How (not) to study insect microbiota in the age of high-throughput sequencing

Piotr Łukasik[1] , Karol H. Nowak[1,2], Raphael Eisenhofer[3,4]

1. Institute of Environmental Sciences, Faculty of Biology, Jagiellonian University, Poland
2. Doctoral School, PhD Programme in Biology, Faculty of Biology, Jagiellonian University, Poland
3. Department of Bioengineering, Technical University of Denmark, Lyngby, Denmark
4. School of Biological Sciences, Adelaide University, Australia

Correspondence: Piotr Łukasik. E-mail: p.lukasik@uj.edu.pl

Highlights

- Insect-associated microbes comprise fundamentally different functional categories that require distinct analytical approaches
- Generic microbiome workflows often fail to account for the low biomass, uneven abundance, and biological features of insect-associated microbes
- Contamination-aware processing, quantitative abundance estimates, and biologically informed analyses substantially improve inference from insect microbiome data
- The choice of analytical workflow should be guided by the biology of the host–microbe association rather than by generic microbiome conventions

Abstract

The recognition of microbiota as a critical component of insect biology and evolution has driven a rapid expansion of insect-microbe interaction research. Yet while high-throughput sequencing has democratized access to microbial community profiling, it has also normalized low-resolution, artifact-prone approaches that often yield limited biological insights.

Insects pose distinct challenges for microbiota characterization. Microbial communities are frequently low in biomass, highly variable across individuals and tissues, and often dominated by one or a few vertically transmitted endosymbionts. The fact that these features violate key assumptions of generic microbiome workflows is commonly overlooked, leading to misleading inferences from compositional data. Here, we summarize the properties of insect-associated microbiota that constrain the use of standard analytical approaches and highlight recurring pitfalls in their study. We emphasize two pervasive issues: (i) contamination, a major and often underappreciated source of bias in low-biomass samples, and (ii) the routine application of off-the-shelf diversity metrics and ordination-based analyses without consideration of their assumptions or biological relevance.

We explain how microbiota quantification, appropriate controls and contamination-aware data filtering approaches, and analytical frameworks that prioritize hypothesis-driven inference, can improve rigor and lead to biologically meaningful conclusions. As data generation becomes cheaper and easier, progress in the field will depend on aligning such methodological and analytical choices with the biology of insect–microbe interactions rather than on increasing sequencing effort alone.

Key words: symbiosis, microbiome, 16S rRNA, metabarcoding, amplicon sequencing, contamination

Introduction

Microbiota are a critical component of the biology of many animal taxa, influencing host physiology, life-history traits, population dynamics, and long-term evolutionary trajectories [1,2]. Recognition of their importance has driven a rapid expansion of host-associated microbiome research, supported by a diverse methodological toolkit. Among these, marker gene amplicon sequencing—most commonly targeting the bacterial 16S rRNA gene—has become the dominant approach for broader microbiota surveys, offering a pragmatic balance between cost, throughput, and taxonomic resolution [3,4].

Among animal groups that attracted substantial attention of microbiota researchers are insects, a hyperdiverse clade that underpins most terrestrial ecosystems and has major agricultural and biomedical relevance. Through their diverse effects, microbial associations frequently shape insect biology and evolution [5–7].

However, insect-associated microbiota differ in fundamental ways from systems in which standard microbiome workflows had been developed. Microbial communities in insects are often low in biomass [8–10], highly variable across individuals and tissues [11,12] and frequently dominated by one or a few heritable symbionts [13]. These features challenge key assumptions underlying commonly used sequencing, processing, and analytical workflows - largely developed and validated for systems such as vertebrate gut and soil microbiota, with high microbial biomass, high microbial taxonomic diversity, and contamination typically representing a minor fraction of recovered DNA [14,15]. Many insect systems are outside of these approaches' domain of validity, increasing the risk of artifacts and misinterpretation [16].

Here, we argue that meaningful progress in insect microbiota research requires aligning methodological choices with the biological properties of these systems. We highlight common pitfalls, discuss their underlying causes, and propose practical guidelines for more robust study design and inference.

The features of insect-associated microbes and microbial communities

Insects have repeatedly associated with taxonomically diverse microbes, at different points in time, resulting in host–microbe systems that vary widely in stability, transmission mode, within-species and tissue distributions, abundance, and biological roles (Table 1) [5,17]. These differences reflect distinct functions and impose important constraints on how insect-associated microbes can be meaningfully characterized.

Functional category	Examples	Prevalence in host sp.	Abundance if present	Transmission	Specificity	Typical research questions
Obligate endosymbionts	*Buchnera* (aphids)	~100%	very high	vertical	very high	evolution, genome reduction
Facultative endosymbionts	*Wolbachia*, *Rickettsia*	intermediate, variable	variable, often high	vertical, sometimes horizontal	medium to high	ecology, distribution
Specialized microbiota	*Snodgrassella*, *Gilliamella* (bees)	very high	variable, often high	social or maternal	high	community assembly
Transient microbiota	*Pseudomonas*	variable, often low	often low	environmental	low	environmental acquisition
Pathogens	*Nosema* (bees), *Phytoplasma*	variable	variable	horizontal, vector-mediated	high	disease ecology, agriculture

