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Author for correspondence:
Rachel L. Vannette
e-mail: rlvannette@ucdavis.edu

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Microbial communities in hummingbird feeders are distinct from floral nectar and influenced by bird visitation

Casie Lee[1], Lisa A. Tell[1], Tiffany Hilfer[1] and Rachel L. Vannette[2]

[1]Department of Medicine and Epidemiology, School of Veterinary Medicine, and [2]Department of Entomology and Nematology, University of California, Davis, CA, USA

RLV, 0000-0002-0447-3468

Human provisioning can shape resource availability for wildlife, but consequences for microbiota availability and exchange remain relatively unexplored. Here, we characterized microbial communities on bills and faecal material of hummingbirds and their food resources, including feeders and floral nectar. We experimentally manipulated bird visitation to feeders and examined effects on sucrose solution microbial communities. Birds, feeders and flowers hosted distinct bacterial and fungal communities. Proteobacteria comprised over 80% of nectar bacteria but feeder solutions contained a high relative abundance of Proteobacteria, Firmicutes and Actinobacteria. Hummingbirds hosted bacterial taxa commonly found in other birds and novel genera including *Zymobacter* [Proteobacteria] and Ascomycete fungi. For feeders, bird-visited and unvisited solutions both accumulated abundant microbial populations that changed solution pH, but microbial composition was largely determined by visitation treatment. Our results reveal that feeders host abundant microbial populations, including some bird-associated microbial taxa. Microbial taxa in feeders were primarily non-pathogenic bacteria and fungi but differed substantially from those in floral nectar. These results demonstrate that human provisioning influences microbial intake by free-ranging hummingbirds; however, it is unknown how these changes impact hummingbird gastrointestinal flora or health.

1. Introduction

Food provisioning by humans can influence availability and composition of resources for wildlife. Although provisioning can positively influence wildlife [1,2], it can also have negative consequences such as suboptimal nutrition [3], novel pathogen exposure [4–6], introduction of antibiotic resistance [7–9], and habitat fragmentation [5,10,11] which can lead to decreased population resilience [4,12,13].

Of particular concern is the potential for provisioning to increase disease transmission through increased population density and shared food resources [14,15]. For birds, human provisioning of food is very common, with nearly 50% of United States households feeding wild bird populations [16]. Bird feeders serve as fomites for exchanging bacteria and fungi within bird populations, which has been linked to disease outbreaks [17]. However, it remains poorly understood (i) to what extent wildlife exchange microbiota with food resources; (ii) if provisioned resources differ from natural food resources in their microbiome composition; and (iii) if the microbiome of such food resources is stable over time. For birds, the few studies performed to date suggest that diet composition is important in determining the assembly and function of the microbiome [18]. As a consequence, understanding how wild birds consume and exchange microbiota with shared food resources, and how human-provisioned food differs from natural sources in its microbiota, is of high importance.

Hummingbirds (family Trochilidae) are one of the world's few avian pollinators, and vector pollen, nutrients and even other organisms among plants [19]. Nearly 15% of hummingbird species are threatened or endangered, so understanding drivers of health and population dynamics may help conservation efforts. Avian microbial associates are just beginning to be studied in depth [18,20,21], and little is known about microbiome assembly in free-ranging birds or its association with avian health. In hummingbirds, some microorganisms have been linked with disease and mortality [22–24], while other microbes may be beneficial [21,25], particularly considering the birds' high-sugar diet [26,27].

Hummingbirds often consume nectar containing high densities of microorganisms [28,29] and introduce microbes to flowers [30] including organisms putatively associated with a disease like *Aspergillus*, *Candida* and *Cladosporium* sp. [22,24,31]. Hummingbirds frequently use feeders that may harbour parasites and pathogens that can be transmitted among hummingbirds [32]. To avoid this, consumers are encouraged to actively maintain their feeders by replacing sucrose solutions and cleaning feeders on a regular basis [33], but in reality, feeder maintenance is likely to vary dramatically. To date, no survey has quantitatively characterized changes to sugar solutions or described the microbes causing these changes. As a result, it is unknown how microbes in hummingbird feeders compare to those found in flowers, and the degree of overlap between hummingbird gastrointestinal microbes and those in human-provisioned sucrose solutions.

