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From pollen to putrid: Comparative metagenomics reveals how microbiomes support dietary specialization in vulture bees

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Abstract

For most animals, the microbiome is key for nutrition and pathogen defence, and is often shaped by diet. Corbiculate bees, including honey bees, bumble bees, and stingless bees, share a core microbiome that has been shaped, at least in part, by the challenges associated with pollen digestion. However, three species of stingless bees deviate from the general rule of bees obtaining their protein exclusively from pollen (obligate pollinivores) and instead consume carrion as their sole protein source (obligate necrophages) or consume both pollen and carrion (facultative necrophages). These three life histories can provide missing insights into microbiome evolution associated with extreme dietary transitions. Here, we investigate, via shotgun metagenomics, the functionality of the microbiome across three bee diet types: obligate pollinivory, obligate necrophagy, and facultative necrophagy. We find distinct differences in microbiome composition and gene functional profiles between the diet types. Obligate necrophages and pollinivores have more specialized microbes, whereas facultative necrophages have a diversity of environmental microbes associated with several dietary niches. Our study suggests that necrophagous bee microbiomes may have evolved to overcome cellular stress and microbial competition associated with carrion. We hypothesize that the microbiome evolved social phenotypes, such as biofilms, that protect the bees from opportunistic pathogens present on carcasses, allowing them to overcome novel nutritional challenges. Whether specific microbes enabled diet shifts or diet shifts occurred first and microbial evolution followed requires further research to disentangle. Nonetheless, we find that necrophagous microbiomes, vertebrate and invertebrate alike, have functional commonalities regardless of their taxonomy.

KEYWORDS
biofilms, carrion, corbiculate apidae, diet, evolution, metagenomics, necrophagy, stingless bees, symbionts

1 | INTRODUCTION

Microbiomes are critical for many animal species, influencing nutrition, metabolism, and immunity (Douglas, 2019). Environments can filter the presence and diversity of microbes present in food resources, subsequently influencing exposure and shaping the microbiome of many species (Disayathanoowat et al., 2020; Gong & Xin, 2021; Hannula et al., 2019). For one ecologically important

group of animals, the bees, the importance of the microbiome is increasingly well recognized, as bee microbiomes contribute to digestion of macromolecules, detoxification of toxins, pathogen defence, and immunity (Engel et al., 2016; Kwong et al., 2014). In wild bees, gut microbiomes are highly variable and at least somewhat shaped by the environment, as opposed to honey bee microbiomes which tend to be more consistent (Kwong et al., 2017; McFrederick & Rehan, 2019). Dietary shifts, and associated shifts in microbiome composition, can also influence the survival and fitness of some stingless bees, such as *Melipona quadrifasciata* (Haag et al., 2023). Specifically, an increase in *Bifidobacteriaceae* and a decrease in *Lactobacillus*, *Candida*, *Zygosaccharomyces*, and *Starmerella* proceeds disease outbreaks in *M. quadrifasciata*, a pattern that was linked to changes in the pollen diet (Haag et al., 2023). Moreover, diverse diets, rich in pollen from many floral resources, have been linked to improved nutrition, fitness, and growth for corbiculate bees (bumble bees, stingless bees, and honey bees) (Alaux et al., 2017; Kaluza et al., 2018; Requier et al., 2015; Smart et al., 2016). Studying how dietary shifts can change microbiomes allows us to understand which microbial members are core members that persist regardless of diet and which are established through diet.

The corbiculate apids share a core microbiome which was previously thought to have persisted for around 80 million years (Kwong et al., 2017). However, recent evidence suggests that the corbiculate core microbiome likely did not result from strict co-diversification, but rather through host switching, where specific members were gained, lost, or retained depending on their host (Sarton-Lohéac et al., 2023). Therefore, stingless bees appear to acquire environmental microbes, and then maintain associations in species-specific ways through vertical and/or social transmission and host filtering (de Paula et al., 2021; Keller et al., 2021). For instance, colonies of *Tetragonula carbonaria* in the same area had similar microbiomes, which changed dramatically following relocation. This example highlights the importance of environment and diet in shaping stingless bee microbiomes (Hall et al., 2020). Thus, there are microbes that all the corbiculate apids share as well as others specialized for specific hosts (and acquired and filtered in specific environments), reflecting the importance of the microbiome for social bees.

There are important differences between the microbiomes of honey bees and bumble bees compared to stingless bees (Kueneman et al., 2023; Kwong et al., 2017). For instance, unlike bumble bees and honey bees, not all stingless bees have *Gilliamella* and *Snodgrassella* and instead have lineage-specific *Acetobacter* species and several acetic and lactic acid fermenters (Figueroa et al., 2021; Kueneman et al., 2023; Kwong et al., 2017). Stingless bees also tend to have more species-level variation compared to honey bees and bumble bees, which likely reflects the diverse ways they supplement their diets (Koch et al., 2013; Kwong et al., 2017; Roubik, 1992). Perhaps the most shocking of these dietary supplements is carrion. When David Roubik first discovered bees ravenously feeding upon a dead lizard carcass, the entire concept of a bee as a vegetarian wasp, that is, obligately pollinivorous, had to be critically reassessed (Roubik, 1982). Since then, three stingless bee species have been identified as obligately necrophagous (*Trigona hypogea*, *T. necrophaga*, and *T. crassipes*), meaning that they exclusively obtain their protein from carcasses, and several others as facultatively necrophagous, which means that they visit both flowers and carrion (Camargo & Roubik, 1991; Dorian & Bonoan, 2021; Figueroa et al., 2021; Roubik, 1982). There are several environmental conditions and pre-adaptations that could have played a role in this extreme dietary transition. First, there is high floral competition in the Neotropics, and neotropical eusocial stingless bees have large numbers of developing brood. Thus, the availability of additional protein sources in non-floral resources such as carrion could be an important contributor to the dietary shift (Baz et al., 2010; Lorenzon & Matrangolo, 2005). Some other pre-adaptations include aggressive behaviour, scent-guided foraging, and sharp mandibular teeth (Camargo & Roubik, 1991). However, only the latter pre-adaptation has been tested, and there turned out to be no association between mandible morphology and facultative necrophagy in swarm-founding neotropical bees and wasps (O'Donnell, 1995; Sarmiento, 2004). Likewise, facultative necrophagy in stingless bees has no phylogenetic signal, suggesting it is evolutionarily labile (Rasmussen & Camargo, 2008). This lability is further supported by the observation that several bee species across the corbiculate apid clade have been found at carrion sources (Dorian & Bonoan, 2021; Figueroa et al., 2021). This lack of phylogenetic signal might imply that something other than the host biology is mediating these frequent dietary transitions and overall dietary flexibility. Despite the myriad possible forces driving bees towards carrion feeding to supplement their diets, the microbiome could enable them to tolerate such a harsh resource. Microbiome-mediated necrophagy could occur through at least two evolutionary paths: gene functionality of certain microbes enabling shifts in diet, or diet shifts changing the microbiome to favour microbes with broad niches and/or able to digest carrion.

