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RESEARCH ARTICLE

BugBook: How to explore and exploit the insect-associated microbiome

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Abstract

Large scale insect farming is exploring routes to enhance the efficiency, stability, and safety of the bioconversion of low-value substrates into insect-derived building blocks for food, feed, and fertiliser. Along with optimising insect rearing conditions and genetics, the insect microbiome is fundamental for the physiology, development, and adaptation of its host to various environmental conditions. To efficiently explore and exploit this ecosystem, a thorough understanding of its composition, function, and dynamics is required. This article aspires to provide a synopsis of the methodologies used to probe the insect-associated microbiome, primarily focusing on industrially relevant insect species. Key considerations for sample timing, selection, storage, and processing are discussed, emphasising the importance of standardised approaches to facilitate cross-study comparisons and enhance reproducibility. Marker gene and shotgun metagenomic sequencing are contrasted as means to investigate microbiome features, touching upon their respective (dis)advantages and potential use cases. Cultivation-based methods are essential for functional characterisation and translating the potential of insect-derived microorganisms for industrial applications. Direct isolation and enrichment cultures, along with anaerobic and aerobic cultivation techniques, are discussed as well. Methods to engineer microbiomes, such as axenic rearing and synthetic community assembly, have developed as powerful tools for exploring the role of specific microbes in host physiology. Beyond these approaches, metabolomics and metaproteomics are emerging as insightful techniques to dig deeper into microbiome functionality and host-microbe interactions. This article provides a multifaceted outline for researchers investigating the insect-associated microbiome and emphasises the importance of standardised methodologies and reporting for advancing the field.

Keywords

Hermetia illucens – *Tenebrio molitor* – microbiome – sequencing – methodology

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1 Why study the insect microbiome?

The study of microbial communities associated with living organisms has gained momentum in recent years, with terms like "microbiome" and "microbiota" becoming central to scientific discourse. While often used interchangeably, these terms carry distinct meanings that are crucial for accurately describing microbial ecosystems and their functions (Berg *et al.*, 2020). Microbiota refers specifically to the collection of microorganisms (bacteria, archaea, fungi, viruses, and protozoa) that inhabit a particular environment, such as the gut, or soil. It emphasises the identity and diversity of these microbial inhabitants. In contrast, microbiome encompasses not only the microbiota but also the genetic material, metabolic products, and interactions within the community, as well as their environmental context. Hence, the microbiome is a more holistic concept spanning both the organisms and their functional capabilities (Berg *et al.*, 2020).

This distinction between microbiota and microbiome is not merely semantic; it also impacts how research approaches the study of microbial communities. When focusing on the microbiota, researchers explore the taxonomic diversity and ecological dynamics of microbial communities, identifying correlations between specific organisms and specific environments. When studying the microbiome, scientists aim to uncover the collective genetic and metabolic potential of these communities, enabling a deeper understanding of their functional contributions. Overall, research on microbial communities is moving from the former, taking stock of the present community, to the latter, trying to understand how these communities function (Gao *et al.*, 2023). The latter does require a more advanced set of methodologies. In fact, it is thanks to technological improvements in several -omics technologies (e.g. genomics, proteomics, metabolomics, and culturomics) (Xu *et al.*, 2024) that it seems within reach to strive for a more in-depth understanding of how microbial communities as a whole function and/or interact with their environment or, in the case of farmed insects, host. Thus, having a consensus on the definitions allows researchers to better frame their questions, methodologies, and interpretations, ensuring clarity and consistency in a rapidly advancing field.

Applications of microbiome research are as diverse as the microbial communities themselves. In medicine, the human microbiome has been linked to numerous physiological and pathological states, from gut health and immune function to conditions such as diabetes and mental health disorders (Hou *et al.*, 2022). In agriculture, the soil microbiome plays a crucial role in nutrient cycling, plant growth promotion, and disease suppression, offering pathways to sustainable farming practices (Banerjee and van der Heijden, 2023). In environmental science, microbiomes are being harnessed for bioremediation, transforming pollutants into less harmful compounds, and even contributing to climate change mitigation through carbon and nitrogen cycling (Silverstein *et al.*, 2023). In insect farming, understanding the microbiome's role in nutrient breakdown and insect health can optimise rearing conditions and productivity (Eke *et al.*, 2023; Jordan and Tomberlin, 2021). Whether we, as a research community, will achieve these envisioned microbiome-based applications for insect farming will not only depend on how the research will be executed, since various parameters will impact the outcome, e.g. sampling site or time, selected target or sequencing methodology, as discussed in detail in the next sections. A perhaps equally important point will be how the results are reported. Since this is key to ensure that the generated data can be pooled to achieve a holistic understanding of what roles microbial communities play during insect farming, and reveal how we can exploit these communities to generate innovative solutions for insect farming.

This article aims to support these efforts by introducing researchers embarking on an exploration of the insect-associated microbial communities to the available methodology, including key caveats and important decisions to be taken when developing an experimental plan. As summarised in Figure 1, it will discuss the state-of-the-art for microbiome sampling (Section 2), -omics techniques to determine the composition of the microbiota (Section 3), methodology to culture and characterise specific members of the microbiota (Section 4) and, finally, how to use microbiota engineering and emerging technologies to assign functions to microbial communities or their members (Section 5).

2 Methodology to sample the insect-associated microbiota

Increased accessibility to faster high-throughput sequencing has brought growing interest in microbiome research. The most frequent approach used is targeted marker genes (e.g. *16S rRNA* and *18S rRNA* genes) amplification sequencing, for which a diverse array of methodologies is available (Section 3). The main steps of a microbiome study always include sampling, storing,

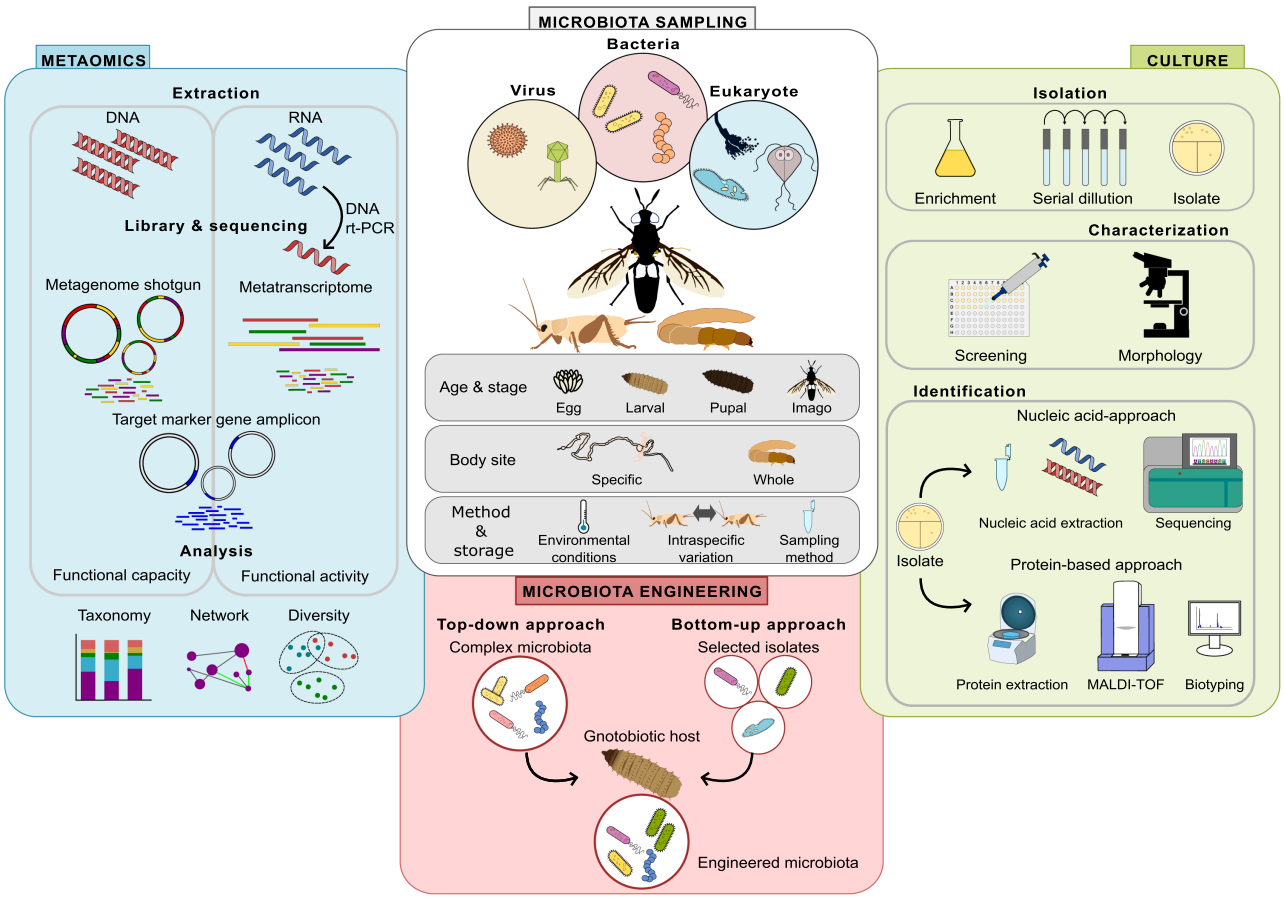

FIGURE 1 Overview of different methodologies available to study insect-associated microbiota.

and processing before sequencing. Key considerations for each of these steps will be described based on the review of pertinent literature. The diversity of protocols and methodologies used across microbiome studies can introduce technical bias at all stages, from sampling to data analysis, potentially leading to discrepancies in reported results. An important part of the conflicting results observed between microbiota studies may originate from methodological differences. It is therefore crucial to document the used methodologies and to consider whether comparison between data obtained using different approaches is relevant, acknowledging also the limitations. Indeed, each approach comes with its own set of advantages and limitations, making the recommendation of a single one useless, as it could not cater to all study-specific concerns. Nonetheless, adopting a more standardised approach would facilitate cross-study comparisons and promote a concerted advance in the field. This section will therefore give an overview of the most common methods and key decision points along the sampling process.

Sample timing: choosing when to sample for microbiome analysis

The results of a study are widely influenced by the timing of sampling. For example, during moulting, the exoskeleton covering the walls of the foregut and hindgut is shed, while the midgut sheds the peritrophic matrix, resulting in a severe disturbance of the microbiota (Engel and Moran, 2013). Sampling directly after moulting may result in an altered community profile. Age is also associated with changes in the microbiome composition and function for the same developmental stage as well as between stages (Dong *et al.*, 2021). Holometabolous insects may change ecological niches between stages and undergo significant reorganisation that modify the microbiome, an opportunity for the host to reconfigure its microbiota communities to better adapt to the new niche (Manthey *et al.*, 2023). The microbiome communities change with developmental stages; and the length of developmental stages will also vary with rearing conditions. An evaluation of the developmental stage should be made alongside the days-post-hatching timing commonly used in the literature, for example by the capsule width method at the larval stage of the black soldier fly (BSF) (Barros *et al.*, 2019) and

morphological cues at the pupal stage (Li *et al.*, 2016). For the BSF, research on the microbiome has predominantly focused on the larval stage, with few attempts to examine other developmental stages (Eke *et al.*, 2023; Querejeta *et al.*, 2023). A great number of studies only focus on the gut microbiota composition across the larval stage (Klammsteiner *et al.*, 2020).

