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Can social partnerships influence the microbiome? Insights from ant farmers and their trophobiont mutualists

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Abstract

Mutualistic interactions with microbes have played a crucial role in the evolution and ecology of animal hosts. However, it is unclear what factors are most important in influencing particular host–microbe associations. While closely related animal species may have more similar microbiota than distantly related species due to phylogenetic contingencies, social partnerships with other organisms, such as those in which one animal farms another, may also influence an organism's symbiotic microbiome. We studied a mutualistic network of *Brachymyrmex* and *Lasius* ants farming several honeydew-producing *Prociphilus* aphids and *Rhizoecus* mealybugs to test whether the mutualistic microbiomes of these interacting insects are primarily correlated with their phylogeny or with their shared social partnerships. Our results confirm a phylogenetic signal in the microbiomes of aphid and mealybug trophobionts, with each species harbouring species-specific endosymbiont strains of *Buchnera* (aphids), *Tremblaya* and *Sodalis* (mealybugs), and *Serratia* (both mealybugs and aphids) despite being farmed by the same ants. This is likely explained by strict vertical transmission of trophobiont endosymbionts between generations. In contrast, our results show the ants' microbiome is possibly shaped by their social partnerships, with ants that farm the same trophobionts also sharing strains of sugar-processing Acetobacteraceae bacteria, known from other honeydew-feeding ants and which likely reside extracellularly in the ants' guts. These ant–microbe associations are arguably more "open" and subject to horizontal transmission or social transmission within ant colonies. These findings suggest that the role of social partnerships in shaping a host's symbiotic microbiome can be variable and is likely dependent on how the microbes are transmitted across generations.

KEYWORDS
ants, aphids, endosymbionts, mealybugs, microbiome, mutualism

1 | INTRODUCTION

Across the tree of life, animals form partnerships with microbes, allowing them to colonize new habitats (Dubilier, Bergin, & Lott, 2008; Mueller, Mikheyev, Hong, et al., 2011), utilize unique metabolic pathways (Pauli et al., 2014; Pinto-Tomas et al., 2009; Raychoudhury et al., 2013), increase protection against natural enemies (Kaltenpoth et al., 2014; Rangan et al., 2016) and even boost their reproductive output under certain ecological conditions (Montllor, Maxmen, & Purcell, 2002; Oliver, Degnan, Burke, & Moran,

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2010). Hosts show a huge range of dependencies on these microbial partners, with some becoming so tightly associated that the formerly independent partners evolve into a single integrated organism (Fisher, Henry, Cornwallis, Kiers, & West, 2017; Gruber-Vodicka et al., 2011; Husnik et al., 2013; Van Leuven, Meister, Simon, & McCutcheon, 2014; West, Fisher, Gardner, & Kiers, 2015). In these cases, physical, genomic and metabolic integration can drive partner interests to be closely aligned, leading to mutual dependence and loss of autonomy (Gruber-Vodicka et al., 2011; Husnik et al., 2013; Kiers & West, 2015; Moran, McCutcheon, & Nakabachi, 2008). Often, these partnerships form the basis of evolutionary innovation, with the microbes' services allowing their hosts to evolve traits and behaviours to tap into novel resources (Joy, 2013; Moran, 2007). This includes farming behaviour, in which hosts promote and control growth, reproduction and often dispersal of the symbiotic microbes or other organisms on which they rely for food (Brock, Douglas, Queller, & Strassmann, 2011; Chomicki & Renner, 2016; Hata & Kato, 2006; Ivens, 2015; Mueller, Gerardo, Aanen, Six, & Schultz, 2005; Pauli et al., 2014).

To successfully access new habitats and resources, however, partnerships need to be reliable (Chomicki, Janda, & Renner, 2017; Meseguer et al., 2017; Mueller, Mikheyev, Solomon, & Cooper, 2011; Simonsen, Dinnage, Barrett, Prober, & Thrall, 2017; Sudakaran, Salem, Kost, & Kaltenpoth, 2012). Especially in cases where microbes do not become physically integrated with their hosts, such as farming mutualisms, environmental context plays a major role in the availability and suitability of particular microbial consortiums (Kaltenpoth et al., 2014; McFall-Ngai, 2008; Poulsen, Fernandez-Marin, Currie, & Boomsma, 2009). While we know that context matters, it is unclear what factors are most important in driving the reliability of particular host–microbe associations. For example, phylogenetic relatedness and associated traits, such as transmission mode and compatibilities, are likely important, such that closely related species have more similar microbiota than distantly related species, (Anderson et al., 2012; Brucker & Bordenstein, 2013; Currie et al., 2003; Groussin et al., 2017; Henry, Maiden, Ferrari, & Godfray, 2015; Sanders et al., 2014). However, social partnerships of the host with other animals, such as intimate mutualistic farming relationships, are also key. It is well known that physical interactions with other organisms can influence an organism's symbiotic microbiome, sourcing and reinforcing specific microbial associations (Gonella et al., 2015; Lax et al., 2014; Macke, Tasiemski, Massol, Callens, & Decaestecker, 2017; Pringle & Moreau, 2017; Sintupachee, Milne, Poonchaisri, Baimai, & Kittayapong, 2006; Song et al., 2013; Stahlhut et al., 2010). Often the relative importance of these factors is difficult to untangle because it is challenging to find examples of distantly related species that share nearly identical social partnerships and physical environments.

The recent characterization of a set of overlapping farming mutualisms allows us to look more closely at the role of phylogeny versus social partnerships in determining host–microbe symbiotic associations. These mutualisms involve two types of honeydew-producing insects that are farmed by several ant species (Figure 1 and Figure S1 in Appendix S1). Subterranean *Lasius* and *Brachymyrmex* ants farm multiple species of aphids and mealybugs (their "trophobionts") often in the same underground root chambers for "milk" (i.e. honeydew) and, occasionally, "meat" for protein (Ellison, Gotelli, Farnsworth, & Alpert, 2012; Ivens, 2015; Ivens, Kronauer, Pen, Weissing, & Boomsma, 2012a; Pontin, 1978). It is likely that the ants depend on the aphid and mealybug honeydew as their sole sugar supply, as well as amino acids via honeydew and predation (Ivens, 2015; Ivens et al., 2012a; Pontin, 1978; Way, 1963). In return for these nutritional benefits, the ants actively protect the trophobionts against predators and provide hygienic services that are key to trophobiont survival (Bach, 1991; El-Ziady & Kennedy, 1956; Ivens, 2015; Paul, 1977; Way, 1963; Zwölfer, 1958).

These farmed mealybugs (Figure 1a) and aphids (Figure 1b) reside in nests of the same host ants and are therefore engaged in similar social partnerships, meaning they are farmed under nearly identical abiotic and biotic conditions (Figures 1c and Figure S1 in Appendix S1). In addition, both groups of organisms rely heavily on bacterial endosymbionts that facilitate their ability to feed off similar sugar-rich, but otherwise nutrient-poor, plant phloem sap (Douglas, 1998; Husnik & McCutcheon, 2016; Oliver et al., 2010). In aphids, the primary, obligate, endosymbiont is *Buchnera aphidicola* (Enterobacteriaceae), which is known to be vertically transmitted and to cospeciate with its insect hosts (Douglas, 1998; Jousselin, Desdevises, & D'acier, 2009; Nováková et al., 2013). *Buchnera* is often complemented by secondary, facultative, endosymbionts such as *Serratia symbiotica* and *Hamiltonella defensa* (both Enterobacteriaceae; Henry et al., 2013, 2015; Russell & Moran, 2006). Similarly, mealybugs harbour the primary, obligate and vertically transmitted endosymbiont *Candidatus* Tremblaya princeps (Betaproteobacteria, hereafter referred to as *Tremblaya*). *Tremblaya*, in turn, often carries another intracellular Gammaproteobacterium such as *Moranella* (von Dohlen, Kohler, Alsop, & McManus, 2001; Husnik & McCutcheon, 2016; Husnik et al., 2013; McCutcheon & von Dohlen, 2011). These Sternorrhyncha endosymbionts typically occur intracellularly. Extracellular (gut) bacteria have so far only been described for a couple of species and are thought to occur as opportunists or pathogens rather than specialised beneficial symbionts (Clark, Daniell, Wishart, Hubbard, & Karley, 2012; Grenier, Nardon, & Rahbé, 1994; Harada, Oyaizu, & Ishikawa, 1996; Sreerag, Jayaprakas, Ragesh, & Kumar, 2014).