Stingless bees are known to rely on microbial symbionts to aid in food storage, preservation, and desiccation prevention (Menezes et al., 2013). Early studies looking into the role of microbes in the meat pots of one obligate necrophagous bee species (*Trigona hypogea*) found a high abundance of *Bacillus* spp. (Gilliam et al., 1985). This genus of bacteria is known to produce dozens of antibiotics and form biofilms that might protect against pathogens or food spoilage microbes (Gupta et al., 2014; Shukla et al., 2018). However, early work used culture-based methods that might not reflect true diversity and abundances of microbes associated with carrion feeders. Figueroa et al. (2021) took a 16S rRNA gene amplicon sequencing approach to characterize the gut microbiomes of another obligately necrophagous bee species (*Trigona necrophaga*) compared to facultatively and obligate pollinivorous bees. They found that there were differences in microbial communities between diet types and hypothesized that lactic acid and acetic acid-producing bacteria were likely playing a role in carrion digestion and pathogen defence (Figueroa et al., 2021). Lactic acid-producing bacteria (LAB) have been found in other non-necrophagous stingless bee provisions and have been hypothesized to play a major role in food preservation (Sarton-Lohéac et al., 2023;

Vit et al., 2013). LAB have also been shown to protect bumble bee workers against pathogens by creating acidic environments that reduce the intensity of infection (Palmer-Young et al., 2019; Vásquez et al., 2012). However, the nature of 16S rRNA gene data limits our understanding of the functional role of these differentially abundant symbionts. Therefore, for this study, we take a shotgun metagenomic approach to assess the functionality of these symbionts through comparing a subset of bees from the same collection points and diet types (obligate pollinivory, facultatively necrophagy, and obligately necrophagy) as Figueroa et al. (2021). We hypothesize that symbionts that are in higher abundance in necrophagous bee guts are enriched with genes for responding to the various stresses associated with carrion feeding, including high microbial competition on carcasses, high-fat and protein content, and osmotic stress from the salts.

2 | MATERIALS AND METHODS

2.1 | DNA extraction and sequencing

We used a subset of the samples collected in Figueroa 2021 for shotgun metagenomic sequencing. Specifically, we analysed 30 bees: four samples of obligate pollinivores (one species: *Tetragonisca angustula*, one of the most abundant stingless bee species in Costa Rica; Slaa, 2006), 12 samples of facultative necrophages (five species: *Partamona musarum*, *Partamona orizabaensis*, *Trigona ferricauda*, *Trigona fulviventris*, and *Trigona silvestriana*), and four samples of obligate necrophages (one species: *Trigona necrophaga*, the only obligately necrophagous bee species found in Central America) (Table S1). Thus, it is important to note that we do not have equal representation in terms of number of species per diet type in our analyses. However, given that there are only three known obligately necrophagous bee species in the world, the one species evaluated represents a third of the total obligately necrophagous bee diversity and the only one occurring in the region.

To preserve DNA while in transit, we collected each bee into a separate, sterile tube filled with 95% ethanol. Upon arrival at UC Riverside, we stored the bees at −80°C until we could proceed with DNA extraction. As described in Figueroa et al. (2021), we used the entire abdomen of each bee for DNA extraction. As insect exoskeletons contain little microbial biomass (Hammer et al., 2015), we did not surface sterilize the abdomens. To minimize contamination, we briefly centrifuged tubes before opening, and we included a blank control to test for contamination in all extraction, library preparation, and sequencing steps. To lyse the samples, we added a small amount (~10 μL each) of 0.1 and 0.07 mm zirconia beads, one 5 mm steel bead, and 700 μL of CTAB extraction buffer (0.1 M Tris HCl, 1.4 M NaCl, 0.02 M EDTA, and 2% CTAB). We bead beat the abdomens at 30 hertz for 1 min, then rotated the tubes and bead beat for an additional minute. To inactivate enzymes and further lyse the cells, we added 10 μL of proteinase K to each sample and incubated the samples at 55°C for 1.5 h. We then washed each sample three times: first with chloroform/isoamyl alcohol (24:1), then with phenol/chloroform/isoamyl alcohol (25:24:1), and then again with chloroform/isoamyl alcohol. We precipitated the DNA overnight at −20°C in 2.5 volumes of ice-cold ethanol and 0.1 volume 3 M sodium acetate. We pelleted and then washed the DNA in ice-cold 80% ethanol, repelleted the DNA, and resuspended the DNA in ultrapure water. We visualized the DNA on a 1.5% agarose gel to verify quality.

To prepare sequencing libraries, we used the resulting DNA and the Qiagen Qiaseq FX DNA library preparation kit. For controls, we included the Zymo Zymobiomics microbial community DNA standard as a positive control and a reagent-only sample as a "blank" negative control. We followed the Qiaseq FX protocol, with an input of 20–100 ng of DNA per library, 20 min of enzymatic fragmentation incubation, and six cycles of library PCR amplification. We purified and size-selected (200–700 base pairs) using dual-sided selection and AMPure magnetic beads. We quantified each library using Pico-Green and diluted the libraries to be equimolar. We quality-checked the resulting libraries on the Agilent 2100 Bioanalyzer in UC Riverside's genomics core. We then sent the resulting libraries to UC San Francisco's genomics core facility for sequencing with S4 2 X 150 paired-end reads on Illumina's NovaSeq.

2.2 | Bioinformatic and statistical analysis

2.2.1 | Quality control and assembly

We quality controlled our raw reads using fastp with default settings to remove paired-end adapters, trim, and filter low-quality reads (Chen et al., 2018). We then assembled each sample into contigs with MEGAHIT (v1.2.9) using the "–presets meta-sensitive" flag and a minimum contig length of 1000 (because this represented the average size of a bacterial gene) (Li, Liu, et al., 2015). We used MEGAHIT because at the time it was comparable to MetaSPAdes, and better than most other assemblers while requiring less computational resources (Saheb Kashaf et al., 2021; van der Walt et al., 2017). For individual samples, around 90% of the reads were unmapped, but when co-assembled we only had 35–63% unmapped, suggesting that there might not be enough coverage in individual samples. For this reason, we created co-assemblies with the same settings by concatenating all the individual fastq files into one file per bee species. For downstream analyses, we used the co-assemblies because they had much higher N50s and were able to map more accurately a higher percentage of reads to taxonomic and protein databases. The total contigs and N50s for our co-assemblies ranged from 8105–98,133 and 1378–23,862 base pairs, respectively (Table S2). Lastly, we evaluated our controls (positive and negative) using phyloFlash (Gruber-Vodicka et al., 2020). We compared the reads from our bee samples to the controls to see whether the samples were sequenced accurately (positive control) and to verify that our top taxa were not contaminants (negative controls). For our blank (or negative control), we had only 15 bacteria reads and two eukaryotic reads map to the SILVA database. None of these were found in high abundance in our

samples indicating that contamination did not influence our results. Specifically, 10 of these reads mapped to Corynebacteriales, four to Chloroplast, one to Lactobacillales, and two for Charophyta. For our positive control, we recovered all of the expected species except *Escherichia coli*. As far as the expected percentage of representation for the other seven bacteria (based on assembled reads per species normalized by total assembled reads), we recovered 4–19.6% versus 12%. For the two fungi, we recovered slightly more than expected (4–8% vs. 2%).