***Table 1**. Main functional categories of insect-associated microbes, and their typical features*

The first, most integrated functional category comprises obligate nutritional endosymbionts that provide essential nutrients lacking in host diets and are transmitted strictly maternally, with near-perfect fidelity [13]. As a result, these symbionts are typically present in all individuals of a host species or clade and often reach high abundance within hosts. They codiversify with their hosts, and due to their elevated evolutionary rates, distinct host lineages may harbor different symbiont genotypes. The second functional category, facultative endosymbionts, known for manipulating reproduction and conferring protection, are heritable but capable of occasional horizontal transmission [18,19]. They typically occur in only a subset of individuals in a species, with prevalence varying in space and time, and when present, they often reach high abundance and strongly influence microbiota profiles. Further, many insects harbor specialized gut or cuticular microbiota that contribute to nutrition or defense [6,20]. These communities, typically transmitted socially or maternally, can comprise multiple taxa and strains that are consistently present but variable in abundance across individuals. In addition, insects frequently associate with transient microbes, many of which represent versatile taxa broadly distributed in the environment, inconsistently present and highly variable in abundance [21]. Some individuals also host pathogens, either infecting the host itself or vectored by it, whose abundance can vary strongly depending on infection stage and context [22,23].

These functional categories differ so fundamentally in their ecology, distribution, evolutionary dynamics, and host effects that they should often be considered as distinct analytical targets rather than components of a single "microbiome" (Table 1).

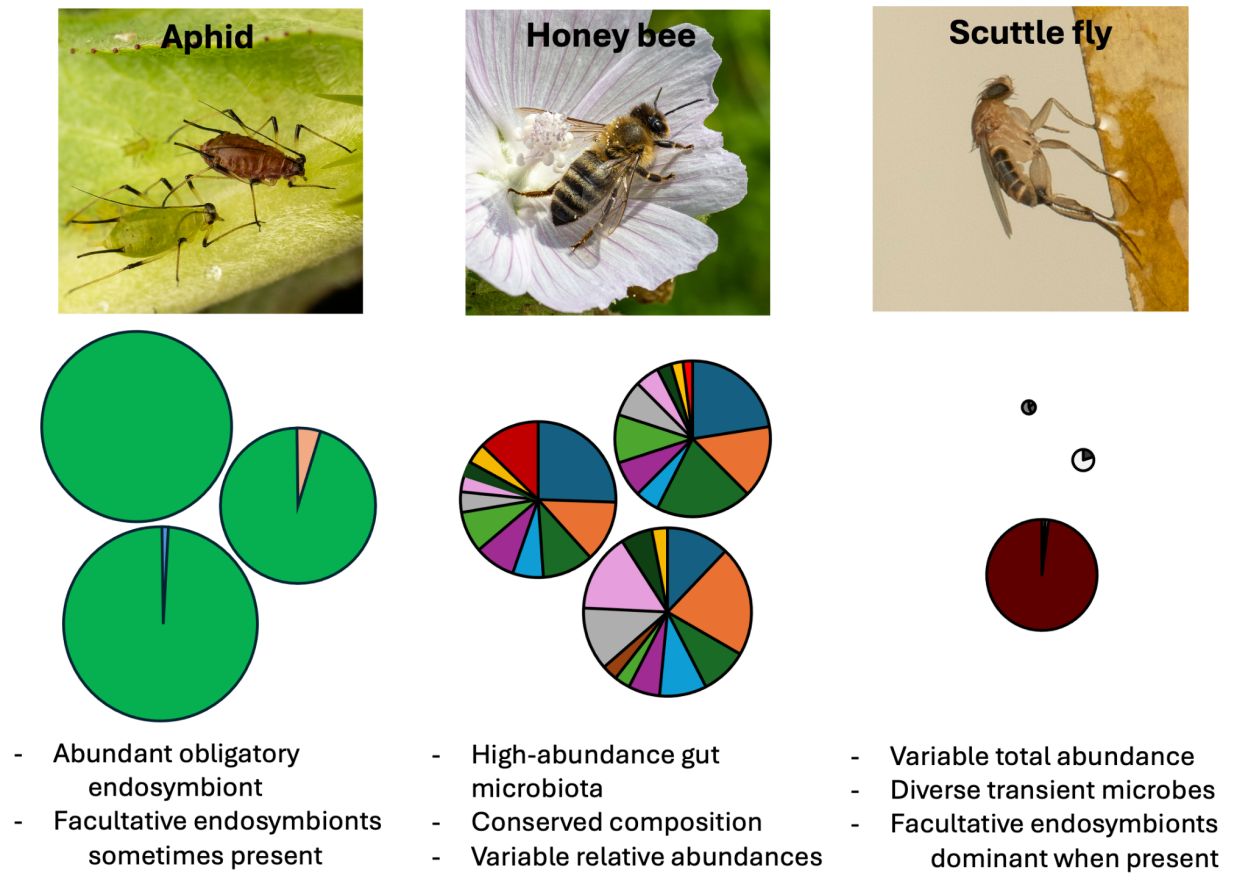

Figure 1*. Schematic representations of contrasting microbial communities in three insect clades, exemplifying fundamentally different host–microbe systems requiring different analytical approaches. Circle size represents approximate microbial abundance, while sectors illustrate community composition. Photos - Piotr Łukasik (aphid, bee), Melinda Fawver / iNaturalist, CC-BY-NC (fly).*

