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SPECIAL ISSUE: THE HOST-ASSOCIATED MICROBIOME: PATTERN, PROCESS AND FUNCTION

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How gut transcriptional function of *Drosophila melanogaster* varies with the presence and composition of the gut microbiota

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Abstract

Despite evidence from laboratory experiments that perturbation of the gut microbiota affects many traits of the animal host, our understanding of the effect of variation in microbiota composition on animals in natural populations is very limited. The core purpose of this study on the fruit fly *Drosophila melanogaster* was to identify the impact of natural variation in the taxonomic composition of gut bacterial communities on host traits, with the gut transcriptome as a molecular index of microbiota-responsive host traits. Use of the gut transcriptome was validated by demonstrating significant transcriptional differences between the guts of laboratory flies colonized with bacteria and maintained under axenic conditions. Wild *Drosophila* from six field collections made over two years had gut bacterial communities of diverse composition, dominated to varying extents by Acetobacteraceae and Enterobacteriaceae. The gut transcriptomes also varied among collections and differed markedly from those of laboratory flies. However, no overall relationship between variation in the wild fly transcriptome and taxonomic composition of the gut microbiota was evident at all taxonomic scales of bacteria tested for both individual fly genes and functional categories in Gene Ontology. We conclude that the interaction between microbiota composition and host functional traits may be confounded by uncontrolled variation in both ecological circumstance and host traits (e.g., genotype, age physiological condition) under natural conditions, and that microbiota effects on host traits identified in the laboratory should, therefore, be extrapolated to field population with great caution.

KEYWORDS
16S rRNA gene amplicon, Acetobacteraceae, functional redundancy, germ-free animals, gnotobiotic, microbiome, transcriptomics

1 | INTRODUCTION

There is now abundant evidence that healthy animals are colonized by microorganisms (collectively known as the microbiota), which influence multiple phenotypic traits of their animal host (Douglas, 2015; McFall-Ngai et al., 2013). Microorganisms in the gut dominate the microbiota of most animals (Engel & Moran, 2013; Sender, Fuchs, & Milo, 2016), and the gut microbiota is generally diverse and variable in composition, both among individual hosts and within one host over time (David et al., 2014; Dethlefsen, McFall-Ngai, & Relman, 2007; Human Microbiome Project Consortium, 2012).

A key unresolved question in microbiome research and, more generally, in microbial ecology is the functional significance of the taxonomic variation. In principle, functional differences among

microbial taxa are predicted to increase with taxonomic distance, but this general pattern can be confounded by horizontal gene transfer, convergent evolution of functional traits and diversifying selection among closely related taxa (Darmon & Leach, 2014; Goberna & Verdu, 2016; Thomas & Nielsen, 2005). A species-rich microbiota may, consequently, display functional redundancy, that is, multiple taxa are equivalent (or nearly so) with respect to a functional trait (Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012; McCann, 2000; Moya & Ferrer, 2016). In addition, the traits of individual microorganisms in the community may also be context-dependent, varying with the presence and abundance of other microorganisms of the same or different species (Fetzer et al., 2015). For example, some traits are only displayed at high density under the control of quorum signalling (Papenfort & Bassler, 2016), and various metabolic functions are the product of syntrophic interactions among microbial taxa with complementary capabilities (Morris, Henneberger, Huber, & Moissl-Eichinger, 2013; Zelezniak et al., 2015). A related issue is the choice of the most appropriate index of function. This problem applies particularly to microbial communities associated with animals or other hosts. Functional annotation of the gene content (or transcripts) of the microbiome, that is, metagenome (or metatranscriptome), is informative for some purposes (Bashiardes, Zilberman-Schapira, & Elinav, 2016; Franzosa et al., 2014; Jovel et al., 2016). Host traits (e.g., host transcriptional profile, phenotypic traits, host fitness) are also used widely, and these indices provide a more direct measure than microbial metagenomes/transcriptomes of how the microbiota composition affects the host (Douglas, 2010; Grote et al., 2017; Westermann et al., 2016).

The association between *Drosophila melanogaster* (henceforth abbreviated to *Drosophila*) and its gut microbiota is an amenable system to investigate the relationship between the taxonomic and functional diversity of the gut microbiota because the microbiota is taxonomically variable and the consequences for the host of eliminating the microbiota are well characterized (Broderick & Lemaitre, 2012; Erkosar, Storelli, Defaye, & Leulier, 2013). Specifically, the dominant microorganisms in this association are bacteria of the Lactobacillales (e.g., *Lactobacillus*, *Leuconostoc*) and Acetobacteraceae (e.g., *Acetobacter*, *Gluconobacter*) and, in some instances, members of the Enterobacteriaceae, as well as yeasts (e.g., *Hanseniaspora*, *Saccharomyces*) (Chandler, Eisen, & Kopp, 2012; Chandler, Lang, Bhatnagar, Eisen, & Kopp, 2011; Chaston, Dobson, Newell, & Douglas, 2015; Staubach, Baines, Kunzel, Bik, & Petrov, 2013; Wong, Chaston, & Douglas, 2013).

Most research on the interactions between *Drosophila* and its gut microbiota has been conducted in the laboratory using *Drosophila* strains that have been in culture for many decades. The taxonomic composition of the gut microbiota in these laboratory *Drosophila* is variable and influenced by many factors including diet, host genotype among-microbe interactions and stochastic processes (Broderick & Lemaitre, 2012; Erkosar et al., 2013). Multiple studies comparing laboratory *Drosophila* with an un-manipulated gut microbiota (conventional flies) and those experimentally deprived of their microbiota (axenic or germ-free flies) concur that the gut microbiota is not essential for the host on nutrient-sufficient diets, but that elimination of the microbiota alters many aspects of host metabolism, immune function, behaviour and cell signalling (Elgart et al., 2016; Jones et al., 2013; Ridley, Wong, Westmiller, & Douglas, 2012; Sharon et al., 2010; Shin et al., 2011; Storelli et al., 2011; Wong, Dobson, & Douglas, 2014). Furthermore, there is evidence that the taxonomic composition of the microbiota can influence host traits, including the gene expression profile of the gut and nutritional status of the insect (Broderick, Buchon, & Lemaitre, 2014; Elya, Zhang, Ludington, & Eisen, 2016; Erkosar et al., 2014; Newell & Douglas, 2014). By comparison, gut microbe–host interactions in field populations of *Drosophila* have received little study, beyond the demonstration of considerable variation in microbial community composition (Chandler et al., 2012; Wong et al., 2013; Staubach et al., 2013).