Sampling site: choosing what to sample for microbiome analysis

In insects, the main sites colonised by microorganisms are the exoskeleton, the digestive tract, and the hemocoel. Symbionts may also colonise specialised structures or organelles; however, these structures have not been identified in the mealworm, the BSF, or the house cricket (i.e. mycangia, mycetangia, mycetocyte, bacteriome, bacteriocyte, crypts and pouches) (Currie *et al.*, 2006; Hulcr *et al.*, 2012; Provorov and Onishchuk, 2018). Microorganism communities are associated with specific environments; the sampling site will significantly influence the composition of sampled microorganisms (Hornung et al., 2019). Since most insects not only consume their diet but also inhabit it during their larval stage (e.g. *Hermetia illucens*, *Tenebrio molitor*), sampling the rearing substrate along the insect should be a standard in microbiome studies to provide an informative context to the assembly and composition of the host microbiome.

While some studies investigate the whole insect microbiome by sampling complete individuals, other studies especially target the microbiota associated with specific structures. IJdema *et al.* (2022) performed a meta-analysis on eight microbiome studies that investigated the BSF whole gut, and two on whole larvae (IJdema *et al.*, 2022). They observed a higher bacterial species diversity in whole larvae compared to in larval gut, which they linked to the residual presence of environmental bacteria that reside on the surface of the insect. Intra-specific variations should also be considered when sampling. Intra-specific variation in this context refers to differences in the microbiome composition between individual insects, even when reared in the same rearing container at the same time, the importance of which remains unclear to date. When the target of the study is the microbiome at the community-level, intra-specific variations may be considered as a kind of background noise introducing bias in results (Lange *et al.*, 2023). This may be addressed by pooling samples from several individuals (often in pools of 3 to 5) (Ray *et al.*, 2019).

Another important consideration when sampling, regardless the tissue, is cross contamination. This is the most preventable source of error that can compromise the validity of research. Measures must be taken to sterilise all equipment, tools, and surfaces between each sample to prevent contamination between the samples and from external sources. Sampling should be done near a flame or inside a laminar flow hood to protect the sample from external contamination. The use of negative control must also be made systematically for each methodological step, and should be processed to sequencing as a baseline for contamination.

How to sample the cuticle? Few studies in the edible insect sector have specifically investigated the cuticular microbiome, despite its known microbial diversity in insects (Suen *et al.*, 2010). In contrast, the skin microbiome has been extensively researched in humans, where it functions as the largest organ and serves as the first physical and biological barrier against pathogens (Byrd *et al.*, 2018). Microorganisms colonising the body surface are thought to be involved in host defence mechanisms, primarily through resource competition and metabolites production. A major challenge of cuticle microbiome study is its low bacterial density compared to other habitats, and its susceptibility to contamination from external sources. A study by Birer *et al.* (2017) investigated the DNA extraction methods used for insect body surface microbiome and found that Qiagen QIAamp DNA mini kit (QIAamp; Qiagen, Carlsbad, CA, USA) resulted in the highest amount of reads and OTUs, as well as having the best consistency and repeatability.

How to sample the insect gut? The gut is a common target in microbiota studies, because gut microbiota is expected to be involved in digestion, xenobiotic metabolism and immune system regulation. For example, gut fungal and bacterial microbiota was exposed as playing an important role in the cellulase digestion of lignocellulose into short-chain fatty acids in higher termites (Marynowska *et al.*, 2020). While the gut is a habitat of abundant microorganisms, it is a subsample of a specific set of communities associated with a particular environment in the insect host and should not be mistaken as the microbiome of the host.

Before sampling the gut (or any other internal part of the insect) for microbiome studies, a body surface disinfection step is needed to prevent external contamination. A common approach is a 70% ethanol wash, followed by two sterile water washes to prevent external contamination (Vandeweyer *et al.*, 2023). Recent literature points toward an insufficient effect of simple

ethanol wash treatment (Chen *et al.*, 2023), indicating sodium hypochlorite (i.e. bleach) wash is a more effective approach (Binetruy *et al.*, 2019). The best method for surface disinfection of the adult lesser mealworm was the subject of a full paper (Crippen and Sheffield, 2006), proposing a serial treatment of ethanol and hydrogen peroxide for the complete removal of surface microorganisms.

Insect's digestive tracts present a wide diversity of morphologies, but are always divided into three main successive regions, the anterior, middle and posterior gut, that reflect the successive functional stages of digestion (i.e. ingestion, digestion and adsorption, and waste elimination). Each region is a physiologically distinct environment, that exert unique selective pressures on the microorganism communities colonising these area (Bonelli *et al.*, 2019). In the BSF larvae, the gut harbour region-specific microbiota assemblies, with the midgut further subdivided into sections (i.e. anterior midgut, middle midgut and posterior midgut) each hosting unique bacterial communities (Bruno *et al.*, 2019; Vandeweyer *et al.*, 2023). These sub-regions of the midgut present impressive variations in their pH, ranging from acidic (5.9), to highly acidic (2.1) to basic (8.3) (Bruno, Bonelli, Cadamuro, *et al.*, 2019). In other insect species, this has been suggested as a sanitation mechanism, preventing colonisation of some regions of the digestive tract (Vogel *et al.*, 2017). For dissection purposes, the division of the gut of the BSF can be made based on visual cues. The foregut ends after the proventriculus, where the midgut starts. The hindgut section is easily recognisable as it begins at the same points where the Malpighian tubules connect to the gut (Gold *et al.*, 2018). For more details on how to sample the alimentary canal, check the Bugbook article "*Hermetia illucens* life cycle, reproduction, and collection of tissues and organs for their morphofunctional charaterisation" (Bruno *et al.*, Bugbook in press).

When sampling whole individuals and whole gut, the sample includes both the resident microbiota (stable microbiota that colonise the surfaces) and transient microbiota (temporarily present, i.e. feed and faeces in the digestive tract). Adding a starvation step is a useful approach to reduce transient microbiota. For this starvation step, larvae are separated from the rearing substrate and starved for 24 hours (X. Li *et al.*, 2021). However, this induces a starvation stress, known to modulate microbiota composition in the BSF, leading to a loss of diversity and a change in the functional profile of the communities (F. Yang *et al.*, 2021). In mealworm, post-harvest starvation was not associated with consistent changes in bacterial community composition (Wynants *et al.*, 2017).

Target selection: going for DNA and/or RNA analysis

Regardless of the tissue selected for the study, it is essential to decide whether to analyse DNA or RNA (or both) before starting the sampling process for metagenomics studies (see section on data analysis). Each nucleic acid material has its own advantages and disadvantages in microbiome composition analysis. DNA is more stable than RNA and allows for the sampling of all microorganisms (except for some viruses) present in the sample. However, it does not distinguish between microorganisms that were dead or alive at the time of sampling. Using RNA offers the advantage of only targeting transcriptionally active microorganisms (Blazewicz *et al.*, 2013). However, not all living bacteria in the microbiome will be active simultaneously, since many bacteria alternate between a state of dormancy and activity. Therefore, using DNA may be considered more exhaustive than RNA to profile the microbiome communities. Inference-based databases such as PICRUST2 can be used to infer the functional repertoire of the microbiome based on 16S rRNA sequencing, but this method only provides potential functionality (see section on data analysis). Only metatranscriptome studies based on RNA sequencing can accurately describe the functional activity of the microbiome. As such studies sequence all present RNA including that of microbes, thus mapping all gene expression within the sampled environment. By sampling at varying environmental conditions or diets, such analysis not only allows the visualisation of shifts in the microbiome composition, but also in its functionality and this enables the correlation of functions to environmental conditions.

Generally, working with RNA is more demanding than working with DNA. RNA is more susceptible to degradation because of its chemical instability and the ubiquity of RNases, which can still retain their activity after conventional sterilisation methods (prolonged boiling and autoclaving). A conscientious effort should be made to remove RNase from the working area and material, using RNase decontamination solutions. The RNA expression profile changes rapidly, experimenters should be aware that the profile starts changing as soon a sampling starts. Rapidity is your best ally to get a representative sample unbiased by the sampling process. Larvae (or any other sampled tissue or matrix) must be flash-frozen with liquid nitrogen to stop the RNA degradation process as fast as possible. Once frozen, samples must stay frozen at –80 °C until sample processing.

Thawing must be avoided as much as possible to prevent degradation of the RNA.

Usually, all processing steps, from sampling to sequencing, are done in aerobic conditions. Researchers should keep in mind that some bacteria are anaerobic, meaning they are killed when exposed to oxygen, therefore normal sampling introduces bias towards strict anaerobic bacteria. To preserve live anaerobic bacteria, dissection should be done in anaerobic conditions (Bellali *et al.*, 2019). This is particularly important for culture-based and RNA-based studies.

Sample storing and processing: how to get your samples ready for sequencing

Samples often must stay in storage for considerable time periods before processing. Proper storing of samples is essential to prevent the loss of information from sample degradation. There is a general consensus that rapid freezing (i.e. flash-freezing) to –80 °C is the best practice (Bahl *et al.*, 2012). When freezing is not easily accessible, microbiome DNA in samples can be preserved using cetyltrimethylammonium bromide or 99% ethanol (Z.-W. Yang *et al.*, 2021). Storing in RNA*later* should be avoided for microbial community analysis, since this introduces divergence in microbiota composition compared to fresh samples (Dominianni *et al.*, 2014).

Before processing, samples should be homogenised mechanically (e.g. bead-beating, pestle crushing or grinding) (Gorzelak *et al.*, 2015). Sample processing includes (i) genetic material extraction, (ii) library preparation and (iii) sequencing. Sample processing is determined by the target (viral, bacterial, archaeal, microeukaryotal, fungal DNA or RNA) and the final analysis desired (see section on method selection). Most studies target the bacterial community, because they are arguably the easiest to investigate and because they are better understood. However, the fungal, viral, archaea, and microeukaryote communities may have just as much influence on the host.