2.2.2 | Gene functional profiling by taxa

We used the HUMAnN3.0 pipeline to functionally profile our co-assembled contigs and assign taxonomy (Beghini et al., 2021). We used the UniRef50 database for the gene annotation and inferred taxonomy using lowest common ancestry (LCAs). We chose UniRef50 over UniRef90 because we were working with microbiomes that are poorly characterized (as recommended by the HUMAnN developers). We then annotated our contigs with the following functional annotation types: Gene Ontology (GO), MetaCyc Reactions, KEGG Orthogroups (KOs), Level-4 enzyme commission (EC) categories, Pfam domains, and EggNOG (including COGs). We then normalized each coassembly by using relative instead of raw abundance. After annotating with HUMAnN3, we used MaAsLin2 (Microbiome Analysis in R, Linear Models 2) models designed for microbiome analyses (Mallick et al., 2021). The model had three diet types: (1) obligate pollinivory, (2) facultative necrophagy, or (3) obligate necrophagy. Diet type was a fixed effect, and we made all comparisons in reference to obligate necrophagy. We took the error-adjusted significant values $q < 0.05$ from the different annotation databases, concatenated them into a file, and then filtered out repeats, host contamination (from animals and plants), and genes that did not have taxonomy assigned. Then to summarize which microbial species were contributing the most differentially abundant genes related to diet, we calculated the relative abundance of each microbial genus for these significant genes. The data were further partitioned into significantly differentially enriched genes that were over- versus under-enriched in the pollinivores and facultative necrophages compared to the obligate necrophage.

2.2.3 | Gene functional profiling

In the HUMAnN3.0 pipeline, we also used a mapping file from the Clusters of Orthologous Groups of proteins (COGs) website that had the functional codes (A–Z) for each COG annotation. We then filtered out the unclassified/ungrouped reads and calculated relative abundance of each functional category for each bee species. These categories did not have taxonomic assignments but were used to assess the high-level functionality of the microbiomes broadly. We then summarized the relative abundance of each COG category by bee species using HUMAnN3.0 and tested, using MaAsLin2, whether there were statistically significant differences (using linear regression) in the COG functions based on diet (the fixed effect) in reference to the obligate necrophagous diet.

2.2.4 | Taxonomic profiling and assembling MAGs

We used the anvio pipeline and kaiju software to assign taxonomy to the co-assemblies (Eren et al., 2021; Menzel et al., 2016). We were able to obtain more reads mapped to the taxonomic reference database with co-assemblies by bee species. We then summarized each species by relative abundance per diet type and plotted the top 15 genera for each bee species.

Beyond understanding which species were most prevalent, we wanted to look at certain bacterial genomes for signatures of selection. To detect signatures of selection, we needed metagenome-assembled genomes (MAGs). To assemble MAGs, we used individual samples, not co-assemblies, and put them through the Autometa pipeline following the bash workflow (Miller et al., 2019). Briefly, this involved filtering out reads under 1000 bp, calculating coverage, delimiting open-reading frames (ORFs) using prodigal, and annotating marker genes. To assign taxonomy for bacteria and archaea, Autometa uses hmmscan and a diamond-formatted database of the NCBI non-redundant protein database, and then determines each ORF's lowest common ancestor (LCA). Then after a majority vote to decide on the taxonomy of each contig, the contigs were split into kingdoms and the k-mer counts were calculated for all bacterial contigs. Lastly, the contigs were binned into MAGs through clustering. We retrieved two *Carnimonas nigrificans* MAGs from the obligately necrophagous bees' guts. There was only one reference genome of *Carnimonas nigrificans* on NCBI, so we could not do selection analyses on this species and instead used CJ Bioscience's online average nucleotide identity (ANI) calculator to look at ANI between our species and the references.

2.2.5 | Selection analyses

We were interested in looking for signatures of positive selection in a couple of the obligately necrophagous bacterial species compared to other bee-associated species in the same genus, specifically *Commensalibacter intestini* and *Lactobacillus acetotolerans* because these genera were found to contribute a high abundance of genes that were significantly different between diets and had enough coverage to assemble into MAGs. To pull other bee-associated species to compare them against, we downloaded the representative genomes from each species on NCBI using the RefSeq fasta files. For the *Lactobacillus* comparison, we found the bee-associated species by referring to (Zheng et al., 2020). For our first comparison, we included *Lactobacillus apis*, *L. bombicola*, *L. helsingborgensis*, *L. kimbladii*, *L. kullabergensis*, *L. melliventris*, *L. panisapium*, *L. acetotolerans*, and *L. acetotolerans* from the obligately necrophagous bee's gut. For the second, we

were interested in bee-associated *Commensalibacter* and included *C. communis*, *C. intestini*, *C. melissae*, and one *C. intestini* from our obligately necrophagous bees' guts and another from one of our facultative necrophages (*Trigona fulviventris*) (Botero et al., 2023; Botero & Vandamme, 2024; Li, Praet, et al., 2015). For both analyses, we used Orthofinder with the flags "-M msa -S blast" to pull single-copy orthologues and create species trees (Emms & Kelly, 2019). We then used EMBOSS to backtranslate the amino acids into nucleotides so, we could complete codon-aware alignments (Rice et al., 2000). We used ete3 build to perform codon-aware alignments of the single-copy orthologues and ete3 evol to run DN/DS analyses (Huerta-Cepas et al., 2016). For the DN/DS analysis, we ran a branch-site model (bsA vs. bsA1) and marked the obligately necrophagous bacterial species as the foreground to compare against the others (Zhang et al., 2005). Then with a custom bash script we pulled *p*-values from the output and then adjusted them to *q*-values in R to account for multiple hypothesis testing. For each gene showing signatures of positive selection (q-val < 0.05), we searched the amino acid sequence of the obligate necrophage's bacterial species with Interpro (Huerta-Cepas et al., 2016; Paysan-Lafosse et al., 2023). We manually verified that the predicted proteins corresponded to the specific areas of the amino acid sequence under positive selection from our query.

2.2.6 | Beta diversity between bees, wasps, and chicken baits

In order to disentangle whether the microbial composition of these bees is indeed unique or identical to the carrion they fed on, and other Hymenopterans in the area, we reanalysed 16S rRNA data from Figueroa et al. (2021). Specifically, we compared the bee samples to chicken baits and wasps collected in the area and calculated their beta diversity. For preprocessing steps and ASV identification in QIIME2, we followed the same workflow as Figueroa et al. (2021). Then, we calculated Bray–Curtis dissimilarities and nonmetric multidimensional scaling using metamds in the Vegan package in R. We tested for differences between bee gut microbiomes (with 47 obligately pollinivorous bees, 91 facultatively necrophagous bees, and 21 obligately necrophagous bees), eight wasps, and five chicken baits. We included location as a block to account for the nonindependence of baits. We then used the betadisper function to test for homogeneity of multivariate dispersion (with location as a block) using the vegan package in R (Dixon, 2003).

3 | RESULTS

3.1 | Gene functional profiling

Overall, amino acid transport and metabolism genes as well as translation, ribosomal structure, and biogenesis genes were abundant across all the bee species compared to the other functional categories (Figure 1). There were also functional categories that differed between diet types. Specifically, there were significantly more coenzyme transport and metabolism genes ($p = .007$) and inorganic ion transport and metabolism genes ($p = .012$) in facultative necrophages compared to the obligate necrophage. For pollinivores, there were also significantly more coenzyme transport and metabolism genes ($p = .037$) and inorganic ion transport and metabolism genes ($p = .007$) compared to the obligate necrophage. In the obligate necrophage compared to facultative necrophages, there were significantly more genes related to translation, ribosomal structure and biogenesis ($p = .006$) and posttranslational modification, protein turnover, and chaperones ($p = .028$). Furthermore, there were significantly more genes related to cell wall/membrane/envelope biogenesis ($p = .022$), cell motility ($p = .016$), and secondary metabolite biosynthesis, transport, and catabolism ($p = .034$) in the facultative necrophages versus the obligate necrophage.

