Ear mite infection restructures otic microbial networks in conservation–reliant Santa Catalina Island foxes (*Urocyon littoralis catalinae*)

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Abstract
Ceruminous gland tumours are highly prevalent in the ear canals of Santa Catalina Island foxes (*Urocyon littoralis catalinae*). Previous work suggests that tumours may result from a combination of ectoparasites, disruption of the host-associated microbiome, and host immunopathology. More specifically, ear mite infection has been associated with broad-scale microbial dysbiosis marked by secondary bacterial infection with the opportunistic pathogen *Staphylococcus pseudintermedius*. Together, ear mites and *S. pseudintermedius* probably sustain chronic inflammation and promote conditions suitable for tumour development. In the present study, we expanded upon this framework by constructing otic microbial community networks for mite-infected and uninfected foxes sampled in 2017–2019. Across sampling years, we observed consistent signatures of microbial dysbiosis in mite-infected ear canals, including reduced microbial diversity and shifted abundance towards *S. pseudintermedius*. Network analysis further revealed that mite infection disrupts overall community structure. In mite-infected networks, interaction strengths between taxa were generally weaker, and numerous subnetworks disappeared altogether. We also found that two strains of *S. pseudintermedius* connected to the main network, suggesting that multistrain biofilm formation may be occurring. In contrast, *S. pseudintermedius* is peripheral in the uninfected network, with its only connections including a second strain of *S. pseudintermedius* and the possible competitor *Acinetobacter rhizosphaerae*. Finally, the lineup of potential keystone taxa shifted across disease states. *Fusobacteria* spp., a carcinogenesis-promoting microbe, assumed a keystone role in the mite-infected community. Considered together, these findings provide insights into how mite infection may destabilize the microbiome and ultimately contribute to tumour development in this island endemic species.

**Keywords**
disease, dysbiosis, host-parasite, mammal, microbiome, wildlife conservation
Island species face elevated risk of disease due to small population size and reduced genomic variation (Frankham, 1997). Particularly when introduced to diseases from invasive species, island endemic species may experience rapid and widespread disease outbreaks, as they are immunologically naïve to novel pathogens (Mooney & Cleland, 2001). As such, disease carried by non-native vectors has been implicated in the population decline and eventual extinction of numerous island species, including endemic rats on Christmas Island that were probably lost to trypanosomiasis (Wyatt et al., 2008) and several Hawaiian bird species lost to avian malaria and birdbox (van Riper III et al., 1986). Given these risks, understanding the pathogenesis of prominent disease threats in island species is an important aspect of wildlife conservation monitoring and management.

Santa Catalina Island foxes (Urocyon littoralis catalinae) exemplify the swift, negative impact that disease can have on island endemic species. In 1999, a population of roughly 1300 foxes crashed by 90% in a single year to around 100 individuals due to a suspected canine distemper virus (CDV) outbreak, probably brought to the island by a stowaway, non-native raccoon from the California mainland (Hamblen & Henk, 2022; Timm et al., 2009). In 2000, the Institute for Wildlife Studies (IWS) and the Catalina Island Conservancy (CIC) responded quickly by launching the Catalina Island Fox Recovery Plan (Timm et al., 2009). Biologists administered CDV vaccinations to at least 85% of the remaining foxes and translocated juvenile foxes to a depopulated end of the island to slow the spread of CDV (King et al., 2014). A captive breeding facility was established and a subset of foxes were monitored. These conservation efforts culminated in the U.S. Fish and Wildlife Service down-listing Santa Catalina Island foxes from “Endangered” to “Threatened” in 2016 (U.S. Department of the Interior, 2016).

As of fall 2021, the foxes have rebounded to a population of approximately 1660 but remain a conservation-reliant species. The CIC continues to regularly document the accidental introductions of raccoons and other non-native species to the island and monitor the population for another decline, conducting health checkups and administering CDV and rabies vaccinations.

Despite these actions, the risk of disease persists on the island. For example, Santa Catalina Island foxes display one of the highest rates of tumour prevalence ever documented in a wildlife population. Present in 52.2% of mature foxes in a 2007–2008 survey, ceruminous gland tumours can occlude the entire ear canal, infiltrate bone, and in severe cases, metastasize to lymph nodes and the lung (Vickers et al., 2015). The likely cause of these tumours is the ectoparasitic ear mite, Otodectes cynotis (which may have been introduced to the island by cats), and subsequent chronic inflammation. Vickers et al. (2015) found that inflammation was strongly linked with ceruminous gland hyperplasia (CGH), an early stage of cancer. They hypothesized that mite infection may be responsible for establishing this landscape of inflammation that ultimately promotes tumour development (Vickers et al., 2015). At the recommendation of wildlife veterinarians, CIC initiated a pilot study in 2008 to test the effectiveness of prophylactic topical ivermectin application on 114 of 304 wild foxes. A year later, the treatment was shown to reduce inflammation and mite burden, and since 2009, this practice has been continued annually for all treated foxes (Hamblen & Henk, 2022).