The broad goal of this study was to extend our understanding of the *Drosophila* gut microbiota and its interactions with the host in natural *Drosophila* populations. Our specific aim was to investigate the relationship between the taxonomic composition of the gut microbiota and host traits in natural *Drosophila* populations. We adopted a molecular approach, using 16S RNA gene amplicons as our taxonomic index and host gut transcriptome, quantified by RNA-Seq, as our index of host traits. The analysis was conducted at the population level, specifically pooling flies to a single sample for each of six separate field collections. Our field analysis was, of necessity, conducted on male *D. melanogaster* because females of *D. melanogaster* and the co-occurring species *D. simulans* cannot be distinguished from external features. This required us to conduct laboratory experiments to validate our proposed use of host gut transcriptome as a molecular index of host traits because published studies of microbiota effects on the gut transcriptome have been conducted exclusively on female *D. melanogaster* (Broderick et al., 2014; Elya et al., 2016; Erkosar et al., 2014). Specifically, we quantified the gut transcriptome of male and female *Drosophila* that were either colonized with a standardized bacterial community or maintained under axenic conditions, and demonstrated significant, but different, effects of eliminating the gut microbiome on the gut transcriptome of the two sexes. The subsequent analysis of wild male *Drosophila* used samples collected in summer and fall in two consecutive years. Despite obtaining high among-collection variation for both the microbiota composition and gut transcriptional profiles, the associations between host transcriptional profiles and taxonomic composition of the microbiota were muted, as compared to experimental manipulations in the laboratory.

2 | MATERIALS AND METHODS

2.1 | The flies

The laboratory-reared flies were *Wolbachia*-free *D. melanogaster* Canton S, reared at 25°C in a 12-h light/12-h dark cycle on a sterile diet of 10% Brewer's yeast (inactive; MP Biomedicals, Santa Ana, CA), 10% glucose (Sigma-Aldrich, St Louis, MO), 1.2% agar (Apex Bio, Houston, TX) and preservatives (0.04% phosphoric acid and

0.42% propionic acid [Sigma]). Axenic insects were generated by dechorionation of eggs in 0.6% bleach, and defined associations with bacteria (gnotobiotic insects) were constructed by adding 5×10^6 cells to each vial containing dechorionated eggs, by the method of Newell and Douglas (2014). The bacteria were, *Acetobacter pomorum* DmelCS_004, *Acetobacter tropicalis* Dmel_006, *Lactobacillus brevis* DmelCS_003, *Lactobacillus fructivorans* DmelCS_002 and *Lactobacillus plantarum* DmelCS_001, administered collectively to generate a five-species association. Guts were dissected from male and female flies at 5 days posteclosion, on glass plates previously cleaned with RNase-AWAY (Thermo Fisher, Waltham, MA) at 20× magnification. Isolated guts (comprising the crop, midgut and hindgut, but excluding the Malpighian tubules) were pooled to give three replicate samples of 30 guts per sample for females and 60 guts per sample for males (which are smaller than females) for each treatment. In all experiments, replicate flies were plated on nutrient agar, and any experiments that yielded bacterial colonies in the axenic treatment or colonies other than the predicted morphology in gnotobiotic treatments (Newell et al., 2014) were discarded.

Adult wild *D. melanogaster* were collected from natural populations at two locations (Ithaca and Geneva, ca. 50 miles apart) in central New York, USA. The habitats comprised mixed fruit orchards, including tree fruit (apples and pears), stone fruit (cherries and plums), and grape, raspberry and blueberry plantings. For each of the six collections (Table 1), 50 traps were set overnight in the canopy of several cultivated and wild host plants. Each trap comprised mashed raspberry bait in a mesh-covered 120-ml container, enclosed within a 1.9-l opaque plastic container (1892.74 ml; Thermo Fisher) ringed with 44 holes (3.175 mm diameter), enabling flies to enter but restricting escape and precluding access to the bait. Flies were collected at two times over 2 years: summer (August) and fall (October). On return to the laboratory, every fly was chill-anesthetized and identified to species and sex: females were discarded because female *D. melanogaster* and *D. simulans* cannot be distinguished from external features. Each male was surface-sterilized in ice-cold 70% ethanol, washed in ice-cold sterile PBS, immersed in RNAlater (Thermo Fisher), and immediately dissected as described above, with 60 pooled guts per collection. The pooled gut samples were transferred to 0.5 ml TRIzol reagent and stored at −80°C for subsequent extraction of DNA and RNA.

TABLE 1 Field collections of *Drosophila melanogaster*

Collection details			Bacterial communities in *Drosophila* guts		
Number	Date	Location[a]	Number of OTUs	Shannon diversity index (H)	Inverse Simpson diversity index (1/D)
1	19/08/2013	Geneva, NY	155	1.45	1.95
2	04/10/2013	Geneva, NY	144	1.52	2.56
3	21/08/2014	Geneva, NY	157	1.84	2.52
4	02/10/2014	Geneva, NY	123	0.68	1.28
5	28/08/2014	Ithaca, NY	121	1.55	2.43
6	07/10/2014	Ithaca, NY	202	1.86	3.49

[a]Geneva: Cornell University New York Agricultural Experiment Station in Geneva NY, USA (42°N, 77°W); Ithaca: Cornell Orchards, Ithaca NY, USA (42°N, 76°W).

2.2 | Nucleic acid extraction

Parallel DNA and RNA extractions were carried out on the pooled gut samples. Tissue samples were homogenized (1:1 by vol) with 0.1-mm glass beads (Scientific Industries, Bohemia, NY) on a MP Fastprep24 at 4.5 M/S for 2 × 30 s. Total nucleic acids were extracted by TRIzol (Life Technologies, Carlsbad, CA) phase separation with chloroform, according to the manufacturer's protocol, yielding RNA in the upper aqueous phase and DNA in the lower organic phase and at the aqueous-organic interphase. The aqueous phase was combined with an equal volume of ice-cold 70% EtOH, and RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), including on-column DNase treatment, and eluted in 50 μl RNase-free water. The DNA was extracted from the interphase-organic layer with back extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate and 1 M Tris) following the TRI Reagent DNA/protein isolation protocol (Thermo Fisher), and DNA was purified from the resulting aqueous phase using the DNAeasy Blood & Tissue kit (Qiagen), following manufacturer's instructions. Purity was checked by Nanodrop and concentration by Qubit (RNA BR Assay Kit and dsDNA HS Assay Kit, Thermo Fisher).