The relative contribution of these functional categories differs markedly among insect taxa, shaping the abundance and composition of their microbial communities (Fig. 1). For example, the microbiota of many aphids are dominated by the ancient, co-diversifying nutritional endosymbiont *Buchnera*, often accompanied by one or more facultative endosymbionts, and generally only minor contributions from other microbes [24,25]. In contrast, adult honey bees harbor conserved multi-partite gut microbiota, reliably transmitted socially among individuals, while exhibiting substantial strain-level diversity and variation in the relative abundance of individual taxa [26,27]. In turn, scuttle flies (Diptera: Phoridae) frequently harbor heritable endosymbionts such as *Wolbachia* and *Rickettsia*, which can be highly abundant when present but vary across species, populations, and sexes. Their other associated bacteria are generally low-abundance and considered environmentally derived and transient [28]. There is substantial variation in microbial associations even among related insects: for example, several ant clades host obligate symbionts, others harbor structured gut communities, some host facultative endosymbionts, and many display low-abundance or highly variable microbiota, or even lack detectable microbial communities [10,29].

Beyond these consistent differences among insect taxa, there is substantial within-taxon variation across multiple scales [11]. Microbial communities can differ among populations, with their different evolutionary histories, but also environmental factors such as climate or predominant diet contributing to the prevalence of different microbes [30,31]. Temporal variation, including seasonal changes in environmental conditions, diet, fluctuating pressure of natural enemies, density-dependent selection, interaction among co-infecting microbes, as well as stochastic effects, can further alter microbial composition and abundance [28,32,33]. Substantial variation also occurs among individuals, influenced by factors such as sex, genotype, physiological state, and stochastic colonization events [27,34]. Additional differences arise across developmental stages and among tissues or body regions within a single host [12,35,36].

Importantly, many insects lack endogenous microbes, and may only form inconsistent, often low-abundance associations with environmentally acquired, transient microbes and occasional pathogens [8,10]. For example, it is plausible that many insects lacking heritable symbionts may emerge with extremely low microbial loads and only later acquire much of their microbiota from food, substrate, or other environmental sources [21]. Other insects may have specific adaptations limiting microbial colonization [8]. Hence, a substantial share of insect individuals in the wild may harbor bacterial amounts near the detection threshold - virtually, no microbiota to speak of. Such absence of a stable microbiota should itself be viewed as a biologically meaningful state rather than simply a failure to detect microbes. Then, in many cases, the biologically relevant question would therefore not be "What is the insect microbiome?", but rather "Which microbial associations are persistent and biologically meaningful?"

Overall, features of insect microbiota such as uneven biomass, strong dominance by a small number of taxa, and discontinuous variation across samples, can strongly skew compositional data and complicate comparisons across samples. These challenges need to be addressed carefully if our goal is to comprehend biological patterns and reconstruct their drivers.

Specific challenges with studying insect microbiota - and how they can be addressed

These properties of insect microbiota create several recurring methodological challenges (Table 2). Many are not unique to insects, but become especially severe in low-biomass systems, or those dominated by few abundant microbes [37]. Importantly, these challenges often interact, amplifying the risk of artifacts and misinterpretation.

First, the taxonomic identity of insects can be difficult to establish, especially in wild samples originating from natural communities. In addition, focal hosts may themselves contain parasites or parasitoids whose own microbes contribute to sequencing profiles. Further, tissue choice fundamentally shapes inferred microbiota composition, and while whole-body homogenization maximizes throughput, it can obscure biologically meaningful spatial structure, and yield high quantities of interfering host DNA. Similarly, pooling individuals may mask substantial inter-individual variation in microbial abundance and composition, while also increasing the risk of inadvertently mixing distinct species.

Second, insects may form taxonomically diverse associations, including with bacteria, Archaea, fungi, and other microeukaryotes [17,38,39]. Capturing these diverse microbial groups often requires different marker genes, primer sets, and analytical workflows. Importantly, commonly used “universal” primers are neither fully universal nor fully specific, potentially biasing amplification toward particular clades while generating substantial off-target amplification [40]. This includes host mitochondrial and plant chloroplast sequences in rRNA amplicon profiles, especially for low-biomass samples [16,36,41].

Third, insect-associated microbiota are characterized by highly variable and often low microbial biomass, with bacterial loads differing by orders of magnitude among individuals, tissues, developmental stages, or environmental conditions [10,28]. Consequently, sequencing-based relative abundance estimates alone may poorly reflect biological reality, particularly when dominant symbionts obscure variation in less abundant microbes, or low-biomass samples produce apparently diverse profiles despite containing little genuine microbial signal. These issues are especially problematic because microbial abundance is rarely quantified directly, even though approaches such as qPCR or quantitative spike-ins can substantially improve data interpretation [16].

Fourth, contamination represents a particularly severe challenge in insect microbiota studies, yet remains inadequately controlled in much of the literature [16]. In low-biomass samples, contaminant DNA originating from reagents, laboratory environment, or index hopping may comprise a substantial fraction of recovered sequences and sometimes dominate microbial profiles [42]. The problem is compounded by the environmental ubiquity of many common contaminants, making them difficult to distinguish from genuine biological associations [42,43]. Importantly, host diet, parasitoids, environmental particles, and surface-associated microbes may also contribute signal unrelated to the focal biological question. Robust microbiota characterization therefore requires extensive controls, including extraction blanks, PCR negatives, mock communities, and ideally microbiota quantification combined with contamination-aware filtering [37,44].

