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Journal

Applied and Environmental Microbiology, 87(7)

ISSN

0099-2240

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Publication Date

2021-03-11

DOI

10.1128/aem.02668-20

Peer reviewed

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MICROBIAL ECOLOGY

Tick Microbiomes in Neotropical Forest Fragments Are Best Explained by Tick-Associated and Environmental Factors Rather than Host Blood Source

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ABSTRACT The composition of tick microbiomes varies both within and among tick species. Whether this variation is intrinsic (related to tick characteristics) or extrinsic (related to vertebrate host and habitat) is poorly understood but important, as microbiota can influence the reproductive success and vector competence of ticks. We aimed to uncover what intrinsic and extrinsic factors best explain the microbial composition and taxon richness of 11 species of neotropical ticks collected from eight species of small mammals in 18 forest fragments across central Panama. Microbial richness varied among tick species, life stages, and collection sites but was not related to host blood source. Microbiome composition was best explained by tick life stage, with bacterial assemblages of larvae being a subset of those of nymphs. Collection site explained most of the bacterial taxa with differential abundance across intrinsic and extrinsic factors. *Francisella* and *Rickettsia* were highly prevalent, but their proportional abundance differed greatly among tick species, and we found both positive and negative cooccurrence between members of these two genera. Other tick endosymbionts (e.g., *Coxiella* and *Rickettsiella*) were associated with specific tick species. In addition, we detected *Anaplasma* and *Bartonella* in several tick species. Our results indicate that the microbial composition and richness of neotropical ticks are principally related to intrinsic factors (tick species and life stage) and collection site. Taken together, our analysis informs how tick microbiomes are structured and can help anchor our understanding of tick microbiomes from tropical environments more broadly.

IMPORTANCE Blood-feeding arthropod microbiomes often play important roles in disease transmission, yet the factors that structure tick microbial communities in the Neotropics are unknown. Utilizing ticks collected from live animals in neotropical forest fragments, this study teases apart the contributions of intrinsic and extrinsic tick-associated factors on tick microbial composition as well as which specific microbes contribute to differences across tick species, tick life stages, the mammals they fed on, and the locations from where they were sampled. Furthermore, this study provides revelations of how notable tick-associated bacterial genera are interacting with other tick-associated microbes as well as the forest animals they encounter.

KEYWORDS anaplasma, endosymbionts, environmental microbiology, Lyme disease, microbiome, neotropical, Panama, *Rickettsia*, tick, tick-borne pathogens

Ticks transmit more pathogenic microorganisms than any other arthropod vector taxon and, therefore, are of considerable human and veterinary medical concern (1, 2). Expanding geographic ranges of important tick vectors and increasing incidences of tick-borne diseases have fueled a growing body of research into the ecology of

Citation Kueneman JG, Esser HJ, Weiss SJ, Jansen PA, Foley JE. 2021. Tick microbiomes in neotropical forest fragments are best explained by tick-associated and environmental factors rather than host blood source. Appl Environ Microbiol 87:e02668-20. https://doi.org/10.1128/AEM.02668-20.

Editor Eric V. Stabb, University of Illinois at Chicago

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Received 29 October 2020
Accepted 13 January 2021
Accepted manuscript posted online 29 January 2021
Published 11 March 2021

tick-borne pathogens (3–8). However, pathogens causing tick-borne diseases represent only a small portion of the microbial flora found in ticks (9). A myriad of other microorganisms can have symbiotic, commensal, or parasitic relationships with their tick host, and we are only beginning to understand the roles that these nonpathogenic microorganisms play in tick development, reproductive fitness, vector competence, and pathogen transmission (10–13). The first studies investigating microbes of ticks are now a century old, but a new period of rapid discovery is underway in part because of recent advancements in high-throughput metagenomic sequencing (14–17).

One key question that has emerged is what factors determine the composition of tick microbiomes. Given the potential role of the microbiome in pathogen transmission processes, such knowledge is highly relevant for developing novel strategies to control ticks and tick-borne diseases (9, 18). For example, *Rickettsia* endosymbionts may hamper the colonization of tick-borne pathogens (13, 19–21) and even displace pathogen-infected ticks in the field (22). However, understanding what factors drive microbiome composition is also of evolutionary and ecological importance, as it highlights coevolutionary processes acting between ticks and their microbes (9). An important step in this direction is to characterize the microbial communities in different species of ticks and to test whether the composition and richness of tick microbiomes are best explained by tick-specific factors (e.g., tick species and life stage) or factors extrinsic to the tick (e.g., host and environmental factors).

Most tick species have a three-host life cycle, in which they take blood meals from a different vertebrate host during each host-feeding life stage (23); small mammals typically are hosts for immature ticks and are reservoirs for numerous tick-borne pathogens (8, 24). These ticks may acquire microorganisms via three different pathways. First, direct contact with vertebrate skin, fur, and blood provides ample opportunity for ticks to become inoculated with a variety of microorganisms, including pathogens (25). Second, spending most of their life off-host, ticks may acquire microorganisms from their environment (e.g., soil and leaf litter) (26, 27). Third, some microorganisms are vertically transmitted from female ticks to their offspring (18, 26) or horizontally from male to female ticks during mating (28, 29). Hence, factors related to the arthropod vector, vertebrate host, and environment all are likely involved in shaping the tick microbiome, but their relative contributions remain poorly studied (30).

Studies have indeed found that tick species (30–35), life stage (17, 27, 31, 36), sex (33–40), host blood-feeding (17, 39–43), and geographic location (35–39) all can affect the microbial composition of ticks. However, only one study, conducted on two temperate tick species, has simultaneously considered tick, host, and environmental factors (30), finding that the bacterial community composition of ticks was influenced by tick species and life stage but not the vertebrate host or environmental conditions. More data sets are needed from diverse locations and tick species to corroborate these findings. Moreover, it remains unclear to what degree microbial communities are shared between closely and more distantly related tick species from the same region, as microbiome surveys typically consider only one or two species of medically important ticks (but see references 31 and 33). Further, past studies were largely restricted to temperate regions, whereas the species diversity and economic impact of ticks is greatest in subtropical and tropical regions (1, 44).