Despite similarities in habitat, overall lifestyle, and their general dependence on endosymbionts, mealybugs and aphids, as well as their respective endosymbionts, are only distantly related in phylogenetic terms. This provides the unique opportunity to explore the influence of phylogeny versus social partnerships. Given their reliance on vertically transmitted endosymbionts, it is expected that the aphid and mealybug microbiota are strongly correlated with the trophobionts' respective phylogenies. However, their overlapping social partnerships could also contribute to shaping the trophobionts' microbiota, for example by increasing opportunities for horizontal transfer by feeding on the same host plants, as has previously been described for transfer of bacterial symbionts among other arthropod

FIGURE 1 *Lasius* ants tending (a) *Rhizoecus* mealybugs, (b) *Prociphilus* aphids and (c) a mixed live stock of mealybugs and aphids on the underside of rocks covering their nests in Millbrook, New York (photos: A.B.F. Ivens) [Colour figure can be viewed at wileyonlinelibrary.com]

taxa (Gonella et al., 2015; Sintupachee et al., 2006; Stahlhut et al., 2010). Because multiple species of both groups reside in the ants' nests, we can begin to tease apart these different factors.

Like the farmed trophobionts, the different species of ants face similar selection pressures from farming the same trophobionts in similar, and rather extreme habitats. For example, they likely harbour gut bacteria that allow them to live off the sugary honeydew of their trophobionts (Russell, Sanders, & Moreau, 2017). We might, therefore, expect the different ant species to show overlap in their core symbiotic microbiota. Alternatively, the symbiotic microbiota of the ants may be correlated with phylogeny, resulting in a unique microbiome in each ant species. Testing this hypothesis is challenging because the exact phylogenetic relationships of the *Lasius* ants have yet to be worked out in more detail. However, if we find different ant species sharing microbiota, then this would suggest a potentially larger role for the ants' social environment.

Here, we test whether the symbiotic microbiomes of interacting aphids, mealybugs and ants are exclusively a function of phylogeny, or whether the social farming partnerships also have some predictive power. To this end, we used a DNA barcoding approach on ants, aphids and mealybugs collected from nests of five North American ant species in Millbrook, New York. We asked (i) do the different trophobionts farmed by ants share a similar microbiome and (ii) do ants that farm the same trophobionts share a core symbiotic microbiome with each other, and even with their trophobionts?

2 | MATERIALS AND METHODS

2.1 | Species diversity and sample collection

The mutualistic network is composed of two groups of insects: the ant farmers in the genera *Lasius* (Formicinae) and *Brachymyrmex* (Formicinae) (five species, *L. claviger*, *L. umbratus*, and *L. nearcticus* [common], as well as *L. flavus* and *B. depilis* [rare]) (Ellison et al., 2012). The group of farmed trophobionts consists of two types of Sternorrhyncha: *Rhizoecus* (Pseudococcidae) mealybugs (unclassified species 1–5) and at least nine *Prociphilus* (Eriosomatinae) (*P. probosceus*, *P. fraxinifolii*, *P. longianus*, *P. erigeronensis* and cryptic species therein) and Pemphigini aphids (very rare, with only a single observation between 2013 and 2017; Figure S1 in Appendix S1, S. A. Schneider, personal communication). Generally, most aphid species are found in nests of all ant species at similar frequencies, given their differences in abundance. Mealybugs *R.* spp. 3 and 4 are also both found in nests of the three most common ants, but the rarer mealybug species *R.* spp. 1, 2 and 5 are restricted to the nests of the rarer ant species *L. flavus* and *B. depilis* (Figure S1 in Appendix S1).

Ants, aphids and mealybugs were collected from 143 unique ant nests between 2013 and 2016, mostly during the months of April—June (Table S1 in Appendix S2). The vast majority of sampling took place in Millbrook, New York, USA (41.767897, −73.750848) with the exception of two sets of samples from Annandale-on-Hudson, New York. All nests were marked, and their GPS coordinates logged for future resampling.

Immediately after collection, we stored most insect samples in absolute EtOH at −30°C awaiting further analysis. Of each trophobiont chamber (defined as a cluster of aphids or mealybugs on a rock surface [Figure 1] or tree root), one individual was stored in 70% EtOH, then heated for 2 minutes at 60°C and stored at room temperature in preparation for slide mounting for taxonomic purposes. Slide mounted specimens are stored at De Vrije Universiteit Amsterdam and are available upon request.

From each sample containing ants, we used one individual worker for species identification, combining *COI* barcoding with morphological identification of the subsequently pinned specimen (Ellison et al., 2012). Specimens stored at The Rockefeller University, New York, are available upon request.

Samples were then subjected to different barcoding approaches as follows: Illumina MiSeq microbiome profiling, Sanger sequencing of insect hosts and specific bacterial endosymbionts, or a combination of both (Table S1 in Appendix S2). Samples that repeatedly failed to amplify or that yielded mixed traces in Sanger sequencing, a sign of contamination, were excluded from further analysis. In total, this study includes data on 602 samples: 21 controls (1 positive, 20 negative), two honeydew samples, 129 ants, 340 aphids and 111 mealybugs (Table S1 in Appendix S2). In total, 446 samples were included for microbiome profiling in one or more of four MiSeq runs (MS1, MS2MS3 [consisting of two MiSeq lanes], MS4, MS5). These

samples included 20 controls, 129 ants (*Brachymyrmex depilis* $n = 2$, *Lasius flavus* $n = 3$, *L. claviger* $n = 55$, *L. nearcticus* $n = 14$ and *L. umbratus* $n = 55$), 76 mealybugs (*Rhizoecus* sp.1 $n = 8$, *Rhizoecus* sp.2 $n = 5$, *Rhizoecus* sp.3 $n = 42$, *Rhizoecus* sp.4 $n = 21$; *Rhizoecus* sp.5 was not included due to its rarity) and 219 aphids (*Prociphilus fraxinifolii* $n = 1$, *P. probosceus* $n = 8$, *P. erigeronensis* $n = 107$ and *P. longianus* $n = 103$; *P. caryae* and the unclassified Pemphigini aphids were not included due to rarity). All mealybug and aphid samples and one worker per ant nest included in the MiSeq runs were subsequently subjected to targeted Sanger sequencing of their mitochondrial DNA (mtDNA) and one or more specific endosymbionts, together with 156 ($n = 1$ negative control, $n = 35$ mealybugs and $n = 121$ aphids) additional samples. Only those ($n = 350$) that successfully yielded high-quality sequences from both insect mtDNA and endosymbiont DNA were included in Figures 2 and 3, and Figures S4–S15 in Appendix S1.

2.2 | DNA extraction

Insect and bacterial DNA for barcoding and microbiome profiling were extracted from whole aphid and mealybug specimens and from ant gasters only. All extractions were performed under the following sterile conditions, with the exception of 30 mealybugs and 114 aphid samples (Table S1 in Appendix S2). In the sterile protocol, all extractions were conducted under a flow hood using sterile consumables, to prevent contamination with environmental bacteria. Prior to extraction, all specimens were surface sterilized by immersing them individually for 30 s in 5% bleach, followed by 30 s in autoclaved 1 × PBS solution. Individual aphids, mealybugs and ant gasters (separated using autoclaved disposable razors) were then placed in sterile 1.5-ml tubes with 180 μl enzymatic lysis buffer (20 mM Tris-CL, PH 8.0, 2 mM Sodium EDTA, 1.2% Triton X100 and 20 mg/ml lysozyme) and a sterile stainless steel bead (5 mm), followed by homogenization in a QIAGEN TissueLyzer II for 3 min at 30 Hz. Next, we extracted insect and bacterial DNA simultaneously using the QIAGEN DNeasy Blood & Tissue kit, using the manufacturer's modified extraction protocol that includes pretreatment for gram-negative bacteria to prevent extraction bias against these bacteria. One negative control was included in each extraction batch. All samples included in MiSeq run MS1 were extracted using the manufacturer's unmodified protocol.

In MiSeq run MS4, two honeydew samples were included, as a pilot for screening for the bacterial presence in the honeydew that is transferred from aphids to ants. The samples were collected from *P. longianus* aphids (nest M172) and obtained by lightly touching the aphid's abdomen with a minute pin, shortly after the aphids had been collected from the field. This "milking" yielded ~0.5 μl honeydew per aphid, taken up in 0.5 μl Drummond Microcaps® microcapillary tubes (Sigma-Aldrich Co. LLC) and stored at −30°C. For DNA extraction, the samples were, after defrosting, each added to 100 μl lysis buffer and further processed using the protocol described above.

For 30 mealybugs and 114 aphids, mostly stemming from sampling events which yielded only a limited number of individuals and therefore stored in 70% EtOH, we used a modified extraction protocol. This "regular" protocol (Table S1 in Appendix S2) allowed for preservation of the sample for future slide mounting for morphological identification if necessary. In this protocol, samples were extracted under standard (nonsterile) conditions, without surface sterilization and without homogenization, to leave the specimen intact for slide mounting. Under these conditions, contamination with environmental bacteria cannot be excluded and, therefore, these samples were not included in MiSeq microbiome profiling and only used for targeted sequencing of specific endosymbionts.