Extraction starts with a cell-lysis step, either chemical or mechanical (or both). The chosen extraction protocol affects the final perception of the microbial community, as certain microorganisms can be more resistant to lysis (e.g. endospores), or the protocol may be better for bacterial cell lysis than archaea or fungi (Gerasimidis *et al.*, 2016). Variations in DNA extraction protocols can significantly affect sequencing outcomes, particularly in low-biomass samples. However, several precautions should be taken to minimise contamination and confounding factors. These include adding negative and positive controls, using multiple extraction kits for comparison, and intentionally introducing batch effects to control for unintentional effects (de Goffau *et al.*, 2018). Unintentional batch effects introduced by confounding factors can complicate data processing and obscure the true signal in data. They can emerge from biological, technical, or computational factors, requiring specific considerations for their prevention, as summarised by Wang and LêCao (2020). Including mock communities in sequencing runs as controls can help to detect and assess variations in DNA extraction protocols and batch effects. Because of their known composition, they can be used to reveal amplification and/or sequencing biases that include the wrong representation of specific taxa (over- or under-representation) but also as a positive control, to ensure that the process from DNA extraction to sequencing has been performed in a reliable way (Schloss *et al.*, 2011). The extraction method should be carefully considered before starting the project, as it can have significant influence on the diversity results of the microbiota (Fernández-Pato *et al.*, 2024; Koorakula *et al.*, 2022). A list of extractions methods frequently used for edible insect microbiota research is presented in Tables 1 and 2.

Insects harbour many compounds, some of which can interfere with the extraction efficiency or the downstream PCR amplification (Schrader *et al.* 2012). In honeybees, the pigmentation of the eyes at the red-eyes pupal stage is known to bind with and prevent PCR and RT-PCR reaction (Boncristiani *et al.*, 2011). This compound is also present in other arthropods at a similar stage. A convenient solution to this problem is to remove the insect head prior to sample processing (Bextine *et al.*, 2004). Alternatively, the PCR protocol can also be adapted using additives to enhance its success rate as discussed in the next paragraph.

Optimisation of PCR for amplicon-based sequencing using additives

DNA extracted and purified form whole insects and, depending on the rearing substrate, from gut content may contain very low amounts of microbial DNA and/or chemical contaminants (e.g. pigments as tannins or melanin, heavy metals, complex polysaccharides) that may persist after DNA purification procedures, leading to failure of target sequence PCR amplification. The latter represents the initial key step for amplicon-based sequencing and its failure can compromise the feasibility of this metataxonomic approach. In the case standard adjustments (e.g. choice of a polymerase with optimal performance in terms of yield, sensitivity, and specificity, optimisation of annealing temperature, cycling

TABLE 1 Methods of nucleic acid extraction in black soldier fly microbiota research

Reference	Nucleic acid	Extraction method	Starvation step	Sample type	Life stage	Primers	Target
Kariuki *et al.* (2023)	RNA	Bioline ISOLATE II RNA Mini Kit (Meridian Bioscience)	No	Gut	Larval	NA	Metatranscriptome
Auger *et al.* (2023)	DNA	Phenol–chloroform–alcohol and chloroform extraction method, precipitated in ethanol	No	Body and gut	Multiple	341F/785R	V3-V4 regions of the16S rRNA gene and V9 region of the 18S rRNA gene
Vitenberg and Opatovsky (2022)	DNA	DNeasy® blood and tissue kit (Qiagen, Germany)	No	Gut	Larval	ITS1/ITS2	ITS
Cifuentes *et al.* (2020)	DNA	DNeasy® blood and tissue kit (Qiagen, Germany)	No	Gut	Larval and pupal	341F/785R	Unspecified region of the 16S rRNA gene
Wynants *et al.* (2019)	DNA	DNeasy® Soil Kit (Qiagen, Germany)	No	Body and frass	Larval	515F/806R	V4 region of the 16S rRNA gene
Li *et al.* (2021)	DNA	E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, USA)	Yes	Gut	Larval	27F/1541R	16S rRNA gene
Vandeweyer *et al.* (2023)	DNA	E.Z.N.A.® Soil DNA kit (Omega Bio-Tek, Belgium)	No	Body and gut	Larval	515F/806R	Unspecified region of the 16S rRNA gene
Van Looveren *et al.* (2024)	DNA	E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, USA)	No	Body and frass	Larval	515F/806R	V4 region of the 16S rRNA gene
Pei *et al.* (2022)	DNA	E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, USA)	No	Gut	Larval	338F/806R	V3-V4 regions of the 16S rRNA gene
Gorrens *et al.* (2022)	DNA	E.Z.N.A.® Soil DNA Kit (Omega Bio-Tek, USA)	No	Body	Larval	515F/806R	V4 region of the 16S rRNA gene
Yu *et al.* (2023)	DNA	E.Z.N.A.® Stool Kit (Omega Bio-Tek, USA)	No	Gut	Larval	338F/806R and ITS1/ITS2	16S rDNA and ITS1
Zhang *et al.* (2022)	DNA	FastDNA SPIN extraction kit (MP Biomedicals)	Yes	Gut and frass	Larval	338F/806R	V3-V4 regions of the 16S rRNA gene
Liu *et al.* (2020)	DNA	FastDNA Spin Kit for faeces (MP Biomedicals, France)	No	Gut	Larval	341F/806R	V4-V5 regions of the 16S rRNA gene
Michishita *et al.* (2023)	DNA	NucleoSpin® DNA Stool kits (Macherey–Nagel, Germany)	No	Frass	NA	Unspecified	16S rRNA gene

TABLE 1 (Continued)

Reference	Nucleic acid	Extraction method	Starvation step	Sample type	Life stage	Primers	Target
Klammsteiner *et al.* (2020)	DNA	NucleoSpin® Soil-Kit (Macherey-Nagel, Germany)	No	Gut	Larval	515F/806R	V4 region of the 16S rRNA gene
Tegtmeier *et al.* (2021a)	DNA	NucleoSpin® Soil-Kit (Macherey-Nagel, Germany)	No	Gut and frass	Larval	U341F/U806R and fITS7/ITS4	V3-V4 regions of the 16S rRNA gene and ITS4
Tegtmeier *et al.* (2021b)	DNA	NucleoSpin® Soil-Kit (Macherey-Nagel, Germany)	No	Gut	Larval	U341F/U806R	V3-V4 regions of the 16S rRNA gene
Klammsteiner *et al.* (2021)	DNA	NucleoSpin® Soil-Kit (Macherey-Nagel, Germany)	No	Gut	Larval	515F/806R	V4 region of the 16S rRNA gene
Cai *et al.* (2018)	DNA	PowerSoil® DNA Kit (MoBio Laboratories, USA)	Yes	Gut	Prepupal	341F/806R and ITS5-1737F/ITS2-2043R	V3-V4 regions of 16S rRNA gene and ITS
Li *et al.* (2023)	DNA	PowerSoil® DNA Kit (MoBio Laboratories, USA)	No	Body and gut	Larval	338F/806R	V3-V4 regions of the 16S rRNA gene
Kooienga *et al.* (2020)	DNA	PowerSoil® DNA Kit (MoBio Laboratories, USA)	No	Gut	Larval	515F/806R	V4 region of 16S rRNA gene
Xiang *et al.* (2024)	DNA	QIAamp® PowerFecal® DNA Kit (Qiagen, Germany)	No	Gut	Larval	NA	Metagenome
Pienaar *et al.* (2024)	DNA and RNA	ZymoBIOMICS DNA and RNA Miniprep Kit (ZYMO Research, Germany)	No	Body	Larval and imago	NA	Metatranscriptome (viral)
De Filippis *et al.* (2023)	DNA	SOP_07 (www.microbiome-standards.org) and NucleoSpin® gDNA Clean up kit (Macherey-Nagel, Germany)	No	Gut	Larval	341F/785R	V3-V4 region of the *16S rRNA* gene and shotgun metagenome
De Filippis *et al.* (2025)	DNA	DNeasy® PowerSoil® kit (Qiagen, Italy) and then NucleoSpin® gDNA Clean up kit	No	Gut	Larval	341F/785R	V3-V4 region of the *16S rRNA* gene

TABLE 2 Methods of nucleic acid extraction in other edible insects microbiota research

Reference	Species	Nucleic acid	Extraction method	Starvation step	Sample type	Life stage	Primers	Target
de Miranda *et al.* (2021))	*Acheta domesticus and Gryllus bimaculatus*	DNA and RNA	DNeasy® Blood and Tissue kit and RNeasy Plant Mini kit (Qiagen, Germany)	No	Body and frass	Multiple	Multiple	Multiple
Fernandez-Cassi *et al.* (2020)	*Acheta domesticus*	DNA	DNeasy® PowerFood Microbial Kit (Qiagen, Germany)	No	Body	Unspecified	Unspecified	V3-V4 region of the 16S rRNA gene
Grispoldi *et al.* (2021))	*Acheta domesticus*	DNA	HipurA™ Insect DNA Purification Kit (HiKedia, India)	NA	Body	Unspecified	Other	Specific genes
Cholleti *et al.* (2022)	*Acheta domesticus*	DNA and RNA	TRIzol™ LS reagent and GeneJet RNA Purification kit (Thermo Fisher Scientific, USA)	No	Body	Imago	NA	Metagenome (viral)
Chen *et al.* (2025)	*Gryllus bimaculatus*	DNA	E.Z.N.A.® kit (Omega Bio-Tek)	Yes	Gut	Larval	338F/806R	V3-V4 regions of the 16S rRNA gene
Savio *et al.* (2024)	*Tenebrio molitor*	DNA	DNeasy® PowerSoil® Pro Kit (Qiagen, Germany)	No	Body and frass	Larval	PCR1F_343/ PCR1_R784	V3-V4 regions of the 16S rRNA gene
Urbanek *et al.* (2024)	*Tenebrio molitor*	DNA	Gene-MATRIX Bacterial and Yeast Genomic DNA Purification Kit (EURx, Poland)	No	Gut	Larval	341F/785R	V3-V4 regions of the 16S rRNA gene
Montalbán *et al.* (2022)	*Tenebrio molitor*	DNA	Maxwell® RSC PureFood GMO and Authentication Kit (Maxwell AS 3000, USA).	No	Gut	Larval	Unspecified	V2–4–8 and V3–6 and V7-9 of 16S rRNA gene
Jung *et al.* (2014)	*Tenebrio molitor*	DNA	NucleoSpin® (Macherey-Nagel, Germany)	No	Gut	Larval	27F/518R	V1-V3 regions of the 16S rRNA gene
Cambon *et al.* (2020)	*Tenebrio molitor*	DNA	Phenol–chloroform–alcohol and chloroform extraction method, precipitated in ethanol	No	Body and gut	Larval	PCR1F_460/ OCR1R_460	V3-V4 regions of the 16S rRNA gene
Osimani *et al.* (2018)	*Tenebrio molitor*	DNA	PowerFood® Microbial DNA Isolation Kit (MoBio Laboratories, USA)	No	Body and frass	Larval and frass	341F/805R	V3-V4 regions of the 16S rRNA gene