DeCandia et al. (2020) investigated how the ear canal microbiome may fit into this mite-induced cascade. Given the low levels of genetic diversity among island foxes (Aguilar et al., 2004; Robinson et al., 2016), variation within microbial communities may serve as an important dimension of host health and disease management (Adams et al., 2021; DeCandia et al., 2018). Microbial symbionts can actively modulate host immune responses (Zheng et al., 2020), and dysbiosis—that is, imbalances within these communities—has been linked to disease. For instance, pandas infected with CDV show altered composition in the gut microbiota (Zhao et al., 2017). Similarly, the skin microbiome of mange-infected coyotes, red foxes, and grey foxes show reduced diversity and distorted community composition (DeCandia et al., 2019). Similar patterns of dysbiosis appeared in mite-infected ear canals of Santa Catalina Island foxes, with the opportunistic pathogen Staphylococcus pseudintermedius overwhelmingly present with mite infection (DeCandia et al., 2020). This finding led to an update of the original mites-to-tumorigenesis framework to now hypothesize that ear mite infection and secondary infection by S. pseudintermedius work in tandem to sustain the inflammation linked to tumour development (DeCandia et al., 2020).

S. pseudintermedius has been a recurring character across a range of canine infections. In domestic dogs, it is a key player in skin, outer ear, urinary tract, respiratory, and reproductive tract infections (Lynch & Helbig, 2021). Among coyotes, red foxes, and grey foxes, it dominates secondary infections that bloom from sarcotic mange (DeCandia et al., 2019), while its resilience against antibiotics like methicillin makes S. pseudintermedius all the more worrisome for the health of both wild and domestic canids (Meroni et al., 2019). Despite the clear role that S. pseudintermedius plays in a number of opportunistic infections, it is also increasingly apparent that this bacterium (and others like it) exist in complex communities of players within the microbiome at large. Innumerable smaller-scale dynamics may also influence host health and immunity. However, in this host–parasite system, it remains unclear why S. pseudintermedius dominates mite-infected ear canals, and the conditions in the microbial community that enable this mite to proliferate are not well characterized.

To bridge this knowledge gap, we performed network analysis to compare the community structures of mite-infected and uninfected ear canals in Santa Catalina Island foxes. This approach enables fine-scale management strategies that target the pathogen and its allies, while supporting the inhibitory work of its antagonists and competitors (Poudel et al., 2016). A comparison of global network statistics can also quantify shifts in overall community structure between disease states. For example, a comparison of gut microbiota between alcoholic and healthy human subjects revealed decreased connectivity in the network topology of alcoholic subjects (Mutlu et al., 2012). These network statistics can aid in describing how microbial community structure differs between mite-infected and uninfected foxes.
Networks can also identify keystone taxa that play crucial roles in shaping the structure of microbial communities, the removal of which may impair normal functioning (Banerjee et al., 2018). For example, network analysis in the context of cystic fibrosis identified anaerobic bacteria as significant taxa associated with declining lung function (Layeghifard et al., 2019), while similar analyses identified candidate taxa that may play important roles in the progression of gastric carcinogenesis (Coker et al., 2018).

In wildlife, numerous microbiome studies have focused on characterizing differences in microbiota composition with respect to diet (Pascoe et al., 2017). For example, network analyses have been used to detect diet-associated shifts in the gut microbial communities of cichlid fishes (Riera & Baldo, 2020), while a comparison of oral yeast communities in vultures revealed distinct co-occurrence networks across species with different feeding and scavenging habits (Pitarch et al., 2020). However, few studies have leveraged network comparisons across disease states, making microbiome network analysis a promising frontier in wildlife research. In one example, network analyses comparing the gut microbiota of healthy and diseased shrimp across species with different feeding and scavenging habits (Pitarch et al., 2020). Yet within free-living wildlife, we are among the first to apply network analysis in the context of disease management.

In the present study, we used network analysis to elucidate drivers of community perturbation following ear mite infection in Santa Catalina Island foxes. Specifically, we sought to identify microbes that may support S. pseudintermedius proliferation, while also identifying taxa that may re-emerge in this opportunistic pathogen under healthy conditions. We began by analysing microbiome samples collected in 2019 to confirm patterns of dysbiosis reported in foxes sampled in 2017–2018 (DeCandia et al., 2020). We then performed network analysis on sequencing data from all three sampling years to characterize how the overall otic microbial community structure shifts across disease states. We hypothesized that mite infection would destabilize community structure by disrupting subnetworks and weakening overall interactions between taxa. We predicted that each network would exhibit distinct keystone taxa and that S. pseudintermedius would exhibit different neighbours and connectedness between mite-infected versus uninfected networks. For example, in the uninfected network, we expected that S. pseudintermedius would co-occur alongside competing bacteria species that may control its numbers; in the infected network, however, we hypothesized that these connections would diminish or disappear, enabling S. pseudintermedius to proliferate.