2.3 | DNA sequencing

Trophobiont and ant mtDNA were amplified using standard primers targeting the *COI* region (mealybugs: primers "Jerry" CI-J-2183 & "Ben" C1-N-2568, 405 bp; aphids and ants: primers LCO1490 & HCO2198, 605 bp and 685 bp, respectively; Table S1 in Appendix S1; Brady, Gadau, & Ward, 2000; Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994; Simon et al., 1994). PCR products were subsequently purified and sequenced in both directions using Sanger sequencing, outsourced to Macrogen Inc. (New York, USA).

We characterized the insects' bacterial communities based on the *V3V4* (initial MiSeq run MS1) or *V4* region (subsequent MiSeq runs MS2MS3, MS4 and MS5) of the bacterial *16S rRNA* gene, using a protocol modified from Caporaso et al. (2011, 2012). Briefly, we amplified the target region using 2.5 μl aliquots of the extractions as DNA template with 0.5 μl of 10 μM primers (*V3V4*: 16S_V3V4F & 16S_V3V4R, *V4*: 16S_V4F & 16S_V4R, each extended with Illumina overhang adapter sequences for later multiplexing using an Illumina Nextera Index kit; Illumina, 2013; Kozich, Westcott, Baxter, Highlander, & Schloss, 2013), 12.5 μl of 2X KAPA HiFi Hotstart Readymix to a total PCR volume of 25 μl, ran at 55°C annealing temperature for 26 cycles (34 cycles in MS4). This amplification PCR was followed by an indexing PCR to allow for multiplexing of all samples in a single MiSeq run. These 50 μl PCR cocktails consisted of 5 μl PCR product taken from the amplification PCR as template and 5 μl of forward and reverse Nextera Index barcodes and were run at 55°C annealing temperature for 8 cycles.

Samples were then purified for final library construction. Because of their higher yield in bacterial DNA, samples in runs containing mostly mealybug and aphid PCR products (MS2MS3, MS5) were purified and normalized with two rounds of the SequalPrep™ Normalization Plate Kit (ThermoFisher Scientific), followed by a final concentration step using 0.6× Agencourt AMPure XP beads (Beckman Coulter) on pools of 24 samples. For samples in MiSeq runs MS1 and MS4, we pooled index PCR products of eight samples of similar PCR product concentration, estimated based on visual inspection of an electrophoresis gel and then used 0.6× Agencourt AMPure XP beads (Beckman Coulter) only for purification. After DNA quantitation of all purified product pools using Qubit™ (ThermoFisher Scientific), they were further pooled and normalized to equimolar concentrations for sequencing on an

FIGURE 2 Mealybugs and aphids host highly specific bacterial endosymbiont strains. (a) Rooted neighbour-joining (NJ) tree (outgroup removed for clarity) based on 405 bp *COI* mitochondrial gene fragments (n = 72) shows five species of *Rhizoecus* mealybugs, each represented by colour-coded clades (original *COI* NJ-tree with sample labels and outgroup in Figure S4 in Appendix S1). Each species harbours a specific strain of *Candidatus* Tremblaya (b) and *Sodalis* (c) endosymbionts, and *Rhizoecus* sp1. and sp2. also harbour specific *Serratia symbiotica* strains (d). (e) The rooted NJ-tree based on 601 bp *COI* mitochondrial gene fragments (n = 218) shows ten clusters (colour-coded) among the root aphids, which all harbour specific *Buchnera aphidicola* strains (d). In addition, most individuals of *Prociphilus longianus* A also harbour a *Serratia symbiotica* strain (d). The mitochondrial phylogeny (e) remains insufficiently resolved for the *P. erigeronensis* clade, and branch location within this clade has been rearranged to reflect haplotype-specificity of *Buchnera* strains within this clade. The original aphid *COI* NJ-tree with sample labels is given in Figure S10 in Appendix S1. Trees in (a) and (e) are based on Tamura–Nei distances (scale bars), only relative distance (not to-scale) of endosymbiont strains is given for clarity (b, c, d and f). For to-scale NJ-trees based on bacterial *16S rRNA*/*rpS15* and *rpS15-16S rRNA* intergenic spacer fragments with sample labels and, for the mealybug endosymbionts, outgroups, see Figures S6, S8, S12, S16 in Appendix S1 [Colour figure can be viewed at wileyonlinelibrary.com]

Illumina MiSeq sequencer using 250 bp (300 bp in MS1), pair-end reads at the Rockefeller University Genomics Resource Center.

To enable construction of higher resolution phylogenies of endosymbionts found with the MiSeq microbiome profiling, we designed novel primers targeting 700+ bp fragments of bacterial *16S rRNA* of *Buchnera aphidicola* and *Serratia symbiotica* in *Prociphilus* root aphids and *Sodalis* in *Rhizoecus* mealybugs. For *B. aphidicola*, we first used universal eubacterial *16S rRNA* primers (10F & 1507R, Munson et al., 1991) to obtain longer sequences based on which we could design primers specific to these strains. For *S. symbiotica* and *Sodalis*, we aligned the OTU sequences obtained during our MiSeq profiling to longer sequences of the five closest related sequences published in GENBANK. Sequences were then aligned, and primers designed using the respective functions in GENEIOUS® 9.1 (Biomatters Ltd.) and targeting regions for primer design that were highly similar across clades and would target regions of maximal possible length. This resulted in primers Buch_proF & Buch_proR for *B. aphidicola*, SerPro2F & SerPro2R for *S. symbiotica* and 16SSodF & 16SSodR for *Sodalis* (see Table S1 in Appendix S1 for primer details). For *Tremblaya*, we amplified fragments of *rpS15* and the adjacent *rpS15-16S rRNA* intergenic spacer using previously published primers C-16S-F & C-16S-R (Baumann, Thao, Hess, Johnson, & Baumann, 2002; Malausa et al., 2011).

We then amplified endosymbiont DNA under PCR conditions optimized for each specific primer pair (see Table S1 in Appendix S1 for PCR conditions). PCR products were then purified and sequenced using Sanger sequencing in both directions by Macrogen Inc. (New York, USA).