Our study entailed a comprehensive survey of tick microbiomes collected directly from small mammals across a series of tropical moist forest fragments in Central Panama. In this area, small mammals such as rodents and opossums are suspected reservoir hosts for a range of tick-borne pathogens (8, 45, 46) and carry many tick species (47). Here, we compared the microbial community composition and richness among 11 tick species collected from eight species of small mammals across 18 tropical forest fragments. Specifically, we assessed the degree to which tick microbiomes varied among tick species and life stages and were influenced by host blood source and collection site, thereby addressing tick, host, and environmental predictors of tick microbial composition and richness. We also examined the presence of bacterial genera that

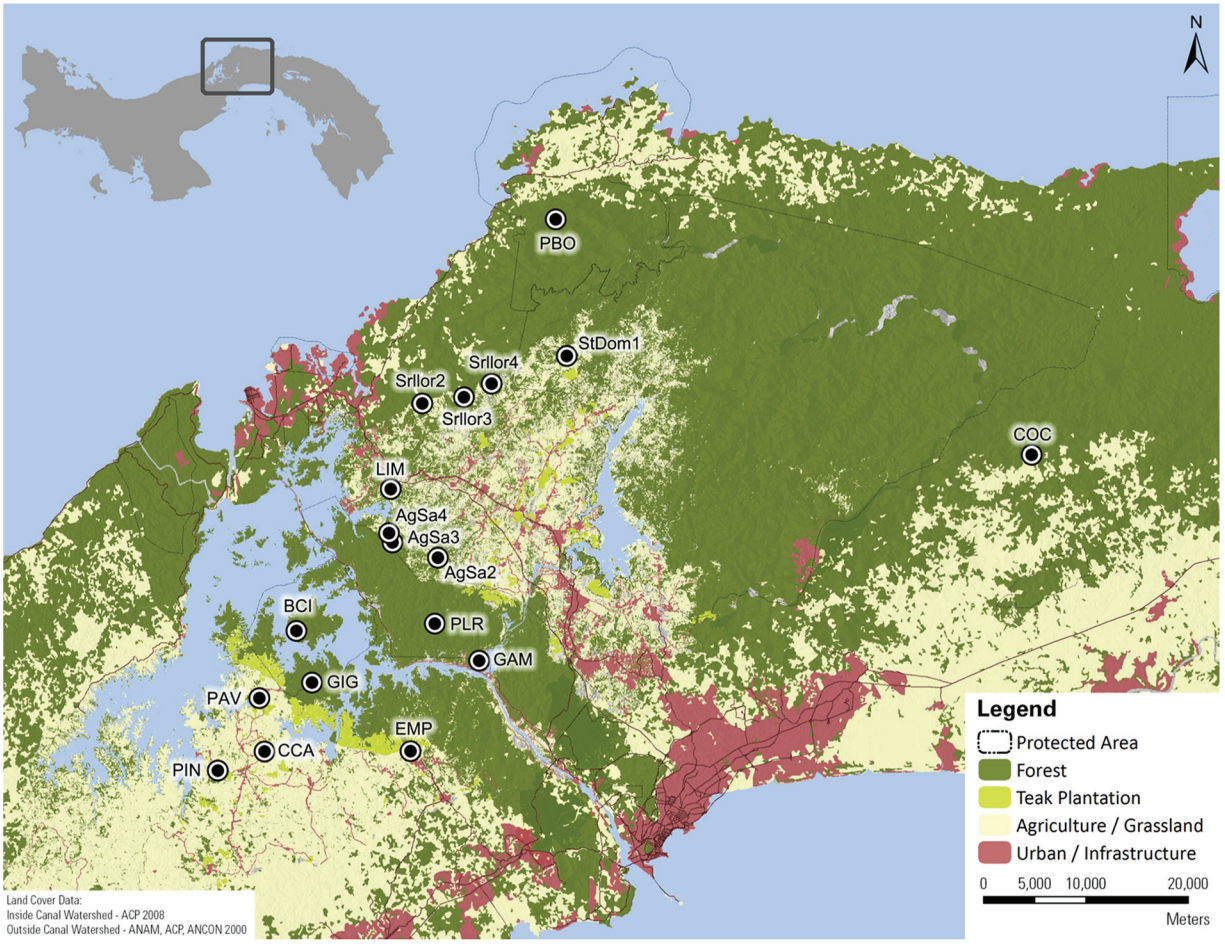

FIG 1 Map of Central Panama showing the locations of the 18 forest fragments that were sampled. Site codes correspond to sample numbers described in Table 1.

contain notable tick endosymbionts and/or pathogenic members (e.g., *Coxiella*, *Francisella*, and *Rickettsia*). Further, we investigated potential interactions among these taxa and other microbes by testing whether they occurred together more or less often than expected by chance. This study provides the most robust data set of tropical tick microbiomes to date and, thus, can help guide our understanding of tick microbiomes from tropical environments more broadly.

RESULTS

Microbiome richness. We obtained data on the bacterial community composition and richness of 733 immature ticks (396 larvae and 337 nymphs) from 11 species of 3 genera, collected from 8 species of rodents and opossums across 18 forest sites in Central Panama (Fig. 1, Table 1). In total, 228 unique ASVs were identified, most of which were sparsely represented or occurred in only a few tick samples. Most individual ticks and tick species had modest bacterial richness (between 10 and 20 bacterial ASVs). Samples typically had several highly common ASVs and a tail of rare ASVs (see Fig. S2A and B in the supplemental material). Species accumulation curves of bacterial ASVs for each tick species reached an asymptote, indicating that the richness of tick bacterial communities was sufficiently captured (Fig. S2C and D). Tick life stage was a significant predictor of alpha diversity, with higher ASV richness in nymphs than in larvae (generalized linear mixed model [GLMM]; $\beta = 0.17$, $P < 0.001$). Likelihood ratio tests indicated that the variability in ASV richness among tick species and collection

TABLE 1 Number of tick samples ($N = 733$) that were sequenced for each tick species, host species, and collection site and per life stage

	No. of:		
Species or site	**Larvae**	**Nymphs**	**Total**
Tick species			
Amblyomma			
A. auricularium	24	47	71
A. dissimile	25	2	27
A. geayi	22	3	25
A. mixtum	66	84	39
A. naponense	11	28	5
A. ovale	4	1	103
A. pacae	24	79	44
A. sabanerae	37	7	232
A. varium	26	1	9
Haemaphysalis			
H. juxtakochi	147	86	150
Ornithodorus			
O. puertoricensis	9	0	28
Total	395	338	733
Host species			
Didelphimorphia			
Didelphis marsupialis ($n = 55$)	199	169	368
Marmosa robinsoni ($n = 3$)	3	0	3
Metachirus nudicaudatus ($n = 5$)	19	10	29
Philander opossum ($n = 12$)	24	17	41
Rodentia			
Hoplomys gymnurus ($n = 23$)	31	24	55
Melanomys caliginosus ($n = 1$)	0	1	1
Oryzomys talamancae ($n = 5$)	2	3	5
Proechimys semispinosus ($n = 75$)	117	114	231
Collection site			
AgSa-2	24	35	59
AgSa-3	2	1	3
AgSa-4	10	24	34
BCI	39	65	104
CCA	11	12	23
COC	2	3	5
EMP	13	20	33
GAM	18	1	19
GIG	33	33	66
LIM	21	1	22
PAV	11	4	15
PBO	25	4	29
PIN	10	7	17
PLR	63	62	125
Srllor-2	74	24	98
Srllor-3	27	8	35
Srllor-4	0	3	3
StDom-1	12	31	43
Total	395	338	733

sites was statistically significant, as removal of these random factors reduced the model fit (likelihood ratio test statistic [LR] = 47.26 and $P < 0.001$ for tick species; LR = 7.76 and $P < 0.01$ for collection site). Alpha diversity for all samples (ASV richness), visualized by tick life stage, tick species, host order, and site, is available in the supplemental material (Fig. S3).