TABLE 2 (Continued)

Reference	Species	Nucleic acid	Extraction method	Starvation step	Sample type	Life stage	Primers	Target
Przemieniecki *et al.* (2020)	*Tenebrio molitor*	DNA	QIAamp® PowerFecal® DNA Kit (Qiagen, Germany)	No	Gut	Larval	Unspecified	V3-V4 regions of the 16S rRNA gene
Yu *et al.* (2024)	*Tenebrio molitor*	DNA	QIAamp® PowerFecal® DNA Kit (Qiagen, Germany)	No	Gut	Larval	Unnamed	V3-V4 regions of the 16S rRNA gene
Cambon *et al.* (2018)	*Tenebrio molitor*	DNA	Quick Extract, Bacterial DNA extraction (TEBU-BIO)	Yes	Gut	Larval	PCRIF_460/ OCRIR_460	V3-V4 regions of the 16S rRNA gene
Mamtimin *et al.* (2023)	*Tenebrio molitor*	DNA	SPINeasy® DNA Kit for Feces (MP Biomedicals, Singapore)	No	Gut	Larval	338F/806R	V3-V4 regions of the 16S rRNA gene

duration, and Mg^{++} concentration) do not improve the PCR efficiency, the latter can be significantly improved by the addition of additives in the reaction mixture (Wilson 1997). Dimethylsulfoxide (DMSO), formamide, and betaine are reported to enhance the amplification effectiveness, especially for G-C rich DNA templates (Wilson, 1997). Namely, DMSO (up to 10% in the reaction mixture) was successfully used to allow the amplification *16S* and *18S rRNA* genes in mosquito-associated microbiota (Thongsripong *et al.*, 2018). Another additive that proved to be effective in enabling or increasing amplification in the presence of low amounts of DNA and/or inhibitors (e.g. phenolic compounds, organic solvents) is bovine serum albumin (BSA), at final concentrations up to 1 μg/μl in the reaction mixture (Farell and Alexandre, 2012; Wilson, 1997). PCR amplification of target *16S rRNA* gene sequence was particularly challenging from DNA extracted from gut content of BSF larvae reared on coffee silverskin, a by-product of the roasting process that is rich in phenolic compounds as lignin, tannins and melanoidins (De Filippis *et al.*, 2025). Despite DNA purification, amplification failed in most samples. The addition of BSA (0.8 μg/μl) to PCR mixture led to good amplification in some samples, and modest or none in others. Optimal results were thus obtained by the addition of co-enhancers to BSA. In particular, although frequently used co-additives as DMSO and formamide did not work, high-yield amplification was obtained using polyethylene glycol at concentrations of 2-5% (v/v) (De Filippis *et al.*, 2025).

Virome sampling and processing

Contrary to the other microorganism groups, the virome does not share a marker gene that can be targeted using universal primers. Virus discovery requires the use of multiple hallmark genes that are not universally found in viruses but enable the discovery of part of the virome (Bonning, 2019). Viruses can infect all walks of life, from bacteria to eukaryotes, making them powerful modulators of the microbiome (L.-Y. Wu *et al.*, 2023). Seven orthologous endogenous viral elements related to the families *Parvoviridae*, *Partitiviridae*, *Rhabdoviridae*, *Totiviridae* and *Ximnoviridae* were identified from screening the BSF genome, and three viruses were discovered in transcriptome datasets, one totivirus (hermetia illucens totivirus 1) and two *Bunyavirales* (Pienaar *et al.*, 2022; Walt *et al.*, 2023). In the mealworm, viral interest was directed toward pathogens, mainly the newly identified *Tenebrio molitor* densovirus (Armién *et al.*, 2023). Alternatively, specific protocols can be used that enable the isolation of viral particles and the extrac-

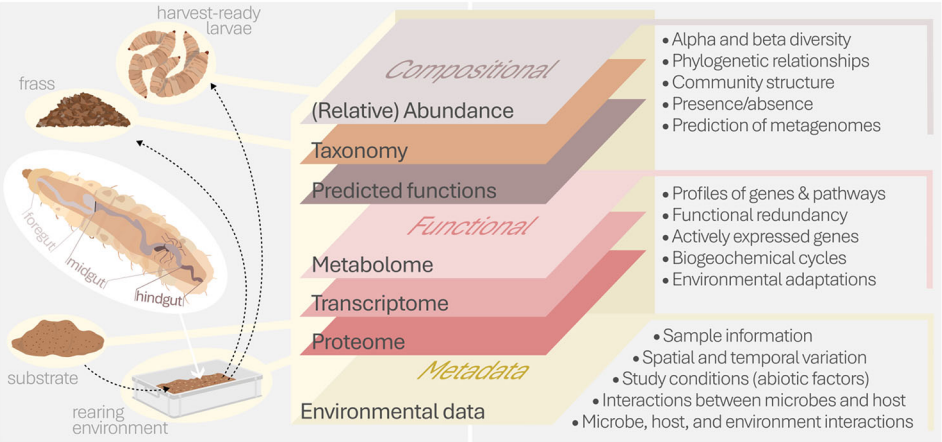

FIGURE 2 Layers of data to consider when investigating microbial communities in the context of insect farming, with metadata as the layer affecting and connecting all other layers.

tion of the encapsulated DNA to allow sequencing of the viral metagenome. One such protocol is the NetoVIR protocol (Conceição-Neto *et al.*, 2018), that can also be used on insect samples, as illustrated by past research on honeybee samples (Deboutte *et al.*, 2020).

Microeukaryotes (protozoa) sampling and processing
Microeukaryotes are largely ignored because they share marker genomic sequences like the 18S with the host, making their detection impossible without first depleting the host genome (Belda *et al.*, 2017). For targeted marker amplification and sequencing, microeukaryotes microbiota research requires an additional processing step, which aims to block host marker gene amplification with blocking primers. Such a technique was implemented with moderate success to characterise the microeukaryote microbiota in black soldier fly (Auger, Deschamps, *et al.*, 2023).

3 Method selection: choosing the best approach to determine the composition of the sampled microbiome

The palette of methods and tools available to explore microbial communities, both compositional and functional, is steadily expanding and becoming increasingly accessible (Figure 2).

In this section, focus will be on the characterisation of microbiome composition, with particular emphasis on marker gene sequencing (often also referred to as 'amplicon sequencing' or 'metabarcoding'), a well-established, cost-effective, and high throughput technique. Some aspects were extended with reference to shotgun metagenomics, which provides a more comprehensive and in-depth analysis of microbial communities. The choice of method largely depends on the research scope and significantly influences the resolution and depth of analysis. Understanding the unique strengths and limitations of each approach is essential for optimising microbiome studies.

Marker gene sequencing vs. metagenome shotgun sequencing

With continuously decreasing costs for sequencing and more accessible tools for the analysis of generated sequence data, sequencing methods targeting the environmental or host microbiota also find auxiliary use in studies that do not necessarily focus on microbial aspects, but use these observations as supportive elements (e.g. assessing the impact of different diets on insect development, evaluation of an insect's waste conversion efficiency, or behavioural studies on insects). As discussed above (see Section 2), selecting, storing, and processing samples for subsequent sequencing requires knowledge of how microorganisms interact with their environment and higher life. This requires incorporating sequencing considerations into the general experimental design from the start. It is important to plan precisely when, which, and how many samples will be collected, the level of replication, and what specific research questions will be addressed by characterising the targeted microbial communities (Knight *et al.*, 2018).

Marker gene sequencing is a targeted approach that aims to amplify hypervariable regions (V1-9) of the bacterial and archaeal 16S rRNA gene (Takahashi *et al.*, 2014; Tegtmeier *et al.*, 2021a) and the internal transcribed spacer (ITS) region in fungi (Abarenkov *et al.*, 2024) (Figure 3A). The 16S rRNA gene is highly conserved, whereas the hypervariable regions vary across

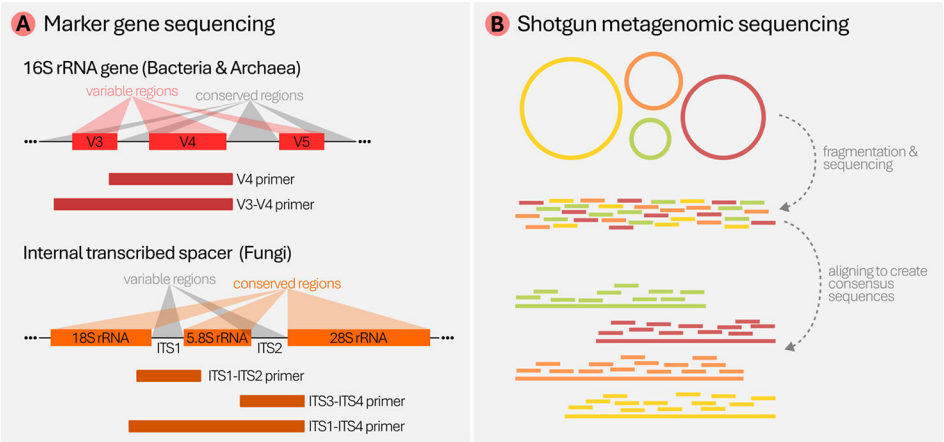

FIGURE 3 Overview of two key methods for characterising microbial communities in terms of composition: (A) marker gene sequencing, targeting hypervariable regions of the 16S rRNA gene of bacteria and archaea, and the internal transcribed spacer (ITS) region in fungi, using the most commonly employed primers in studies of farmed insects. (B) Shotgun metagenomic sequencing as a method for the comprehensive and in-depth analysis of microbial diversity and function.

TABLE 3 Key features and potential use cases for marker gene sequencing and shotgun metagenomic sequencing

Technology	Key features	Potential use cases
Marker gene sequencing (16S rRNA gene and ITS)	Low-cost Fast turnaround times Low computational requirements More accessible in terms of data processing and interpretation	Monitoring microbial diversity over time Assess the effect of various substrates on the gut microbiota Detect shifts in microbial communities that could indicate contamination or disease outbreaks in populations (dysbiosis) Compare microbiome composition across studies / farming environments
Shotgun metagenomic sequencing	High taxonomic resolution and coverage Allows functional characterisation Wider applicability (captures all microorganisms)	Understand functional roles of microbiomes Identify genes involved in insect nutrient digestion, feed conversion, and immune responses In-depth exploration of microbe-host relationships

taxa and allow for economic and efficient taxonomic identification at the genus level (Table 3) (Bharti and Grimm, 2021). In fungi, the ITS regions are more conserved within species and allow for taxonomic characterisation. A higher taxonomic resolution down to the species level using amplicon sequencing variants (ASV) has been proposed but should be applied and interpreted carefully (Callahan *et al.*, 2017; Watson *et al.*, 2021).