3.2 | Taxonomy

3.2.1 | Functional taxonomy

Commensalibacter, *Lactobacillus*, and *Zymobacter* appear to contribute significantly more genes in the obligately necrophagous bee compared to the pollinivore and facultatively necrophagous bees (Figure 2). *Gluconobacter* appears to contribute to the genes that are significantly over-abundant in the obligate pollinivorous and facultatively necrophagous bees compared to obligately necrophagous bees, followed by *Acetobacter* and *Bombella* (previously *Parasaccharibacter*) (Figure 2). Given the high abundance of genes of *Lactobacillus* and *Commensalibacter* in the obligately necrophagous bee, we wanted to determine whether these genes were showing signatures of positive selection compared to other obligate pollinivorous bee associates in the same genera. There were nine genes showing signatures of positive selection in the obligately necrophagous bee gut for *Lactobacillus acetolerans* and 14 for *Commensalibacter intestini* (Appendix S2 and Table A2). We also found two MAGs isolated from the obligate necrophage that had high similarity to each other (97.6% identical). From our ANI analysis, the closest match was to *Carnimonas nigrificans*. However, when compared to the reference genome on NCBI, we only found a 77.59% and 78.48% similarity, indicating that these MAGs in the obligately necrophagous bee likely represent a new species (Kim et al., 2014).

3.2.2 | Marker taxonomy

There is strong partitioning of the top taxa by diet type (Figure 3). For obligate pollinivorous bees, there are abundant species likely acquired from fruits and flowers such as *Neokomagataea* sp., *Acetobacter* sp., and *Gluconobacter* sp. (Yamada et al., 1999; Yukphan et al., 2011). The facultative necrophages' top taxa reflect

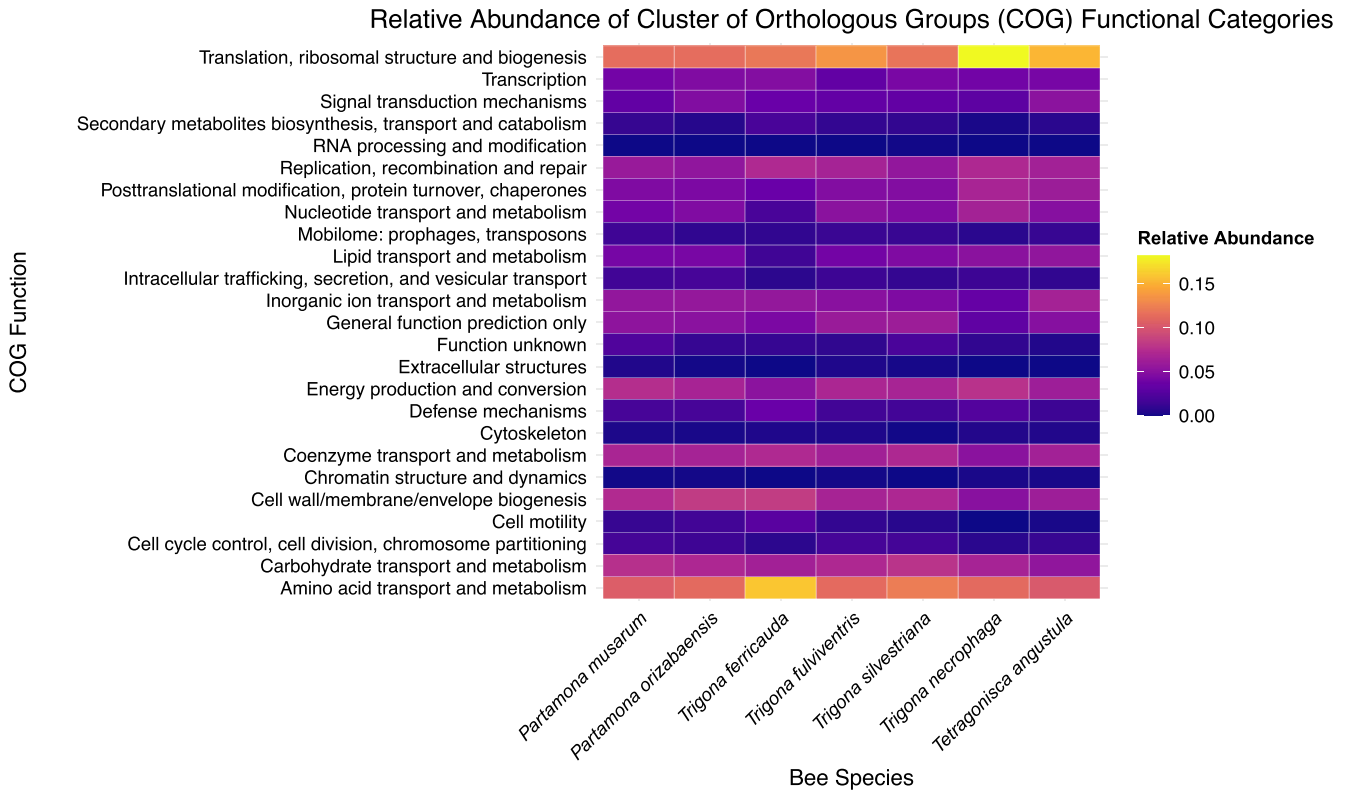

FIGURE 1 Relative abundance of different functional categories (COGs) of genes for each bee species. The first five species are facultatively necrophagous, *Trigona necrophaga* is obligately necrophagous, and *Tetragonisca angustula* is obligately pollinivorous.

their diverse environmental associations as they contain microbes from genera known to be acquired from flowers such as *Pectinatus* sp. and *Acinetobacter* sp. but also other microbes that are found on vertebrate bodies or mud such as *Salmonella enterica*, *Escherichia coli*, *Thermophillibacter immobilis*, *Pseudomonas* sp., *Olsenella uli*, *Paralysiella testudinis*, *Comamonas* sp., and *Klebsiella* sp., several of which are considered opportunistic human pathogens (Busse et al., 2021; Lu et al., 2021; Ryan et al., 2022). Likewise, facultative necrophages have some bacteria known to be linked to pollinivorous diets such as *Prevotella* sp. and others known to be linked to high-fat animal diets like *Bacteriodes* sp. (Wu et al., 2011). Another likely environmentally acquired microbe that to our knowledge has never been found in association with insects yet is in high abundance in the obligately necrophagous bee, is *Spinellus fusiger* (mycoparasitic filamentous fungi). While the functional relationship with this specific fungus is unknown, it is not uncommon for stingless bees to associate with other filamentous fungi (Eltz et al., 2002; Oliveira & Morato, 2000). There is also a high abundance of *Lactobacillus* sp. and *Chlamydia abortus*, which are perhaps acquired environmentally. For the obligately necrophagous bee, there are several fatty food and/or salted meat-associated taxa such as *Carnimonas nigrificans*, *Halomonas* sp., and *Halotalea alkalilen*ta (Ntougias et al., 2015). Overall, we found that several bacteria are likely acquired from the environment but are filtered based on dietary needs.