2 | MATERIALS AND METHODS

2.1 | Sample and data collection

We collected samples from Santa Catalina Island foxes during three consecutive field seasons (2017–2019) under the following permits, authorizations, and approvals: U.S. Fish and Wildlife Service (USFWS) Federal 10(a)(1)(A) permit (no. TE 090990-2), a Memorandum of Understanding with the California Department of Fish and Wildlife (CDFW), Scientific Collecting Permits (no. 005821 and no. 009858) issued by CDFW, and Princeton University Institutional Animal Care and Use Committee (Princeton IACUC no. 3073).

Sample collection procedures are detailed in DeCandia et al. (2020). Briefly, we placed welded-wire box traps (model no. 106 Tomahawk Live Trap Company) baited with loganberry lure, cat kibble, and canned cat food along roadside transects that were checked four days in a row. We used anti-bite protective eye covers and manually restrained foxes during sample collection and recorded metadata such as unique passive integrated transponder tag number, sex, weight, and body condition. We used tooth wear patterns to estimate age (Wood, 1958), and documented ectoparasite burden by combing the fur for fleas, ticks, and lice while using an otoscope to assess ear mite infection status (Figure 1). To sample otic microbial communities, we rotated sterile BBL culture swabs inside the ear canal 10 times in each direction, before storing them frozen until DNA extraction. All foxes were subsequently treated with the acaricide Ivermectin (IVOMEC injection, 0.05 ml of 1% solution, Merial Ltd.) before being released at the capture site.

2.2 | DNA extraction and amplicon sequencing

We extracted DNA from negative controls and samples collected in 2019 using the same protocol implemented for 2017–2018 samples (DeCandia et al., 2020). Briefly, we used a modified DNeasy PowerSoil Kit protocol that included two disruptions on a Qiagen TissueLyser II (both 12 min at 20 shakes/s) and an extended 15 min elution in 60 µl buffer C6 preheated to 70°C. We quantified DNA extract using a high-sensitivity Qubit fluorometer and standardized samples to 2.5 ng/µl. We then used barcode primers (forward GTGCCAGCMGCCGCGGTAA; reverse TAATCTWTGGGVCATCAGG) to target and amplify the 16S ribosomal RNA (rRNA) hypervariable 4 (V 4) region (Caporaso et al., 2011). The PCR recipe included 5 µl HiFi HotStart DNA polymerase mixture, 2.5 µl of each primer, 2 µl of 20X PCR buffer, 0.2 µl of each dNTP, and 1 µl of template DNA. The PCR reaction was then run using the following conditions: initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 60 s, and a final extension at 72°C for 5 min. The PCR products were then purified and sequenced using Illumina MiSeq platform.
ReadyMix (KAPA Biosystems), 3.2 μl primer mix (1.25 μM), and 1.8 μl DNA extract. The cycling protocol included: initial denaturation (94°C/3 min), 30 touchdown cycles decreasing 1°C each cycle (94°C/45 s, 80°C–50°C/60 s, 72°C/90 s), 12 additional cycles (94°C/45 s, 50°C/60 s, 72°C/90 s), and final extension (72°C/10 min). We quantified PCR product using Quant-iT PicoGreen dsDNA assays, pooled libraries, and size selected for fragments between 300–400 base pairs (bp) in length using Agencourt AMPure XP magnetic beads. We sequenced final pooled libraries (2 x 150 bp) on the Illumina MiSeq sequencer at the Princeton University Genomics Core Facility. A total of 132 samples collected in 2019 were newly sequenced for this study (ear canal, n = 101; perianal area, n = 22; negative controls, n = 9), representing 66 unique foxes. The 2017–2018 and 2019 data have been deposited in the NCBI Sequence Read Archive (BioProjects PRJNA580023 and PRJNA876281).

2.3 | Bioinformatics and data processing

We used a paired-end, dual-indexed barcode splitter in Galaxy to demultiplex raw sequencing reads (Afgan et al., 2018). One nucleotide mismatch was permitted between expected and observed barcode sequences. We then filtered reads with the dada2 denoise-paired plugin implemented in QIIME 2 version 2020.8 (Bolyen et al., 2019), which removed likely sequencing errors and trimmed 13 low-quality bases from the front end of each read. We retained 4,553,426 sequences after denoising, which contained 9409 amplicon sequence variants (hereafter ASVs), or unique taxonomic features, across 123 samples and nine negative controls. We then examined the ASVs present in negative controls and found that the most abundant ASV in any one control appeared at a frequency of 554. We therefore filtered the data set to exclude ASVs with frequencies less than 560 to discard potential contaminants while avoiding the removal of potentially meaningful taxa (Eisenhofer et al., 2019; Salter et al., 2014). This retained 1201 ASVs for downstream analysis. We then filtered the data set to only include samples from the right ear canal (i.e., our body site of interest). We additionally removed one sample due to low read counts. The final data set contained 1,341,433 sequences, which represented 887 ASVs from 50 unique foxes (mite-infected, n = 32; uninfected, n = 18) sampled in 2019.