Microbiome composition. The tick microbiomes were predominantly (more than 93%) composed of Gram-negative bacteria of the phylum *Proteobacteria* (21). The most abundant bacterial sequences represented the genera *Francisella* (42%), *Rickettsia* (9%),

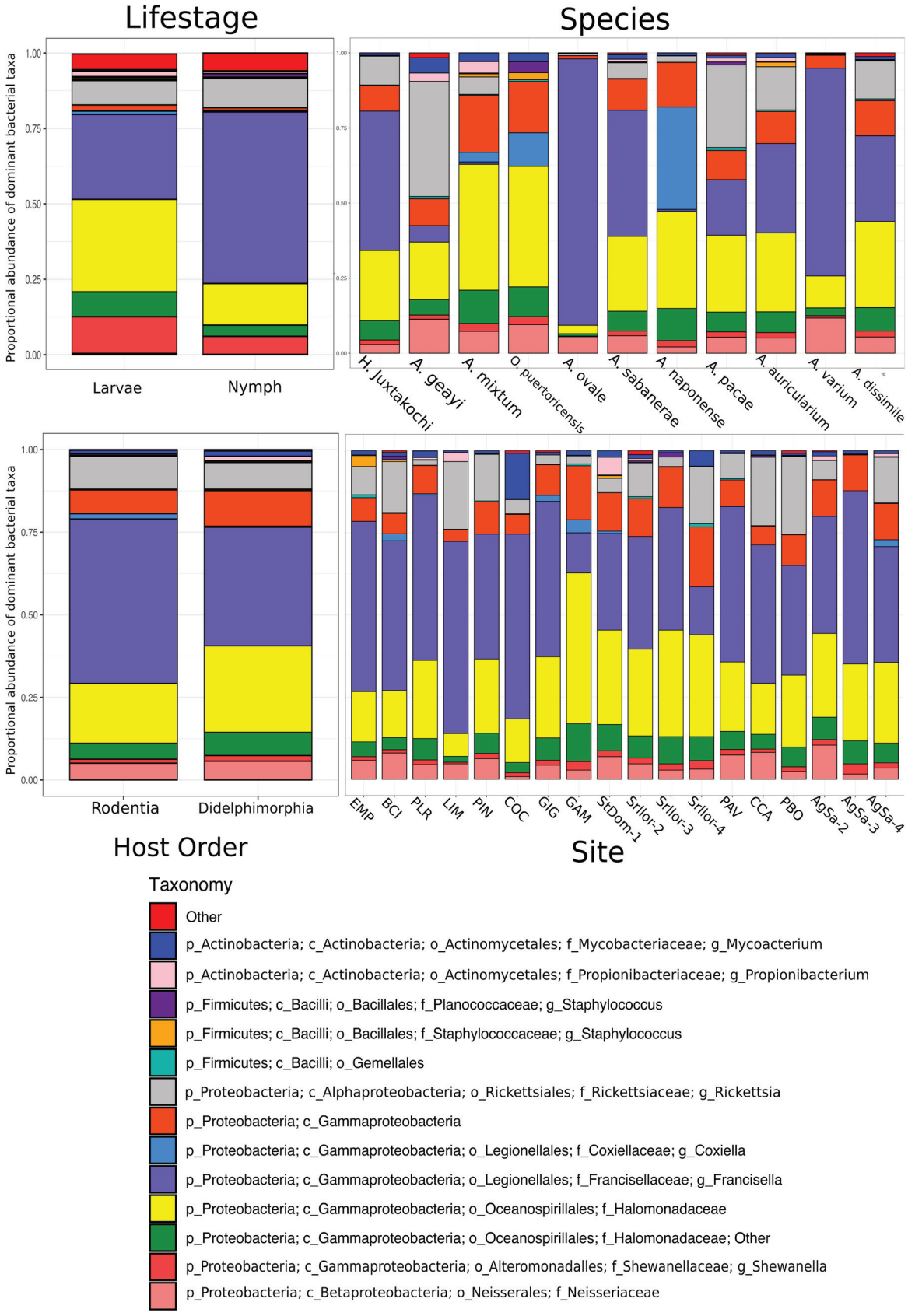

FIG 2 Proportional abundance of amplicon sequence variants (ASVs) in the microbiome of ticks collected from small mammals in forests across Central Panama compared between tick life stages, tick species, host orders, and collection sites. ASVs with an average relative abundance of >20% of total sequences are displayed individually, and the remainder are grouped under Other (red).

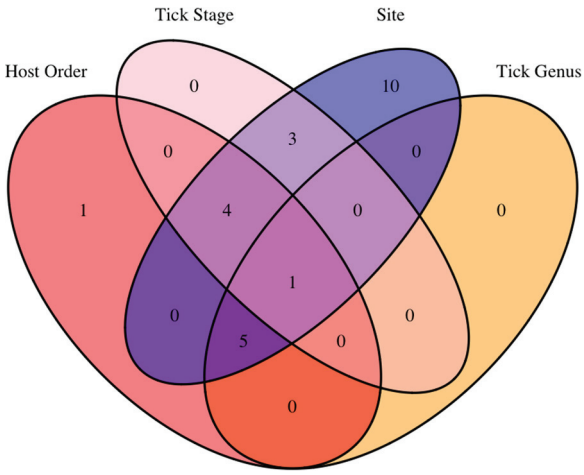

FIG 3 Number of differentially abundant microbial taxa as uncovered by ANCOM, summarized for tick species, life stage, host order, and collection site. Only four microbes are differentially abundant in all four metadata categories.

and *Shewanella* (9%) and the family *Halomonadaceae* (29%) (Fig. 2). While these dominant taxa were found across nearly all samples, we found considerable variation, measured by beta dispersion, within tick life stages, species, host order, and collection sites (Fig. S4).

Multivariate regression run on the balance trees indicated that tick life stage, species, site, and host order all explained some variation in bacterial composition ($P < 0.05$ by false discovery rate) but had small fold changes, a metric similar to effect size. Tick life stage was the intrinsic factor with the largest fold change (2-fold; Fig. S5). Using analysis of composition of microbiomes (ANCOM) (based on 719 ticks of 9 species), we identified four bacterial taxa that were differentially abundant across tick life stage, species, site, and host order (Fig. S6). In addition, there were five bacterial ASVs solely differentiated by site, zero ASVs solely by tick life stage, one ASV solely by host order, and two ASVs solely by tick species (Fig. 3). We did not detect a strong signal of spatial autocorrelation of bacterial groups by collection site ($r = 0.043$ and $P = 0.002$ by Mantel-Bray Curtis; $r = 0.033$ and $P = 0.123$ by weighted UniFrac), suggesting that nearby sites were not more similar to each other than far-away sites.