The only genera that all the diet types share in high abundance are *Monosiga brevicollis* and *Salpingoeca rosetta*. Otherwise, there are some microbes shared between the obligate pollinivores and facultative necrophages and found in high abundance, such as the "core" corbiculate members *Snodgrassella* and *Gilliamella*. The facultatively necrophagous bees also share a few genera in high abundance with the obligately necrophagous bees such as *Commensalibacter* sp., *Pseudomonas*, and *Escherichia coli*. Beyond those similarities, the most abundant microbes are unique to each diet type. The top 15 microbes for each bee species can be found in Appendix S2 and Table A1.

3.2.3 | Beta diversity between bees, wasps, and chicken baits

The composition of the bees, wasp, and chicken bait microbiomes and their beta dispersion differed significantly (adonis $F=1.53$, df$=4$, $p<.001$). Pairwise comparisons in beta dispersion indicate that the chicken bait microbiomes were significantly different from all of the bee microbiomes but not the wasp microbiome (Obligate pollinivore×chicken bait $p=.005$; Facultative necrophage×chicken bait $p<.001$; Obligate necrophage×chicken bait $p=.008$; wasp×chicken bait $p=.720$; Table S4). The difference between bee-associated microbiomes and bait microbiomes indicates that there may be some bee-specific filtering or bee-associated microbes dominating their gut.

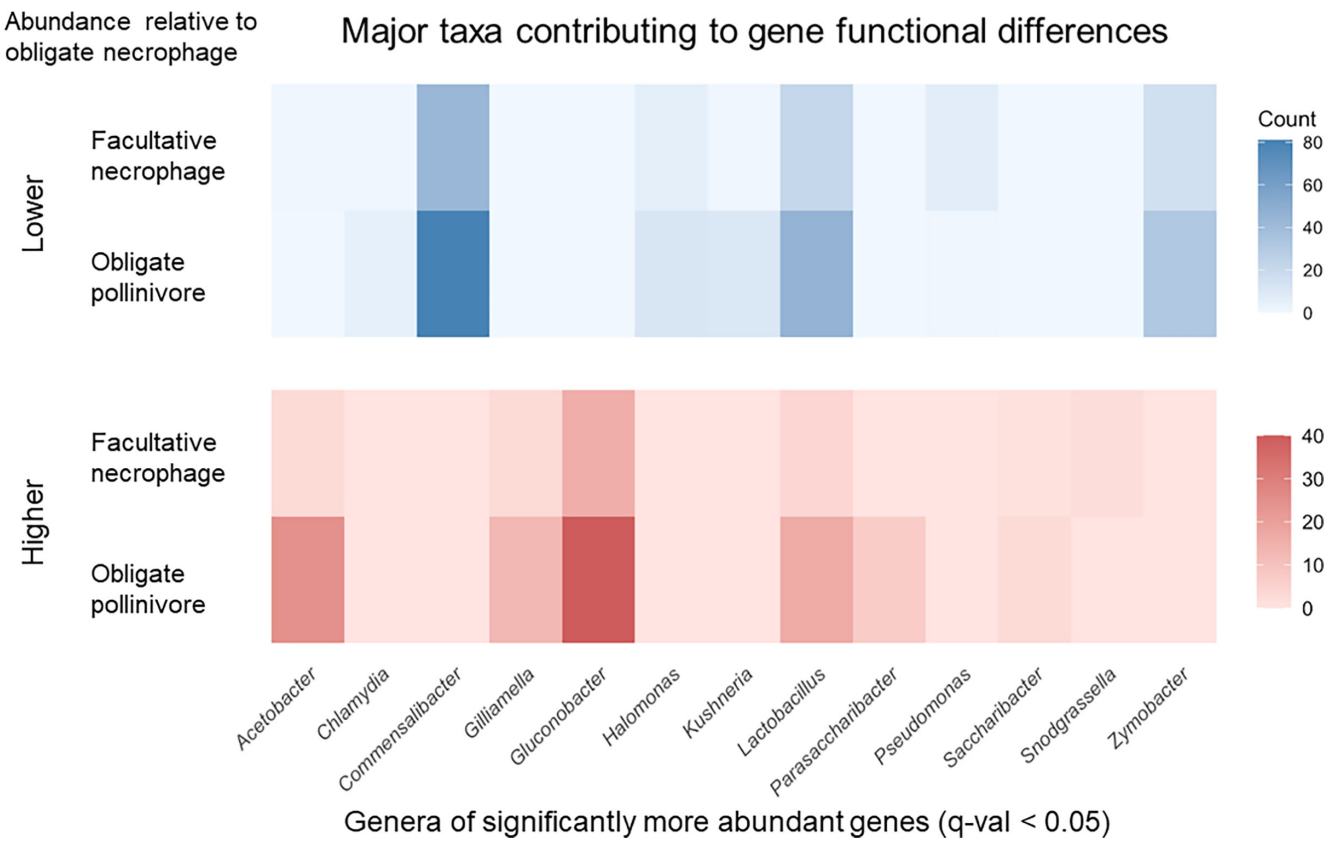

FIGURE 2 Summary of microbial taxa underlying the significantly differentially abundant genes between diet types. This figure represents how often there was a specific taxa's genes in lower abundance compared to the obligate necrophage (blue) and higher abundance compared to the obligate necrophage (red).

4 | DISCUSSION

Decomposition is a complex and dynamic process, and thus carrion-feeder microbiomes need to be adapted to a variety of stresses including nutrient variability, toxic compounds, microbial competition and interactions, osmotic stress, low pH, and temperature and oxygen fluctuations (Figure 4) (Benbow et al., 2015; Pechal et al., 2013). Our study suggests that the obligately necrophagous bee microbiome may be adapted to protect the host from opportunistic pathogens found on carcasses while overcoming novel nutritional challenges, cellular stress, and microbial competition (Figure 4). One of the most important mechanisms underlying bees' defence against pathogens involves the formation of strong biofilms in their guts (Martinson et al., 2012; Powell et al., 2014). These biofilms are typically formed by *Gilliamella apicola* and *Snograssella alvi* (key members of the corbiculate core) as well as *Frischella perrara* (Dixon, 2003; Engel et al., 2015; Kim et al., 2014; Kwong & Moran, 2013; Powell et al., 2016; Zhang et al., 2022). However, in the obligately necrophagous bee (*T. necrophaga*), there is a much lower abundance of *Gilliamella* and *Snodgrassella* than in the facultatively necrophagous and pollinivorous bees (and none have *Frischella* in high abundance). Thus, this protective biofilm might be derived from other microbes. All three diet types share a high abundance of the choanoflagellates *Monosiga brevicollis* and *Salpingoeca rosetta*, which are model systems for studying the evolution of multicellularity. A key protein thought to be involved in the transition to multicellularity in the choanoflagellates was found under positive selection in the obligate necrophage's gut symbiont *Commensalibacter intestini* – C-type lectin fold, which is involved in cell adhesion and signalling (Booth & King, 2020; King et al., 2008; Levin et al., 2014). Thus, perhaps adaptations for forming collective phenotypes that can line the bee gut and outcompete pathogens from the carcass is a key feature of the stingless bee microbiome.