2.3 | 16S amplicon sequencing

DNA extracted from the pooled gut samples was amplified with a panel of barcoded universal bacterial primers 319F (5′ Illumina linker - Index seq. 1 - spacer – ACTCCTACGGGAGGCAGCAG) and 806R (3′ Illumina linker - Index seq. 2 - spacer - GGACTACHVGGGTWTC TAAT), using the primers, unique barcodes and amplification procedure of Fadrosh et al. (2014). Briefly, the 25 μl reaction comprised 0.25 μl Phusion polymerase (Thermo Fisher), 5 μl HF buffer, 0.5 μl 10 mM dNTPs, 0.75 μl DMSO, 8.5 μl water, 2.5 μl each 1 μM primer and 5 μl template DNA. The cycling conditions were modified from Fadrosh et al. (2014): 98°C for 60 s, followed by 35 cycles of 98°C for 15 s, 58°C for 15 s and 72°C for 15 s, and a final elongation of 72°C for 60 s then held at 4°C. Amplicons were cleaned and normalized using the SequalPrep 96-well plate kit (Life Technologies), pooled and concentrated with the AMPure kit (Beckman Coulter Inc., Brea, CA), according to manufacturer's instructions. Multiplexed, paired-end sequencing was performed on the Illumina MiSeq platform (2 × 300 bp reads) at the University of Rochester Genomics Research Center.

Sequence processing was conducted with Qiime 1.9.1 (Caporaso et al., 2010). Paired-end reads were joined with fastq-join (Aronesty, 2013). Joined reads were demultiplexed and quality filtered with default parameters. Potentially chimeric sequences, identified by de novo chimera detection with USEARCH6.1 (Edgar, 2010), were

removed. Sequences were assigned to operational taxonomic units (OTUs) via open-reference OTU picking (Rideout et al., 2014). The ten OTUs assigned to the genus *Wolbachia* (highlighted in Data S1a) were excluded from analysis as these taxa are endosymbionts and not expected in the gut lumen. After quality filtering and removal of reads assigned to endosymbiont taxa, the data set comprised 646,578 reads (Data S1b). Reads were evenly subsampled to a depth of 35,960 reads per sample (Data S1c), which was sufficient to cover the diversity present in all samples (Figure S1).

2.4 | Gut transcriptome analyses

Transcriptome libraries were generated using a protocol modified from Wang et al. (2011). Using 2.5 μg total RNA per sample, poly-A+ RNA was purified with DynabeadsOligo (dT) 25 (Life Technologies) according to the manufacturer's instructions, and the poly-A+ fraction was fragmented by incubation at 94°C for 5 min to generate 200- to 250-bp fragments. Double-stranded cDNA was synthesized using the Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, cA) following manufacturer's instructions. Resulting cDNA was cleaned using RNA Clean XP magnetic beads (Agencourt, Brea, CA). Second-strand synthesis was performed providing dUTP instead of dTTP to generate strand-specific libraries. Double-stranded cDNA was end-repaired using the End Repair Mix LC (Enzymatics, Beverly, MA) and resulting blunt-end fragments were A-tailed using the Klenow 3′-5′ exo-enzyme (Enzymatics). Universal Y-shaped adapters were ligated using A-T ligation. Adapter ligated DNA was purified and size-selected using Ampure XP beads (Agencourt). Finally, the uracil-containing second strand was digested using uracil DNA glycosylase (Enzymatics) and DNA was subjected to 15 cycles of PCR amplification using barcoded Illumina index primers (Table S1). Final DNA was purified using Ampure XP beads (Agencourt) and eluted in 12 μl Qiagen Buffer EB. The DNA concentration was determined on a Qubit 2.0 fluorometer (as above) and sample concentrations ranged from 4.1 to 19.7 ng/μl. Library quality was assessed on Bioanalyzer, and equimolar pools were subjected to 50-bp single-end sequencing for laboratory flies, and 150-bp paired-end sequencing for wild-caught flies, both by Illumina HiSeq at the Biotechnology Resource Center, Cornell University.

The resulting data were analysed using the Galaxy bioinformatic platform (Afgan et al., 2016). Raw reads were uploaded, and sequence quality was screened using FastQC. Reads were then quality filtered using the "Filter by quality" (version 1.0.0) tool with default parameters (quality ≥30 for at least 90% of bases in the sequence). Filtered reads were aligned to the *D. melanogaster* reference genome (dm3) using TopHat version 2.1.0 (Trapnell, Pachter, & Salzberg, 2009) implemented in Galaxy. Default parameters were used, except that "library type" was switched to "FR first strand" for strand-specific sequencing. TopHat-accepted hits were subjected to Cufflinks (version 2.2.1) (Trapnell et al., 2010), using the *D. melanogaster* dm3 reference annotations. FPKM (fragments per kilobase of transcript per million mapped fragments) values were downloaded (file: gene expression), normalized to one million total fragments, and subsequently analysed with reference to Flybase (St Pierre, Ponting, Stefancsik, McQuilton, & FlyBase, 2014), Flymine (Lyne et al., 2007) and designated with Gene Ontology (GO) terms via DAVID (Huang, Sherman, & Lempicki, 2009). Only genes that differed significantly between treatments and had both ≥10 FPKM in one sample and greater than twofold difference were considered for subsequent analyses.

2.5 | Statistical analysis

Statistical analyses were conducted principally in R (R Core Team, 2015). For sequence coverage plots, reads were subsampled at evenly spaced intervals and the number of OTUs detected at each step was calculated with the iNEXT package (Hsieh, Ma, & Chao, 2016). Bacterial diversity in each fly was calculated as the number of OTUs (OTU richness) and the Shannon–Weaver (H') and inverse Simpson (1/D) diversity indices. Relationships among collections were visualized with principal coordinates analysis (PCoA) plots based on distance calculated from relative abundance (Bray–Curtis dissimilarity) and presence/absence (Jaccard Index) of bacterial OTUs (Figure S2b,c), and FPKM values for the transcriptome data sets. To assess overall relationships between the fly transcriptome and composition of the microbiota, Procrustes rotation (Peres-Neto & Jackson, 2001) was performed to compare principal coordinate analysis ordinations of the individual transcripts and GO terms to the microbiota at the taxonomic levels of OTU and order (relative abundance and presence/absence). Permutation tests were conducted with the "protest" function in the "vegan" package to test the significance of these relationships (Oksanen et al., 2016). Spearman's ρ was used for analysis of the correlations between relative abundance of bacterial 16S reads and FPKM of host gut transcripts, with $p = .05$ significance cut-off indicated by $|\rho| > 0.885$. Only those transcripts or OTUs detected in at least four of the six samples were considered in the correlation analysis to avoid the zero-inflated data.