Bacterial genera that include notable tick endosymbionts and pathogens. We used the unrarefied microbial data set to assess the presence of bacterial genera that harbor well-known tick endosymbionts and/or pathogens. We detected ASVs belonging to *Anaplasma* ($n = 1$), *Bartonella* ($n = 1$), *Coxiella* ($n = 4$), *Francisella* ($n = 13$), *Rickettsia* ($n = 5$), and *Rickettsiella* ($n = 3$). *Francisella* and *Rickettsia* were detected in each of the 11 tick species, with *Francisella* being present in 711 samples (97%) and *Rickettsia* in 398 (54%). While these two genera were highly prevalent, their proportional abundance differed greatly among tick species (Fig. 4). *Anaplasma* was detected in 16 samples of 8 species, including *A. auricularium* (2/71), *A. dissimile* (1/27), *A. geayi* (1/25), *A. ovale* (3/103), *A. pacae* (1/44), *A. sabanerae* (4/233), *H. juxtakochi* (3/150), and *O. puertoricensis* (1/27). Of these tick species, *Anaplasma* was proportionally most abundant in *A. sabanerae*. *Bartonella* was detected in 2 samples of 2 different species, i.e., *A. ovale* (1/103) and *O. puertoricensis* (1/27), both collected from Tome's spriny rat *Proechimys semispinosus*. *Coxiella* was detected in 64 samples of 6 species, including *A. mixtum* (33/39), *A. naponense* (3/5), *A. ovale* (3/103), *A. sabanerae* (1/233), *H. juxtakochi* (2/150), and *O. puertoricensis* (22/27). The proportional abundance of *Coxiella* varied considerably between tick species, being most abundant in *A. mixtum*, *A. naponense*, and *O. puertoricensis* (Fig. 4). *Rickettsiella* was detected in 22 samples of 6 species, including *A. auricularium* (3/71), *A. mixtum* (2/39), *A. ovale* (3/103), *A. pacae* (1/44), *A. sabanerae* (12/233), and *H. juxtakochi* (1/150).

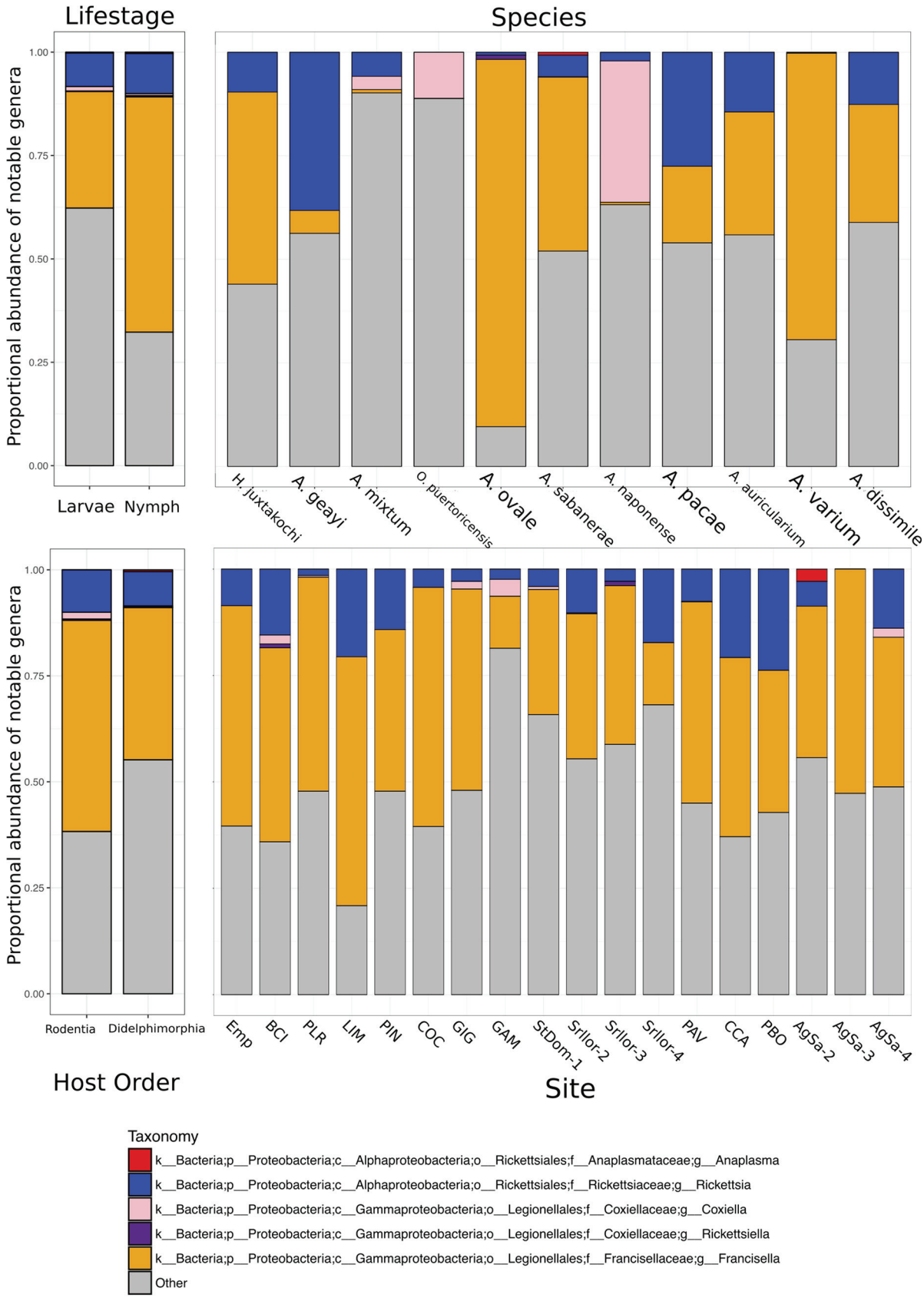

FIG 4 Proportional abundance of notable bacterial genera that include tick endosymbionts and pathogens (i.e., *Anaplasma*, *Coxiella*, *Francisella*, *Rickettsia*, and *Rickettsiella*), compared between tick life stages, tick species, host orders, and collection sites. Bacterial taxa that do not match these genera are assigned as Other and are shown in gray.