Here, we examined the bacteria and fungi in hummingbird feeders and compared their composition to hummingbird bill and faecal samples, and to floral nectar. We experimentally manipulated bird visitation to feeders and compared microbial communities and sucrose solution characteristics over time. By comparing bird-visited feeders to feeders where birds or all visitors were excluded, we assessed which microbes in feeder solutions were vectored by birds.

2. Material and methods

(a) Hummingbird and flower sampling

Calypte anna (Anna's hummingbird) and *Archilochus alexandri* (black-chinned hummingbird) were captured using Hall feeder traps at a private residence in Winters, CA (38°32′ N and 121°51′ W). Urine and faecal samples from the cloaca were collected into haematocrit tubes and bill samples collected by allowing birds to drink from 1.5 ml tubes of sterile sugar solution. Sampled birds were then identified (age, sex and species), banded and released. Floral nectar was collected from plant species including *Salvia microphylla*, *Salvia greggii*, *Salvia darcyii*, *Leonotis loenurus* and *Kniphofia* spp. in Winters, CA at nearby sites and nectar from 6–10 flowers were pooled by plant species for DNA extraction.

(b) Hummingbird feeder experimental set-up

To examine if birds deposit specific microorganisms in feeder solutions, feeders were manipulated to experimentally control visitation. Hummingbird feeders (Perky Pet®, Model bb209b, Woodstream Corp., Lititz, PA 17543) were set up at two locations in Winters, CA.

Three trials were conducted in June and July 2017. Hummingbird feeders were sterilized and filled with a 20% (1 part sugar to 4 parts water) sucrose-water solution using commercial processed white sugar and bottled water (Nestlé purified water, Nestlé Waters North America, Stamford, CT 06902). Feeders were assigned to one of three treatments including (i) access by both hummingbirds and insects (open feeders), (ii) restricted access by birds but access by insects allowed (caged feeders, 1.5 cm square mesh), or (iii) restricted access by both birds and insects (feeders bagged using gallon paint strainer bags), with two replicates of each treatment set up at each site. Bird point counts were performed at 06.00 and 18.00 for 15 min and hummingbird visits (bill inserted into the feeder port) counted.

During the third and final trial, we also assessed the effect of water type on bird visitation and microbial growth [34–36], including commercial water (Nestlé brand water purified via reverse osmosis), deionized water and tap water sourced from Davis, CA. Each water type had two replicates of open and bagged (control) treatments.

(c) Sucrose solution sample collection and analysis

At 06.00 on days 0, 1, 2, 4 and 7 post-study initiation or when feeder reservoirs were near empty (feeder weight less than 800 g), feeders were weighed and 20 ml sucrose solution samples were collected. For each sucrose solution sample, pH was determined using a pH test strip (Fisherbrand 0.0–6.0 Plastic pH Indicator Strips, Cat. no. 13-640-520). Optical density was quantified at 600 nm (Molecular Devices SpectraMAX 190).

(d) Culture-based assessment of microbial abundance in sucrose solution samples

Sucrose solutions were plated on four media types to isolate fungi, bacteria including potential pathogens in the Enterobacteriaceae (electronic supplementary material, methods S1), colony forming units (CFUs) were counted and the most common morphotypes on each media were identified using MALDI-TOF and BIOTYPER software (electronic supplementary material, methods S1).