Fifth, downstream processing, analysis, and presentation of insect microbiota data often rely on workflows developed for high-biomass, high-diversity microbial communities [14,15]. In practice, analyses are frequently dominated by diversity indices, ordination plots, and averaged relative abundance summaries that may have limited interpretability in systems dominated by a few symbionts. Presence–absence variation of microbes such as *Wolbachia* can strongly distort alpha- and beta-diversity patterns, while averaging across heterogeneous individuals may obscure ecologically meaningful variation. Similarly, comparisons performed exclusively at high taxonomic levels may mask strain-level specificity central to insect–microbe interactions. In many insect systems, microbe-centric approaches focused on the distribution and function of specific symbionts would therefore be more informative than community-centric analyses. More generally, analytical choices should follow biological questions rather than conventions of the microbiome field. Hence, in systems characterized by strong hierarchical structure, uneven microbial abundance, and substantial among-individual variation, model-based approaches that explicitly incorporate host traits, spatial structure, or species co-occurrence patterns may provide substantially more informative inference than generic diversity-based analyses. Such

frameworks, including hierarchical models of species communities (HMSC), are increasingly proving their worth for insect microbiota research [45,46].

Challenge	Why it matters in insects	Recommended approaches
Host identity and sample definition	Wild-collected insects may include cryptic species, parasites, and parasitoids. Microbes are unevenly distributed across tissues. Whole-body homogenization and specimen pooling can obscure biologically meaningful variation.	Verify host identity where relevant; sequence host marker genes; standardize tissue and life stage; avoid pooling specimens into one sample
Capturing diverse microbial groups	Insect-associated microbiota may include bacteria, fungi, microeukaryotes, and host-derived sequences that differ greatly in abundance and detectability; no single primer set captures all groups reliably	Match marker systems and workflows to the focal microbial groups; assess amplification biases and off-target amplification; avoid interpreting non-detection as absence
Low and variable microbial biomass	Biomass may differ by orders of magnitude among individuals, tissues, and life stages; relative abundances alone may poorly reflect biology	Quantify microbial load (e.g., qPCR, spike-ins); interpret low-abundance signal cautiously
Contamination	Samples, especially low-biomass, are highly susceptible to reagent, environmental, and cross-contamination that may dominate sequence profiles if not anticipated and controlled	Include extraction and PCR negatives; use mock communities; apply contamination-aware filtering; be cautious about presence-absence comparisons
Bioinformatic data processing	Alternative analytical approaches provide different results. Arbitrary filtering and compositional effects may distort microbiota structure, particularly in systems dominated by few symbionts; incomplete reference databases hinder taxonomy reconstructions	Use transparent, reproducible pipelines and up-to-date tools and databases; set meaningful thresholds; consider dominant symbiont effects explicitly
Statistical analysis and biological interpretation	Diversity metrics and ordinations are determined by analytical thresholds and uneven data distributions. Averaging across heterogeneous individuals or using only high-level taxonomy may obscure biologically relevant patterns.	Match analyses to biological questions; focus on reliably detected, biologically relevant microbes; avoid overinterpreting exploratory diversity analyses. Show individual-level variation; report prevalence and abundance distributions; consider strain-level resolution.

Table 2. *Major methodological challenges in insect microbiota studies and recommended approaches to address them*

Collectively, these challenges highlight that rigorous insect microbiota research requires more than simply adapting existing microbiome workflows. Methodological and analytical choices should be guided by the biology of the focal host–microbe system and the questions being addressed. In many cases, biologically meaningful insight may require simpler but system-specific analyses rather than increasingly complex generic workflows.

Towards biologically meaningful insect microbiota research

Insect-associated microbiota, often uneven and discontinuous, are prone to methodological artifacts including the conflation of methodological noise with biological signal and the overinterpretation of compositional patterns [15,16,37]. As such, they differ fundamentally from the high-biomass, relatively homogeneous systems such as vertebrate gut or soil microbiota for which standard microbiome workflows were originally developed. Consequently, challenges inherent to microbiota of most insects - microbial abundance variation, contamination, striking differences among microbial functional categories in their distribution, dominance by a small number of symbionts, or heterogeneity among samples - are not normally addressed. Hence, the routine application of generic microbiome workflows to insect systems can lead to biologically uninformative—or worse, biased and misleading—conclusions. One striking example is the control of contamination: it is one of the most severe challenges in insect microbiota research, but barely one-sixth of published studies sequence negative controls and attempt contamination-aware filtering [47].

At the same time, we do not advocate another universal workflow for insect microbiota analysis. Different insect–microbe systems follow different biological rules, requiring different analytical strategies. Rather than advocating a universal workflow, we argue that analytical choices should be tailored to the biology of the focal host–microbe association and the biological question being addressed.