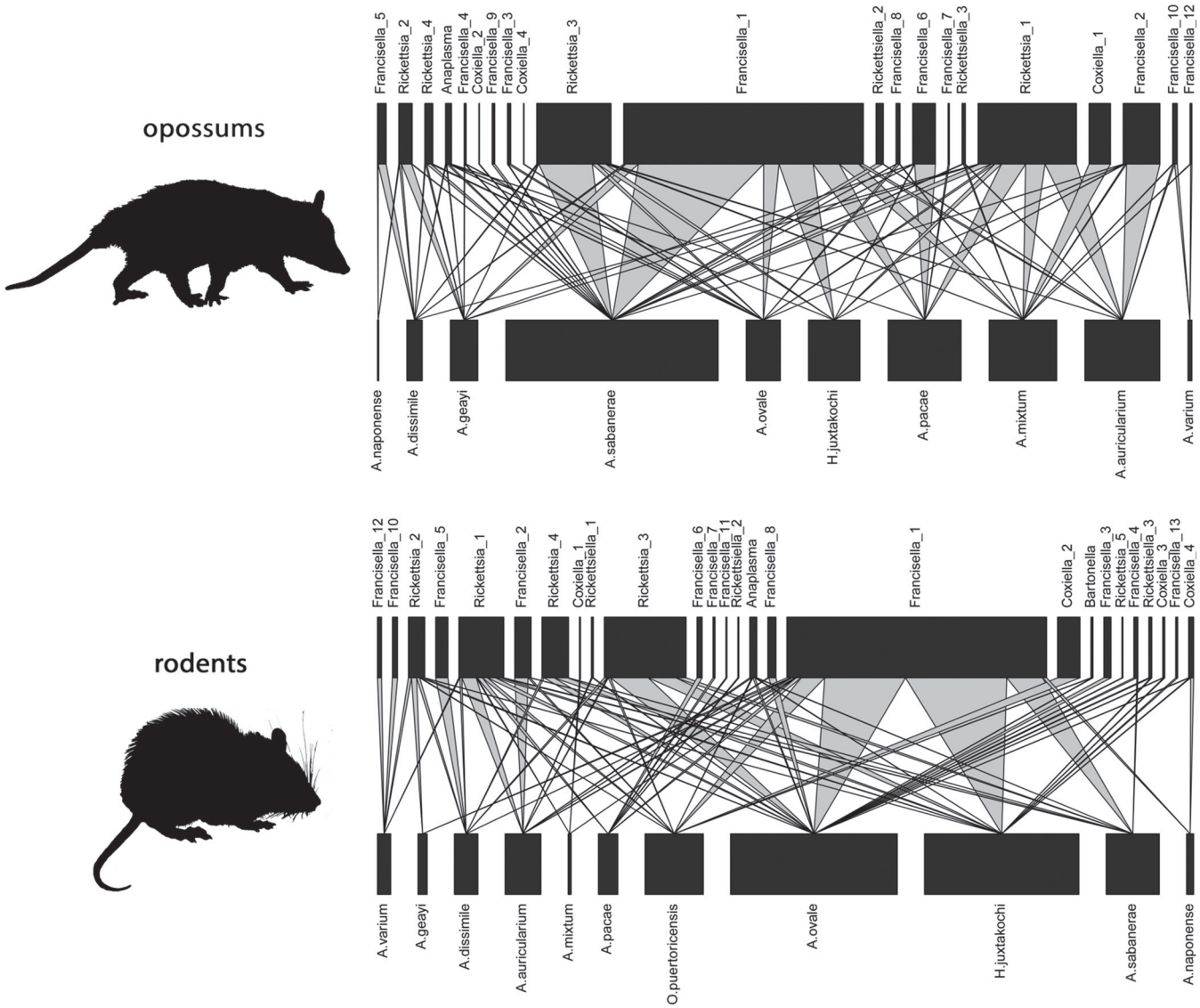

FIG 5 Bipartite network of notable ASVs (*Anaplasma*, *Bartonella*, *Coxiella*, *Francisella*, *Rickettsia*, and *Rickettsiella*) and the tick species in which they were detected, separated by the host (rodent or opossum) from which these ticks were collected. The width of the bars is proportional to the number of individuals sampled per tick species (lower black bars) and the total number of ticks in which each ASV was detected (upper black bars). The width of the gray links connecting the ticks and bacterial ASVs is proportional to the number of ticks of a specific species in which an ASV was detected.

We visualized the detection of *Anaplasma*, *Bartonella*, *Coxiella*, *Francisella*, *Rickettsia*, and *Rickettsiella* per tick species and by host order using bipartite interaction networks (Fig. 5). These networks show the degree to which ASVs from the aforementioned bacterial genera are shared among tick species or whether they are specific to certain tick species. We see similar patterns for ticks collected from either opossums or rodents, i.e., the most abundant ASV (*Francisella_1*) is shared among a wide range of taxonomically diverse tick species, as are 4 out of 5 *Rickettsia* ASVs as well as *Anaplasma*. In contrast, most other ASVs belonging to *Coxiella*, *Francisella*, and *Rickettsiella* were detected in very few tick species, and some were even associated with only one tick species (e.g., *Coxiella_1* occurred only in *A. mixtum*), suggesting that these are species-specific endosymbionts.

All *Rickettsia*-positive *A. mixtum* organisms were PCR tested using conventional PCR and DNA sequencing, and all came back as *Rickettsia amblyommatis*, a suspected cause of a mild spotted fever-like illness in humans (48). We acknowledge that OmpA is missing in some *Rickettsia* species, so some rickettsial species could have been missed by