We used this 50 sample data set for downstream diversity and taxonomic analyses to investigate whether 2019 samples recapitulated patterns observed in 2017–2018 (DeCandia et al., 2020). For the subsequent network analysis, we pooled samples from all three sampling years (2017–2019). ASVs were again filtered to account for possible contamination, and low-quality samples, duplicates, and blanks were removed. Two uninfected ear canal samples were removed randomly to equalize sample sizes for the two infection states because differences in sample size may impact network structure (Peschel et al., 2021). The final 2017–2019 network analysis data set contained 3,083,026 sequences that contained 1340 ASVs from 118 unique foxes (mite-infected, n = 59; uninfected, n = 59).

2.4 | Alpha and beta diversity analyses

We used the QIIME2 align-to-tree-mafft-fasttree pipeline to align representative sequences, remove highly variable positions, and construct a rooted phylogenetic tree, where the root was planted midway between the longest branches. This tree was then fed into the core-metrics-phylogenetic and alpha-rarefaction functions to calculate phylogenetic diversity metrics and alpha rarefaction curves, respectively.

We assessed alpha diversity differences by evaluating species richness and species evenness. Expressed as the number of observed ASVs (Hagerty et al., 2020), species richness is a measure that tallies up the number of unique taxa present in each sample. Species evenness considers how close taxon counts are to one another as a measure of species equitability (Pielou, 1966). We conducted Kruskal-Wallis tests using alpha-group-significance to gauge whether there were significant differences between mite-infected and uninfected samples using both alpha diversity measures. We additionally used Bray-Curtis dissimilarity to consider beta diversity differences in species abundance between samples (Bray & Curtis, 1957). We performed analysis of variance with permutation (PERMANOVA) tests using diversity adonis to determine the significance of dissimilarity (Anderson, 2001; Oksanen et al., 2022).

2.5 | Taxonomic analyses and differential abundance testing

We trained a Naïve Bayes classifier on reference sequences stored in the Greengenes 13_8 database to determine the taxonomic composition of each sample (Bolyen et al., 2019; DeSantis et al., 2006). Reference sequences were trimmed to only include bases from the 16S V4 region and clustered at 99% similarity. We deployed this classifier via the QIIME2 function classify-sklearn to match taxonomic labels to ASVs.

By nature, microbiome data is compositional with taxon presence reported as a relative abundance value; for a given sample, all these values sum up to one. Processing this type of data comes with statistical hurdles, especially when we identify taxa responsible for driving changes in abundance. An uptick in the proportion of one taxon will change the proportions of other community members. To confidently determine significant differences in the microbial compositions of mite-infected and uninfected samples, we ran an analysis of composition of microbes (ANCOM). This method calculates log-transformed ratios between pairs of taxa within samples in each infection group. The null hypothesis assumes that both infected and uninfected groups have the same mean values for these calculations (Mandal et al., 2015). The ANCOM test statistic, W, represents the number of times this
null hypothesis is rejected for any given ASV. Thus, taxa with high W values are considered significantly different in abundance between infection groups. We queried significant ASVs in the NCBI BLASTn database to assign fine-scale taxonomy.

To detect additional microbial players that differ between the uninfected and infected groups, we implemented linear discriminant analysis (LDA) effect size (LEfSe) in Galaxy (Segata et al., 2011). The LEfSe method uses a combination of significance testing and analyses for biological consistency and effect size to pinpoint taxa that underlie differences between phenotype groups of mite-infected and uninfected ear canals (Segata et al., 2011). For this analysis, we grouped sequences at the genus level (when known), and we used default parameter settings with the one-against-all strategy for between-group analysis.

2.6 Network construction and comparison

To explore changes in microbial community structure, we constructed co-occurrence networks for each infection state using the full 2017–2019 data set. We first created separate feature tables for mite-infected and uninfected samples and ran downstream analyses in parallel for each phenotype group. We used the QIIME2 plug-in Sparse Co-occurrence Network Investigation for Compositional data (SCNIC) to construct two networks: one for the infected samples and another for the uninfected samples (Shaffer et al., 2020).

SCNIC begins the network construction process by generating a matrix of pairwise correlations between each pair of microbial taxa in a given phenotype group (Shaffer et al., 2020). We used the sparCC metric to generate this matrix, as it has been recommended for use with compositional data sets (Friedman & Alm, 2012). Before calculating correlation coefficients, we implemented the sparcc-filter method to remove all features with an average abundance less than two. This step is recommended because correlation analyses are hampered by a multitude of zeros, which signify the absence of a given taxa (Friedman & Alm, 2012). Using this filtered data, we calculated pairwise correlations between taxa via the sparCC method (Friedman & Alm, 2012). For correlations that cleared a significance threshold of \( r = .35 \), a common cutoff when working with 16S rRNA data, we then used the make-modules-on-correlations function in SCNIC to detect modules, which are strongly interconnected areas of a network (Friedman & Alm, 2012). To determine whether the distributions of correlation strengths for each network were significantly different, we used a two-sample F-test for variances due to the right-skewed distribution of these metrics.