Several of the bacteria we found in high abundance in the facultative necrophages are considered opportunistic human pathogens that are known to be antibiotic resistant and biofilm forming such as *Pseudomonas*, *Myroides*, *Bacteroides*, *Serratia*, *Enterococcus*, *Klebsiella*, *Salmonella*, and *Enterobacter* (Busse et al., 2021; Goullet & Picard, 1997; Ryan et al., 2022; Theocharidi et al., 2022) (Figure 3). Perhaps this ability for opportunistic pathogens to respond socially (as biofilms or swarming phenotypes) to stress or antibiotics produced by competing microbes during decomposition makes them beneficial symbionts for the bees. In fact, most other carrion-feeding insects including carrion beetles (Silphidae), blowflies (Calliphoridae), and flesh flies (Sarcophagidae) share the same microbial genera in high abundance (along with a few other biofilm formers) (Deguenon et al., 2019; Gupta et al., 2014; Shukla et al., 2018; Vogel et al., 2017). Specifically, the groups that almost all carrion-feeding insects shared

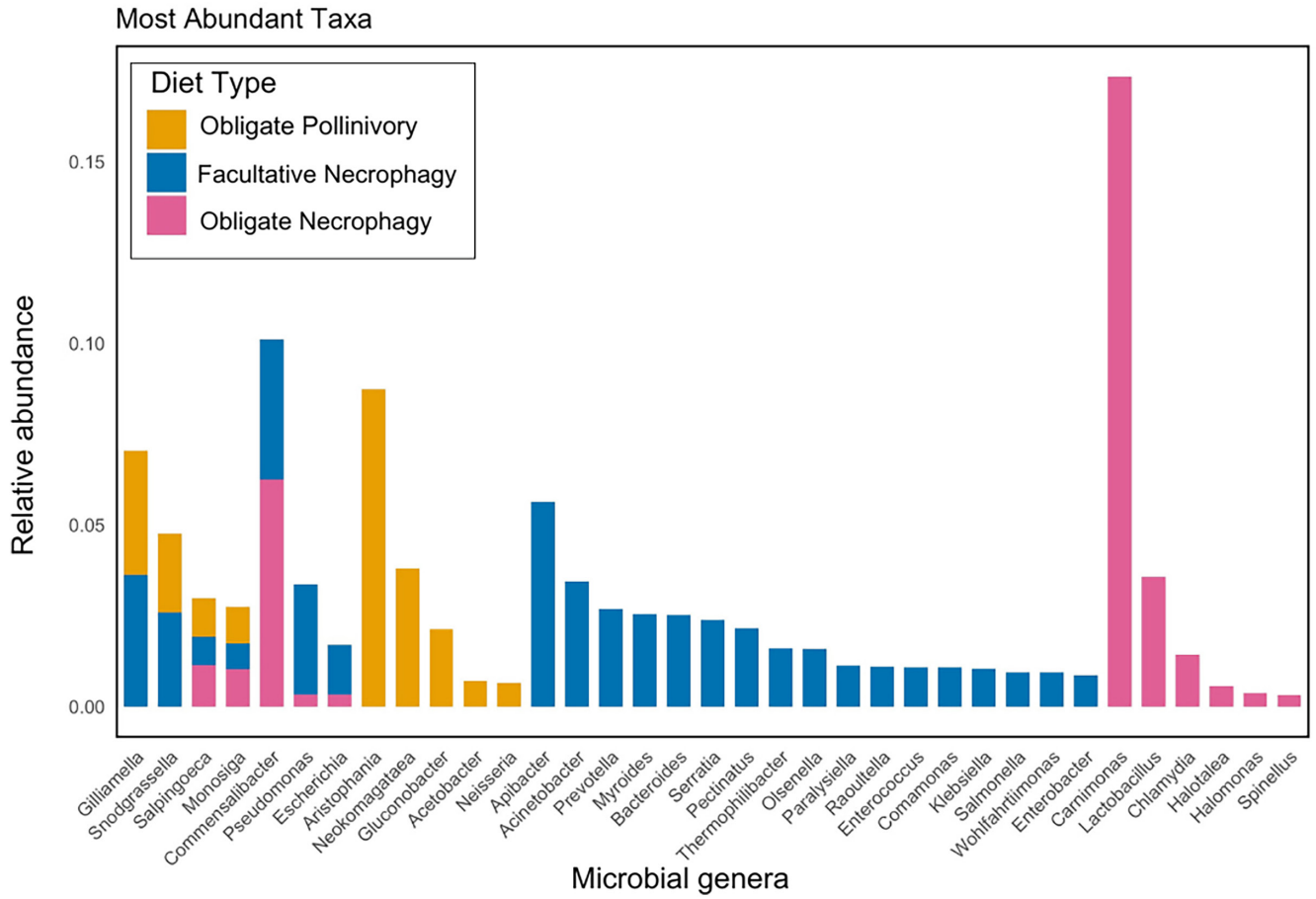

FIGURE 3 Relative abundances of the top 15 most abundant microbes for bee species grouped by diet type. The *x*-axis was sorted based on diet type (decreasing relative abundance within each group). The first seven genera are shared by two or more diet types, and the rest are unique to each diet type. When these taxa were mapped to the species level, we discuss them in a species-specific manner in the discussion (more information in Appendix S2 and Table A1 about exact counts per species). Otherwise, we identify and discuss the top taxa at the genus level. When summarizing the top 15 taxa in this figure, the species within the same genus are grouped together, which is why there appears to be less than 15 for some. There are more than 15 for the facultative necrophages because there were more bee species summarized compared to the obligate necrophage and pollinivore. More information about the functional role of each microbe can be found in Table S3.

with the facultative necrophages in high abundance were *Myroides*, *Wohlfartiimonas*, and *Acinetobacter*. *Myroides* and *Acinetobacter* are found in several different environments and are known to secrete antimicrobial substances and form biofilms that likely protect insects from pathogens (Deguenon et al., 2019; Dharne et al., 2008; Maeda & Morihara, 1995). Similarly, in vultures, the necrophagous bees' namesake, the most abundant members of the microbiome are also opportunistic human pathogens that form biofilms and show antibiotic resistance/production (Goullet & Picard, 1997; Roggenbuck et al., 2014). However, they have different microbes dominating their guts, mainly *Clostridium* and *Fusobacteria* (Goullet & Picard, 1997; Roggenbuck et al., 2014). In the vulture microbiome, it is thought that these predominant members outcompete other gut bacteria and allow the vultures to tolerate toxins and digest meat (Goullet & Picard, 1997; Roggenbuck et al., 2014). Thus, necrophagous microbiomes tend to have these functional commonalities regardless of their taxonomy, including vertebrates and invertebrates alike. To corroborate this hypothesis, our results show an overabundance of genes related to secondary metabolite biosynthesis, transport, and catabolism in the facultative necrophages, which may indicate microbial competition via excretion of antimicrobials. Likewise, the abundance of cell wall/membrane/envelope biogenesis genes in the facultative necrophage microbiome likely reflects the need to respond to microbial threats targeting cell walls.