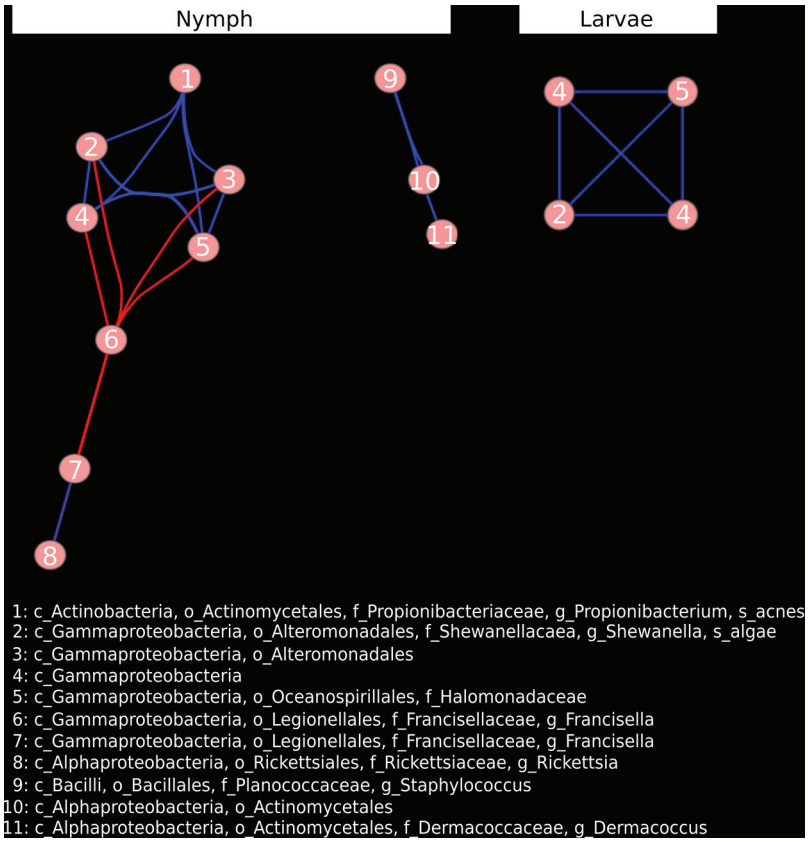

FIG 6 Network of bacterial cooccurrences within tick nymphs and larvae for all bacterial ASVs found in ticks ($n = 733$) from all sites. Circular nodes represent bacterial ASVs with significant correlation with other ASVs in the network. Red lines indicate negative correlations, whereas blue lines indicate positive correlations between two ASVs.

this analysis. In addition, we provided a BLAST search that best matches our microbial sequences from *Anaplasma*, *Coxiella*, *Francisella*, *Rickettsia*, and *Rickettsiella* to longer read archived sequences, for which a more detailed context is available in Table S2.

Patterns of microbial cooccurrence. Network analysis was done to detect whether the abundance of tick microbial ASVs was correlated (positively or negatively) with bacterial genera that include known tick endosymbionts and/or pathogens. Because tick life stage was the intrinsic factor with the largest effect in the ANCOM and balance tree analysis, separate correlation networks were constructed for larval and nymphal life stages (Fig. 6). Significant correlations can be interpreted as a potential interaction between members of the microbial metacommunity. For nymphal ticks, we identified 11 ASVs that were either positively or negatively associated with each other, and these ASV correlations occurred in two distinct correlation networks. Within the nymphal network, two distinct ASVs from the genus *Francisella* were negatively correlated with each other, while one *Francisella* and one *Rickettsia* ASV were positively correlated with each other. For larval ticks, we identified four positively associated ASVs and no negative associations (Fig. 6).

DISCUSSION

Microbiomes of ticks contain elements that are directly responsible for infection and can mediate tick-borne pathogen transmission in mammals, yet little is known about what determines these communities. We assessed the degree to which intrinsic and extrinsic factors explain the composition and richness of microbiomes found in ticks in the species-rich tropical moist forests of Central Panama, using a robust

analysis of 733 ticks of 11 species (719 ticks of 9 species for ANCOM) collected directly from small mammals at 18 lowland forest sites. Tick life stage best explained variation in microbiome composition. Microbiome richness was greater in nymphs than in larvae and varied between tick species and collection site. Microbiome composition was best explained by tick life stage, although collection site best explained differentially abundant taxa across intrinsic and extrinsic factors.

Our finding that tick life stage is a major driver of the microbial composition and richness in ticks is supported by previous studies (17, 27, 31, 36). The microbiome of larvae was a subset of those of nymphs, suggesting that nymphs had more opportunities to acquire bacterial microorganisms from vertebrate hosts and their off-host environment. Another possible explanation for the role of life stage in structuring tick microbiomes is a colonization "priority effect." A tick's initial microbiome, in some cases derived from its mother, could determine its microbiome throughout development. For example, some bacteria in the genus *Rickettsia* are transmitted transovarially and can inhibit subsequent colonization with other *Rickettsia* species (20). *Rickettsia* was a dominant component of tick microbiota for several species in this study, including *A. geayi* and *A. paca*, and contributed greater than 5% of microbiome composition to *H. juxtakochi*, *A. mixtum*, *A. sabanerae*, *A. auricularium*, and *A. dissimile*. If priority effects play a key role in driving tick microbiome composition, then differences across life stages may be predictable from knowledge of initial conditions. Priority effects may be maintained through tick development by competition for space and resources as well as chemicals produced during microbe-microbe interactions.

Tick microbiome richness differed among collection sites, which is also in line with previous findings (36). In addition, we found that some bacterial taxa were differentially abundant across sites. However, collection site was only a weak predictor of overall microbiome composition, and sites close together were not more similar than sites further away. These results contrast with studies that identified strong spatial autocorrelation of tick microbiome composition (35, 38, 49). One explanation may be that these studies assessed ticks from areas that were much further apart than our collection sites. However, other studies also failed to find strong geographic differences in tick microbiome composition (31, 50–52) or only detected these after all *Francisella* and *Rickettsia* components were excluded from the analysis (37). This emphasizes the importance of spatial scale when considering environmental impacts on tick microbiome composition (53).

Host blood source did not explain variation in tick microbial richness and had only limited influence on tick microbiome composition. This finding is in line with Hawlena et al. (30), who also simultaneously considered tick, host, and environmental factors. However, studies that specifically focused on the role of the host blood source did find strong effects on tick microbiome composition and richness (40–42). For example, *Ixodes pacificus* fed on lizards had lower bacterial richness than those fed on rodents, and the authors suggested that this reduction affects the future acquisition of *Borrelia burgdorferi*, the causative agent of Lyme borreliosis (41). Likewise, the microbiome composition of *Ixodes scapularis* varied widely among ticks fed on six mammalian host species (42). One explanation for the discrepancy between our findings and these studies is that we collected and analyzed ticks that were still feeding, whereas these other studies analyzed ticks after molting (41, 42). Further, our ticks had various stages of engorgement, which might influence tick microbial composition (40). On the other hand, studies comparing the microbial composition of ticks and that of their host's blood did not find any clear correlations (17, 32, 54), and tick microbial richness has been found to increase (40, 43), decrease (17), or not change at all after host blood feeding (55). Clearly, the role of the vertebrate host in shaping tick microbial composition and richness warrants further investigation.

To date, few studies have compared the microbiomes of multiple tick species collected from the same geographic region. Most comparative microbiome studies focused on *Ixodes scapularis* and one other tick species (30, 32, 35, 50, 52) or considered only three

species (56, 57). Nakao et al. (33) found evidence for species-specific effects on the microbiome composition of seven tick species. However, as these species were collected in Japan, the Netherlands, and Gambia, it is unclear to what degree the differences among species could be attributed to the diverse environments from which they were collected. To the best of our knowledge, only Chicana et al. (31) considered how tick microbiomes varied among a range of sympatric species with diverse host feeding preferences and life histories. They found significant differences in microbial richness and composition among the six tick species (31). The results of our study, in which we considered 11 sympatric tick species, partly agree with these findings. We found that microbial richness was significantly different among tick species, as was microbial composition when we used more traditional analyses (permutational analysis of variance [PERMANOVA]), as in Chicana et al. (31) (see the supplemental material). However, when we used compositionally aware balance trees to explore this relationship, we found that life stage explained more of the variation in microbiome composition. Moreover, ANCOM revealed that only two bacterial taxa were statistically associated with a tick species. This underlines the importance of considering differences in sequencing depth and to account for the compositional nature of the data in microbiome analyses (58).