Shotgun metagenomic sequencing, as an untargeted approach, offers high taxonomic resolution down to the strain level, along with the possibility of functionally characterising microbial communities by fragmenting and sequencing the total DNA and building consensus sequences instead of investigating only the targeted region (Figure 3B). However, this method is more complex in terms of data processing and interpretation and requires more extensive computational power (Table 3). In samples obtained from e.g. the insect gut, contamination with host DNA poses a challenge, as the sequencing efficiency of microbial DNA can be reduced if large amounts of host DNA are present (Pereira-Marques *et al.*, 2019). Nevertheless, starting from midgut content enclosed in peritrophic matrix allows to minimise or even avoid contamination by host DNA. This strategy has been successfully applied for the characterisation of BSFL midgut microbiome by shotgun metagenomics (De Filippis *et al.*, 2023).

In summary, the choice of approach depends on the goals of the study (community profiling vs. functional profiling), costs and (computational) resources, and available bioinformatical expertise. The key features

and potential use cases of the two methods are listed in Table 3.

Method variables to consider when selecting the most suited sequencing platform

The platform used to generate sequence data affects the output and features of the data. For marker gene sequencing, Illumina MiSeq (San Diego, CA, USA) has long been the gold standard, and many sequencing providers perform analyses on this platform. It is cost-effective for smaller projects and offers fast turnaround times, while generating relatively long reads of up to 2 × 300 bp. However, the Illumina NovaSeq6000 platform is steadily gaining more users as it offers much higher throughput while maintaining cost efficiency and generates more data (higher sequencing depth); however, at the price of slower turnaround times, increased computational requirements, and typically shorter read lengths (up to 2 × 250 bp). For sequencing of longer reads and shotgun metagenomic sequencing, Illumina HiSeq and NovaSeq platforms, PacBio's Sequel (Pacific Biosciences, Menlo Park, CA, USA), and Oxford Nanopore (Oxford, UK) platforms, including MinION, GridION and PromethION, are frequently used.

For marker gene sequencing, the choice of suitable primers targeting one or more specific hypervariable regions is more important than the sequencing platform. For bacteria, primer pairs amplifying the V3-V4 (e.g. 341F/805R; Takahashi *et al.*, 2014) or V4 (e.g. 515F/806R; Caporaso *et al.*, 2011) region are most frequently used. However, opinions differ regarding which pairs should be preferred. While V3-V4 primers cover a longer stretch on the 16S rRNA gene and, thus, provide longer contigs, concerns have been raised that the much shorter overlap between forward and reverse reads, compared to the reads generated using V4 primers, leads to more erroneous contigs (Kozich *et al.*, 2013). When working in a specific environment or host, it is advised to consult the available literature and opt for the primers most used in this area of research, as it drastically enhances the comparability across studies and facilitates subsequent meta-analyses of collected datasets. However, users should be aware that a given targeted 16S rRNA regions will exhibit differences in their detection ability of some bacterial taxa and in the assessment of community diversity (Soriano-Lerma *et al.*, 2020).

The same applies to studies focusing on the mycobiome (i.e. the fungal communities) using primers that target the internal transcribed spacer (ITS) regions (e.g. the ITS1-ITS4 primers covering the ITS1 and ITS2 regions; Gardes and Bruns, 1993). However, analysing data from fungal communities poses certain challenges. There are less well-curated and standardised databases available than for 16S rRNA data, with the most prominent being the UNITE database (Abarenkov *et al.*, 2024). The ITS region is also less conserved even within closely related species, potentially leading to amplification biases, and ITS sequences vary in length across taxa which leads to variable lengths in the generated sequences (Kauserud, 2023).

Data analysis

Bioinformatics is a fast-growing field, and efforts to make bioinformatics tools more accessible to a wider audience are constantly developing. Established methods such as marker gene and shotgun metagenomic sequencing can now also be employed without knowledge of programming languages or command-line applications (Abdelsalam *et al.*, 2023). Sequencing providers often process sequencing data using in-house bioinformatics pipelines and deliver publication-ready figures. However, it is strongly advised to only use these outputs to gain a first insight into the generated data and subsequently conduct your own processing, filtering, statistical testing, and visualisation of data, as each dataset comes with its own peculiarities (outliers, varying sequencing depth, batch effects) that require customised processing.

Before data analysis, the raw sequence data needs to be pre-processed, which includes various filtering steps (low quality bases, adapter removal, dereplication, chimera removal, length filtering, low abundance filtering), clustering in operational taxonomic units (OTUs) or identification of amplicon sequence variants (ASVs), and taxonomic alignment (Knight *et al.*, 2018). Whether to rarefy uneven sequencing depths is an ongoing debate; however, recent research indicates that rarefaction is still the most robust approach to manage differences in sample read numbers (McMurdie and Holmes, 2014; Schloss, 2024). Among the most widely used tools for sequence data processing are mothur (Schloss *et al.*, 2009), QIIME 2 (Bolyen *et al.*, 2019) and dada2 (Callahan *et al.*, 2017). Each approach has its advantages and disadvantages and requires specific considerations. Data analysis tools that are free of charge often come with the downside that they require at least an intermediate understanding of how to use the command line or work in one of the programming languages popular for bioinformatics (R, Python and, more recently, Julia). However, with the increased use of marker gene sequencing, more

TABLE 4 Non-exhaustive list of frequently used alpha (α) and beta (β) diversity indices

Type	Measure	Description
α	Observed species (S_{obs})	Total number of species observed in a sample.
	Shannon index (H') (Shannon, 1948)	Accounts for species richness and evenness, considering also rare species. Increases with increased abundance and evenness of species.
	Chao1 index (S_{chao1}) (Chao, 1984)	Estimate of species richness that takes into consideration the number of rare species in a sample.
	Simpson's diversity index (D) (Simpson, 1949)	Calculates the probability that two randomly selected individuals from a sample belong to the same species.
	Pielou's evenness index (J) (Pielou, 1966)	Measures how even species are distributed, i.e. how equal the abundances of species are within a sample. The higher J', the more even the species abundance are.
	Abundance based coverage estimator (ACE) (Chao and Yang, 1993)	Indicator for species richness sensitive to rare OTUs.
β	Bray–Curtis dissimilarity (Bray and Curtis, 1957)	Measures community dissimilarity based on relative abundance of species and ranges from 0 (identical) to 1 (completely different).
	Jaccard similarity coefficient (Jaccard, 1912)	Measures community similarity based on presence/absence data
	Weighted UniFrac (Lozupone *et al.*, 2007)	Phylogenetic metric that includes both the evolutionary distances between species and their relative abundance.
	Unweighted UniFrac (Lozupone *et al.*, 2007)	Phylogenetic metric based on presence/absence of species, focusing on the proportion of unique branch lengths in a phylogenetic tree between communities.

The term 'species' is used interchangeably with OTUs (Operational Taxonomic Units) or ASVs (Amplicon Sequence Variants).

accessible point-and-click solutions, such as MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/) or QIIME2view (https://view.qiime2.org/), have emerged, allowing even complete beginners to explore their amplicon data, perform preprocessing steps, and visualise data (Lu *et al.*, 2023). Popular packages for statistical analysis and visualisation of microbiome data in R include phyloseq (McMurdie and Holmes, 2013), mia (Borman *et al.*, 2024), and vegan (Oksanen *et al.*, 2001). Similar Python packages include QIIME 2 (Bolyen *et al.*, 2019) and scikit-bio (Rideout *et al.*, 2023).

Statistical analysis of microbial community data typically begins by calculating alpha and beta diversity indices to describe within-sample diversity and diversity between samples, respectively (Table 4). Alpha diversity describes community richness and evenness within a sample, whereas beta diversity facilitates the comparison between samples or groups of samples to outline patterns of (dis)similarity. Ordination methods, including non-metric multidimensional scaling or principal coordinate analysis, are used to reduce multidimensional datasets (samples × features (OTUs, ASVs, species, etc.)) into a two (or three)-dimensional coordinate system to visualise relationships between samples based on their community profiles. In addition, tools such as PICRUSt2 (Douglas *et al.*, 2020) can infer, to some extent, metabolic functions from marker gene data, offering a cheaper, but prediction based, alternative to the more expensive shotgun metagenomic sequencing by providing a rough insight into microbial community functions. To investigate differentially abundant taxa between groups, statistical methods such as linear discrimination of effect size (Segata *et al.*, 2011), ANCOM-II (Kaul *et al.*, 2017), and ALDEx2 (Fernandes *et al.*, 2013) are frequently used.

Standardisation of method reporting

Good reporting starts with careful recording and notetaking of all the data processing and analysis steps. As the time spent analysing a dataset increases, it becomes progressively more difficult to track the changes made, making thorough documentation essential for maintaining a clear overview (Table 5). Python and R are widely used for data analysis and statistics in micro-

TABLE 5 Minimum information to be provided for sequencing projects

Analysis step	What to include in the description
(1) Sample collection and DNA extraction:	(a) How were the samples processed? (b) Which DNA extraction kit was used?
(2) Sequencing and library preparation:	(a) Which platform was used? (b) What hypervariable region was targeted using which primer pair? (c) What library preparation protocol was used (d) What is the sequencing depth per sample?
(3) Bioinformatics analysis	(a) Which tools and versions thereof were used? (b) Which reference databases and versions thereof were used? (c) What settings were applied (e.g. for quality filtering)?
(4) Statistical testing	(a) What tools/packages and versions thereof were used? (b) Which tests were conducted and what were their settings? (c) What significance thresholds were set? (d) How were samples grouped? (e) What method was used, if any, to correct for multiple testing?
(5) Data availability	(a) How can the data stored in a public repository be accessed? (b) Where can additional metadata be found (e.g. GitHub)

biome studies, owing to their readability, versatility, and extensive toolsets. Both languages are also used in powerful tools for documentation such as Jupyter Notebooks and R Markdown documents, which provide flexible possibilities for interactive, automated, and reproducible reporting. Reproducible documentation and storage of sequence data and corresponding metadata in publicly accessible repositories is essential (Huttenhower *et al.*, 2023). Emerging from the field of human microbiome research, international consortia have elaborated on best practice approaches in conducting microbiome studies and sharing, analysing, and storing generated sequence data (Bharti and Grimm, 2021; Huttenhower *et al.*, 2023; Knight *et al.*, 2018). Metadata must be carefully linked to raw sequence data to facilitate sample identification and integrate environmental information (Figure 3). This can be achieved using unified sample identifiers at the replicate level, ensuring consistency and traceability across datasets. Several standardised formats for metadata compiled by the Genomic Standards Consortium are available (https://genomicsstandardsconsortium.github.io/mixs/). Moreover, Findable, Accessible, Interoperable, Reusable (FAIR) data principles should be followed to maximise reproducibility. This necessitates that the generated sequence data is deposited in public archives such as the National Center for Biotechnology Information (NCBI) or the European Nucleotide Archive (ENA).