3 | RESULTS

3.1 | Transcriptional responses of laboratory *Drosophila* to gut bacteria

Our first analysis compared the gut transcriptome of laboratory *Drosophila* Canton S that had been reared either under microbiologically sterile conditions (axenic flies) or colonized with five bacterial strains of the species, *Acetobacter pomorum*, *Acetobacter tropicalis*, *Lactobacillus brevis*, *Lactobacillus fructivorans* and *Lactobacillus plantarum*, originally derived from this *Drosophila* culture (gnotobiotic flies). By colonizing the *Drosophila* with a standardized microbiota, we ensured that the microbiota content of replicate samples was uniform, avoiding the variability in microbiota composition of conventional (i.e., unmanipulated) *Drosophila* (Chandler et al., 2011; Wong et al., 2013). Previous studies have shown that when colonized with this standardized set of bacteria, this *Drosophila* strain displays multiple microbiota-dependent metabolic and performance traits that are

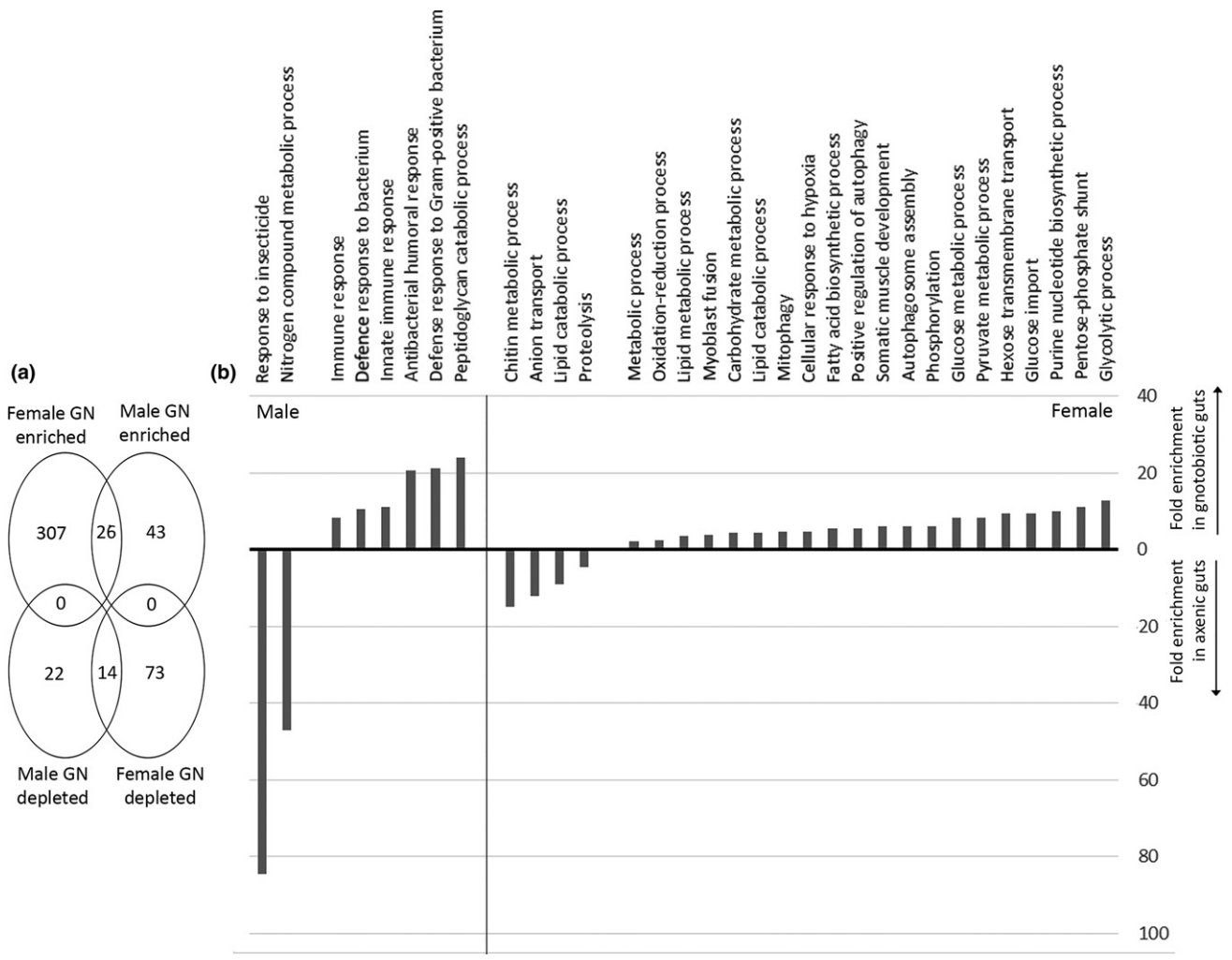


FIGURE 1 The gut transcriptomes of male and female laboratory *Drosophila*. (a) The number of genes with significantly enriched or depleted expression in gnotobiotic flies (GN) relative to axenic flies ($p < .05$ and >2-fold difference). (b) GO terms (Biological Process) that were significantly over-represented in the differentially expressed genes ($p < .05$) relative to the entire gut transcriptome, with male and female flies displayed separately

quantitatively comparable to conventional flies (Dobson et al., 2015; Newell & Douglas, 2014).

The transcriptome sequencing of dissected *Drosophila* guts yielded 12–38 million reads for each of the three replicate samples of axenic and gnotobiotic flies for each sex, with transcripts of 5,602 genes detected across the full data set (Data S2a, Table S2). In total, 485 (8.7%) of the 5,602 genes met our criterion for significant differential expression between axenic and gnotobiotic flies ($p < .05$, greater than twofold difference, and FPKM ≥10 in at least one sample) (Figure 1a; Data S2b,c). Overall, more genes were significantly differentially expressed in females (419 genes) than males (105 genes) and the transcripts of more genes were enriched in gnotobiotic flies relative to axenic flies (374 genes) than the reverse (111 genes). In the complementary principal components analysis (PCoA) of the transcriptomes (Figure S3), the expression patterns of the two sexes were nonoverlapping on the first PCo axis, consistent with published evidence for major transcriptional differences between male and female *Drosophila* (Ayroles et al., 2009; Huang et al., 2015; Hudry, Khadayate, & Miguel-Aliaga, 2016); additionally, the among-sample variation was considerably greater for the females than males, but the magnitude of the difference between the sexes did not vary by microbiota status (Figure S3). None of the significantly differentially expressed genes had discordant patterns between the two sexes.