Of particular interest is the role of tick microbiomes in mediating pathogen acquisition and transmission (59). Endosymbionts can interact with tick-borne pathogens both directly and indirectly, and these interactions can be either facilitative or competitive (13). For example, the endosymbiont *Rickettsia peacockii* blocks transovarial transmission of *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever, in *Dermacentor andersoni* ticks (11). In that same tick species, the endosymbiont *Rickettsia bellii* was negatively correlated with the pathogen *Anaplasma marginale*, whereas positive correlations were found for *Francisella* endosymbionts and the pathogen *Francisella novicida* (13). Several other studies have also found positive or negative cooccurrence between bacterial genera in tick microbiomes (19, 34, 36, 37, 51, 52), suggesting that endosymbiont-pathogen interactions are common.

In our study, *Francisella* and *Rickettsia* were the most prevalent bacterial genera in the microbiomes of 8 out of 11 tick species, suggesting that these are endosymbionts. The three tick species in which these genera were not as prevalent include *A. mixtum*, *A. naponense*, and *O. puertoricensis*. Instead, *Coxiella* was proportionally more abundant in these tick species than in others. Based on the seemingly inverse relationship between *Francisella* and *Coxiella*, we expected a negative cooccurrence between the two. Surprisingly, we did not detect such a relationship, although one *Francisella* sample showed negative cooccurrence with another *Francisella* member while at the same time showing positive cooccurrence with a *Rickettsia* member. An inverse relationship between *Francisella*-like endosymbionts and spotted fever group *Rickettsia* was previously documented in *Dermancentor occidentalis* (37). Whether these correlations reflect actual microbial interactions (i.e., competition or facilitation) or rather arise due to confounding factors (e.g., shared or opposite ecological niches) remains to be elucidated.

Several limitations systemic to this field of study influence our ability to draw conclusions. First, there is limited knowledge of the broader bacterial communities circulating in the blood of neotropical small mammals, as most studies so far have focused on pathogens (45, 60–62). The bloodmeal is not believed to be sterile; the feeding lesion within the skin may have contamination from dermal bacteria that would be ingested and, thus, become represented within the tick's microbiome. Some of these host-derived microbial agents may be retained transstadially to become a stable part of a tick's microbiome. To truly study the effect of host feeding on tick microbiomes, one should compare nonengorged ticks with fully engorged and recently molted individuals of the same species (17, 40, 43, 55). Future work could specifically address this challenge. Second, although not an explicit goal of this study, we cannot interpret how much risk of tick-borne disease there is in these communities without better characterization of the bacterial species and pathogenicity of all of the components of the tick

microbiomes. This is particularly true for *Anaplasma*, *Coxiella*, *Francisella*, and *Rickettsia*, which we detected in our samples. We address this limitation to the best of our data's ability by providing an ASV BLAST best percent match to sequences in GenBank. However, we stress that detection of bacterial DNA in a tick does not indicate that these microbes will be maintained transstadially or that they can be transmitted to another host. For example, we detected *Bartonella* in two of our samples, but this pathogen is primarily vectored by fleas (but see reference 63). Third, our data generated from the first two life stages do not address adult tick microbiomes or tick sex, which may also contribute to differences in tick microbiomes (33–40), since adult females need a large blood meal compared to males to develop eggs. Finally, our tick sequence data cannot address potential seasonal or climatic effects on tick microbiomes.

Our study indicates that intrinsic tick factors (i.e., life stage and species) and environmental factors (i.e., collection site) explain variation in the composition and richness of neotropical tick microbiomes. Further research evaluating how ticks acquire microbiomes and how stable microbiomes are over tick development is needed. This is important, because defining the tick microbiome in this way and deciphering the associations between the tick and its symbiotic bacteria, in the context of tick development and pathogen transmission, can inform novel approaches to control tick-borne diseases. An important next step in this direction is to increase our understanding of negative microbial interactions, particularly between endosymbionts and pathogens, as these may provide new management strategies to mitigate the medical and veterinary impacts of ticks (51).

MATERIALS AND METHODS

Tick sampling. Ticks were collected at 18 sites in Central Panama between 2012 and 2014 (Fig. 1, Table 1). All sites were lowland tropical rainforest but varied in elevation from 30 to 460 m (Advanced Spaceborne Thermal Emission and Reflection Radiometer [ASTER] Global Digital Elevation Model [GDEM], V2) and annual rainfall from 1,825 to 3,975 mm $year^{-1}$ (64). The study sites also differed in disturbance histories and patch sizes, ranging from small forest fragments to large national parks, and, therefore, varied widely in wildlife community composition (65). Ticks were collected directly from small mammals during the dry season from January to May and were trapped using 100 Tomahawk live traps (40 by 13 by 13 cm) per site that were placed in a grid with 10-m interspacing and baited with ripe banana. Trapped mammals were identified to species according to Reid (66) and subjected to extensive search over their entire body for ticks. We collected and analyzed ticks from 179 individual small mammals of eight species, including four species of opossums and four species of rodents (Table 1). All ticks collected were larvae or nymphs.

Ticks were stored in 95% ethanol and identified to species level either morphologically or molecularly. Larvae and nymphs of *Amblyomma ovale*, *Haemaphysalis juxtakochi*, *Ixodes affinis*, and *Ornithodoros puertoricensis* were morphologically identified using an extensive reference collection at the Gorgas Memorial Institute and taxonomic keys (67). All other immature *Amblyomma* ticks were molecularly identified using either the 16S rRNA gene or the mitochondrial DNA cytochrome *c* oxidase subunit 1 (COI) barcode fragment (68). We also determined the DNA sequence of the 16S rRNA gene for a subset of the morphologically identified ticks to confirm correct species identification. In total, we identified 815 ticks (456 larvae and 359 nymphs) to 15 species: *Amblyomma auricularium* ($n = 82$), *A. dissimile* ($n = 31$), *A. geayi* ($n = 28$), *A. longirostre* ($n = 1$), *A. mixtum* ($n = 44$), *A. naponense* ($n = 5$), *A. oblongoguttatum* ($n = 1$), *A. ovale* ($n = 103$), *A. pacae* ($n = 45$), *A. sabanerae* ($n = 257$), *A. tapirellum* ($n = 1$), *A. varium* ($n = 9$), *H. juxtakochi* ($n = 165$), *I. affinis* ($n = 2$), and *O. puertoricensis* ($n = 41$). In addition, one *Amblyomma* larva yielded a sequence that did not match to any known species in GenBank.