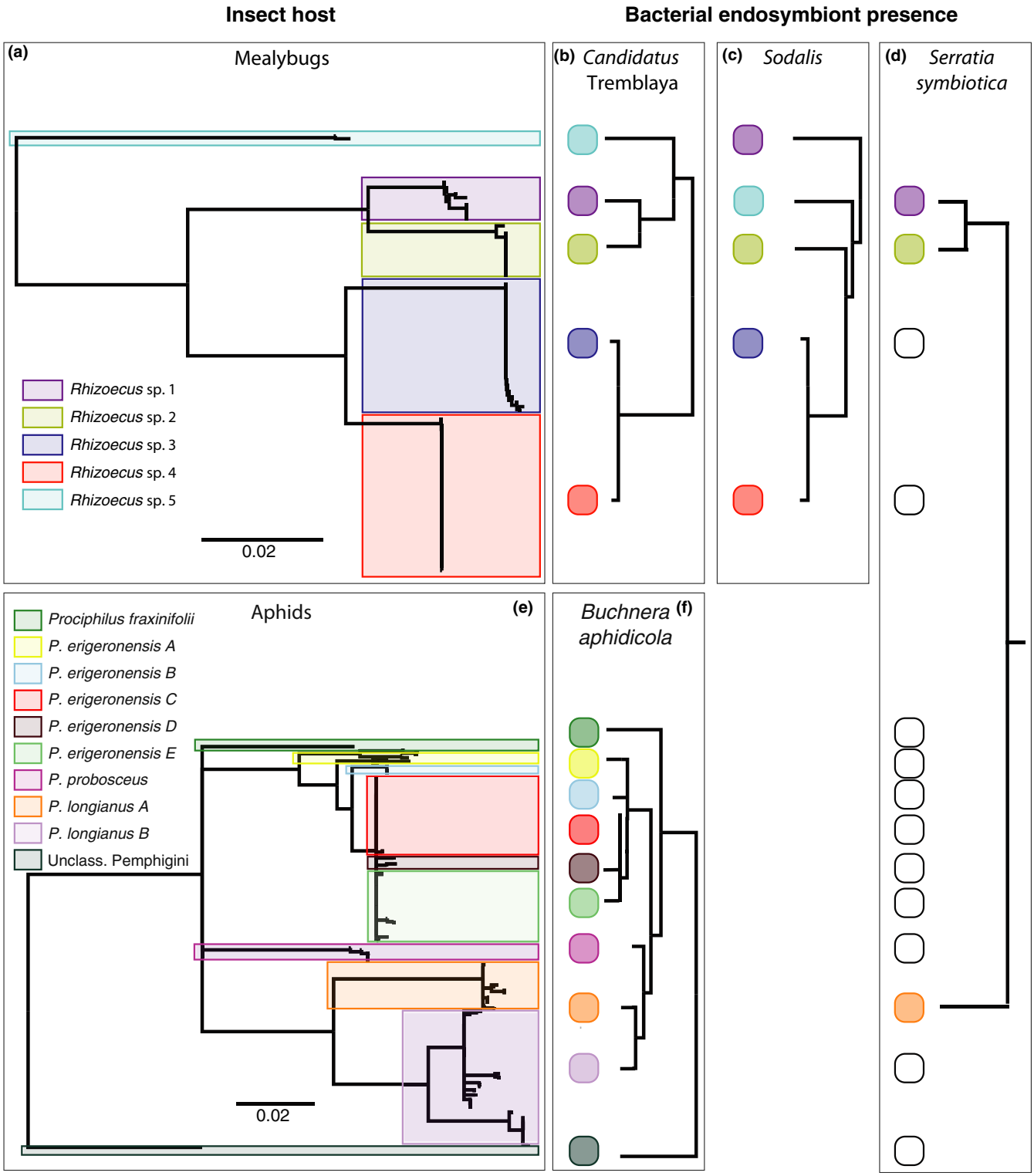
2.4 | Sequence processing and curation

All MiSeq results were analysed using the most recent release of the software package MOTHUR and reference database RIBOSOMAL DATABASE PROJECT (RDP) at the time of analysis (MS2M3: MOTHUR v1.35.1, RDP14; MS4: MOTHUR v1.36.0, RDP14; MS5: v1.39.1, RDP14 and MS1: MOTHUR v1.39.5, RDP16) (Cole et al., 2014; Schloss et al., 2009). We used a MOTHUR pipeline modified from (Kozich et al., 2013; Lukasik et al., 2017). The full, annotated script of the most recent analysis (MS1) can be found in Appendix S2. Briefly, pair-end reads were first joined into contigs. Then, all sequences were curated from sequencing errors by removing all sequences that were 50 bp shorter or longer than the expected product size, showed homopolymers longer than 8, did not well align to the targeted *16S rRNA* reference region or were estimated to be chimeras by UCHIME (Edgar, Haas, Clemente, Quince, & Knight, 2011). In addition, all singletons (or, in MS1, sequences with copy number <3) were removed, assuming these were sequencing artifacts. This curated set of sequences was then clustered into operational taxonomic units (OTUs) at the 97% level using the "average neighbour" algorithm as implemented in MOTHUR and identified taxonomically using the RDP reference. After final removal of all sequences derived from chloroplasts, mitochondria, Archaea or Eukaryota, this analysis resulted in one table per MiSeq run, with read counts per OTU for each multiplexed sample.

The purpose of our MiSeq screens was detection of endosymbiont presence in each of our screened host species. After the initial sequence curation, we therefore curated the data further to minimize false positive OTU calls for our samples, which could be caused by one of three technical issues: (i) the presence of contaminants in extraction and amplification reagents (Russell et al., 2017; Salter et al., 2014), (ii) sequencing errors resulting in novel OTUs and (iii) "leakage" between multiplexed samples due to sequencing errors in the Illumina overhang adapter sequences (see Appendix S2 for definitions and curation details for each issue). Lastly, samples were omitted as "failed" when their total read number was lower than 10% of the average read number for their type of sample (Figure S2 in Appendix S1).

All runs included blank negative controls, technical replicates and biological replicates (samples collected in the same sampling event). MS1 also included a *Cephalotes* ant worker as positive control (not shown). As additional quality check, we confirmed that in all runs, negative controls were mostly blank except for contaminant and "leakage" reads, and technical and biological replicates yielded very similar results. Lastly, we verified successful normalization by checking read number distribution per sample and removed the sample with least reads of each replicate pair from the final results (Figures S2 and S3 in Appendix S1).

All forward and reverse sequence pairs generated by the sequencing of insect mtDNA and targeted sequencing of specific endosymbionts were joined into contigs and then manually curated for sequencing errors, trimmed and aligned in GENEIOUS®10.2.2 (Biomatters Ltd.).



2.5 | Data analysis

Sequence consensus alignments generated in GENEIOUS® were then used to construct neighbour-joining distance trees, using the built-in tree builder function of GENEIOUS® with the Tamura–Nei distance model and 1,000 bootstrap replicates. In addition, RAXML trees were constructed using nucleotide model GTR gamma, the rapid-hill climbing algorithm and 100 bootstrap replicates using the RAXML 8.2.11 plug-in in GENEIOUS® (Stamatakis, 2014). Estimating the phylogenies using RAXML instead did not qualitatively alter the conclusions (see Figures S5, S7, S9, S11, S13, S15 in Appendix S1). Trees were edited for readability in FIGTREE v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Consensus sequences for each alignment as well as the most abundant genotype observed for each reported OTU in our MiSeq