To investigate the differentially expressed genes further, we determined the functions represented by genes that were differentially expressed between gnotobiotic and axenic flies using Gene Ontology (GO). There was no overlap in the GO terms enriched in male and female *Drosophila* (Figure 1b). This result is consistent with the low number of individual genes that are differentially expressed in both sexes (Figure 1a), and is indicative of major differences between the sexes in transcriptional responses to elimination of the gut microbiota.

Transcripts enriched in the guts of gnotobiotic male flies were dominated by immunity-related genes, including genes encoding receptors and modulators of the humoral immune-deficiency (IMD)

pathway (*PGRP-SC* and *PGRP-LB*) and multiple antimicrobial peptides (e.g., *Attacin-A*, *Attacin-B*, *Drosomycin*, *Diptericin-B*). The guts of axenic males had significantly elevated expression of genes coding broadly defined predicted enzymatic functions (nitrogen compound metabolic process) and two cytochrome P450 genes (*cyp12d1-p* and *cyp12d1-d*: response to insecticide), perhaps indicative of a greater detoxification function in the guts of axenic flies than gnotobiotic flies. By comparison, the GO terms enriched in the differentially expressed genes in gnotobiotic females were dominated by metabolism genes, especially central carbon metabolism and nucleotide and lipid biosynthesis, all consistent with the metabolic demands for egg production in females. These functional groups include genes coding key enzymes in the pentose-phosphate pathway, notably the rate-limiting enzyme glucose-6-phosphate dehydrogenase (Zw) and almost every enzyme involved in glycolysis, including the rate-limiting enzyme phosphofructokinase (Pfk), as well as the lipase Lip3 and the α-glucosidase target of brain insulin (Tobi) (Data S2c). Genes enriched in axenic female flies included several *Jonah* genes encoding proteolytic enzymes and genes with undefined functions in chitin metabolism and transport of anions (Data S2c).

In summary, our transcriptional analysis of laboratory *Drosophila* demonstrates multiple genes with microbiota-dependent expression in the gut, and also reveals major differences between male and female flies in the transcriptional response to elimination of the microbiota. This analysis provided the experimental basis for our subsequent investigation of how the gut transcriptome of male *Drosophila* in natural populations varies with the composition of the bacterial communities.

3.2 | The taxonomic composition of the gut microbiota in field-collected *Drosophila*

We used high-throughput sequencing of 16S rRNA gene amplicons to characterize the taxonomic composition of bacteria in guts dissected from six collections of field-collected male *Drosophila* (Table 1). Overall, 433 bacterial OTUs were detected, with 121–202 OTUs present in a single collection (Data S1c, Figure S2a). The samples were dominated by bacteria of the orders Rhodospirillales (specifically the family Acetobacteraceae) and Enterobacteriales (Figure 2a). A single unidentified Acetobacteraceae OTU (ID 2499164) was particularly abundant, comprising more than 40% of the reads in five of the six collections (Data S1c), and all collections bore a further 28–41 additional OTUs assigned to the Acetobacteraceae (Figure 2b). The proportion of Enterobacteriales varied considerably among collections, ranging from less than 2% of the reads in collections 1, 4, and 5, to more than 30% of the reads in collections 2, 3 and 6 (Figure 2a). Collections with higher relative abundance of Enterobacteriales had a greater Enterobacteriales diversity (Figure 2b). The Enterobacteriales genera *Providencia*, *Pantoea* and *Erwinia* (all members of the family Enterobacteriaceae) were present in at least five of the six collections, but the most abundant Enterobacteriales could not be identified to genus (Data S1c). The Lactobacillales were represented by multiple OTUs, including the genus *Weisella*

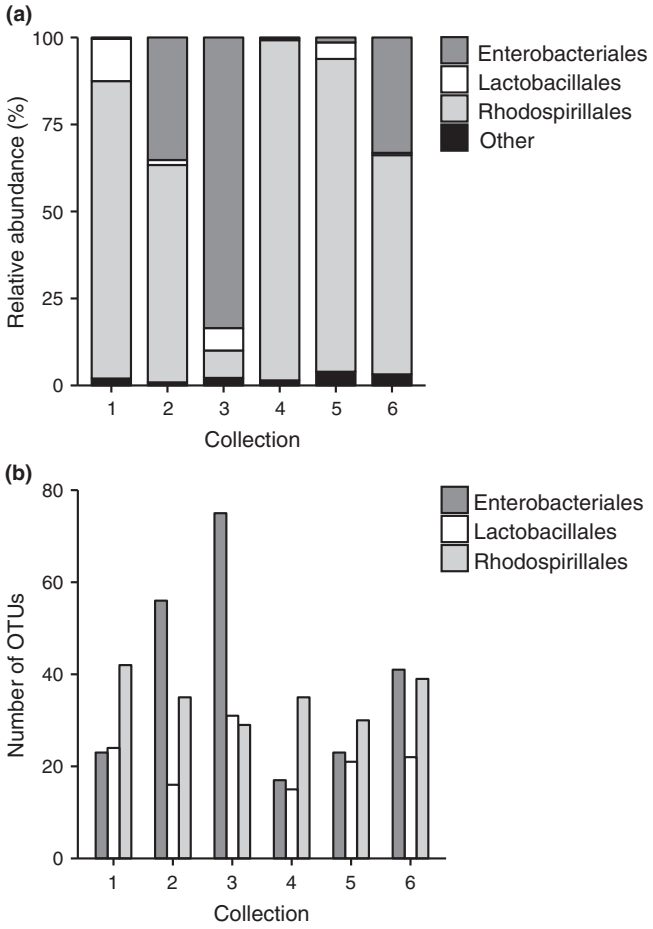
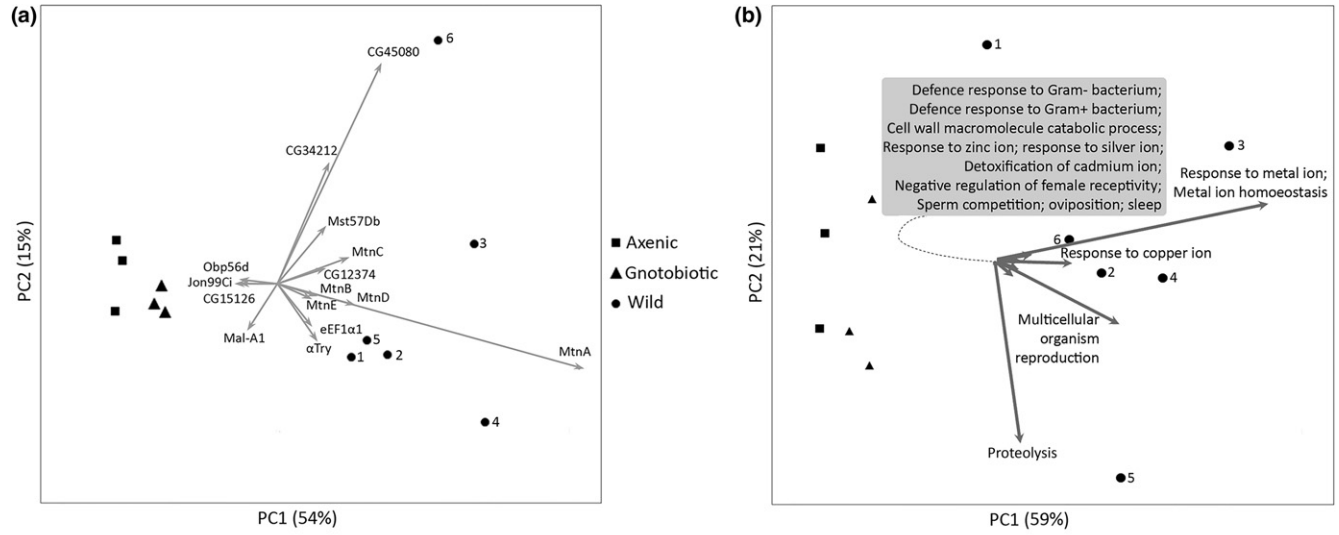