We visualized mite-infected and uninfected networks in Cytoscape version 3.8.2 (Shannon et al., 2003). Nodes represent individual taxa, and edges depict correlations between nodes. With Cytoscape’s analysis tools, we calculated global summary statistics for each network. These summary statistics included: number of nodes, number of edges, clustering coefficient, and number of connected components, among others.

We also identified potential keystone taxa by calculating weighted node connectivity (WNC) values for each node using the program WiPer (Azuaje, 2014). The WNC metric is a promising strategy for identifying biologically meaningful nodes in gene coexpression networks, and it has also been adapted for the same purpose in microbiome co-occurrence networks (Coker et al., 2018). We used permutation tests to calculate the statistical significance of WNC scores for each network, and nodes with WNC scores within the top 95th percentile demonstrating a p-value above .05 were designated as potential keystone taxa. We assigned taxonomy to these taxa using the NCBI BLASTn online tool.

3 RESULTS

3.1 Across sampling years, mite infection significantly altered microbial diversity and taxonomic composition

Ear canal samples collected in 2019 recapitulated the patterns observed between mite-infected and uninfected ear canals in 2017–2018 (DeCandia et al., 2020). Mite-infected ear canals exhibited decreased alpha diversity when examining species richness (Kruskal-Wallis test, observed ASVs, \( H = 12.875, df = 1, p < .001 \); Figure 2a) and evenness (\( H = 19.236, df = 1, p < .001 \); Figure 2b). Regarding beta diversity, we observed significantly altered species abundance between infection groups (PERMANOVA, Bray-Curtis dissimilarity, \( R^2 = 0.197, df = 1, p = .001 \); Figure 2c). Samples appeared to separate by mite infection status along PC1, which accounted for 28.8% of the variation. This result was supported by the taxonomic composition of mite-infected versus uninfected ear canals (Figure 3), where we observed a large proportion of Bacilli in mite-infected ear canals (73.4%) compared to uninfected ear canals (14.0%). ANCOM performed at the ASV-level supported two ASVs as significantly different in abundance between groups: 9f8ba41e6a4cf9b9299d-c2155b7a34 (\( W = 885, clr = 3.863 \)) and 3f0449c545626dd-14b585e9c7b2d16f4 (\( W = 885, clr = 5.158 \); Figure 4). NCBI BLASTn results returned Bacilli species S. pseudintermedius with high similarity for both ASVs.

3.2 Mite-infected networks exhibit fewer taxa and weaker edges than uninfected networks

We observed differences in feature table and network properties between infection groups, despite the same number of samples in each data set (\( n = 59 \) each). After implementing sparcc-filter to remove zeros, the infected feature table contained more sequences yet fewer ASVs than the uninfected table feature. Infected foxes yielded 1,700,277 sequences representing 374 ASVs from 59
foxes, whereas the uninfected foxes yielded 1,337,993 sequences representing 724 ASVs from 59 foxes. The infected network similarly contained fewer nodes \((n = 193)\) and edges \((n = 304)\) than the uninfected network, which contained 464 nodes and 857 edges (Figure 5). Additional summary statistics calculated by Cytoscape are presented in Table 1. Overall, the edges in the infected network were built from weaker sparCC correlations than those in the uninfected network \((F\text{-test, } F = 1.987, df_{between\ groups} = 1, df_{within} = 1159, p < .001)\).

There are two taxa of interest in the neighbourhood of the original strain of \(S.\ pseudintermedius\) (3f0449c545626dd14b585e9c7b2d16f4). In both networks, \(S.\ pseudintermedius\) shared an edge with feature 9fd8ba416a4cffe92999dc2155b7a34 (infected, \(r = .387\); uninfected, \(r = .395\)), which was identified as another strain of \(S.\ pseudintermedius\) (also observed in ANCOM). In the uninfected network, \(S.\ pseudintermedius\) also shared an edge with feature 3c1ea9842554f3dbd2da9885a8b7999 \((r = .404)\), which was identified as \(Acinetobacter\ rhizosphaerae\). However, this edge with \(A.\ rhizosphaerae\), disappeared in the infected network. Additional taxa of interest include possible keystone taxa. Based on significant WNC scores in the top 95th percentile, the uninfected network contained...
seven potential keystone taxa, and the infected network contained two (Figure 5). The taxonomies of these hubs were identified using NCBI BLASTn and are summarized in Table 2.

### 3.3 Additional microbial taxa differentiate mite-infected versus uninfected ear canals

We detected additional bacterial players that differentiate mite-infection groups using LEfSe (Figure 6). We performed this analysis at the genus level and included the full 118 fox data set from 2017–2019 (mite-infected, $n = 59$; uninfected, $n = 59$). LEfSe detected six taxa characteristic of the mite-infected group and 53 taxa characteristic of the uninfected group (all logarithmic LDA scores $>3.00$, all $p < .05$). Among the infected group, *S. pseudintermedius* was identified as a dominant taxon with the highest LDA score of 5.335 ($p > .001$). Additional taxa detected in this group included genera *Paraprevotella*, *Devosia*, and *Streptococcus*, family Enterobacteriaceae, and order Bacteroidales. Among the uninfected group, an order of magnitude more taxa were identified as characteristic of healthy microbial communities. Numerous taxa identified through network analysis – such as edge genus *Acinetobacter* and keystone genera *Bacteroides*, *Blautia*, *Methylobacterium*, and *Sphingomonas* – were similarly detected by LEfSe as significant players in uninfected ear canals.