Figure 2 illustrates how successive analytical choices can fundamentally change the biological conclusions that can be drawn from the same insect microbiota dataset [28]. Contamination-aware filtering removes nearly half of recovered reads on average (Fig. 2A), showcasing how technical artifacts can comprise a major fraction of apparent microbial communities. Standard microbiome summaries—including averaged relative abundances, alpha diversity and ordination analyses (Fig. 2B–D) — provide only limited insight into this symbiont-dominated system. By contrast, incorporating spike-in based estimates of bacterial abundance reveals four orders of magnitude variation among individuals and fundamentally changes data interpretation. Combining absolute abundance with relative abundance of dominant taxa uncovers biologically meaningful differences among populations and sexes that remain largely hidden in compositional analyses alone (Fig. 2E).

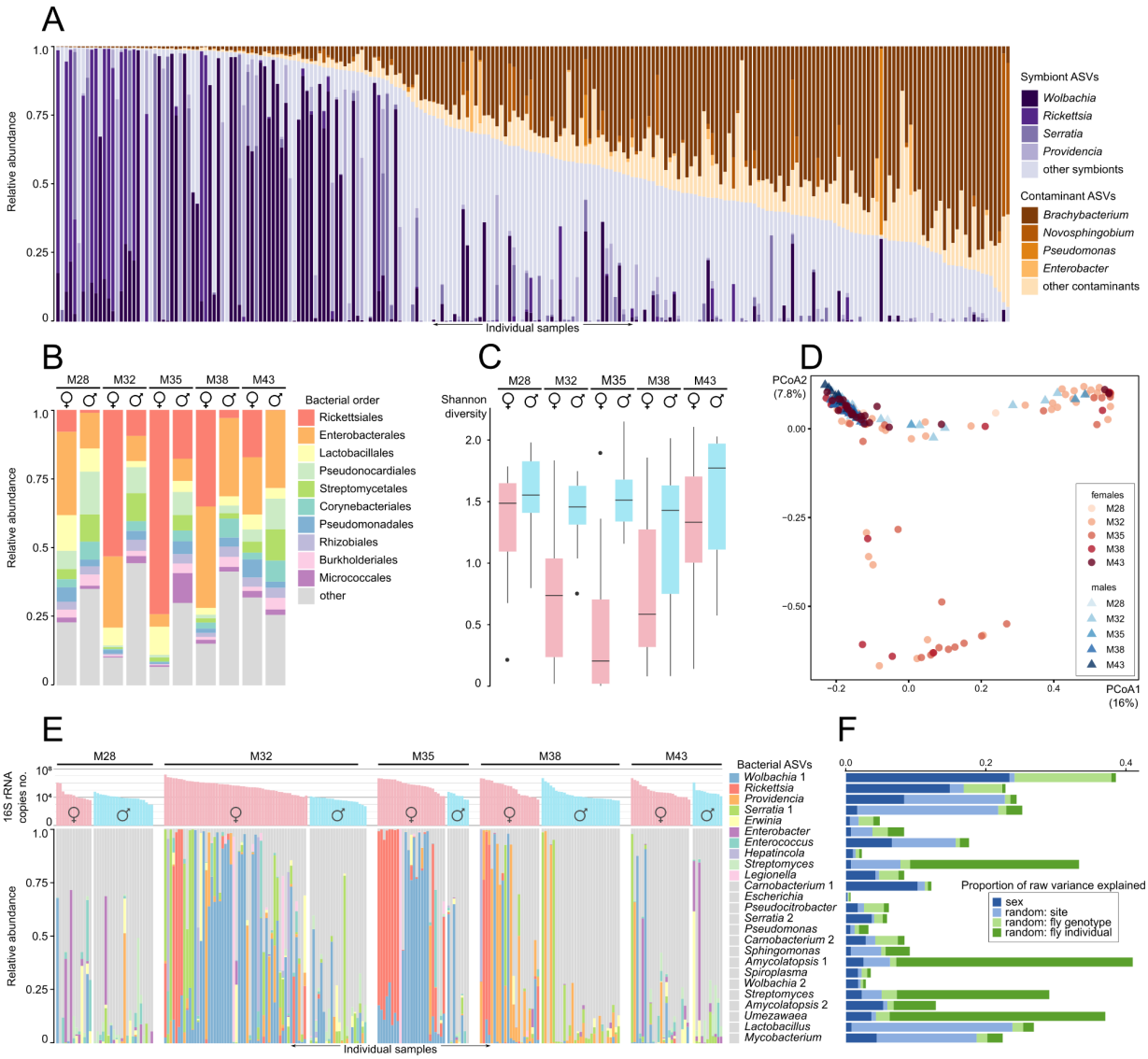

Figure 2. Different analytical workflows applied to the same insect microbiota dataset provide markedly different levels of biological insight. *The figure illustrates alternative perspectives on bacterial 16S rRNA amplicon data for 226 individuals of a single scuttle fly species,* Megaselia sp. *(sp. 1 in Nowak et al. 2025 [28]).* ***A.*** *The upper panel highlights the variable abundance of dominant symbionts and common laboratory contaminants identified through comparisons with negative controls - illustrating the importance of contamination-aware data processing in low-biomass insect samples.* ***B-D.*** *The middle panels show results routinely reported in insect microbiota studies, including averaged relative abundance summaries, alpha diversity comparisons, and PCoA ordination analyses. Applied to contamination-filtered data for females and males from five sites (MXX), they provide limited biological insights in this host-microbe system.* ***E.*** *The combined estimates of bacterial abundance with the relative contribution of dominant bacterial ASVs reveal biologically meaningful variation among host populations and sexes that was largely obscured by generic community-level summaries.* ***F.*** *Hierarchical modelling of species communities (HMSC) provides an integrated framework for partitioning the effects of selected host traits and environmental variables on the presence of selected ASVs, with abundance-if-present and microbial associations modelled simultaneously (see Ovaskainen et al., this issue). Together, the panels illustrate that biologically meaningful inference depends on choosing analytical approaches that match the biology of the focal host–microbe system.*