FIGURE 2 Composition of gut microbiota in collections of wild male *Drosophila*. (a) Relative abundance of Enterobacteriales, Lactobacillales, Rhodospirillales and other bacterial orders. (b) Number of OTUs assigned to Enterobacteriales, Lactobacillales and Rhodospirillales

(family Leuconostocaceae), which contributed more than 10% and 3% of the reads in collections 1 and 5, respectively, and the genus *Enterococcus* (family Enterococcaceae), which was the dominant Lactobacillales in collection 3 (Data S1c). Collections did not cluster based on sampling date or location for both presence/absence and relative abundance of bacterial OTUs (Figures S2b,c).

3.3 | The gut transcriptome of wild male *Drosophila*

A total of 6-15.9 million mapped reads were obtained for the gut transcriptomes from the six collections of wild male *Drosophila* (Table S2). Expression of 6,414 genes was detected in this data set, of which 4,886 (76%) were also detected in the laboratory male *Drosophila* (Data S2a). The wild fly transcriptomes separated from the laboratory flies on the first axis of the PCoA plots for both individual genes and GO categories (Figure 3a,b). The key genes driving this difference were metallothionein (*Mtn*) genes, which function in protection against metal toxicity, especially copper (Andrews, 2000; Egli et al., 2006; Petering & Fowler, 1986), as well as genes involved in reproduction, including the male-specific gene *Mst57Db*, proteolysis