### 4 | DISCUSSION

In the present study, we found that otic microbial dysbiosis consistently accompanies *O. cynotis* ear mite infection in Santa Catalina Island foxes. This imbalance was reflected in reduced microbial species richness and evenness, and altered community abundance in mite-infected ear canals. Across all three sampling years, the opportunistic pathogen *S. pseudintermedius* drove differences in taxonomic abundance between infection groups. These findings lend confidence to the framework proposed by DeCandia et al. (2020) and support the hypothesis that mite infection and secondary bacterial infection by *S. pseudintermedius* together sustain inflammation in the ear canal. This landscape of inflammation may, in turn, facilitate ceruminous gland hyperplasia, which sets the stage for tumour development (Moriarty et al., 2015; Vickers et al., 2015).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Global network properties calculated by Cytoscape between mite-infected and uninfected networks ($r = .35$ for both)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Global network property</strong></td>
<td><strong>Uninfected network</strong></td>
</tr>
<tr>
<td>Number of nodes</td>
<td>464</td>
</tr>
<tr>
<td>Number of edges</td>
<td>857</td>
</tr>
<tr>
<td>Average number of neighbours</td>
<td>4.370</td>
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<tr>
<td>Network diameter</td>
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</tr>
<tr>
<td>Network radius</td>
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</tr>
<tr>
<td>Characteristic path length</td>
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</tr>
<tr>
<td>Clustering coefficient</td>
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</tr>
<tr>
<td>Network density</td>
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</tr>
<tr>
<td>Network heterogeneity</td>
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<tr>
<td>Network centralization</td>
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<tr>
<td>Connected components</td>
<td>56</td>
</tr>
</tbody>
</table>

**Figure 5** Co-occurrence networks were constructed for mite-infected and uninfected microbial communities in Cytoscape. Nodes represent individual taxa, and edges represent sparCC correlations greater than 0.35. Significant nodes ($p > .05$) in the top 95th percentile of WNC scales were identified as potential keystone taxa. Annotated with BioRender.com [Colour figure can be viewed at wileyonlinelibrary.com]
In addition to supporting the temporal stability of past findings, the present study expanded our understanding of mite-induced dysbiosis by considering additional players in the otic microbial community. For example, network analysis unspooled the complexities of ear canal dysbiosis to reveal how mites reshuffle overall microbial community structure. Here, we found that the mite-infected network was noticeably more compact than the uninfected network, as it was constructed from fewer nodes and

**TABLE 2** Potential keystone taxa for the infected and uninfected networks, calculated from WNC scores

<table>
<thead>
<tr>
<th>Feature ID</th>
<th>WNC</th>
<th>p</th>
<th>Percentile</th>
<th>Order</th>
<th>Family</th>
<th>Genus/species</th>
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<tbody>
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<td><strong>Potential keystone taxa in uninfected ear canal communities</strong></td>
<td></td>
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<td></td>
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<td></td>
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<td>559f3e11f6a315687a5a0cd4d7c95579</td>
<td>6.092</td>
<td>.007</td>
<td>1.000</td>
<td>Bacteroidales</td>
<td>Prevotellaceae</td>
<td>Prevotella spp.</td>
</tr>
<tr>
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<td>.007</td>
<td>0.989</td>
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<td>Methylobacteriaceae</td>
<td>Methylobacterium spp.</td>
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<td>.049</td>
<td>0.985</td>
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<td>Lachnospiraceae</td>
<td>Blautia</td>
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<td>.024</td>
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<td>Prevotellaceae</td>
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<td>&lt;.001</td>
<td>0.976</td>
<td>Sphingomonadales</td>
<td>Sphingomonadaceae</td>
<td>Sphingomonas spp.</td>
</tr>
<tr>
<td>fb7f790b257d0a9e8a66f3f62223560c</td>
<td>4.865</td>
<td>.007</td>
<td>0.970</td>
<td>Chlorophyta</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>13a41136781896f29585f67741ebae</td>
<td>4.317</td>
<td>.041</td>
<td>0.955</td>
<td>Bacteroidales</td>
<td>Bacteroidaceae</td>
<td>Bacteroides spp.</td>
</tr>
<tr>
<td><strong>Potential keystone taxa in infected ear canal communities</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e45cf3e00e83ca181c695beac8e59f44</td>
<td>5.7095</td>
<td>.021</td>
<td>1</td>
<td>Neisseriales</td>
<td>Neisseriaceae</td>
<td>Neisseria</td>
</tr>
<tr>
<td>1c136d6cd31c8cddf17c838d574143</td>
<td>5.2674</td>
<td>.024</td>
<td>0.995</td>
<td>Fusobacteriales</td>
<td>Fusobacteriaceae</td>
<td>Fusobacterium</td>
</tr>
</tbody>
</table>

**FIGURE 6** Linear discriminate analysis effect size (LEfSe) identified six taxa characteristic of mite-infected ear canals (in red) and 53 taxa characteristic of uninfected ear canals (in tan). Asterisks indicate duplicate taxa, and letters denote taxonomic level where c, class; f, family; g, genus; o, order. Note that *Staphylococcus* had the largest LDA score (log10) calculated. [Colour figure can be viewed at wileyonlinelibrary.com]
weaker edges. This reflects the reduced species richness detected in our alpha diversity analysis.