(e) Culture-independent microbial community analyses

Microbial communities from combined urine and faecal samples, feeder solutions from days 2, 4 and 7 and nectar samples were characterized using metabarcoding. Briefly, DNA was extracted using the MoBio PowerSoil Kit (Qiagen, Hilden Germany), the V4–V5 and the ITS2 region of the 16S rRNA gene amplified and sequencing using Illumina MiSeq to characterize bacteria and fungi, respectively (see full methods in the electronic supplementary material, methods S2; performed by Dalhousie IMR facility). Reads were error-corrected and assembled into amplicon sequence variants (ASVs) using *DADA2* V1.6 [37], representing microbial taxa present in and across samples.

Taxonomy was assigned using SILVA V. 128 for bacteria [38] and UNITE database (2017 release) for fungi [39]. We recovered 2 824 074 bacterial sequences after non-target sequences were removed, averaging 34 865 $\pm$ 2572 s.e. per sample from six bill, eight faecal, 48 feeder and nine nectar samples. We recovered 1 015 007 fungal sequences, averaging 12 229 sequences $\pm$2052 s.e. per sample from five bill samples, eight faecal, 44 feeder and three nectar samples. Sequence abundance was converted to normalized counts [40] and analysed using the R package *phyloseq* [41].

(f) Statistical analysis

To compare microbial communities across sample types, Bray–Curtis dissimilarities were calculated and visualized using non-metric multidimensional scaling (NMDS) and analysed using permutational ANOVA ('adonis' in vegan [42]). Subsequent analyses to determine which ASVs were differentially abundant between sample types were performed using *DESeq2* [43].

To examine if feeder treatment or solution age determined (i) visitation rate, (ii) sucrose solution pH, or (iii) optical density