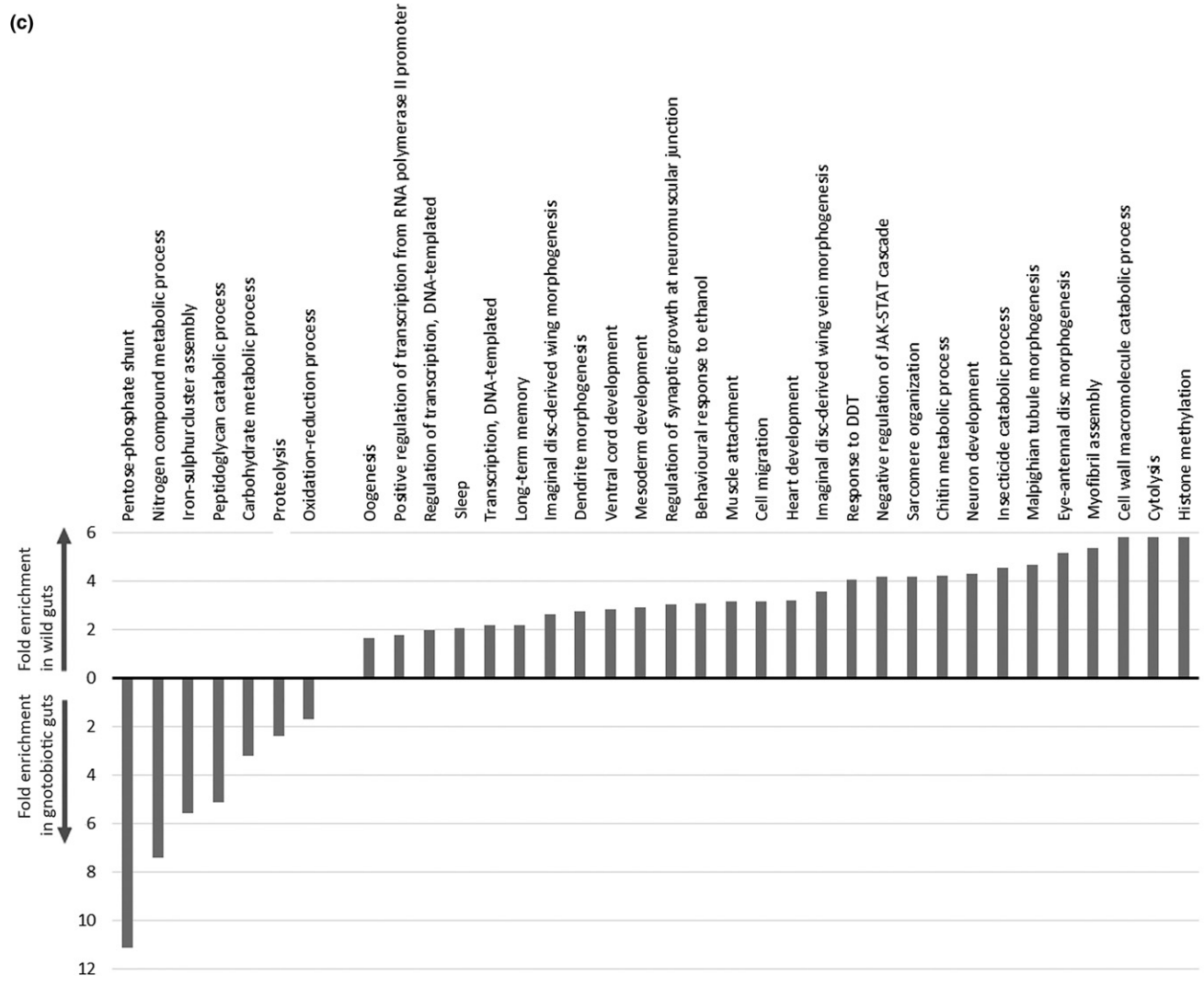


FIGURE 3 Gut gene expression patterns of wild and laboratory male *Drosophila*. (a) PCoA plot of expressed genes (with FPKM>10 in at least one sample). Each point represents a gut sample of wild or laboratory *Drosophila*, with collection number (1–6) of wild samples and loading scores for the top 15 genes contributing to PC1 shown. (b) PCoA plot of GO terms (Biological Process) represented by the transcriptomes, with collection number and loading scores for the top 15 GO categories (the GO categories contributing to the central arrows are listed in the grey box). (c) Enrichment of GO terms (Biological Process) in transcriptomes of wild and laboratory *Drosophila*, based on differentially expressed genes ($p < .05$, >2-fold difference between mean expression level)

(e.g., CG12374 and the digestive trypsin α-*Try*) and immune responses (Figure 3a–c).

Visual inspection of the data (Figures 2 and 3) was not indicative of a relationship between the abundant bacterial taxa in the gut microbiota and ordinations of the gut transcriptomes for the six wild *Drosophila* collections. Consistent with this observation, we detected no significant association between the fly transcriptome and taxonomic composition of the gut microbiota (Procustes permutation tests; $p > .05$ of the individual transcripts and GO terms to bacterial OTUs and orders in the microbiota [relative abundance and presence/absence]). As a complementary analysis, we quantified the pairwise correlations between the relative abundance of bacterial taxa and the host gut transcriptome, using multiple taxonomic levels for the bacterial communities and either gene or GO category for the host transcriptome. Confirming the Procrustes analyses, these analyses yielded very few significant correlations at $p < .05$ and no detectable pattern of correlations with respect to bacterial taxonomy or host function. However, the pairwise correlations between abundance of bacterial OTUs and host transcripts, which collectively had just 1.8% significant correlations (Data S3), included three OTUs correlated with the expression of a relatively large number of host genes that encompassed multiple GO categories. These three taxa were all members of the Acetobacteraceae: OTU156232 with significant correlations to 11.5% of host genes, and New.Reference_OTU285 and New.Reference_OTU293, both with significant correlations to 10.6% of the host genes (Data S2d). These OTUs were present in all collections apart from OTU285, which was not detected in Collection 3, and they contributed <1% of the total reads in every sample.

4 | DISCUSSION

This study has demonstrated that the pattern of gene expression in the *Drosophila* gut is far more responsive to the perturbation of eliminating the gut microbiota in the laboratory than to natural variation in microbial community composition in the field. Here, we discuss three issues arising from these results: the pattern of variation in taxonomic composition of the gut microbiota in laboratory and field *Drosophila*; the use of gut transcriptome as an index of microbiota-dependent host function; and the biological importance of the independence between taxonomic composition of the gut microbiota and gut gene expression in the wild *Drosophila*.

The taxonomic diversity of bacteria detected in wild *Drosophila* is fully consistent with the evidence from multiple laboratory and field studies that *Drosophila* can associate with a wide range of bacteria (Chandler et al., 2011; Chaston, Newell, & Douglas, 2014; Corby-Harris et al., 2007; Staubach et al., 2013); and similar results have been obtained for other drosophilid species, for example, Brooks, Kohl, Brucker, van Opstal, and Bordenstein (2016), Chandler, James, Jospin, and Lang (2014), Martinson, Douglas, and Jaenike (2017), Vacchini et al. (2017) and Wong et al. (2013). The Acetobacteraceae, which are obligately aerobic bacteria that exploit sugar-rich habitats (Crotti et al., 2010), are strongly represented in most laboratory and field *Drosophila* (including this study). Interestingly, *Lactobacillus* (Lactobacillales) that dominate some laboratory cultures of *Drosophila* are at low abundance in wild *Drosophila*, which mostly contain Lactobacilli of the genera *Weisella*, *Leoconostoc* and *Enterococcus* (Chandler et al., 2011; Storelli et al., 2011; Wong et al., 2015; this study). Presumably, some *Lactobacillus* species, especially *L. brevis* and *L. plantarum*, have greater tolerance of the laboratory environment than the Lactobacillales associated with wild *Drosophila*, but the mechanisms underlying this difference remain to be established. Members of the Enterobacteriales have been reported to dominate some laboratory *Drosophila* cultures and field populations (including in this study) but with much taxonomic variation (Chaston et al., 2015; Staubach et al., 2013; Wong et al., 2013). In summary, this study is fully consistent with multiple published reports, collectively indicating that *Drosophila* is adapted to associate with bacterial communities of diverse and variable taxonomic composition.

Transcriptomic data provide a valuable index of host response to the gut microbiota in *Drosophila* and other animals. Although the gut microbiota status of the animal can affect the gene expression of multiple organs (Clemente, Ursell, Parfrey, & Knight, 2012; Erkosar et al., 2013), the *Drosophila* gut displays more extensive responses than other organs to the gut microbiota (Dobson, Chaston, & Douglas, 2016), consistent with the proximity of the gut epithelium to the microbial cells in the gut lumen. Several studies have demonstrated substantial effects of eliminating the gut microbiota on gut gene expression in female *Drosophila* (Broderick et al., 2014; Elya et al., 2016; Erkosar et al., 2014). Our finding that axenic rearing has sex-specific effects on the gene expression profile of *Drosophila* (Figure 1) demonstrates that, although the microbiota composition does not generally differ between male and female *Drosophila* (Chandler et al., 2011; Wong, Ng, & Douglas, 2011), many of the genes with microbiota-dependent expression differ between the sexes. Sex-specific host responses to microbiota perturbations have been reported in other animals (Human Microbiome Project Consortium, 2012; Jasarevic, Morrison, & Bale, 2016; Markle et al., 2013), but the underlying mechanisms are largely unknown. Interestingly, the genes and functional categories enriched in the gut transcriptome of *Drosophila* colonized with a standardized 5-member *Acetobacter/Lactobacillus* association (this study) overlap strongly with published studies on conventional flies (Broderick et al., 2014; Erkosar et al., 2014) and flies colonized with the yeast *Saccharomyces cerevisiae* (Elya et al., 2016). Taken together, these data suggest that the *Drosophila* gut gene expression is responsive to the presence of microbes, but without a strong taxon-specific signal. Exceptionally, certain single strains of *Acetobacter* and *Lactobacillus* induce a very weak host transcriptional response (Elya et al., 2016), consistent with other studies demonstrating that some individual bacterial taxa do not significantly affect host traits (Chaston et al., 2014; Newell & Douglas, 2014).

