biofilm production, bioluminescence	35,43
Xenorhabdus nematophila	nematodes	*nilABC* locus	40

animal gut microbiotas and other types of host-microbe associations (**Table 1**).

Methodologies to probe the genomic underpinnings of specialization are becoming ever more accessible due to advances in sequencing technologies. Genome-wide association,[38] RNAseq,[36] and TnSeq[48] are now effective ways to quickly screen for candidate genes. Meanwhile, the toolbox for organismal genetic manipulation is also increasing rapidly.[49,50] We anticipate that the development of new model systems, such as the bee gut community, will continue to accelerate in the years to come, and will provide much needed context toward understanding the diversity of gut microbial symbioses.

Conclusion and Perspective

Mounting evidence suggests that many gut microbes are host-specific,[54-56] preferentially associating with a particular species over any other potential host or environment. Thus far, however, correlational data is in much greater abundance than elucidated causal mechanisms. Are these host-specific microbes really *specialized* to their hosts, or have circumstances simply produced the observed associations? In other words, given the chance, are these microbes able to colonize a range of other hosts? Specialization should be tested by transplantation and competition assays, and mechanisms need to be deduced from '–omics' approaches and verified experimentally. Given the enormous plasticity of microbial genomes and propensity for horizontal gene transfer, greater scrutiny of strain-level variation at a genome-wide scale will also be essential to explain the evolution and diversification of gut microbes.

As a whole, gut microbes already comprise a highly derived group of organisms, distinct from their free-living predecessors. The forces driving ever-increasing specialization, down to the strain level, have yet to be clarified, but we predict that transmission mode and relative fitness benefits to the host and/or the microbe play a large part. Quantifying the contribution of fitness, over long time scales, to the development of specialization remains a challenge for the study of symbioses from an evolutionary perspective. Another open question is whether specialization destines microbes to an evolutionary dead-end due to the increased risk of extinction that result from highly restricted host ranges and the loss of functional capabilities from genome erosion. Intracellular symbionts can degenerate to the point where they are replaced,[57] but for gut microbes, the prospect of gene flow may prevent this outcome.

The bee gut microbiota represents a system in which bacterial lineages have diversified within hosts and have evolved to specialize to distinct host species. These features parallel those apparent in the more complex microbiotas of mammals including humans, and the parallels reflect the fact that both are transmitted directly among individual hosts through social contact. The extent and nature of within-host and between-host diversification of such symbionts may have major implications for hosts.[58] Thus, the bee gut community offers a simple model for investigating how coevolution of host-specialized gut symbionts affects host health and disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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