DNA extraction and sequencing. We used molecular methods to assess tick microbiomes. All identified ticks ($n = 815$) were individually processed under open benchtop conditions. Prior to DNA extractions, we surface sterilized ticks in 10% bleach and then rinsed them with sterile molecular-grade water, as described by Clay et al. (26). Genomic DNA was extracted from whole ticks using the Qiagen DNeasy kit (Valencia, CA, USA) by following the manufacturer's protocol. PCR products were obtained targeting the V1 to V3 hypervariable regions of the 16S rRNA gene using the forward primer 5′-ATTACCGCGGCTGCTG-3′ and reverse primer 5′-GTTTGATCCTGGCTCAG-3′, following Hawlena et al. (30). These hypervariable regions have previously been shown to be suitable for distinguishing bacterial species to the genus level (69). DNA extraction and PCR control samples showed no isolation or significant amplification and were subsequently excluded. The 16S rRNA PCR products of the 815 samples were multiplexed and sequenced on an Illumina MiSeq sequencer. The data set was deposited in Dryad (https://doi.org/10.5061/dryad.h9w0vt4h0).

Sequence and sample processing. The 16s rRNA sequences were demultiplexed and quality-filtered using Quantitative Insights Into Microbial Ecology (QIIME) 1.9 (70). All sequences were trimmed to 150 bp. Amplicon sequence variants (ASVs), previously described as suboperational taxonomic units (sOTU), were determined using the deblur workflow (minimum q = 19), which allows for unique taxa to be differentiated based on single-nucleotide differences (Deblur 1.1.0) (71). Bacterial taxonomic

assignments were made using the Ribosomal Database Project Classifier (72). All chloroplast and mitochondrial sequences were removed, and a phylogenetic tree was built from representative sequences in QIIME using fasttree2 (73). After filtering, the data set comprised 10,689,269 high-quality 16S rRNA sequence reads from 733 tick samples of 11 species, with a median of 8,414 sequences per sample. ASVs with fewer than 10 sequence reads and/or fewer than 0.05% of reads across all samples reads were removed (74). Samples were subsequently rarefied (without replacement using single_rarefaction.py in QIIME) at 2,014 sequences per sample to account for variable numbers of raw sequences. Analyses of alpha and beta diversity utilized the rarefied data sets, whereas correlation networks, analysis of composition of microbiomes (ANCOM) (75), and balance trees (76) were done on the unrarefied data set after any samples from libraries of sizes less than 1,000 were excluded. In the analysis of the unrarefied data set, ASVs with low prevalence (total count less than half the number of samples) and presence (in less than 5% of samples) were also removed as described by McMurdie et al. (77).

Presumptively *Rickettsia*-positive samples were subjected to conventional PCR and DNA sequencing. Amplification of the rickettsial outer membrane protein A (*ompA*) gene was performed using primers R190-70 and R190-602 (78), modified to use GoTaq Green master mix (Promega, Madison, WI, USA) in 25-μl reaction mixtures containing 1.0 M each primer and 3 μl of template DNA. Results of PCR were assessed by electrophoresis and UV transillumination of GelStar (Lonza, Rockland, ME USA)-stained 1% agarose gels. Bands of the expected size were excised and cleaned with a QIAquick gel extraction kit (Qiagen) per the manufacturer's instructions. Products were sequenced in the forward direction in an ABI Prism 3730 Genetic Analyzer (UC DNA Sequencing Facility, Davis, CA, USA). DNA sequences were manually trimmed and corrected if the nucleotide could be unambiguously determined and then compared with sequences in a large database (GenBank; NCBI, Bethesda, MD, USA) by BLAST search.

Statistical analyses. We characterized within-community (α) ASV diversity (richness) and between-community (β) diversity of tick microbiomes in R, version 3.3.3 (79). We included tick species, tick life stage, host blood source, and collection site as predictors for tick microbial diversity and composition. We first summarized the taxonomic composition of all bacteria at the genus level by each of these predictors (Fig. 2). We also summarized the composition of bacterial genera that harbor well-known tick endosymbionts and/or pathogenic members (i.e., *Anaplasma*, *Bartonella*, *Coxiella*, *Francisella*, *Rickettsia*, and *Rickettsiella*) by each of these predictor variables (Fig. 4). Additionally, we summarized the bacterial phyla present in each tick (see Fig. S1 in the supplemental material).

To assess which factors best explain ASV richness, we performed a generalized linear mixed model (GLMM) with a Gaussian distribution and log link function using the *glmmADMB* package (80). Tick life stage was modeled as a fixed factor, while host species, tick species, and collection site were modeled as random factors. We used likelihood ratio tests to test for significance of the random factors.

We used balance trees to investigate the groups of bacteria that change with intrinsic and extrinsic factors (76). Specifically, we ran a multivariate regression on balances to test the relative importance of each factor. Balance trees naturally correct for differences in sequencing depth and use nonoverlapping subcommunities to account for the compositional nature of the data (58). Accounting for compositional data is not possible with previously common methods for analyzing tick microbial composition (e.g., PERMANOVA). Thus, for ease of comparison with previously published tick microbiome studies, we provide the results of PERMANOVA analyses (999 permutations) in the supplemental material (supplemental Discussion, Table S1, Fig. S7).

ANCOM was used to investigate ASVs that were significantly over- or underrepresented in each tick life stage, species, host order, and collection site (75). Because machine learning within ANCOM requires greater that 25 samples for sufficient data training and testing, two tick species with fewer than 25 samples (*A. varium* and *A. naponense*) were removed prior to ANCOM analysis. Additionally, host species were summarized at the order level (i.e., Rodentia or Didelphimorphia), because several host species had fewer than 25 samples.

To test for pairs of ASVs that occurred together significantly more or less often than expected by chance, we performed a Sparse Correlations for Compositional Data (SparCC) network analysis using PySurvey (81). Using a Mantel test, we examined spatial autocorrelation of bacterial ASV by collection site. Bipartite interaction networks were constructed using the R package bipartite (82) to visualize notable bacterial genera (i.e., *Anaplasma*, *Bartonella*, *Coxiella*, *Francisella*, *Rickettsia*, and *Rickettsiella*) that were detected per tick species and host order (Didelphimorphia and Rodentia).

Data availability. Data for all newly sequenced samples are available in Dryad (https://doi.org/10.5061/dryad.h9w0vt4h0). All figures include associated raw data, and there are no restrictions on data availability.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.6 MB.

ACKNOWLEDGMENTS

This study was supported by the Smithsonian Institution Grand Challenges Award (47BIODIVERSITY), the graduate school of Production Ecology and Resource Conservation of Wageningen University (PE&RC A30), the De Vos Fund for Vector-Borne Diseases (2015), and the Simons Foundation (429440, WTW).

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