We obtained major transcriptional differences both between the wild and laboratory *Drosophila* and among the six collections of wild *Drosophila* (Figure 3). This is fully expected, given the many

differences with respect to diet, abiotic conditions, *Drosophila* genotype and microbiota composition across the different samples. The key genes driving the difference between the wild and laboratory *Drosophila* in the ordination plots are the fivefold to 15-fold greater expression of metallothionein genes (*MtnA-E*) in the wild flies (Figure 3). Metallothionein proteins protect animals against toxic levels of metals (Andrews, 2000; Petering & Fowler, 1986), with evidence that the *Drosophila* Mtn proteins play critical roles in *Drosophila* resistance to copper, zinc and cadmium (Egli et al., 2006). Copper-based fungicides, for example, Bordeaux mixture comprising copper sulphate and lime, have historically been used in vineyards and fruit farms in the region of this study, and have been demonstrated to cause the accumulation of copper in soils (potentially contaminating fallen fruit) often for many years after applications have ceased (Dumestre, Sauvé, McBride, Baveye, & Berthelin, 1999; Pietrzak & McPhail, 2004). We hypothesize that this environmental contaminant may confer a strong selective advantage for high *Mtn* gene expression in the flies. This hypothesis this can be tested by analysis of the scale of exposure of wild *Drosophila* populations to toxic levels of metals and the fly *Mtn* expression response to environmentally relevant doses of these contaminants. Other transcriptional differences between the laboratory and field *Drosophila* may also be indicative of differences in ecological circumstance. For example, the enrichment of nitrogen metabolism and proteolysis in the laboratory samples may reflect differences in the availability of nitrogen resources between the two conditions, while the enrichment of "behavioural response to ethanol" in the field flies may relate to higher ethanol levels in natural diets than in laboratory conditions. However, many of the differences, especially those with developmental or loosely defined functions, may reflect the greater variation in age and physiological condition of the field flies than those in the laboratory.

The substantial among-collection variation in both host gut transcriptome profiles and microbiota composition provided the basis to interrogate the data sets for associations between bacterial taxonomy and host function. Such analyses can be confounded by multiple caveats, including poor statistical power, weak taxonomic resolution of 16S analyses and transcriptional noise; and future studies would benefit from more extensive sampling, to include a wider range of sites and longer timescales. Nevertheless, the magnitude of the difference in patterns of ordinations obtained for the bacterial communities and gut transcriptomes (Figures 2 and 3) indicate that these technical limitations would be unlikely to mask strong, systematic correspondence between the taxonomic composition of the bacterial community and host gut transcriptional profile at the population level. The low incidence of correlations suggests that, although the architecture of the *Drosophila* transcriptome is strongly dependent on the presence of gut microorganisms (Dobson et al., 2016), it is relatively insulated from variation in bacterial composition, relative to other environmental factors encountered by natural *Drosophila* populations. This conclusion is broadly consistent with the conclusions from laboratory studies that gut microbial communities of different compositions can have similar effects on the gut transcriptome (see above). Despite this, the data sets include a small number of taxa that contribute <1% of the 16S rRNA gene reads and support a relatively large number of significant correlations, raising the possibility that these few taxa may play a role disproportionate to their abundance in shaping host transcriptional function. These traits are indicative of candidate keystone taxa in the gut microbiome (Berry & Widder, 2014). However, this interpretation is tentative and requires validation by isolation and characterization of the bacterial taxa, including analysis of their impact on the *Drosophila* gut transcriptional profile under defined conditions.

A further important extension of this study is to investigate the relationship between microbiota composition and gut transcriptome in individual flies from a single collection. The among-sample variation in transcriptional signal attributable to ecological factors would likely be smaller than for the population-level comparison used in this study; but the relationship between microbiota and host transcriptome may be complex because the microbiota composition varies over time within an individual fly, and the gut transcriptome may be influenced by both the current microbiota and history of interactions with taxonomically different microorganisms (which, in field populations, is unknown).

The factors driving among-collection variation in the gut gene expression of wild *Drosophila* remain to be determined; our data suggest that these factors are not strongly correlated with either season or location (although our study was not designed explicitly to test for these factors). Linked to its ecology as a denizen of rotting fruit and plant material, wild *Drosophila* may encounter a wide diversity of microorganisms, including yeasts and protists which were not analysed in this study, and are colonized by microbial communities that are taxonomically diverse in comparison to laboratory flies (Chandler et al., 2011; Wong et al., 2013; Staubach et al., 2013). In the context of the ecology of natural *Drosophila* populations, the robustness of the gene expression profile of the *Drosophila* gut to diverse microbial communities may be adaptive. This is in contrast to the taxon-specific transcriptional responses that mediate the antagonistic interactions with specific co-evolved pathogens in *Drosophila* (Buchon, Broderick, Poidevin, Pradervand, & Lemaitre, 2009; Dutta et al., 2015).

An important open question arising from this study is the generality of our finding that, in natural *Drosophila* populations, the taxonomic composition of the gut microbiota is not a key driver of host gut gene expression. Further research is required to assess the extent to which this effect may be a specific adaptation linked to the nutritional ecology of *Drosophila*, common to animals utilizing microbe-rich diets, or a general feature of animal interactions with their gut microbiota.

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DATA ACCESSIBILITY

16S amplicon sequences and transcriptome sequences are deposited in NCBI SRA under Access ion Numbers PRJNA381755 and PRJNA393828, respectively.

AUTHOR CONTRIBUTIONS

A.B., S.F. and G.L. conducted the field work; S.F. conducted the laboratory experiments; A.B., S.F., K.L.A. and V.G.M. did the molecular biology and analysis; A.B., S.F., K.L.A. and A.E.D. wrote the manuscript; all authors participated in discussions and revised the manuscript.

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SUPPORTING INFORMATION

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