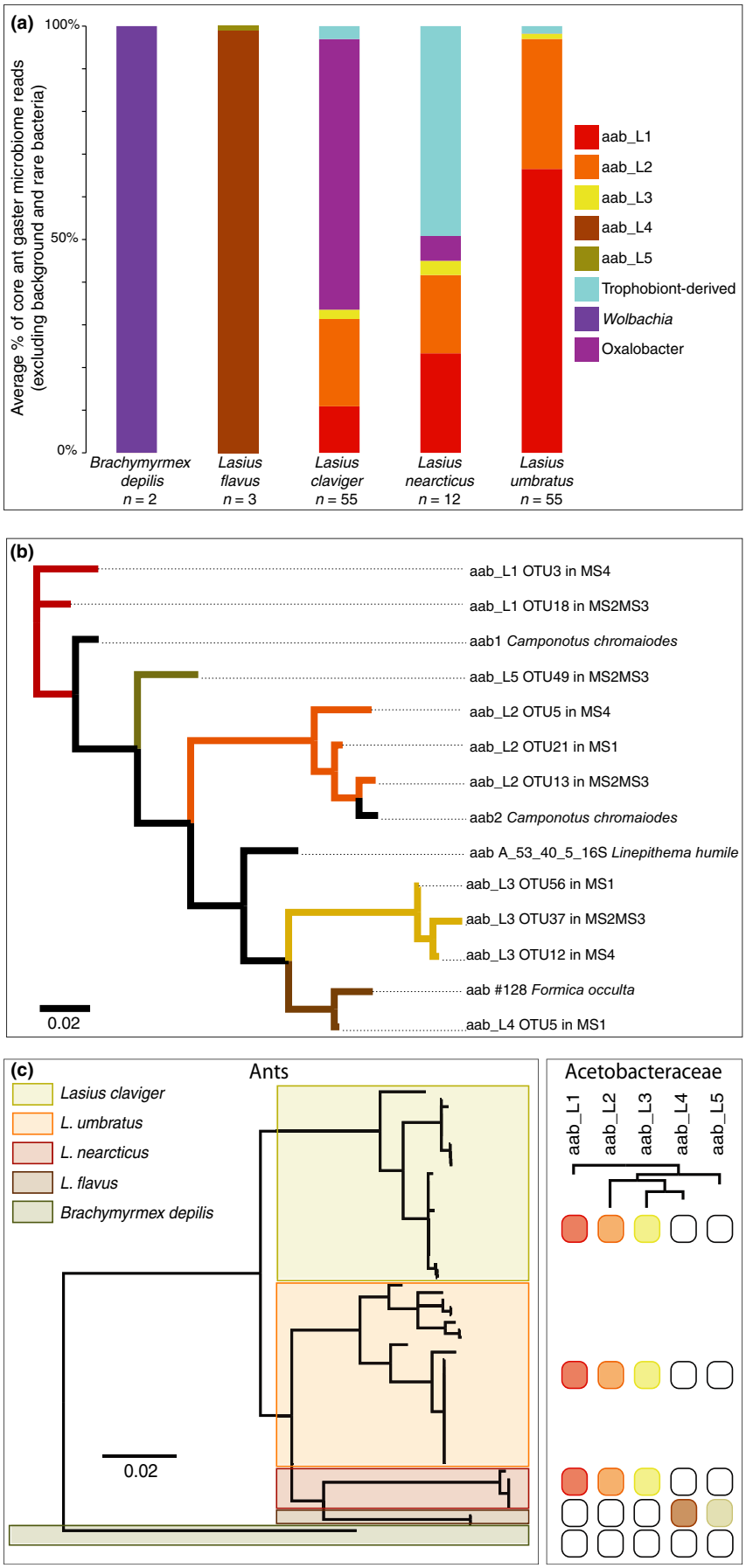


FIGURE 3 Microbiome of five species of trophobiont-farming ants. (a) Microbiome sequencing results from the gasters of the ants *Brachymyrmex depilis*, *Lasius flavus*, *L. claviger*, *L. nearcticus* and *L. umbratus*. Results are given as the average percentage of microbiome reads for each of the Acetobacteraceae strains (colour codes match those in Figure 3b), trophobiont-derived bacteria (in blue, including *Buchnera aphidicola*, *Serratia symbiotica* and unclassified Enterobacteriaceae) and potential core bacteria *Wolbachia* (purple) or *Oxalobacter* (pink) over all individuals per ant species. Only bacteria belonging to the core microbiome were included in this analysis. For complete reads of individual ant workers and separate OTUs, see Figures S2 and S3 in Appendix S1. (b) NJ-tree of Tamura–Nei distances (scale bar) between 293 bp and 1,426 bp bacterial *16S rRNA* fragments showing close phylogenetic relationship between the five clusters of Acetobacteraceae OTUs (here named strains aab_L1-L5, colour coded) found during three MiSeq runs (MS1, MS2MS3, MS4) in ants in this study, and those previously described from other ant guts (aab1 and aab2 from *Camponotus chromaiodes*, A_53_40_5_16S from *Linepithema humile*, Hu et al., 2017; #128 from *Formica occulta*, Russell et al., 2009). (c) Rooted NJ-tree (outgroup removed for clarity) based on Tamura–Nei distances (scale bar) of 685 bp *COI* mitochondrial gene fragments (n = 60) shows five species of ants (four *Lasius* and one *Brachymyrmex*), each represented by colour-coded clades (original *COI* NJ-tree with sample labels and outgroup in Figure S14 in Appendix S1). Four of these species harbour Acetobacteraceae strains aab_L1-aab_L5 [Colour figure can be viewed at wileyonlinelibrary.com]

analyses were matched against sequences previously deposited in NCBI GENBANK using their BLAST® search. The sequences with the maximum "total score" were reported as "closest match," provided the information stored under their accession number was still available. If not, we reported the next sequence listed. For each matching sequence, we reported its source, as well as the % of sequence identity to the queried sequence and its Expect (E)-value. The E-value gives the likelihood of the match having occurred by chance given the database size. The closer to 0, the more significant the match is.

We estimated host–microbe specificity using the H'_2 network specificity metric, adopted from ecological network theory (Blüthgen, Menzel, & Blüthgen, 2006; Ivens, von Beeren, Blüthgen, & Kronauer, 2016). This metric estimates the specificity of a bipartite network of two interacting species groups based on the number of times each species-to-species interaction is observed, taking into account the total number of possible interactions. H'_2 values can range from 0 (generalist network) to 1 (specialist network). We estimated H'_2 values for each host–bacteria bipartite network by compiling a host-by-bacterium matrix of the observation numbers of each possible combination. Network metrics were then calculated using the software R version 3.4.1 (R Development Core Team, 2011) and R package BIPARTITE 2.08 (Dormann, Gruber, & Fründ, 2008). We tested for statistical significance by comparing the observed H'_2 values to those of 10,000 randomized networks of equal size (using http://rxc.sys-bio.net; Blüthgen et al., 2006; Patefield, 1981).

3 | RESULTS

3.1 | Specificity of mealybug symbiotic microbiomes

First, we asked whether the symbiotic microbiomes of the five *Rhizoecus* species were unique and best explained by phylogeny, or whether their microbiomes were correlated with overlapping social partnerships with farming ants. Using Illumina MiSeq sequencing of *16S rRNA*, we first screened the four most common mealybug species for the presence of internal bacteria. This broad-scale screen showed that these *Rhizoecus* mealybugs harbour a simple microbiome, solely consisting of endosymbionts. We found three groups of bacterial operational taxonomic units (OTUs) at the 97% level: those belonging to *Tremblaya*, those of *Sodalis* (Gammaproteobacteria) and a single OTU of *Serratia symbiotica* (Figure S2, Tables S2 and S3 in Appendix S1). The closest matching bacterial sequences to longer fragments of these three OTUs (obtained with targeted sequencing, see below) currently included in the NCBI nucleotide database all stem from associates of other insects. The closest *Tremblaya* match is from the mealybug *Planococcus ficus* (at 90% identity, E-value = 0.0), *Sodalis* matches *Sodalis glossinidius* found in the Tsetse fly (95% identity, E-value = 0.0) and *Serratia symbiotica* matches a known secondary endosymbiont of aphids (99% identity, E-value = 0.0) (Figure S2, Tables S2 and S3 in Appendix S1; Chen, Wang, Chen, & Qiao, 2015; López-Madrigal, Latorre, Moya, & Gil, 2015; Matthew, Darby, Young, Hume, & Welburn, 2005).

The MiSeq results already showed variation at the ~300 bp resolution, with several different OTUs belonging to the same bacterial taxon. To examine our question further at the genotype level, we used custom-designed primers targeting ~700–1,000 bp *16S rRNA* fragments of *Sodalis* and *Serratia*, and *rpS15* and the *rpS15-16S rRNA* intergenic spacer in *Tremblaya*. The targeted sequencing showed that each of the five mealybug species harbours their own genotype (hereafter referred to as strain) of *Tremblaya* (n = 61 mealybugs) and *Sodalis* (n = 59 mealybugs; Figure 2b,c and Figures S6–S9 in Appendix S1). In addition to these well-known mealybug endosymbionts, mealybugs belonging to *Rhizoecus* sp. 1 and 2 also invariably harbour species-specific strains of *Serratia symbiotica* (Figure 2c, Figure S16 in Appendix S1). These data demonstrate that the endosymbiotic microbiomes of all five mealybug species are highly species-specific (mealybug-endosymbiont bipartite network specificity $H'_2 = 1$, $p < .001$). Thus, we found no evidence of a shared symbiotic microbiome among species, nor was there any clustering according to which ant species farmed the mealybugs. This suggests that indeed phylogeny rather than social partnership is correlated with microbiome diversity in mealybugs.

3.2 | Specificity of aphid symbiotic microbiomes

Second, we asked whether the symbiotic microbiome of the farmed aphids followed a similar pattern of species-specificity or whether social partnerships with the same farming ants played a role. Our aphid MiSeq screening covered the eight most common *Prociphilus* root aphids found in the ants' nests. The results showed that these aphids harbour a maximum of two endosymbionts: *Buchnera aphidicola*, the primary endosymbiont of all aphids and also, like the mealybugs, *Serratia symbiotica*, the secondary endosymbiont known from several other aphids (Henry et al., 2015). The closest matching sequences of these OTUs stem from other aphids: *Prociphilus ligustrifoliae* (*B. aphidicola*, 98% identity, E-value = 0.0) and *Stomaphis longirostris* (*S. symbiotica*, 99% identity, E-value = 0.0; Figure S2, Tables S2 and S3 in Appendix S1). The single, rare, sample of *Prociphilus fraxinifolii* harboured an unclassified Enterobacteriaceae, a potential endosymbiont as its closest match is a bacterial endosymbiont of the scale insect *Coelostomidia pilosa* (98% identity, E-value 1×10^{-137}; Figure S2 and Table S3 in Appendix S1). Lastly, we observed a handful of isolated cases of likely pathogenic infections with Microbacteriaceae and other actinomycetes (Figure S2 and Table S3 in Appendix S1).

Mapping the aphid microbiome to its phylogeny at strain level resolution was again achieved by targeted sequencing of *B. aphidicola* and *S. symbiotica* using custom-designed primers. The results show that each strain of *B. aphidicola* (n = 218) was limited to a single *COI* clade or species of aphid (Figures 2f and Figure S10–S13 in Appendix S1). Currently, the mitochondrial phylogeny of *P. erigeronensis* (Figure 2e) remains insufficiently resolved to assess clade-specificity of *Buchnera* strains. However, each aphid in this clade has one of three types of *Buchnera*, and importantly, each of the aphid mitochondrial haplotypes is always associated with the same

Buchnera haplotype, suggesting that these might, in fact, represent separate aphid lineages as well. More data are needed to verify this.

All included aphids were also screened for the presence of *S. symbiotica*, but only aphids in the clade *P. longianus* A tested positive for this endosymbiont, confirming the initial results of our MiSeq screen (Table S1 in Appendix S2, Figures S2 and S16 in Appendix S1). *Serratia symbiotica* was found in the majority of the aphids in this clade, but not in all of them (22 of 28 screened *P. longianus* A aphids) (Table S1 in Appendix S2, Figures 2d and Figures S2, S16 in Appendix S1).

Together, this suggests that also the aphid endosymbiotic microbiome is characterized by a strong phylogenetic signal, with one-to-one clade-specificity of endosymbiont genotypes (aphid endosymbiont bipartite network specificity $H'_2 = 1$, $p < .001$) and no evidence for an effect of social partnerships.