Globally, the infected network also had a smaller clustering coefficient and fewer connected components, suggesting that mite infection disrupted and, in some cases, eliminated subnetworks otherwise found in healthy microbiomes. Each taxon, on average, had fewer neighbours in the infected network, and overall, we observed weakened pairwise interactions within the mite-infected community, compared to the uninfected communities. Taken together, mite infection seemed to destabilize the pre-existing community structure of the ear canal—which may give \textit{S. pseudintermedius} an opening to proliferate unchecked. This result mirrors similar studies in the literature that examine microbial community networks in the context of disease. For example, the scalp microbiota co-occurrence network of human subjects with seborrhoeic dermatitis exhibited a collapse of microbial community equilibrium, indicated by lower network density in the diseased group when compared to the healthy group (Park et al., 2017). Similarly, in a salmon study, a network analysis of the mucosal microbiomes in salmon revealed distinct pathogenic and nonpathogenic “guilds” among fish with high and low levels of lice infection, respectively (Llewellyn et al., 2017).

In the absence of mites, \textit{S. pseudintermedius} levels may have been limited by its “neighbours” in the uninfected network. We zeroed in on the \textit{S. pseudintermedius} node and surveyed how its neighbouring interactions shifted across infection states. Under uninfected conditions, \textit{S. pseudintermedius} shared an edge with \textit{Acinetobacter rhizosphaerae}, a phosphate-solubilizing microbe. However, in the infected network, the node representing \textit{A. rhizosphaerae} vanished altogether. \textit{Acinetobacter} was also detected in the LEfSe analysis as a significant taxon differentiating uninfected from infected ear canals. While the role of \textit{A. rhizosphaerae} in microbial communities (much less its relationship with \textit{S. pseudintermedius}) has not been extensively studied, two related species—\textit{Acinetobacter baumannii} and \textit{Staphylococcus aureus}—have been reported to occupy the same niche in human diabetic infections (Castellanos et al., 2019). Furthermore, \textit{A. baumannii} has demonstrated inhibitory activity against \textit{S. aureus}, implying competition between these two species (Hardy et al., 2020).

Guided by this context, we hypothesize that under uninfected conditions, \textit{A. rhizosphaerae} and \textit{S. pseudintermedius} may compete for a shared niche, potentially keeping \textit{S. pseudintermedius} levels in check. However, mite infection may create conditions unfavourable for \textit{A. rhizosphaerae}, allowing \textit{S. pseudintermedius} to proliferate and mount a secondary bacterial infection.

In the uninfected network, \textit{S. pseudintermedius} was connected to one other node—another \textit{S. pseudintermedius} strain. This relationship was maintained in the infected network, suggesting a cooperative relationship, possibly representing the formation of a multistrain biofilm. \textit{S. pseudintermedius}, in particular, has demonstrated a robust capacity for biofilm formation, which can trigger a host inflammatory response via the toll-like receptor pathway (Arima et al., 2018; Singh et al., 2013). A multistrain biofilm may play a role in sustaining the inflammatory conditions linked to tumour development in Santa Catalina Island foxes, as well as conferring additional antibiotic resistance to \textit{S. pseudintermedius}.

Across infection states, we observed a shift in which microbes were detected as potential keystone taxa. In the uninfected network, genera \textit{Bacteroides}, \textit{Blautia}, \textit{Methylobacterium}, \textit{Prevotella}, and \textit{Sphingomonas} were identified as significant taxa, with four out of five (i.e., all except \textit{Prevotella}) emerging as significant players in the LEfSe analysis. In the infected network, only genera \textit{Neisseria} and \textit{Fusobacterium} were identified as potential keystone taxa. This is further evidence of community-wide structural changes brought about by mite infection: mite-induced dysbiosis may disrupt the microbial neighbourhoods surrounding keystone taxa. The roles of the keystone taxa in the uninfected network are unclear, but they may serve as indicators of a healthy microbiome. Their abundance levels can potentially be monitored as markers of a microbial community in balance.