When the aim is to identify ecological drivers rather than simply describe microbial communities, model-based approaches provide a natural next step. Hierarchical models of species

communities (HMSC), for example, simultaneously incorporate host traits, environmental variables and microbial associations, allowing variation to be partitioned among competing ecological drivers [45,46,48]. In our example (Fig. 2F), HMSC shows that the occurrence of facultative endosymbionts *Wolbachia* and *Rickettsia* is largely explained by host sex and genotype, whereas some other bacteria vary primarily among sites and many remain largely unexplained, highlighting opportunities for further ecological investigation.

While our example focuses on a symbiont-dominated microbiota in a fly species, the broader message extends across insect systems that may require different types of evidence. In systems dominated by heritable symbionts, estimating microbial prevalence, abundance, and interactions may be considerably more informative than community-level diversity metrics. In structured gut communities, temporal dynamics, strain turnover and functional redundancy become central questions. Conversely, in insects lacking stable resident microbiota, distinguishing biologically meaningful associations from transient environmental microbes and contamination becomes the primary analytical challenge. Comparative analyses across host taxa further require explicit consideration of host phylogeny and microbial transmission and function. Recent studies of honey bees [27], solitary bees and wasps [47,49], and Greenland mosquitoes [50] illustrate these contrasting analytical priorities.

Overall, the most informative analyses are not necessarily the most computationally sophisticated, but those that explicitly incorporate biological knowledge about the focal host–microbe system. As sequencing technologies continue to improve, progress in insect microbiota research will depend less on generating ever larger datasets than on selecting methods that match the biology of the system and the questions being asked. Ultimately, the key question is no longer simply how microbial communities compare, but which microbial associations are persistent, functional, and evolutionarily or ecologically meaningful.

Acknowledgements

PŁ and KHN were supported by the Polish National Science Centre grants 2018/31/B/NZ8/01158 and 2021/43/B/NZ8/03376. We thank Diana Rojas Guerrero for help with the HMSC analysis.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process: During the preparation of this work, the authors used ChatGPT (OpenAI GPT-5.5) to assist with language editing, improving clarity of expression, developing the manuscript structure, and identifying relevant literature. All generated content was critically reviewed, verified, and edited by the authors, who take full responsibility for the content of the published article.

References

1. McFall-Ngai M, Hadfield MG, Bosch TCG, Carey HV, Domazet-Lošo T, Douglas AE, Dubilier N, Eberl G, Fukami T, Gilbert SF, et al.: **Animals in a bacterial world, a new imperative for the life sciences**. *Proc Natl Acad Sci* 2013, **110**:3229–3236.

2. Perreau J, Moran NA: **Genetic innovations in animal–microbe symbioses**. *Nat Rev Genet* 2021, doi:10.1038/s41576-021-00395-z.

3. Weinroth MD, Belk AD, Dean C, Noyes N, Dittoe DK, Rothrock MJ Jr, Ricke SC, Myer PR, Henniger MT, Ramírez GA, et al.: **Considerations and best practices in animal science 16S ribosomal RNA gene sequencing microbiome studies**. *J Anim Sci* 2022, **100**:skab346.

4. Thompson LR, Sanders JG, McDonald D, Amir A, Ladau J, Locey KJ, Prill RJ, Tripathi A, Gibbons SM, Ackermann G, et al.: **A communal catalogue reveals Earth's multiscale microbial diversity**. *Nature* 2017, **551**:457–463.

5. Douglas AE: **Multiorganismal insects: diversity and function of resident microorganisms**. *Annu Rev Entomol* 2015, **60**:17–34.

6. Engel P, Moran NA: **The gut microbiota of insects – diversity in structure and function**. *FEMS Microbiol Rev* 2013, **37**:699–735.

7. Łukasik P, Kolasa MR: **With a little help from my friends: the roles of microbial symbionts in insect populations and communities**. *Philos Trans R Soc B Biol Sci* 2024, **379**:20230122.

8. Hammer TJ, Sanders JG, Fierer N: **Not all animals need a microbiome**. *FEMS Microbiol Lett* 2019, **366**.

9. Hammer TJ, Janzen DH, Hallwachs W, Jaffe SP, Fierer N: **Caterpillars lack a resident gut microbiome**. *Proc Natl Acad Sci* 2017, **114**:9641–9646.

10. Sanders JG, Łukasik P, Frederickson ME, Russell JA, Koga R, Knight R, Pierce NE: **Dramatic differences in gut bacterial densities correlate with diet and habitat in rainforest ants**. *Integr Comp Biol* 2017, **57**:705–722.