3.3 | Specificity of ant symbiotic microbiomes

We next looked within ants and asked whether the five trophobiont-farming subterranean ant species *Brachymyrmex depilis*, *Lasius flavus*, *L. claviger*, *L. nearcticus* and *L. umbratus* each harbour their own species-specific microbiome or whether farming of the same aphids and mealybugs results in a shared microbiome across ant species, or with their trophobionts. To address this question, we amplified bacteria from individual ant worker gasters of all species for subsequent *16S rRNA* MiSeq sequencing. Amplification rates varied widely across samples, both between species (Figure S2, S3 and S17 in Appendix S1) and within- and between ant colonies (Figure S3 in Appendix S1), suggesting that none of the species consistently contained symbiotic bacteria at significant levels. These generally low bacterial densities were confirmed visually by fluorescent microscopy on SYBR-green-stained ant gut extracts in a limited number of samples, following Sanders et al. (2017). Owing to these varying amplification rates, we were only able to sequence the microbiome of 129 of the 160 ant workers initially sampled, with the species *Lasius claviger* and *L. umbratus* best represented (Figure 3a).

The symbiotic microbiome sequencing revealed the presence of a relatively simple set of OTUs at the 97% level with significant presence in one or more individuals (Figures S2, S3 and Table S3 in Appendix S1). After contaminant removal, the remaining OTUs grouped into four categories: noncore OTUs, which were observed only occasionally, potential core OTUs (overall rare OTUs showing significant presence in certain species or ant colonies), core OTUs (making up the majority of reads across species and colonies) and lastly, trophobiont-derived bacteria.

The first and second categories of noncore and potential core OTUs occasionally infect individual workers or ant colonies. The noncore OTUs mostly included *Streptococcus*, *Diplorickettsia*, *Entomoplasmatales*, *Spiroplasma* and *Lactobacillus* (Figures S2, S3 and Table S3 in Appendix S1). All microbiome reads belonging to this category were excluded from further analysis. We also found significant presence of *Wolbachia* (exclusive to both included *B. depilis* workers) and *Oxalobacter* (four *L. claviger* workers from two nests), which were therefore deemed potential core OTUs (Figures 3a and Figures S2, S3 in Appendix S1).

The third category, the core OTUs, contains members of the sugar-processing Acetobacteraceae (aab). These bacteria were consistently found in *Lasius* ant gasters, in all three MiSeq runs that included ant samples (MS1, MS2MS3, MS4) (Figure 3). Phylogenetic comparison of all Acetobacteraceae OTUs found in the various runs shows that these OTUs cluster together into five groups, hereafter called strains aab_L1-L5 (Figure 3b). The closest NCBI GENBANK matches to these OTUs are to Acetobacteraceae found in other ants with sugar-rich diets. Inclusion of these sequences in our phylogeny reveals close relationship between Acetobacteraceae found across these different ants, with aab1 and aab2 from *Camponotus chromaiodes* matching closer than 97% to our observed strains aab_L1 and aab_L2, respectively, and thus being the same strains, under the 97% identity definition, as those found in *Lasius* ants (Figure 3b and Table S3 in Appendix S1; Brown & Wernegreen, 2016; Hu et al., 2017; Russell et al., 2009). Interestingly, the three most common ant species *L. claviger*, *L. nearcticus* and *L. umbratus* share the same three Acetobacteraceae strains (aab_L1, aab_L2, aab_L3), while the three analysed individuals of *L. flavus* exclusively harboured strains aab_L4 and aab_L5 (Figure 3a,c).

The fourth and final category of bacteria observed in ant gasters is that of potentially trophobiont-derived bacteria (Figure 3a, in blue). This category included the occasional observation of an unclassified Enterobacteriaceae, which matches closest to a secondary endosymbiont of giant scale insect *Coelostomidia pilosa* and was also found in the single sample of *P. fraxinifolii* we screened (Table S2 and Figure S2 in Appendix S1; Dhami, Buckley, Beggs, & Taylor, 2013). Whether this is indeed a trophobiont–ant-transferred endosymbiont merits further study.

The most notable two bacteria in this category are *Buchnera aphidicola* and *Serratia symbiotica*, which we found at significant levels in at least three ant workers. These were included in MiSeq run MS4, which did not include any aphid or mealybug samples, so the possibility of these reads being the result of "leakage" because of multiplexing can be ruled out. In addition, the presence of *S. symbiotica* was confirmed in these ant samples using our specific *Serratia* primers and the sequence matched those observed in *P. longianus* A samples most closely (Figure S16 and Table S3 in Appendix S1). Whether these are transient bacteria that the ants obtained from their trophobionts directly, either by preying on them or from transfer via honeydew, or whether these observations indicate colonization of ants by these bacteria is unknown. The two honeydew samples, collected from *P. longianus* B, included in our microbiome analysis did not show significant presence of any of the OTUs observed in the ants or their trophobionts (Figure S2 in Appendix S1). With *B. aphidicola* being an obligate endosymbiont of aphids, its occasional occurrence in ant workers is therefore best explained by recent consumption of aphids.

Thus, we observe indications of both microbiome species-specificity (the H'_2 of the ant-microbiome network being 0.421 ($p < .0001$),

which is mostly driven by the apparent exclusive association of the two rare ant species *B. depilis* and *L. flavus* with *Wolbachia* and two unique strains of Acetobacteraceae, respectively) and influence of social partnerships (e.g. three Acetobacteraceae strains shared among the other three *Lasius* species) on the microbiome composition of these five ant species (Figure 3a,c). Interestingly, the three *Lasius* species that share Acetobacteraceae strains have also been found to farm the same two mealybug species (*Rhizoecus* spp. 3 and 4), indicating more similar social partnerships among these species than between them and *B. depilis* and *L. flavus*, which have never been found to farm *Rhizoecus* spp. 3 and 4 (Figure S1 in Appendix S1). The small sample sizes of the latter two ant species, however, do not allow us to draw any firm conclusions regarding this potentially interesting correlation.

4 | DISCUSSION

We took advantage of a recently characterized subterranean symbiosis in which five species of ants farm multiple species of trophobionts (mealybugs and aphids) inside their nests, often simultaneously (Figure 1 and Figure S1 in Appendix S1). We first asked whether trophobionts that are farmed by similar hosts have similar microbiomes, or whether their microbiomes are correlated with their respective phylogenies. This latter hypothesis is expected given the ancient relationships between the trophobionts and their endosymbionts (Douglas, 1998; Husnik et al., 2013). Our results confirmed that in the trophobionts, phylogeny is the key correlate. Both mealybugs and aphids harbour phylosymbiotic microbiomes that are highly species-specific, composed of multiple endosymbionts that closely diverged with their insect hosts (Figure 2). None of the observed endosymbiont strains are shared among different groups or species of trophobionts. Despite the similarity in habitat and available food sources due to being farmed by overlapping ant species in similar, isolated underground environments, we found that the trophobionts' symbiotic microbiome is exclusively correlated with their phylogeny.

Next, we asked whether this is also the case for the farming ants. Do the symbiotic microbiomes of different ant species that farm the same trophobionts share core components with each other and their trophobionts, or is microbiome composition species-specific and correlated with ant phylogeny? We found that the three most common *Lasius* ant species, which all farm the same trophobionts, share a trio of sugar-processing bacteria (Figure 3), suggesting a potential role of social partnerships. In addition, a limited data set on two additional ant species, *Lasius flavus* and *Brachymyrmex depilis*, which farm a slightly different set of trophobionts (Figure S1 in Appendix S1), indicates that these may harbour bacteria exclusively associated with them, including sugar-processing bacteria. Despite the potential microbial transfer between the trophobionts and the ants via the trophobionts' honeydew, we also did not find any evidence for consistently shared microbiomes between ants and their trophobionts.

The high level of species-specificity in the trophobionts' symbiotic microbiome is most likely explained by two characteristics: (i) the highly specialized metabolic function of these microbes within their unique hosts and (ii) the predominant, if not exclusive, vertical mode of transmission of the endosymbionts.

The majority of the bacterial endosymbionts observed in the trophobionts are closely related to those previously found in related hosts. In these well-studied examples, the endosymbionts provide vital metabolic functions specific to their hosts (Douglas, 1998; Husnik & McCutcheon, 2016; Husnik et al., 2013; Oliver et al., 2010). For example, *Tremblaya* and *Sodalis* endosymbionts were found in all *Rhizoecus* mealybug species. *Tremblaya* is known to perform indispensable metabolic roles, such as production of essential amino acids (Husnik et al., 2013). In other systems, both *Tremblaya* and its mealybug host have undergone genome reduction over evolutionary time, making them codependent due to complementary genome function (Husnik et al., 2013; McCutcheon & von Dohlen, 2011). This mealybug–*Tremblaya* partnership, in fact, can constitute a hierarchical symbiosis, in which the *Tremblaya* endosymbiont contains its own endosymbiotic Gammaproteobacterium. The best-studied example is *Morenella*, which fulfils several essential genome functions of its own (von Dohlen et al., 2001; Husnik et al., 2013; McCutcheon & von Dohlen, 2011). Over evolutionary time, these Gammaproteobacteria have repeatedly been replaced, also with *Sodalis*-like bacteria (Husnik & McCutcheon, 2016). The trio of perfectly matching mealybug, *Tremblaya*, and *Sodalis* phylogenies reported here is therefore in line with a nested symbiosis of *Rhizoecus* mealybugs and *Tremblaya* endosymbionts, which in turn contain *Sodalis* endosymbionts. While we would need to confirm this physical nestedness using for example fluorescent in situ hybridization (FISH) (von Dohlen et al., 2001), our results are in accordance with a long history of co-evolution, with little potential for colonization and sharing of endosymbionts.