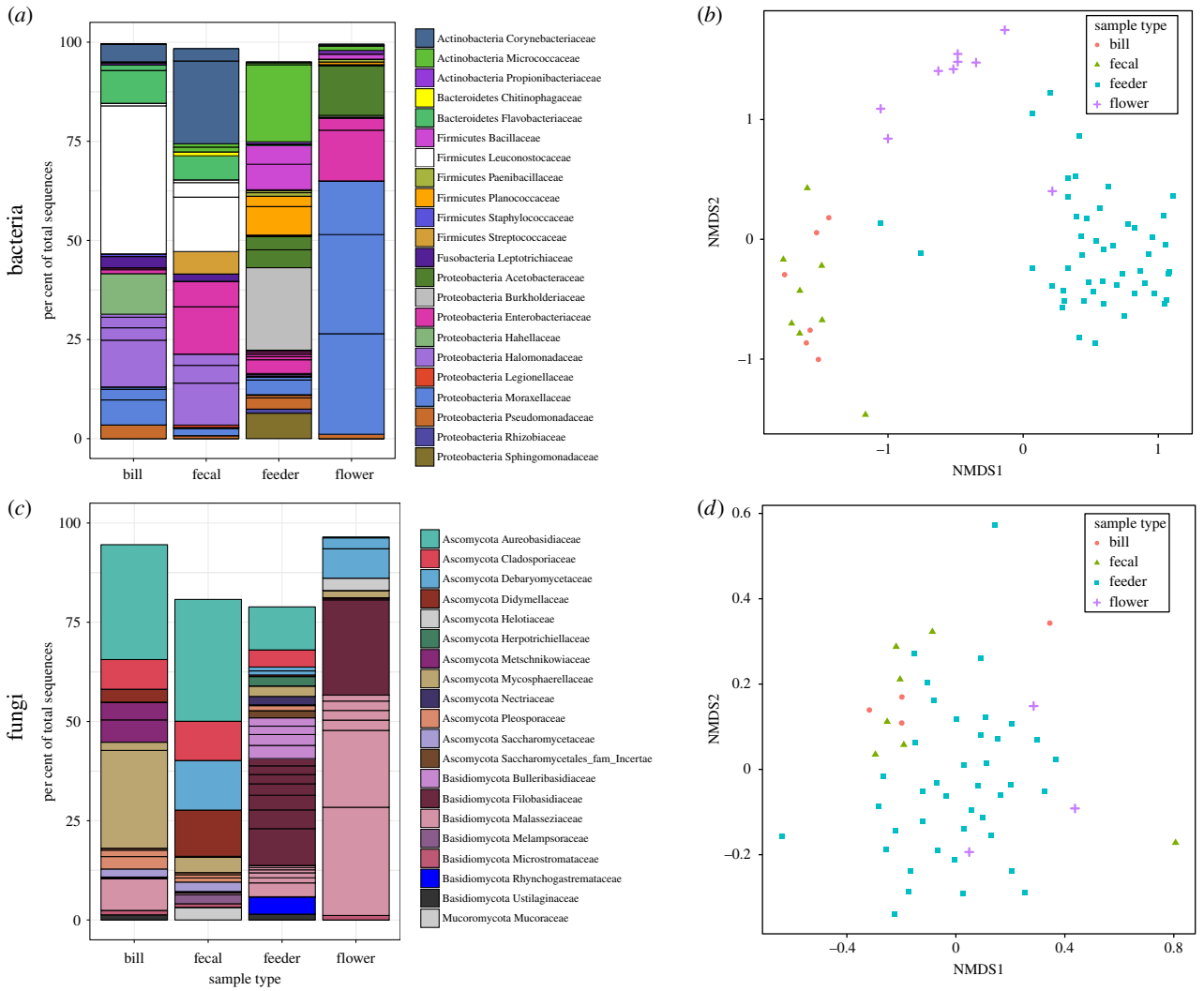

Figure 1. Microbial communities associated with hummingbird (*Calypte anna* and *Archilochus alexandri*) bills, faecal samples, sucrose solutions from hummingbird feeders and floral nectar. In (*a*) average composition of 50 most abundant bacterial phyla and families characterized using 16S sequencing and (*b*) bacterial species composition visualized using NMDS based on Bray–Curtis dissimilarity. In (*c*) average composition of most abundant fungal families characterized by ITS2 and (*d*) NMDS of fungal species composition based on Bray–Curtis dissimilarity. In (*a*,*c*), black lines within each colour (family) indicate the number of ASVs represented and the difference between taxa shown and 100% is comprised by low abundance taxa.

(OD_{600}), linear mixed models were implemented in *nlme* [44]. Main effects included feeder treatment, sucrose solution age and age^2 and all two-way interactions, with site and trial number as random effects. Feeder treatment or sucrose solution age were associated with $\log_{10}$-transformed (CFU $\mu l^{-1} + 1$) in feeders using mixed models, with predictors as above. Separate models were used for each media type, with feeder site and trial number included as random effects. Culturable microbial taxa identified using BIOTYPER software with a score greater than 1.6 or high repeatability (poor score overall, but replicates were identically scored) were tabulated and compared across sample types using chi-square tests, with alpha-values adjusted for multiple comparisons.

3. Results

(a) Community composition

Hummingbird faeces and bills, feeders, and floral nectar differed in the composition of bacteria and fungi (figure 1, bacteria $p < 0.001$, $R^2 = 0.26$; fungi $p < 0.001$, $R^2 = 0.09$). Bacteria detected on the bills or faeces of *C. anna* and *A. alexandri* differed from nectar which were both largely distinct from feeder communities. Bird-associated bacterial communities were comprised of approximately 40% Proteobacteria, 30–40% Firmicutes, 15–25% Actinobacteria, with a smaller contribution by Fusobacteria and Bacteroidetes. Nectar communities were dominated by Proteobacteria, with 80% of total reads contributed by genera *Acinetobacter* and *Rosenbergiella*, whereas feeders contained mainly *Ralstonia* and *Pseudoarthrobacter*. Feeder and bird samples hosted higher bacterial species richness and Shannon diversity than did nectar (richness $F_{2,68} = 6.36$, $p = 0.003$; Shannon $F_{2,68} = 5.41$, $p = 0.006$). Fungal communities associated with bird samples were comprised of approximately 75% Ascomycetes, whereas feeders and nectar contained less than 50% Ascomycetes (figure 1). Fungal diversity did not differ significantly between feeders, birds and nectar samples (richness $F_{2,58} = 2.09$, $p = 0.13$; Shannon $F_{2,58} = 0.07$, $p = 0.97$). Birds, feeders and nectar were each characterized by distinct bacterial and fungal taxa (figure 2). Birds were characterized by bacterial genera *Corynebacterium*, *Lonsdalea* and *Zymobacter*; and fungal genera including Curvularea and *Lachancea* (figure 2), distinct from taxa characterizing either feeders or nectar samples.