11. Lange C, Boyer S, Bezemer TM, Lefort M-C, Dhami MK, Biggs E, Groenteman R, Fowler SV, Paynter Q, Verdecia Mogena AM, et al.: **Impact of intraspecific variation in insect microbiomes on host phenotype and evolution**. *ISME J* 2023, **17**:1798–1807.

12. Lanan MC, Rodrigues PAP, Agellon A, Jansma P, Wheeler DE: **A bacterial filter protects and structures the gut microbiome of an insect**. *ISME J* 2016, **10**:1866–1876.

13. Moran NA, McCutcheon JP, Nakabachi A: **Genomics and evolution of heritable bacterial symbionts**. *Annu Rev Genet* 2008, **42**:165–190.

14. Knight R, Vrbanac A, Taylor BC, Aksenov A, Callewaert C, Debelius J, Gonzalez A, Kosciolek T, McCall L-I, McDonald D, et al.: **Best practices for analysing microbiomes**. *Nat Rev Microbiol* 2018, **16**:410–422.

15. Gloor GB, Macklaim JM, Pawlowsky-Glahn V, Egozcue JJ: **Microbiome datasets are compositional: and this is not optional**. *Front Microbiol* 2017, **8**.

16. Williamson EM, Hammer TJ, Hogendoorn K, Eisenhofer R: **Blanking on blanks: few insect microbiota studies control for contaminants**. *mBio* 2025, **16**:e02658-24.
* *A literature review of contamination-aware approaches in insect microbiome research,*

demonstrating that most published studies do not adequately control for DNA contamination. *

17. Kaltenpoth M, Flórez LV, Vigneron A, Dirksen P, Engl T: **Origin and function of beneficial bacterial symbioses in insects**. *Nat Rev Microbiol* 2025, doi:10.1038/s41579-025-01164-z.
** *A recent synthesis of the diversity, evolution, transmission, and functions of beneficial bacterial symbioses across insects, providing a comprehensive framework for understanding insect–microbe associations.* **

18. Kaur R, Shropshire JD, Cross KL, Leigh B, Mansueto AJ, Stewart V, Bordenstein SR, Bordenstein SR: **Living in the endosymbiotic world of *Wolbachia*: A centennial review**. *Cell Host Microbe* 2021, **29**:879–893.

19. Oliver KM, Degnan PH, Burke GR, Moran NA: **Facultative symbionts in aphids and the horizontal transfer of ecologically important traits**. *Annu Rev Entomol* 2010, **55**:247–266.

20. Moran NA, Ochman H, Hammer TJ: **Evolutionary and ecological consequences of gut microbial communities**. *Annu Rev Ecol Evol Syst* 2019, **50**:451–475.

21. Ravenscraft A, Coon KL: **Transient microbes in insects: fleeting but functional**. *Annu Rev Entomol* 2026, **71**:253–273.
** *Synthesizes current evidence that environmentally acquired microbes can make important functional contributions to insect biology, broadening the concept of insect microbiota beyond stable symbiotic associations.* **

22. Laroche M, Raoult D, Parola P: **Insects and the transmission of bacterial agents**. *Microbiol Spectr* 2018, **6**:10.1128/microbiolspec.mtbp-0017–2016.

23. Li C, Holmes EC, Shi W: **The diversity, pathogenic spectrum, and ecological significance of arthropod viruses**. *Trends Microbiol* 2025, **33**:826–838.

24. Russell JA, Weldon S, Smith AH, Kim KL, Hu Y, Łukasik P, Doll S, Anastopoulos I, Novin M, Oliver KM: **Uncovering symbiont-driven genetic diversity across North American pea aphids**. *Mol Ecol* 2013, **22**:2045–2059.

25. Yang Q, Gill A, Robinson KL, Umina PA, Ross PA, Zhan D, Brown C, Bell N, MacMahon A, Hoffmann AA: **A diversity of endosymbionts across Australian aphids and their persistence in aphid cultures**. *Environ Microbiol* 2023, **25**:1988–2001.

26. Engel P, Kwong WK, McFrederick Q, Anderson KE, Barribeau SM, Chandler JA, Cornman RS, Dainat J, de Miranda JR, Doublet V, et al.: **The bee microbiome: impact on bee health and model for evolution and ecology of host-microbe interactions**. *mBio* 2016, **7**:10.1128/mbio.02164-15.

27. Kešnerová L, Emery O, Troilo M, Liberti J, Erkosar B, Engel P: **Gut microbiota structure differs between honeybees in winter and summer**. *ISME J* 2020, **14**:801–814.

28. Nowak KH, Hartop E, Prus-Frankowska M, Buczek M, Kolasa MR, Roslin T, Ovaskainen O, Łukasik P: **What lurks in the dark? An innovative framework for studying diverse wild insect microbiota**. *Microbiome* 2025, **13**:186.

* *Demonstrates how contamination-aware processing, microbial abundance quantification, and model-based analyses can reveal biologically meaningful patterns that remain obscured by standard community-level summaries. The study illustrates the analytical framework advocated in this review and forms the basis of Figure 2.* *

29. Moreau CS: **Symbioses among ants and microbes**. *Curr Opin Insect Sci* 2020, **39**:1–5.

30. Lemoine MM, Engl T, Kaltenpoth M: **Microbial symbionts expanding or constraining abiotic niche space in insects**. *Curr Opin Insect Sci* 2020, **39**:14–20.