Similarly, all root aphids in the system contain species-specific genotypes of *Buchnera aphidicola*, a well-studied, obligate primary endosymbiont of aphids, well-known for its vital metabolic functions, most importantly in the production of essential amino acids (Douglas, 1998). The long-term symbiosis between *Buchnera* and its aphid hosts is further characterized by the evolution of a specific organ inhabited by *Buchnera*, the bacteriocyte (Douglas, 1998; Jousselin et al., 2009). Like in the mealybugs, this integrated, physically "closed" symbiosis leaves little room for frequent exchange or colonization by shared symbionts.

The high congruence between host phylogenies, based on mitochondrial *COI* sequences, and the symbiont *16S rRNA* phylogenies, is likely the result of transmission mode. Vertical transmission of the endosymbionts from mother to daughter trophobionts drives the evolution of species-specific high-dependency symbiosis (Fisher et al., 2017). Vertical transmission has long been established as the sole transmission mode in both the mealybug–*Tremblaya* complex and for *Buchnera* in aphids (von Dohlen et al., 2001; Douglas, 1998; Jousselin et al., 2009).

In addition to strong endosymbiont-specificity at the mitochondrial level, species-specificity even at the host nuclear genetic level

is likely aided by predominant, if not exclusive, asexual reproduction in all focal species of mealybugs and aphids. This is because the absence of a sexual cycle will reduce nuclear genetic recombination levels and, therefore, preclude novel endosymbiont-nuclear gene combinations. During our 5 years of field work (2013–2017), trophobiont males were never observed (A. B. F. Ivens, personal observation). Mealybugs often reproduce exclusively clonally, with males being only rarely observed or absent (Ross & Shuker, 2009). Likewise, aphids are cyclic parthenogens, which commonly become fully parthenogenetic when they forego their annual sexual cycle (Ivens, Kronauer, Pen, Weissing, & Boomsma, 2012b; Simon, Rispe, & Sunnucks, 2002). The subterranean lifestyle provides a relatively stable environment, and being associated with ants may have further facilitated the loss of the sexual cycle in aphids (Ivens, 2015; Ivens et al., 2012b; Law & Lewis, 1983; Wulff, 1985).

Surprisingly, we observed an additional species-specific endosymbiont, *Serratia*, to be present in some clades of both aphids and mealybugs. While *Serratia symbiotica* is a known secondary, facultative, endosymbiont of other aphids, most notably of Lachnids (Burke, Normark, Favret, & Moran, 2009; Henry et al., 2015; Russell, Latorre, Sabater-Muñoz, Moya, & Moran, 2003), our study is the first to observe *S. symbiotica* in mealybugs. The three observed *Serratia* strains cluster together, away from *Serratia* known from five other aphid hosts (Figure S16 in Appendix S1). Importantly, our finding of all individuals of *Rhizoecus* spp. 1 and 2 invariably harbouring species-specific *Serratia* genotypes indicates a potential long-term association of these mealybugs with this third endosymbiont, meriting further exploration.

In some aphids, this bacterium has become an obligate endosymbiont, providing metabolic functions complementary to the resident *Buchnera* strain and potentially being on its way to replacing *Buchnera* (Burke & Moran, 2011; Meseguer et al., 2017; Pérez-Brocal et al., 2006). *Serratia* has been shown to confer several ecological advantages such as heat-stress tolerance and nutritional benefits (Koga, Tsuchida, & Fukatsu, 2003; Montllor et al., 2002; Russell & Moran, 2006). It is overrepresented in monophagous aphid species and in those feeding on specific *Acer* trees (Henry et al., 2015). In our study, we found *Serratia* to be exclusively associated with *Prociphilus longianus* clade A aphids, although not all individual aphids belonging to this clade contained *Serratia*. Field observations and preliminary root barcoding results suggest that *Prociphilus longianus* aphids primarily feed on *Quercus*, possibly also *Acer* (A. B. F. Ivens, personal observation). It is conceivable that in this species, *Serratia* confers an ecological benefit in this feeding niche, but more work is needed to clarify the exact root feeding niches of these organisms.

One potential explanation for the presence of *Serratia* in both aphids and mealybugs is that it can be horizontally transmitted, even via host plants (Burke et al., 2009; Henry et al., 2013, 2015; Oliver et al., 2010). With aphids and mealybugs frequently sharing host ant nests, and even nest chambers and, consequently, feeding niches, *Serratia* could then even be transmitted across taxon boundaries (Figure 1c). However, the strong mitochondrial clade-specificity of *Serratia* in both aphids and mealybugs (Figure 2 and Figure S16 in Appendix S1) suggests that if there ever was horizontal transfer, it must have been historical, and since that time, there has been a transition to vertical transmission and strict host-specificity. Such historic transfer has been shown before in whiteflies harbouring aphid endosymbiont-like, but diverged, symbionts (Darby, Birkle, Turner, & Douglas, 2001). In a recently published survey of endosymbionts in above-ground aphids, there was a trend towards a higher *Serratia* prevalence in ant-farmed aphid species coinciding with a marked absence of two other facultative aphid endosymbionts, *Hamiltonella defensa* and *Regiella insecticola* (Henry et al., 2015). This pattern can potentially be explained by *Serratia* conferring nutritional benefits complementary to the protective benefits provided by farming ants, while the other endosymbionts confer benefits redundant with ant protection, such as protection against parasitoids (Henry et al., 2015). While their natural enemies remain unknown at this point, the matching results of our survey indicate that the same mechanisms may be at play in root aphids, but this would need to be further verified by broader screening of both ant-tended and nonant-tended root aphids.

In contrast to these highly specific and somewhat diverse microbial associations in the trophobionts, we find little diversity and specificity in the microbiomes of the ant farmers. Instead, the microbiomes of the focal ant species *Brachymyrmex depilis*, *Lasius flavus*, *L. claviger*, *L. nearcticus* and *L. umbratus* are simple, with limited diversity. Many of our screened ant workers lacked detectable levels of bacteria, and overall, we only observed seven core OTUs (Figure 3). The observed low bacterial density and diversity in *Lasius* ants were expected given past work on other ant microbiomes, which display similar patterns (Hu et al., 2017; Moreau & Rubin, 2017; Ramalho, Bueno, & Moreau, 2017; Russell et al., 2009, 2017). While there are exceptions representing a number of highly specialized ant–microbe associations in a handful of specific ant clades (Anderson et al., 2012; Lukasik et al., 2017; Russell et al., 2009, 2017; Sanders et al., 2014), overall this low density and diversity is in line with previous work (Sanders et al., 2017).

We identified the most prevalent bacteria in the surveyed *Lasius* species as belonging to the Acetobacteraceae, a family of acetic acid-producing bacteria that thrive in sugar-rich environments (Figure 3). These bacteria are generally found in hosts with sugar-rich diets, such as the honeydew on which these *Lasius* ants predominantly feed (Ano, Toyama, Adachi, & Matsushita, 2008). Indeed, the observed OTUs are closely related to those previously published from the guts of other ants feeding on carbohydrate-rich diets, including honeydew-feeding *Camponotus* carpenter ants, *Formica* wood ants and *Linepithema* Argentine ants (Brown & Wernegreen, 2016; Hu et al., 2017; Russell et al., 2009) (Figure 3, Tables S2 and S3 in Appendix S1). In addition, these bacteria are related to *Asaia* bacteria, recently found in *Pseudomyrmex* and *Tetraponera* ants, which both feed on another sugar-rich diet of extra floral nectar (Kautz, Rubin, & Moreau, 2013; Samaddar et al., 2011). Their prevalence among these specific ants, in combination with shown experimental increase in bacterial density with sugar-rich diets, suggests that Acetobacteraceae aid in the ants' digestion of sugary honeydew,

although more functional work is needed (Hu et al., 2017). In addition to sugar-processing abilities, *Asaia* found in *Tetraponera* ants has been suggested to play a role in nitrogen fixation (Samaddar et al., 2011). This additional functionality could also be present in their relatives colonizing the *Lasius* ants in this study.

The ant–Acetobacteraceae symbiotic relationship has previously been suggested to be quite old and specialized (Brown & Wernegreen, 2016). *Camponotus* strains aab1 and aab2 were shown to be members of a monophyletic Acetobacteraceae clade that is highly specific to ants, in particular the subfamily Formicinae, to which also *Lasius* ants belong.