4 Culturing and identifying insect-associated microorganisms

Functional characterization and subsequent industrial applications of insect-associated microorganisms such as bacteria, archaea and fungi rely on pure cultures. Microbial isolates with beneficial functions can further be developed as probiotics for insect farming as well as for various other fields such as biotechnology, medicine and agriculture. Therefore, we evaluate several common cultivation methodologies and strategies and how they can be implemented for the isolation and characterisation of insect-associated microorganisms.

Sampling from insects

In order to culture gut microorganisms, insects have to be dissected. Usually, the insects are first immobilised by cooling on ice and subsequently killed by decapitation. After surface sterilisation of the insect body, guts are dissected using sterile forceps and micro-scissors. For culturing anaerobic microorganisms, insects have to be dissected under nitrogen atmosphere in an anoxic glove box or intact guts have to be placed immediately after dissection in culture tubes with anoxic media containing a reducing agent. Gut homogenates can be generated by grinding with a mortar or by vortexing with glass beads in a culture tube with the respective cultivation medium and oxygen-free headspace (Tegtmeier *et al.*, 2016a,b). Homogenates from whole insects can be generated by a similar procedure. However, depending

on the specimen, insects may be cut into smaller pieces before the homogenisation step. Ideally the samples are immediately used for subsequent cultivation in order to recover as many viable cells as possible. A short storage in the fridge is possible, but this might affect the microbial community in the samples. For a long-term storage whole guts and their microbiomes can be cryopreserved by freezing at –80 °C in glycerol. Compared to normal freezing this method is intended to prevent the formation of ice crystals that damage cell membranes.

Direct isolation vs. enrichment cultures

The approach of direct isolation allows the isolation of the most abundant culturable microorganisms. For this purpose, the gut homogenates are serially diluted and directly plated onto solid agar media aiming to obtain separate colonies, which can be picked with an inoculation loop. Pure cultures can be obtained after two to three consecutive transfers to fresh media. This approach is the most suitable for culture-dependent analysis of the insect gut microbiome, as the abundant culturable microbes which are present in the gut at the sampling time are targeted. This method has been used by Gorrens *et al.* (2021b) to obtain a culture collection of several of the most abundant bacteria form whole *Hermetia illucens* larvae. A large proportion of bacteria found by amplicon-sequencing in *H. illucens* guts has already been brought into pure culture by direct isolation using a variety of culture media as well as aerobic and anaerobic cultivation techniques. By including several selective and low nutrient media in this cultivation approach, also less abundant taxa have been isolated (Tegtmeier, Hurka, Mihajlovic, *et al.*, 2021). As part of direct microbial isolation, also a microbial count can be performed either by recording the total viable count (TVC) on non-selective media or by using selective media targeting specific groups. This is of particular interest for the assessment of potential foodborne pathogens in whole edible insects. Selective growth media for several microbial target groups such as Enterobacteriaceae, *Salmonella* spp., *Staphylococcus* spp., *Bacillus cereus*, *Listeria monocytogenes* and fungi have been used by Fröhling *et al.* (2020) to determine microbial counts during the processing chain of *Acheta domesticus* and by Wynants *et al.* (2018) for microbial counts from whole *Alphitobius diaperinus*.

The approach of enrichment cultures can be used either to isolate abundant microorganisms or fastidious, slow-growing or less abundant microorganisms. To isolate the most abundant microorganisms the "dilution to extinction" method is commonly used. Less abundant microbes are excluded by the dilution steps and only the abundant microbes will be enriched by incubation and can subsequently be isolated by streaking. Using this method Cifuentes *et al.* (2022) obtained a variety of aerobic heterotrophic bacteria, including several genera, which have been discussed as part of the core microbiome of *H. illucens* in previous studies.

In order to enrich a specific group of bacteria, selective media for the desired microbes have to be prepared. This can be achieved by using special substrates which cannot be utilised by the majority of microbes, except the desired ones. For example, cellulose-degrading bacteria present in insect gut samples can be enriched by culturing in basal medium with cellulosic materials such as filter paper, carboxymethylcellulose (CMC) or ball milled-cellulose (Cruden and Markovetz, 1979; Gupta *et al.*, 2012; McDonald *et al.*, 2012). Callegari *et al.* (2020) used different enrichment media (e.g. uric acid, CMC and filter paper medium) to selectively enrich uricolytic and cellulolytic gut bacteria from *H. illucens*. Media with polystyrene as sole carbon source have been applied to selectively enrich potential polystyrene degrading bacteria from *Tenebrio molitor* guts (Lin *et al.*, 2024; Machona *et al.*, 2022). Methanogenic archaea can be enriched by applying hydrogen and CO_2 or methanol and CO_2 as the only substrates to the medium (Paul *et al.*, 2012). Furthermore, antibiotics can be used to eliminate certain bacteria and enrich methanogenic archaea (Carson *et al.*, 2019) or bacteria resistant to the used antibiotic in the culture.

A pasteurisation step at 70 to 90 °C can be applied, to kill vegetative cells and select for spore-forming or heat-resistant bacteria. This method has been applied by Tegtmeier *et al.* (2021b), to isolate spore-forming *Bacillus* and *Clostridia* species from *H. illucens* guts.

For the enrichment of a specific group of bacteria also buffer systems and the choice of media additives have to be considered. High concentrations of phosphate can inhibit some microorganisms such as methanogenic archaea (Conrad *et al.*, 2000). Therefore, special media with alternative buffer systems such as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) or bicarbonate buffer (Tegtmeier *et al.*, 2016a,b) might be applied when culturing certain insect gut microorganisms. As host-associated environments often harbour more auxotrophic taxa then other environments (Ramoneda *et al.*, 2023) certain media additives such as vitamins, casamino acids and yeast extract should be provided in low amounts when culturing insect gut bacteria (Tegtmeier *et al.*, 2016a,b).

In order to design targeted enrichment and isolation strategies, information about the microbial community present in the gut sample and the potential metabolism of the desired microbes are needed. Therefore, a culture-independent microbiome analysis (e.g. amplicon sequencing, metagenomics or metatranscriptomics), as described in the previous section is highly recommended prior to setting up enrichment cultures and complex cultivation processes.

Anaerobic vs. aerobic cultivation

Prior to the development of anaerobic culturing, *E. coli* was thought to be the main inhabitant of the animal intestine since early microbiologists commonly grew bacteria under air. However, *E. coli* is often only a minor inhabitant in most gastrointestinal ecosystems (Ingerson-Mahar and Reid, 2011). Besides the vertebrate gut also the guts of many insects are partly anoxic environments (Appel and Martin, 1990; Brune, 1998; Johnson and Barbehenn, 2000; Schauer *et al.*, 2012; Tegtmeier *et al.*, 2016a,b). Next-generation sequencing techniques have shown that insect guts commonly harbour obligately anaerobic bacteria, especially in the orders Blattaria, Megaloptera and Coleoptera they are found in high relative abundance (Yun *et al.*, 2014). The guts of termites, cockroaches and beetle larvae are colonised by methanogenic archae and obligately anaerobic members of different phyla: Bacteroidota, Spirochaetota and Bacillota. Examples of the latter are Lachnospiraceae (family) and Ruminococcaceae (family) of the Clostridia (class) (Brune, 2018; Ozbayram *et al.*, 2024; Schauer *et al.*, 2012). Also, *H. illucens* guts can be colonised by obligately anaerobic bacteria in higher relative abundance, depending on the type of diet used for the larval feeding (Klüber *et al.*, 2022). Furthermore, in *T. molitor* obligately anaerobic bacteria such as *Selenomonas*, *Clostridium* and *Ruminococcus* have been detected by amplicon sequencing (Przemieniecki *et al.*, 2020). However, very few of these anaerobic bacteria have been cultured so far and anaerobic microorganisms are often underrepresented in cultivation-dependent studies. As oxygen is toxic to obligately anaerobic microorganisms, they require special anaerobic cultivation techniques. Anoxic media can be prepared by boiling, evacuation and applying inert gases such as nitrogen, CO_2 or helium to the headspace of the culture via the Hungate technique (Hungate, 1947). Furthermore, reducing agents such as thioglycolate, cysteine-HCl, sodium sulfide and sodium dithionite can be used to generate anoxic cultivation media (Wagner *et al.*, 2019).

Culture vessels have to be airtight e.g. glass serum bottles or test tubes sealed with butyl rubber stoppers. Anoxic glove boxes can be used to culture strict anaerobes without exposure to oxygen. Anaerobes which tolerate a short exposure to air (e.g. some spore-forming Clostridiales and Lachnospiraceae) often grow on the agar surface and can be isolated by plating dilution series on plates with agar medium, followed by subsequent incubation in an anoxic jar. Using this approach Tegtmeier *et al.* (2021b) and Klüber *et al.* (2024) obtained pure cultures of the respective anaerobic taxa form guts of *H. illucens* reared on chicken feed and different industrial side streams. An anoxic atmosphere can be created by flushing the culture vessels with nitrogen or by using gas packs, which bind oxygen and release CO_2 (e.g. via the reaction of Na_2CO_3 with citric acid). Isolation of strict anaerobes can be achieved by deep agar dilution series (Pfennig and Trüper, 1981). Using anaerobic cultivation techniques both obligately and facultatively anaerobic microorganisms can be cultured, thus covering a large proportion of insect gut microbes which can provide new insights into microbial communities.

However, the cultivation under oxic conditions is less time consuming and often allows a high yield of aerobic and facultatively anaerobic microbial isolates. For example, Oberpaul *et al.* (2020) used a specialised high-throughput cultivation platform for the isolation of Acidobacteriota from termite nests. Callegari *et al.* (2020), Cifuentes *et al.* (2022) and Gorrens *et al.* (2021b) obtained larger strain collections form *H. illucens* guts by aerobic cultivation. Aerobic or microaerophilic cultivation can also be used to isolate microbes which colonise the gut wall or the microoxic gut periphery. Potential lignin- and cellulose-oxidizing Actinomycetota that colonise the microoxic periphery of the hindgut, have been found in termites (Salgado *et al.*, 2024). Such microbes could be cultured under microoxic conditions with a reduced oxygen atmosphere of 1–8% and an elevated CO_2 atmosphere in the headspace.

Identification of microorganisms

Currently, sanger sequencing of phylogenetic maker genes such as the 16S rRNA gene of bacteria and archaea and the Internal Transcribed spacer (ITS) of fungi is the gold standard to identify microorganisms up to species level. Genomic fingerprinting can further be used to differentiate between different strains of the same species. Genomic fingerprinting of larger bacterial strain collections from *H. illucens* has been used by Tegtmeier *et al.* (2021b) and Cifuentes (2022) for genotyping and to exclude redundant stains from further analysis.