31. Sudakaran S, Kost C, Kaltenpoth M: **Symbiont acquisition and replacement as a source of ecological innovation**. *Trends Microbiol* 2017, **25**:375–390.

32. Smith AH, Łukasik P, O'Connor MP, Lee A, Mayo G, Drott MT, Doll S, Tuttle R, Disciullo RA, Messina A, et al.: **Patterns, causes and consequences of defensive microbiome dynamics across multiple scales**. *Mol Ecol* 2015, **24**:1135–1149.

33. Zhao D, Zhang Z, Niu H, Guo H: **Pathogens are an important driving force for the rapid spread of symbionts in an insect host**. *Nat Ecol Evol* 2023, **7**:1667–1681.

34. Jones JA, Newton IG, Moczek AP: **Microbiome composition and turnover in the face of complex lifecycles and bottlenecks: insights through the study of dung beetles**. *Appl Environ Microbiol* 2024, **91**:e01278-24.

35. Michalik A, Franco DC, Deng J, Szklarzewicz T, Stroiński A, Kobiałka M, Łukasik P: **Variable organization of symbiont-containing tissue across planthoppers hosting different heritable endosymbionts**. *Front Physiol* 2023, **14**:1135346.

36. Williamson E, Hill K, Hogendoorn K, Eisenhofer R: **The bacterial community associated with the solitary resin bee *Megachile tosticauda* throughout its life cycle**. *FEMS Microbiol Ecol* 2025, **101**:fiaf023.

37. Fierer N, Leung PM, Lappan R, Eisenhofer R, Ricci F, Holland SI, Dragone N, Blackall LL, Dong X, Dorador C, et al.: **Guidelines for preventing and reporting contamination in low-biomass microbiome studies**. *Nat Microbiol* 2025, doi:10.1038/s41564-025-02035-2.
* *A comprehensive overview of DNA contamination in microbiome studies, including its sources, consequences, and practical recommendations for contamination-aware study design and analysis.* *

38. Arora J, Kinjo Y, Šobotník J, Buček A, Clitheroe C, Stiblik P, Roisin Y, Žifčáková L, Park YC, Kim KY, et al.: **The functional evolution of termite gut microbiota**. *Microbiome* 2022, **10**:78.

39. Protasov E, Nonoh JO, Kästle Silva JM, Mies US, Hervé V, Dietrich C, Lang K, Mikulski L, Platt K, Poehlein A, et al.: **Diversity and taxonomic revision of methanogens and other archaea in the intestinal tract of terrestrial arthropods**. *Front Microbiol* 2023, **14**.

40. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO: **Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies**. *Nucleic Acids Res* 2013, **41**:e1.

41. Hanshew AS, Mason CJ, Raffa KF, Currie CR: **Minimization of chloroplast contamination in 16S rRNA gene pyrosequencing of insect herbivore bacterial communities**. *J Microbiol Methods* 2013, **95**:149–155.

42. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, Turner P, Parkhill J, Loman NJ, Walker AW: **Reagent and laboratory contamination can critically impact sequence-based microbiome analyses**. *BMC Biol* 2014, **12**:87.

43. Eisenhofer R, Minich JJ, Marotz C, Cooper A, Knight R, Weyrich LS: **Contamination in low microbial biomass microbiome studies: issues and recommendations**. *Trends Microbiol* 2019, **27**:105–117.

44. Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ: **Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data**. *Microbiome* 2018, **6**:226.

45. Ovaskainen O, Tikhonov G, Norberg A, Blanchet FG, Duan L, Dunson D, Roslin T, Abrego N: **How to make more out of community data? A conceptual framework and its implementation as models and software**. *Ecol Lett* 2017, **20**:561–576.

46. Ovaskainen O, Łukasik P, Roslin T: **Joint species distribution modelling of insect microbiota: Time to jump onto a new opportunity**. 2026, doi:10.32942/X2M388
* *Introduces hierarchical modelling of species communities (HMSC) as a flexible framework for disentangling host, environmental, and microbial drivers of insect microbiota, facilitating the transition from descriptive community analyses to hypothesis-driven ecological inference.* *

47. Williamson E, Hill K, Eisenhofer R, Hogendoorn K: **Experimental evidence for the absence of a functional gut microbiome in the solitary bee *Megachile tosticauda***. *FEMS Microbiol Lett* 2026, **373**:fnag012.

48. Minard G, Tikhonov G, Ovaskainen O, Saastamoinen M: **The microbiome of the *Melitaea cinxia* butterfly shows marked variation but is only little explained by the traits of the butterfly or its host plant**. *Environ Microbiol* 2019, **21**:4253–4269.

49. Saiyawong JNS, Watrous KM, Buchmann SL, Melin A, Hammer TJ: **Sparse gut microbiomes in solitary bees and wasps**. 2026, doi:10.64898/2026.07.02.736198.

50. Rojas-Guerrero D, Roslin, Tomas, Ovaskainen O, Kolasa M, Gårdman, Viktor, Nowak KH, Michalik A, Prus-Frankowska M, Buczek M, Łukasik P: **Cold and lonely: low microbial abundance and no core microbiota in mosquito populations across Greenland**. 2025, doi:10.1101/2024.08.27.609970.