Our observation of these same strains in *Lasius* ants suggests a more wide-spread distribution than hitherto thought, spanning across several genera within the Formicinae.

We did not observe any Acetobacteraceae in the two screened workers of *Brachymyrmex depilis*. Instead, both workers, collected from the same nest, showed exclusive colonization by *Wolbachia*, constituting the only two observations of this bacterium in our study. *Wolbachia* is a common symbiont of ants (Brown & Wernegreen, 2016; Kautz et al., 2013; Russell, 2012), and its potential roles include manipulation of the ants' reproduction as well as beneficial roles such as nutritional aid and protection (Russell et al., 2017). With *Wolbachia*'s prior record as an ant symbiont with a large potential to impact the ecology and evolution of its hosts, a possible *Brachymyrmex*–*Wolbachia* relationship merits further study. In addition, we cannot exclude the possibility that the presence of *Wolbachia* in relatively high abundance (making up around 50% of the sequencing reads, Figure S3 in Appendix S1) in these two ant workers precluded detection of any Acetobacteraceae present at lower abundance. Future experiments using Acetobacteraceae strain-specific primers to screen larger sample numbers of each of the five focal ant species will help resolve this issue. A second observed potential core bacterium was *Oxalobacter*, with significant presence in workers of two *L. claviger* colonies (Figure S3 in Appendix S1). The function of *Oxalobacter* in ants remains unknown, but it has been attributed a beneficial role in humans (Barnett, Nazzal, Goldfarb, & Blaser, 2016).

Contrasting previous findings of strong phylogenetic signal of ant–microbe associations at higher taxonomic levels (Anderson et al., 2012; Russell et al., 2009, 2017), our survey shows only weak phylogenetic signal of microbial occurrence in these trophobiont-farming ants. Although we find differences at the genus level, with the limited sample of two *Brachymyrmex* workers exclusively containing *Wolbachia*, all ants surveyed within the genus *Lasius* harbour Acetobacteraceae, with three of these strains being shared by several ant species. The only species-specific microbiome observed within *Lasius* is that of the three screened *L. flavus* ant workers containing two additional Acetobacteraceae strains, while the other species share their three strains. Because of the rarity of this species, our sample size was limited. This finding needs further confirmation using the approach of targeted Acetobacteraceae sequencing described above, but it points to a potentially interesting association. Rather than a phylogenetic signal, this distribution pattern may be best explained by the ants' social partnerships. The three most common *Lasius* species not only share three strains of Acetobacteraceae but also exclusively farm *Rhizoecus* spp. 3 and 4 mealybugs, while *L. flavus* and *Brachymyrex* have only been found associated with *R.* spp. 1, 2 and 5 (Figure S1 in Appendix S1). These differences in food source resulting from different honeydew produced by different mealybugs could then possibly be driving maintenance of different symbiotic microbiomes.

In summary, our results confirm a predominant correlation with phylogeny over one with social partnerships in the trophobiont microbiomes and, in contrast, point to a potentially important effect of social partnerships in the formation of the *Lasius* ant microbiomes. These findings are both supported by recent work on the microbial communities of a tropical ant–plant–hemipteran symbiosis in which two different ant species farm the same two scale insect trophobiont species and house them in domatia of the same species of ant–plant (Pringle & Moreau, 2017). The effect of social partnerships on trophobiont microbiomes was limited, with the scale insects both harbouring their own microbiome. In contrast to our findings, however, the microbiomes of both ant species were very species-specific (Pringle & Moreau, 2017). This is likely because one of the two focal species belongs to the genus *Cephalotes*, which is known for the strong phylogenetic signal of its microbiome (Sanders et al., 2014). In contrast, the microbiome of the other ant species, *Azteca*, seems to be more transient and displays lower bacterial abundances. This is more in line with the microbiomes observed in the *Lasius* and *Brachymyrmex* ants in our study (Pringle & Moreau, 2017; Sanders et al., 2014).

These differences in phylogeny-correlated microbiomes versus social partnership-correlated microbiomes have been described as "closed" versus "open" symbioses. In "closed" symbioses, the start of an association coincides with birth (e.g. vertical transmission of a symbiont). In "open" symbiosis, on the other hand, microbial colonization and birth are decoupled (e.g. horizontal transmission) (Douglas, 2015). In open cases, the hosts may be colonized by novel microbial partners over their life time, providing scope for a large influence of social partnerships, but perhaps less strict dependency (Fisher et al., 2017). Yet, even in a system that is mostly marked by closed symbioses, Pringle and Moreau (2017) also observed "microbial leakage," where bacterial OTUs overlap between samples originating from the different organisms. This "leakage" can either be explained by a technical issue, namely mistakenly assigning sequencing reads in multiplexed MiSeq runs which include samples stemming from different organisms, or alternatively by ecological microbial transfer in species interactions among different taxa (Pringle & Moreau, 2017). Our employed stringent sequence curation protocol (Appendix S2) was designed to prevent the former technical issue in all multiplexed runs. We could therefore only observe true ant–trophobiont overlap in microbiomes in the single "ant-only" run MS4, and likely because of this reason, we observed it to a very limited extent only. These observations may indeed be explained by ecological microbial transfer among the focal organisms. For example, transfer can happen when ants prey on their trophobionts. This mode of transfer likely resulted in the remnants of trophobiont-derived

Buchnera and *Serratia* we observed in several ant workers (Figure 3, Figures S2 and S3 in Appendix S1). Likewise, previous observations of *Serratia* in honeydew-feeding *Formica* ants were best explained by the ants' predation on their aphid livestock (Sirviö & Pamilo, 2010). Alternatively, transfer could occur when ants consume trophobiont-produced honeydew that contains bacteria (Leroy et al., 2011). We did, however, not detect any bacteria in the two screened honeydew samples (Figure S2 in Appendix S1). The predominant absence of trophobiont-derived symbionts in the screened ants matches previous findings in which a suite of trophobiont (lycaenids, Sternorrhyncha) symbionts was found to be absent from ants (Russell et al., 2012). Regardless of the mode of transfer, merely observing bacterial presence by *16S rRNA* sequencing does not distinguish passive transfer of bacteria through consumption from active colonization of the host ant's gut by bacteria. Future studies employing dietary manipulation of ants in combination with FISH microscopy could aid distinguishing between these two possibilities. Interestingly, Pringle and Moreau (2017) observed most OTU overlap between one species of scale insects and the plant domatia it was housed in, suggesting that the physical environment can play a role in microbiome formation. In future, we will include samples from trophobiont chamber walls in *Lasius* nests to further test this idea.

This study suggests that the role of social partnerships in shaping a host's symbiotic microbiome is variable and likely dependent on whether the partnership is a "closed" symbiosis, with strict vertical transmission (Douglas, 2015; Pringle & Moreau, 2017). This is in line with previous studies finding a large effect of social partnerships on microbiome formation in the case of "open" symbioses where microbes are acquired from the environment (Lax et al., 2014; Pringle & Moreau, 2017; Song et al., 2013). The species-specific patterns we observed may also be the result of historic events derived from shared social partnerships. Because in ant–trophobiont relationships, both animal hosts are nutritionally so intricately connected, it is potentially the microbiome that sets the boundaries of viable mutualisms, with only those ant–trophobiont partnerships persisting that harbour complementary microbiomes. Future studies, including those employing experimentally manipulated ant and trophobiont microbiomes, will be able to shed further light on the emerging question of whether it is, in fact, the microbes that indirectly govern maintenance of the higher level animal-animal social partnerships in which they play such essential nutritional roles.

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DATA ACCESSIBILITY

All DNA sequences of OTUs and strains as reported in Table S3 in Appendix S1 are accessible via GENBANK, and accession numbers are provided in the respective table.

Unique microbial DNA sequences from the NJ-trees in Figure 2 are accessible via GENBANK, and accession numbers are provided in Table S1 in Appendix S2 with representative samples.

Complete MiSeq count tables, taxonomies, fasta sequence files and MOTHUR scripts are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.t2q12.

All curated sequences, sequence alignments and original NJ and RaxML trees from Geneious are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.t2q12.

Network tables and R scripts for the network specificity analyses of the aphid–*Buchnera* and ant–Acetobacteraceae networks are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.t2q12.

All raw MiSeq sequencing reads are accessible via the Sequence Read Archive at https://www.ncbi.nlm.nih.gov/sra/SRP128691.

AUTHOR CONTRIBUTIONS

A.B.F.I. and D.J.C.K. designed the study, with input from E.T.K.; A.B.F.I. and A.G. collected field samples and performed laboratory analyses; A.B.F.I. performed data analysis; A.B.F.I. wrote the manuscript with support from E.T.K. and D.J.C.K.; D.J.C.K. supervised the project.

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