MALDI-TOF mass spectrometry, which is based on the measurement of species-specific protein spectra from ribosomal proteins is also commonly used for microbe identification, especially in clinical samples (Wieser *et al.*, 2012). This method has been used by Gorrens *et al.* (2021b) to identify abundant bacteria isolated from *H. illucens* and by Fröhling *et al.* (2020) to identify bacteria in *Acheta domesticus* along the processing chain. The advantage of this method is that it is less time-consuming than sanger sequencing as bacterial cells can be directly applied and no DNA extraction and amplification are needed. However, the databases used for identification via MALDI-TOF might not be sufficient for identification of several insect gut microbes.

In vitro characterisation

Commonly the *in vitro* characterisation of microbial isolates involves the analysis of metabolic properties such as substrate spectrum, enzymatic activity and the formation of fermentation products as well as physiological properties such as pH-and temperature range, salinity and oxygen requirement and other growth conditions. Furthermore, for the description of novel taxa a phenotypic characterisation including cell morphology (e.g. by electron microscopy), gram determination, catalase and oxidase activity and chemotaxonomic markers (e.g. fatty acid profiles, polar lipid composition and quinone types) are required by several journals such as International Journal of Systematic and Evolutionary Microbiology (IJSEM).

Microbial isolates from farmed insects have been screened mostly for properties useful for potential industrial applications. Bacterial isolates from *H. illucens* guts have been characterised in regard of their hydrolytic abilities (Callegari *et al.*, 2020). Cellulolytic activity has been screened by Jeon *et al.* (2011), Callegari *et al.* (2020) and Klüber *et al.* (2024) by growing the strains on media with carboxymethylcellulose and subsequent staining with Congo Red. Pectinase, esterase, lipase, and protease activity has been tested by similar approaches using different cultivation media and staining methods (Callegari *et al.*, 2020; Jeon *et al.*, 2011). Furthermore, *H. illucens* gut isolates have been screened for antimicrobial activity using an inhibition zone assay (Tegtmeier *et al.*, 2021b). Klüber *et al.* (2022) screened *H. illucens* gut isolates for their putative entomopathogenicity by intracoelomal injection into *H. illucens* larvae. Isolation and screening strategies of bacteria from *Tenebrio molitor* guts mostly focused on polystyrene degradation by using emulsion and agar containing polystyrene (Machona *et al.*, 2022) as well as by scanning electron microscopy and observation of polystyrene film degradation (Lin *et al.*, 2024).

5 Methodology to assess the role of (single) microorganisms during insect ontogeny *in vivo*

Generating axenic larvae as tools to explore the link between microbes and host phenotype

Axenic models have been used since the 1960s as a tool to characterise and quantify the impact of the microbiota on its host (Gustafsson and Lanke, 1960). The term Axenic is used to describe metazoan exempt of any microorganisms. Rearing axenic individuals may not be possible for all species, as some depend on obligate symbionts for vital functions in the host. Development may also be inhibited in absence of the microbiome, and axenic individuals may have abnormal physiology and morphology. Axenic models are often used to study gnotobiotic models (i.e. individuals colonised only by specific microorganisms), as to understand the role of a specific microorganism in the host metabolism (Schaedler *et al.*, 1965). Since microbiota are complex and dynamic, identifying specific members of the community that have the greatest impact on the host is an attractive approach to characterise the host-microbiota ecosystem. Metagenomic plasticity and functional redundancy makes it difficult to clearly attribute metabolic functions or responses to environmental stimuli between the host and the microbiota. An emergent field of research to better understand the relative role of each member of the holobiont is the use of synthetic assembly of specific microorganisms (SynCom). SynCom are engineered by co-culturing specific taxa as a simplified version representative of the natural microbiota functional repertoire, see the next section. This SynCom is usually administered to axenic individuals (Großkopf and Soyer, 2014). This approach is used to reduce the complexity of the microbiota as well as the variability of metagenomic plasticity, while increasing the community stability. Ideally, host with an adapted SynCom should have similar growth and development as naturally reared individuals (Bolsega *et al.*, 2021). Syncoms are useful tools to study the function and interactions of the microbiota with their host, identifying essential species and functions in host ontogeny and systems.

Axenic rearing can be challenging since contaminants are easily introduced in the environment. Usually, axenic insects are produced by removing the microbiota

on the surface of the eggs by treatment washes (without killing the egg) before hatching and rearing in sterile conditions (J. Wu *et al.*, 2023). The treatment solution and duration must be optimised for each species. Gold *et al.* (2020) compared protocols to produce surface sterilised BSF eggs for axenic rearing. They found that a four-step treatment immersing eggs in 70% ethanol and 0.6% sodium hypochlorite alternatively wielded the best results (Gold *et al.*, 2020). Axenic BSF have been used to investigate the influence of the microbiome on host metabolic pathways (Auger, Bouslama, *et al.*, 2023), the functional activity of specific symbionts through gnotobiotic models (X. Li *et al.*, 2023; Y. Yu *et al.*, 2023), the effect of probiotics (Pei *et al.*, 2022) and the contribution of the microbiome in tetracycline degradation (Cai *et al.*, 2018). *Tenebrio molitor* larvae were also successfully reared in axenic condition to understand the functional role of the microbiota in host digestion (Genta *et al.*, 2006).

The sterilisation process of surface sterilisation of the eggs induces stress on the eggs, associated with a high mortality rate before hatching (Gold *et al.*, 2020). Another approach to rearing axenic individuals is the use of wide spectrum antibiotics in feed. However, this may induce significant stress on the host, complete sterilisation may also be difficult and the modulation of the microbiota on the host prior to removal cannot be identified. Recolonisation of axenic individuals also causes a great stress on axenic insects (Vera-Ponce de León *et al.*, 2021).

The most difficult aspect of axenic insect rearing may be sterilisation of the rearing substrate. Autoclaving may change the physicochemical properties of the rearing substrate, making it unusable as feed. The density and matrix of the rearing substrate can also hinder the efficiency of autoclaving. Sterilisation by irradiation is more expensive but avoids the problems of autoclaving. Periodic microbiological testing is needed in axenic experiments to ensure that the condition stays truly sterile throughout the experiment (Dremova *et al.*, 2023). The rearing substrate should be monitored with regular samples taken to inoculate all-purpose microbial enriched culture medium and incubated for at least 72 h. Microbiological culture should be repeated on fungal appropriate media, such as Brain-Heart Infusion medium. Molecular methods can be used to confirm sterility, such as amplification of 16S rRNA with PCR. These methods are more sensitive, but have a higher rate of false positive, because of the presence of mitochondrial 16S rRNA, which can also be derived from dead microbes.

Using microbiome engineering to explore microbe or microbiome functionality within insects

Microbiome engineering is a research area that is gaining more and more interest due to advances in our ability to track and characterise microbial communities. In essence, microbiome engineering is the research field aiming to modify microbial communities to alter ecosystems of interest and/or to restore ecological balance (Albright *et al.*, 2022; Foo *et al.*, 2017). However, the applied methods can also be used to explore the role of specific microbes for more fundamental research.

There are two main approaches towards microbiome engineering. The bottom-up strategy starts with isolates that are identified, and purposefully assembled into a synthetic community. These strains are often selected because they have known benefits to the host or functions that are desirable. The bottom-up strategy's goal is mainly to change or to explore microbial and host-microbial interactions of specific microorganisms (J. Wu *et al.*, 2023). The top-down approach uses selective pressure from environmental conditions to select an unidentified collection of microorganisms. For example, a microbiota sample will be put into an environment with an antibiotic, and the microorganisms that still thrive are collected and used as a synthetic microbiota assembly (Bolsega *et al.*, 2021).

Several studies have used the bottom-up strategy to supplement the insect-associated microbiome with either detrimental or beneficial microbes to explore their dynamics within the insect, their effect on the host phenotype, and/or their functionality within insect physiology. For example, a large number of so-called 'challenge experiments' have been conducted to explore the fate of, mainly food pathogenic, inoculated microorganisms (e.g. *Escherichia coli*, *Salmonella* sp. and *Staphylococcus aureus*) during insect rearing, as summarised in Table 6. The fate of these microbes is best investigated in both the insect rearing substrate and the insects themselves. Another important point of attention is the availability of a functional method to specifically retrieve and count the inoculated microorganisms from the complex insect-associated microbiome. Such methods often include the use of a selective medium combined with the use of a genetically engineered microorganisms that contains an additional selection marker (e.g. antibiotic resistance or fluorescent tag). Details on these protocols are included in Table 6.

To test suspected probiotic microorganisms, inoculation trials are used and the effect on insect performance is evaluated. A thorough evaluation on such studies can be found in the review of Gorrens *et al.* (2023)

TABLE 6 Culture-dependent studies of microbiota of farmed insects

Reference	Species	Feed source	Sample type	Life stage	Cultivation method	Identification method	In vitro characterisation
Jeon *et al.* (2011)	*Hermetia illucens*	Food waste, calf forage, cooked rice	Gut	Larval stage (unknown)	Aerobic, direct isolation	Sanger sequencing (16S)	Protease, lipase, cellulase and amylase activity
Callegari *et al.* (2020)	*Hermetia illucens*	Wheat germ 50%, alfalfa 30%, corn flour 20%	Gut	Larval stage (unknown)	Aerobic, enrichment cultures	Sanger sequencing (16S), genotyping	Amylase, cellulase, pectinase, esterase, lipase, protease and urease activity, ammonia and exopolysaccharides production
Tegtmeier *et al.* (2021)b)	*Hermetia illucens*	Chicken feed	Gut	Larval stage 5	Aerobic and anaerobic, direct isolation	Sanger sequencing (16S), genotyping	Antimicrobial activity
Gorrens *et al.* (2021a,b))	*Hermetia illucens*	Chicken feed, 5 high fiber diets (pectin, keratin, cellulose, hemicellulose, lignin)	Whole insect	Larval stage (16 days old)	Aerobic, direct isolation	MALDI-TOF	
Cifuentes *et al.* (2022)	*Hermetia illucens*	Chicken feed	Gut	Larval stage (4 weeks old)	Aerobic, dilution to extinction, enrichment cultures	Sanger sequencing (16S), genotyping	
Klüber *et al.* (2022)	*Hermetia illucens*	Palm kernel meal	Gut	Larval stage 5	Aerobic, direct isolation	Sanger sequencing (16S and ITS)	Entomopathogenic activity
Raimondi *et al.* (2020)	*Hermetia illucens*	Vegetable substrate (25% cornflour, 15% wheat bran, 10% alfalfa flour)	Whole insect	Larval stage 2 and 3	aerobic and microaerophilic, direct isolation	Sanger sequencing (16S)	
Klüber *et al.* (2024)	*Hermetia illucens*	Empty fruit bunches, cottonseed press cake, potato pulp	Gut	Larval stage 5	Aerobic and anaerobic, direct isolation	Sanger sequencing (16S)	Cellulase activity