Microbial species composition of bird samples did not differ between species or geographical sampling site (PerMANOVA bacteria: bird species $p = 0.41$, $R^2 = 0.05$; site $p = 0.36$,

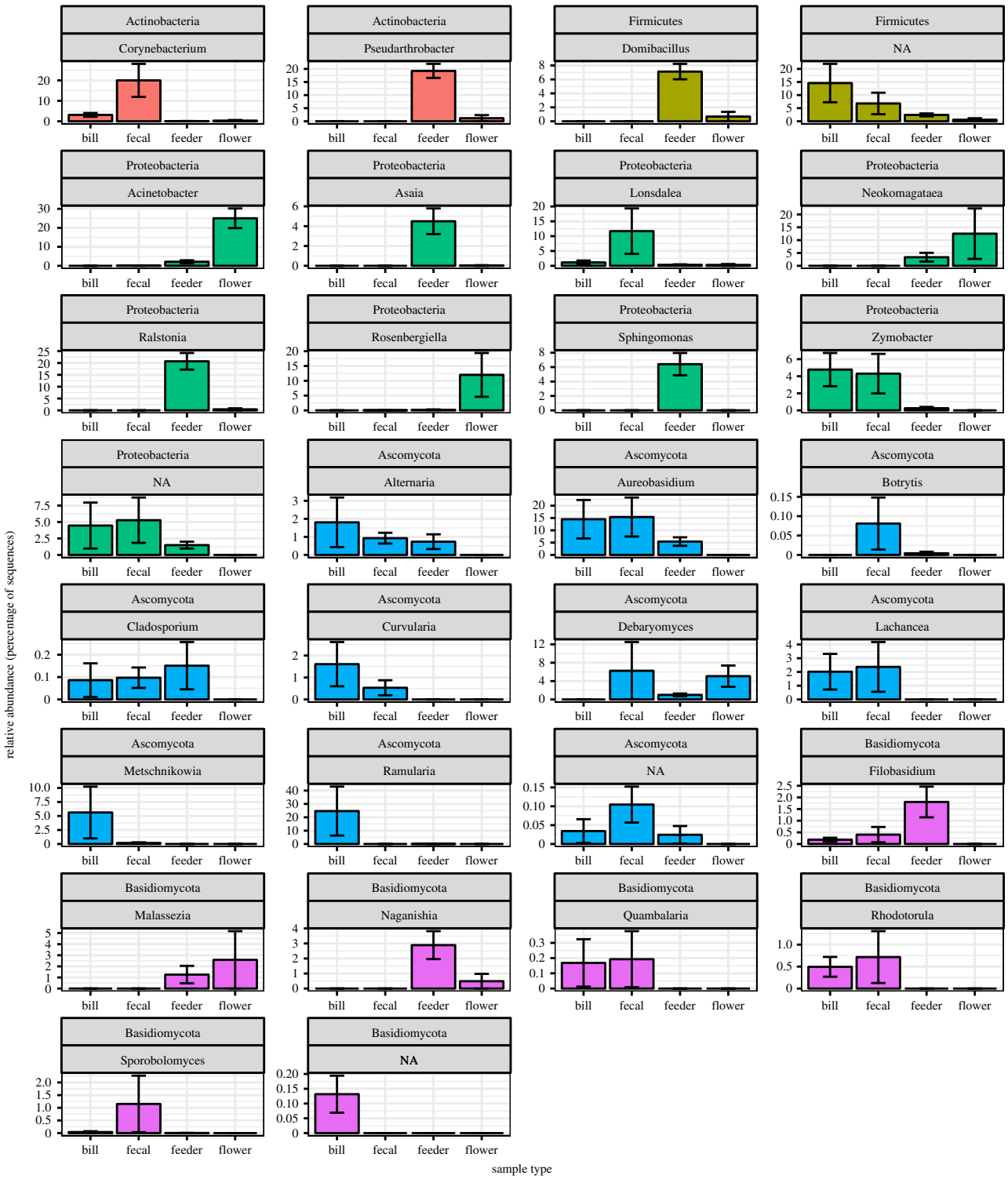

Figure 2. Bacterial and fungal genera found to be differentially abundant among sample types (DESeq2 false discovery rate less than 0.05, performed separately for bacteria and fungi). Bars indicate average per cent of reads within a particular sample type comprised by the focal ASV (of bacterial or fungal reads) ± 1 s.e. NAs indicate that genus could not be assigned with confidence. Flower indicates floral nectar samples. Top labels for each panel and bar colour indicates microbial phyla and bottom indicates genera of the ASV.

$R^2 = 0.07$; fungi: species $p = 0.43$, $R^2 = 0.07$; site $p = 0.59$, $R^2 = 0.06$) but male and female birds differed in microbial species composition (PerMANOVA sex: bacteria $p = 0.02$, $R^2 = 0.12$; fungi $p = 0.03$, $R^2 = 0.16$).

(b) Feeder experiments

Hummingbirds visited open but not bagged or caged feeders. Small arthropods were found in feeder ports in open and caged feeders but not observed feeding.

Bird visitation rate differed among treatments (ANCOVA treatment × time $F_{2,336} = 4.0$, $p = 0.019$), increasing from day 0 to day 2, but decreased for solutions more than 2 days old (figure 3*a*) and the rate of sucrose solution loss mirrored visitation patterns (figure 3; treatment $F_{2,214} = 