Several proteins exhibiting signatures of positive selection in the obligately necrophagous symbionts *Lactobacillus acetotolerans* and *Commensalibacter intestini* suggest a link between biofilm formation, cell wall protection, and antibiotic resistance. These positively selected proteins include Cyclic-di-AMP phosphodiesterase, ABC transporters, penicillin-binding protein 2, and Type 1 protein exporters. These proteins hint at microbial competition as they not only regulate uptake and efflux of virulence factors but defend against microbial attacks damaging peptidoglycan (the primary target of most beta-lactam antibiotics) (Bai et al., 2013; Commichau et al., 2015; Georgopapadakou, 1993; Holland et al., 2005; Vanderlinde et al., 2010). There are also several genes showing

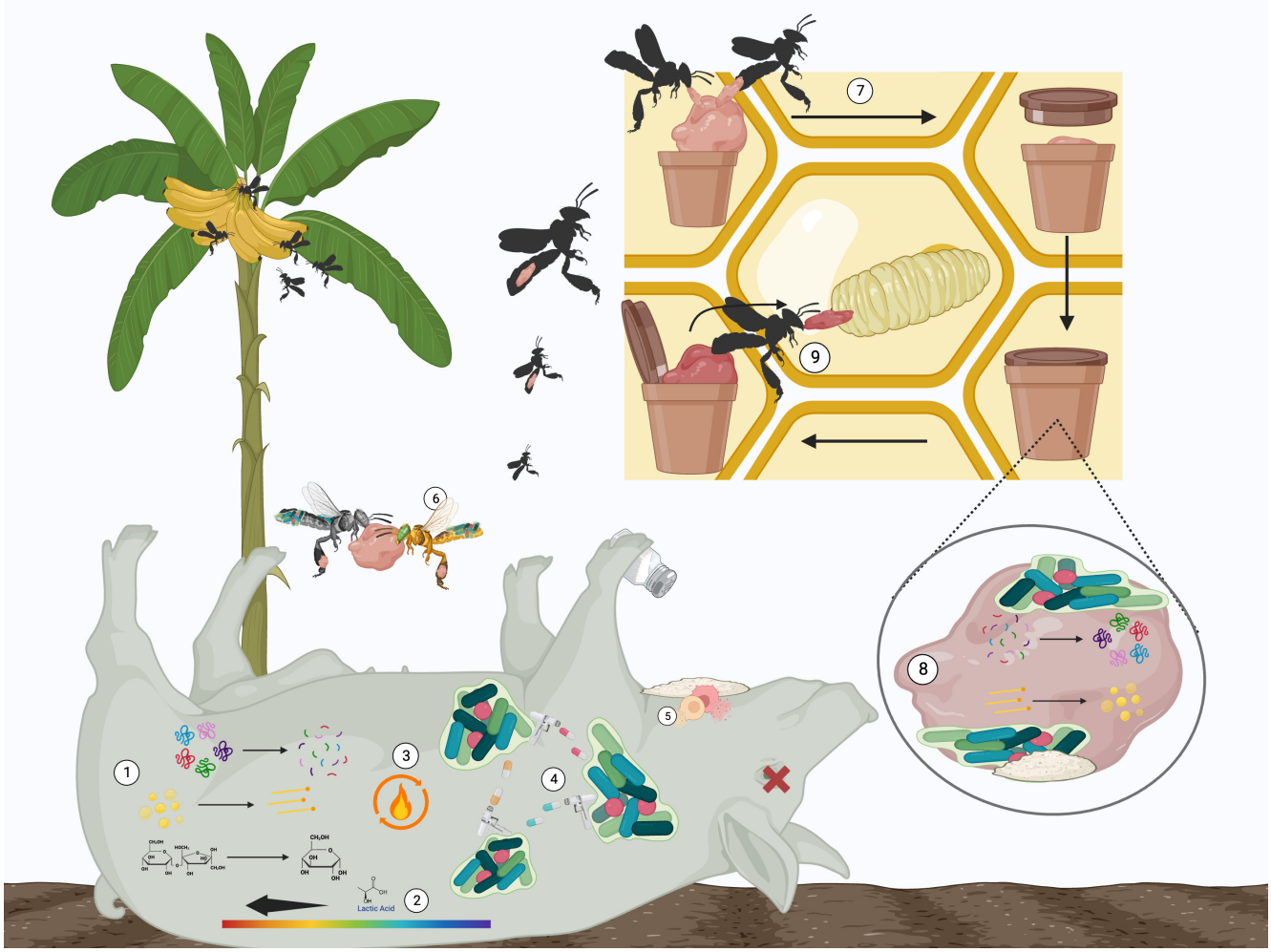

FIGURE 4 Graphical summary of our conclusions contextualized with natural history as well as hypotheses of the mechanisms taking place in the system. The microbiome of both obligate and facultatively necrophagous bees is likely adapted to decomposition stresses such as (1) nutrient variability, (2) decreasing pH, (3) temperature increases, (4) microbial competition, (5) high salinity. (6) The facultative necrophages' microbiomes have a high abundance of environmental bacteria known to be associated with antibiotic resistance, biofilm formation, and opportunistic pathogenicity. These likely protect the bees against pathogens on the carcass through forming biofilms in the gut. (7) The obligate necrophages deposit meat (seeding it with their gut microbiome) into cerumen pots that they seal off for ~14 days before feeding the brood. (8) Symbionts such as *Lactobacillus* and *Commensalibacter* aid in biosynthesis of fats and proteins and are adapted to cellular and DNA stress. Halophilic bacteria create biofilms in high salinity to outcompete spoilage microbes. (9) Then the processed meat can be fed safely to the larva. These hypotheses should be tested by manipulative experiments in future research (visual made with Biorender).

signatures of selection in these two obligately necrophagous bee symbionts that stabilize cell membranes, such as CDP-alcohol phosphatidyltransferase, hopanoid biosynthesis-associated radical SAM protein (HpnH), and DegV (Landgraf et al., 2016; Nogly et al., 2014; Zhong et al., 2020). DegV likely responds to cellular stress associated with carcasses through transcriptional modification and binding fatty acids (Broussard et al., 2016; Zhao et al., 2010). Perhaps these microbes are sensing and responding to variable stress signals epigenetically, especially given that the microbiome of the obligately necrophagous bee tends to have an overabundance of genes related to translation, ribosomal structure and biogenesis and posttranslational modification, protein turnover, and chaperones (Figure 1).

Biofilms often form in response to stress signals and can induce an SOS response leading to more mutations and horizontal gene transfer of antimicrobial-resistant genes (Penesyan et al., 2020; Rice et al., 2000). We found several genes related to epigenetics and DNA repair under positive selection in both *Lactobacillus acetotolerans* and *Commensalibacter intestini* in the obligately necrophagous bee gut such as rRNA small subunit methyltransferase G, Methylthiotransferase tRNA-2-methylthio-N(6)-dimethylallyladenosine synthase MiaB, acetyltransferase, GNAT family, UvrABC system, subunit B, and DNA recombination and repair protein RecA (Adami & Bottai, 2020; Burckhardt & Escalante-Semerena, 2020; Dash & Modak, 2021; Goosen et al., 1998; Xie et al., 2014). The microbiome is likely protecting the bees by responding to carrion stresses, perhaps through biofilm formation.