TABLE 6 (Continued)

Reference	Species	Feed source	Sample type	Life stage	Cultivation method	Identification method	In vitro characterisation
Machona *et al.* (2022)	*Tenebrio molitor*	Expanded polystyrene (EPS)	Gut	Larval stage	Aerobic, enrichment cultures	Sanger sequencing (16S)	
Wang and Zhang (2015)	*Tenebrio molitor*	Wheat bran and various vegetables	Gut	Larval stage	Aerobic, direct isolation	Sanger sequencing (16S)	
Lin *et al.* (2024)	*Tenebrio molitor*	Expanded polystyrene (EPS)	Gut	Larval stage	Aerobic, enrichment cultures	Sanger sequencing (16S)	Polystyrene degradation
Fröhling *et al.* (2020)	*Acheta domesticus*	Unknown	Whole insect	Adult	Aerobic, microbial counts direct isolation	MALDI-TOF	
Li *et al.* (2024)	*Locusta migratoria*	Wheat seedlings	Gut	Nymphal stage 4 and 5	Aerobic, direct isolation	Sanger sequencing (16S)	Cellulase activity
Wynants *et al.* (2018)	*Alphitobius diaperinus*	Vegetable raw materials	Whole insect	Larval stage	Aerobic, microbial counts, direct isolation	Sanger sequencing (26S)	
Cucini *et al.* (2022)	*Alphitobius diaperinus*	Polystyrene	Gut	Larval stage	Aerobic, enrichment cultures	Sanger sequencing (16S)	Phenotypic characterisation, sensitivity to antibiotics

for the BSF. This review primarily focuses on the large variation in set-up parameters between protocols that exist in literature and the need for standardisation or at least adequate reporting on the methodology. Parameters that can hinder cross-study comparison include: rearing substrate type, substrate pretreatment, environmental conditions, insect age, inoculation strategy and methodology to track the colonisation potential of the tested microorganisms. When setting up such inoculation trials, it is important to have a thorough evaluation of each parameter and ensure your ability to keep it constant to minimise variability and maximise repeatability.

Also, the top-down approach can lead to valuable insights into the functionality of the insect microbiome and/or the identification of microorganisms with interesting properties, even in a broader industrial scope. As mentioned, these approaches not aim to directly affect the microbiome, but allow selective pressure to modulate the microbiome to become enriched with specific, desired microbes. Recent example in literature is the identification of plastic-degrading microbes by placing BSF larvae on a diet containing solely plastics as nutrient source De Filippis *et al.* (2023) or the unveiling of lignocellulose degrading enzymes from larvae fed with lignocellulose-enriched diets (Kariuki *et al.*, 2023). In both cases, the diet was altered in such a way that it allows the proliferation of those microbes present that are able to take advantages of the created niche. Similar approaches could also be used to explore how the microbiome reacts to other variations in key nutritional elements.

Beyond DNA or RNA, using metabolomics or (meta)proteomics to explore microbe functionality

To date, most microbiome screening studies executed on industrially produced insects have relied on the already described DNA or RNA sequencing methods. A similar pattern can be seen more generally in microbiome studies as they allow to generate an inventory of the present organisms and/or genes. Yet in for example human gut microbiome studies, these analyses are more and more supported by evaluations at the proteome or metabolome level. Due to the challenges of linking DNA level data to the functional activity of a microbiome in a specific condition (Zhang and Figeys, 2019). RNA sequencing methods are already an improvement as they only detect the actively transcribed genes, i.e. transcripts. However, not all mRNA transcripts will be translated into proteins, causing discrepancies between both profiles (Liu *et al.*, 2016). Measured proteins and metabolites in microbiome studies, using metaproteomics and metabolomics, respectively, will provide the required additional functional information to gain a mechanistic understanding of the microbiome functioning. While the two methods complement each other, proteomics targets proteins and provides complex and high-dimensional data that helps to study the underlying functional mechanisms. In contrast, metabolomics targets small molecules such as amino acids, sugars, or lipids that provide a snapshot of an organism's metabolic state through less structurally complex but chemically diverse data.

Recent advances in the field of metaproteomics, including higher resolution mass spectrometers and better quantitative proteomics techniques and more efficient bioinformatic tools to study the complexity of microbiomes, are providing the necessary tools to execute more extensive coverage of metaproteomes in larger studies, including microbiome analysis (Kolmeder and de Vos, 2014). Ultradeep metaproteomics has been used for example to explore the functional redundancy in the human gut microbiota, a key property of ecosystems, and could reveal that xenobiotics have a more pronounced negative effect on functional redundancy at the proteome level, then at the taxonomic level (Li *et al.*, 2023). It will be exciting to see, where future developments in this field will take our understanding of insect microbiome functionality. The first articles implementing this methodology on BSF already show great promise, but also reveal that standardised protocols will need to be optimised at sampling, detection and analysis level in the upcoming years (Bose *et al.*, 2023; L. Lu *et al.*, 2021).

The second major mass spectrometry-based -omics technique, that could prove a game changer is metabolomics, which offers a direct readout of the function of a system, making it the closest representation of phenotype. As such it can yield insights into the cellular processes in response to stimuli or host-microbe interactions. A prerequisite to obtain a mechanistic understanding of how chemical environments shape microbial communities and the function of the microbial-derived molecules on ecosystems (Bauermeister *et al.*, 2022). Theoretically, a metabolomics experiment detects all small molecules, more specifically, chemicals with molecular weights of <2000 Da; more realistically, it is a partial picture defined by the extent to which molecules can be extracted, ionised and detected. Since mass spectrometry (MS) is often used for detection, given its good sensitivity and its capacity to detect and quantify a large diversity of molecules in

complex biological samples (Aksenov *et al.*, 2017). As for metaproteomics, there are only a few studies published that implement this methodology on BSF or mealworm, yet the potential support of such studies for the ongoing efforts to understand which microbiome functions are key for insect performance is evident. Ho *et al.* (2021) extracted metabolites from bacterial immunised BSF larvae using a methanol:chloroform:water (1:1:0.7, v/v/v) mixture and analysed the aqueous phase using ^{1}H-NMR spectroscopy, revealing the triggering effect of the presence of these bacteria on amino acid (more specific arginine and proline) and nucleotide metabolism. Mani *et al.* (2023) used a methanol extraction and an untargeted LC-MS metabolomics approach to compare two wild and one domesticated population, showing how wild populations produce more aromatic amino acids, possible to support higher levels of defence mechanisms under natural conditions.

Aside from the above sequencing, culturing or mass spectrometry-based technologies, there is a final methodology worth mentioning, which is the use of imaging to explore the spatial organisation of microbial communities within the insect (gut) (Earle *et al.*, 2015; Zhu *et al.*, 2023). This localisation within, for example, the insect gut is crucial information as it adds another layer of complexity to understand the functioning of the microbial community as a whole. Specific community members need to be in close proximity to execute their predicted functions and it is thus important to confirm this is the case. One example in insect research of the use of imaging involves the use of an engineered insect symbiont, *Burkholderia*. Its expression of GFP enabled the researcher to track its colonisation of the gut of its host, the Bean bug, *Riptorus pedestris* (Kikuchi and Fukatsu, 2014). In general, such imaging analyses can tell us a lot about whether specific microbes can persist and colonise specific micro-environments along the intestinal tract and shed light on the best strategies to ensure swift and robust colonisation of the insect gut.

By now, it must be clear that there is a plethora of techniques to study the microbiome at different levels, yet what also became clear is that no single level analysis will suffice to answer intricate questions on the microbiome functioning. This is where systems biology comes into play, as it aims to integrate these observations from the -omics methodologies into a comprehensive predictive model that allows researchers to simulate how a biological system would react under various conditions (Kirschner, 2005). This approach has been gaining traction to study alterations of the human gut microbiome in relation to underlying health and disease mechanisms (Narad *et al.*, 2024). One specific approach that adheres to this system-level approach is genome-scale metabolic modelling (abbreviation: GSMM). These models computationally describe gene-protein-reaction associations for entire metabolic genes in an organism. Such models are more and more being applied to infer diet–microbiome, microbe–microbe and host–microbe interactions under physiological conditions (Sen and Orešič, 2019). In fact, such a model has already been generated for *Drosophila melanogaster*, and used to investigate metabolic responses to genetic/environmental perturbations (Cesur *et al.*, 2023). A recent study by Baa-Puyoulet *et al.* (2025), further showcases the power of metabolic modelling to understand microbe-host interactions, by developing a metabolic network database to study the interactions between an aphid, *Sipha maydis*, and two of its obligate bacterial associates.

6 Outlook

The methodologies available to study microbiomes are continuously advancing and evolving, enabling researchers to uncover both the composition and functionality of microbial communities. High-throughput sequencing techniques, such as 16S rRNA gene sequencing and shotgun metagenomics, enable an extensive cataloguing of microbial diversity and its correlation to changing environmental conditions, while advances in transcriptomics, proteomics, and metabolomics are shedding light on the functional potential and ecological roles of specific microbes or whole communities. Especially with the latter, there is still much ground to be covered in studying those microbial communities associated with industrially produced insects. The next decade promises exciting advancements as research delves deeper into these microbial ecosystems, potentially illuminating insights that could drive substantial improvements in the productivity and sustainability of the sector. Indeed, these tools, combined with culture-dependent approaches and emerging imaging technologies, offer a comprehensive toolkit to understand the insect microbiome in unprecedented detail.

However, this article also highlighted significant challenges and opportunities that lie ahead. While current methodologies provide detailed snapshots of microbiome composition, putting these findings into practice in an industrial setting remains complex. The functional redundancy observed in microbial communities and the dynamic interplay between host and microbiota

will require more sophisticated experimental designs that integrate temporal and spatial dynamics. Developing standardised protocols for microbiome research in insects, taking into account the key parameters (e.g. sampling time, sampling site, selected target) and following the guidelines offered from sampling to data analysis, will be critical to ensure reproducibility and comparability across studies. This reproducibility will also be key for the next big advance in the field, which will be the integration of multi-omics data with machine learning and systems biology approaches to unravel the intricate relationships between insects, their microbiota, and their environments.

A better understanding of the whole picture, including all relationships, could guide microbiome engineering efforts and translate the involved microbes into tools to industrial insect farming through tailored microbial consortia or probiotics. As such, exploring the functional microbiome under mass-rearing conditions and on unconventional substrates (keeping in mind the caveats listed above), will pave the way for enhancing process sustainability and broader applications in bioconversion and waste valorisation. Indeed, by combining basic research with applied science, the study of industrial insect microbiomes can drive innovation in agriculture, waste management, and beyond.

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