Of the two potential keystone taxa in the infected network, \textit{Fusobacterium} is of particular interest. Species within the phylum \textit{Fusobacteria} are commonly found in predatory mammals (Nishida & Ochman, 2018), including numerous Canidae species (DeCandia et al., 2019, 2021). But the relevance of \textit{Fusobacterium} extends beyond this commensal connection due to its possible role in carcinogenesis. In human colon cancer, \textit{Fusobacterium} has been shown to inhibit natural killer cells from targeting tumours (Gur et al., 2015), and in oral cancer, \textit{Fusobacterium} may promote cancer cell invasiveness and survival (Herstad et al., 2018). These studies suggest that mite-induced dysbiosis may support a shift to carcinogenesis-promoting keystone taxa. There is limited research surrounding how \textit{Neisseria} may contribute to this pattern, representing a frontier for future research. However in humans, \textit{Neisseria meningitidis} has been reported to cause bacteremia (Mahmoud & Harhara, 2020). We suspect that \textit{Neisseria} species in the fox population may also be contributing to secondary infection alongside \textit{S. pseudintermedius}.

Considered together, these findings support the addition of two new players to the framework proposed by DeCandia et al. (2020) regarding ceruminous gland tumour development in Santa Catalina Island foxes. First, we hypothesize that mite infection alters community structure such that \textit{A. rhizosphaerae} no longer shares the same niche as \textit{S. pseudintermedius}. Facing reduced competition, this may allow \textit{S. pseudintermedius} to take over as the dominant pathogen and drive inflammation. Second, mite infection reshuffles the lineup of keystone taxa in the ear canal microbiome, allowing carcinogenesis-promoting microbes like \textit{Fusobacterium} to take up a newfound keystone role. Amid the backdrop of wide-scale otic microbial change, these results suggest that mite infection may facilitate tumour development both indirectly, via \textit{S. pseudintermedius}-driven inflammation, and directly, via the establishment of \textit{Fusobacterium}.

Ear canal tumours are only found in Santa Catalina Island foxes, even though conspecific foxes on San Nicholas and San Clemente islands also host ear mites. Future studies will focus on inter-island comparisons of microbiome communities across fox subspecies,
with the goal of determining whether differences in the host-associated microbiome may contribute to differing susceptibilities to mite-induced tumorigenesis. Examining broad-scale community structure through network analysis may provide critical insights into the relationships between the keystone taxa, allies, and competitors that may inhibit tumorigenesis in other island fox species, even in the presence of mite infection. Specifically, we are interested in characterizing the keystone taxa and topology of the microbiome networks of the ear canals of San Nicholas and San Clemente Island foxes. We suspect these tumour-free populations may have network features that are distinct from those seen in the tumour-susceptible Santa Catalina Island foxes. These features may serve as a protective mechanism against tumorigenesis in San Nicholas and San Clemente foxes.

Network analyses are needed in this and other wildlife systems to identify microbiome-associated protective mechanisms, which in turn enables us to further our understanding of how disease unfolds differently across related species. Within the Channel Island fox system, this information will allow us to survey island fox populations naive to ear mites (namely, Santa Rosa, San Miguel, and Santa Cruz Island foxes) to see which network structure they resemble. If networks mirror Santa Catalina, these foxes may be susceptible to mite-induced tumorigenesis; if networks mirror San Clemente and San Nicolas, these foxes may face little to no threat from the accidental introduction of ear mites. The patterns uncovered may therefore lead to very different management strategies should ear mites ever be detected in the northern islands.

Network analysis grants us a bird’s-eye view of important microbial interactions. Using this tool, we were able to uncover new insights into the Santa Catalina Island fox and O. cynotis host–parasite system. S. pseudintermedius was previously identified as the primary driver of disease (DeCandia et al., 2020), but co-occurrence networks enabled us to expand this understanding by identifying newfound markers of a healthy microbial community at equilibrium. Using global network statistics, we were also able to gauge how disease may have disrupted the microbial community within the island fox ear canal. Although interest in the microbiome has been rapidly growing within wildlife disease management, microbial network analyses remain limited. This technique can – and should – be applied to other systems. Network analyses can unlock additional insights into disease pathogenesis and thereby help us better monitor and mitigate disease pressures threatening island and mainland species worldwide.

AUTHOR CONTRIBUTIONS
Jasmine Lu, Bridgett M. vonHoldt, and Alexandra L. DeCandia designed the study. Emily E. Hamblen, Lara J. Brenner, and Julie L. King collected the samples. Alexandra L. DeCandia conducted the laboratory work. Jasmine Lu processed and analysed the data. Jasmine Lu, Emily E. Hamblen, Lara J. Brenner, Julie L. King, Bridgett M. vonHoldt, and Alexandra L. DeCandia prepared, contributed to, and approved the final manuscript.

CONFLICT OF INTEREST
All authors declare that they have no conflicts of interest.

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DATA AVAILABILITY STATEMENT
Sequencing data analysed in this study have been made publicly available through the NCBI Sequence Read Archive under BioProject PRJNA580023 (2017–2018 data) and PRJNA876281 (2019 data) (Lu et al., 2022). Demultiplexed forward and reverse reads are available for each sample (BioSamples SAMN13149394 to SAMN13149650; SAMN30652412 to SAMN30652462), and SRA accession numbers range from SRR10358741 to SRR10358997 (2017–2018 data) and from SRR21408517 to SRR21408567 (2019 data).

BENEFIT-SHARING STATEMENT
Benefits from this research accrue from the sharing of our data on the aforementioned NCBI database.

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