Several Halomonadaceae genera are known to be biofilm forming, opportunistic pathogens that produce antimicrobial substances

and resist antibiotic treatments such as vancomycin (Heyrman et al., 2002; Tahrioui et al., 2013). Several of such Halomonadaceae were either in high abundance in the obligate necrophages (such as *Halotalea*, *Halomonas*, *and Carnimonas*) (Figure 3) or provided a large portion of their differentially more abundant genes (such as *Halomonas*, *Kushneria*, and *Zymobacter*) (Figure 2). Thus, these salt-tolerant microbes may be important for overcoming the high fat and salinity of carcasses, as has been found in halomonads isolated from salted meat products (Singh et al., 2022; Ventosa, 2013; Ventosa et al., 2011). The obligately necrophagous bee *Trigona hypogea* deposits their foraged meat into a cerumen pot, seeding it with their own microbiome, and sealing it off for 14 days before feeding the brood (Noll et al., 1996; Yukphan et al., 2011). Perhaps these halomonads in *T. necrophaga* can also be found in the bees' storage pots, curing or processing the stored meat. These halophilic bacteria are known to produce exopolysaccharides (EPS) that form a protective coat around the cells in response to extreme conditions associated with carrion such as high salt, low nutrient availability, high temperatures, and pH imbalance (Singh et al., 2022). These EPS might protect vulture bees and their food through a range of adaptive roles to retain nutrients and water, create biofilms or cell aggregations that establish symbiotic or syntrophic interactions, and produce toxins and antibiotics (Singh et al., 2022). However, we were not able to assemble MAGs for these halomonads so we cannot determine which genes might have been specifically selected for overcoming the nutritional challenges of meat.

Carnimonas has been found in bee bread of other stingless bees, so we expect it plays an important role in the meat pots of obligate necrophages (Tang et al., 2021). We were able to assemble MAGs of the potentially new species of *Carnimonas* we found in the obligately necrophagous bee gut but were not able to apply the same comparative method because there are currently no available genomes of closely related bee-associated species. We also speculate that *Spinellus fusiger* may be preventing the overgrowth of spoilage fungi in the meat pots through parasitism and antifungal resistance (Boddy, 2016; Peters et al., 2008). Other Hymenoptera are known to rely on symbionts to produce antifungal protection to their brood, such as the beewolf and its symbiotic *Streptomyces* and honey bees and their *Bombella apis* symbiont (Kaltenpoth, 2016; Miller et al., 2021). Thus, future research looking at the role of *Spinellus* in vulture bee meat pots might prove to be an important avenue into understanding the role of symbiotic fungi in protecting the brood.

Given that excess fat can be detrimental to bee health, perhaps the necrophagous bee microbiome aids in breaking down fat into a less detrimental form (Manning et al., 2007; Ruedenauer et al., 2020; Vaudo et al., 2016). No studies have directly addressed the fat content of the vulture bee storage pots, but Noll et al. (1996) examined protein content in *T. hypogea*. During the 14 days of maturation, the meat in the storage pots had an increased availability of free amino acids and a decreased level of soluble protein. Future research should look more into the nutritional content of meat pots to understand how vulture bees regulate their fat levels and how the fat/protein ratio in their meat pots compares to bee pollen stores. Given that the meat deposited into these pots comes directly from foragers, after being chewed and transported in their crops in *T. hypogea* (Noll et al., 1996), perhaps their microbiomes seed the meat pots with an inoculum that can aid in meat digestion. We were able to identify several genes under positive selection in the obligately necrophagous bee's symbiont *Commensalibacter intestini* that are involved in amino and fatty acid metabolism and biosynthesis, such as biotin synthase/biotin biosynthesis bifunctional protein (BioAB), dihydroxy-acid/6-phosphogluconate dehydratase, phosphoribosylglycinamide formyltransferase, peptidase, and putative sodium bile acid cotransporter (Flint et al., 1993; Hagi et al., 2020; Paetzel, 2019; Wallace-Povirk et al., 2021; Westby & Gots, 1969). These genes may play a role in overcoming nutritional challenges associated with meat digestion such as high bile salts, fat, biotin, iron, and protein.

In contrast, we found several carbohydrate fermenting, osmotolerant microbes that thrive in sugar and pollen dominating the microbiome of the obligate pollinivore such as *Neokomagataea*, *Gluconobacter*, and *Acetobacter* (Charoenyingcharoen et al., 2022; Khan et al., 2020; Vannette & Fukami, 2018; Yukphan et al., 2011). The latter two, as well as *Gilliamella*, *Bombella* (formerly *Parasaccharibacter*), and *Saccharibacter*, were contributing several differentially more abundant genes in the pollinivores versus the obligate necrophage (Figure 2). The facultatively necrophagous bees had a high abundance of anaerobic microbes known to be environmentally acquired such as *Pectinatus* (involved in beer spoilage) in addition to microbes associated with vertebrates (*Paralysiella testudinis* – only ever isolated from a turtle cloaca) and even mud (*Thermophilibacter immobilis*) (Busse et al., 2021; Lu et al., 2021; Rodríguez-Saavedra et al., 2021). In addition to the environmental microbes, the facultative necrophages also maintain a high abundance of the corbiculate and Meliponini core such as *Gilliamella* and *Snodgrassella*, *Apibacter*, and *Commensalibacter*. Thus, the facultatively necrophagous bee microbiome reflects their diverse lifestyle as they maintain the typical bee-associated microbes important for a pollinivorous lifestyle as well as vertebrate-associated microbes that are typically found in other carrion-feeding insects.

5 | CONCLUSIONS AND FUTURE DIRECTIONS

The microbiomes of each diet type are likely adapted to survive the nutritional challenges associated with the specific diet (i.e. high sugar for obligate pollinivores or high salt and fat for obligate necrophages). In this sense, our results corroborate the general trend that stingless bees tend to acquire environmental microbes readily and then filter them in species-specific ways (Kueneman et al., 2023; Kwong et al., 2017). Species-specific filtering is further supported by the different microbial compositions between bees, wasps, and chicken baits. However, more research is needed to understand the origin of these microbial associations, whether functional adaptations of certain microbes enabled the dietary shift, or whether the dietary shift to necrophagy resulted in changes to the bees'

microbiome. Experiments that switch the microbiome of obligately necrophagous bees into non-necrophagous bees and vice versa will help us tease apart just how influential the role of the microbiome is as opposed to bee adaptations alone for carrion feeding. While diet type influences the abundance of coenzyme transport and metabolism genes, differences in gene abundances related to microbial competition and cellular stress suggest that the major differences in the microbiomes between diets relate to pathogen defence.

In this study, we assessed one of the three species of obligate necrophages and one species of obligate pollinivore. Thus, future research should experimentally test the hypothesized functional role of the specific genes and microbes posited here with a more balanced and larger sample size. Experiments testing the hypotheses generated by the data presented here will identify the specific mechanisms underlying the microbiome's role in pathogen defence and carrion digestion. Explicitly assessing whether novel necrophagous bee-associated microbes are bee-adapted or transient in the bee gut is also necessary. These exciting research avenues can help us not only understand how the microbiome plays a role in the evolution of diets but also potentially find microbes useful for new antibiotic production.

AUTHOR CONTRIBUTIONS

JJM manuscript writing, bee identification, bioinformatic, and statistical analysis. QSM conception, design of the work, the acquisition of data, and manuscript editing. LLF conception, design of the work, the acquisition of data, and manuscript editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The dataset supporting the conclusions of this article will be publicly available in the Sequence Read Archive (SRA) repository upon publication under bioproject PRJNA749807 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA749807) (Maccaro et al., n.d.). This includes all raw fastq metagenomic sequences for samples and controls (including the sample metadata). The code supporting the conclusions of this article is included as Appendix S1 and S2.

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SUPPORTING INFORMATION

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