114.9$, $p < 0.001$; time $F_{1,214} = 44.11$, $p < 0.001$; treatment × time $F_{2,214} = 22.07$, $p < 0.001$). Feeder solutions became more acidic over time, particularly for the open treatment (figure 3*c*; pH treatment $F_{2,159} = 4.9$, $p = 0.008$; time $F_{1,159} = 94.07$, $p < 0.001$; treatment × time $= F_{2,159} = 8.79$, $p < 0.001$). Solution

cloudiness (OD_{600}) increased over time, but did not vary among feeder treatments (figure 3*d*; OD treatment $F_{1,158} = 0.46$, $p = 0.63$; time $F_{1,158} = 103.4$, $p < 0.001$; treatment × time $F_{2,158} = 0.56$, $p = 0.56$).

(c) Culture-based assessment of microbial abundance in feeder sucrose solution samples

The density of culturable bacteria and fungi in feeders increased over time and varied with feeder treatment on most media types (electronic supplementary material, figure S1). Feeders visited by birds had higher bacterial CFU densities on lysogeny broth (electronic supplementary material, figure S2; time $F_{1,160} = 311.21$, $p < 0.001$; treatment $F_{2,160} = 5.87$, $p = 0.003$), MacConkey's agar (electronic supplementary material, figure S1a; all MacConkey's time $F_{1,161} = 9.33$, $p = 0.0026$; treatment $F_{12,161} = 4.7$, $p = 0.0096$; lactose-fermenting: time $F_{1,42} = 10.86$, $p = 0.002$; treatment $F_{2,42} = 1.48$, $p = 0.23$) and higher fungal CFU densities on yeast media (YM) (time $F_{1,159} = 41.93$, $p < 0.001$; treatment $F_{2,159} = 5.07$, $p = 0.007$). Bacterial CFU density on R2A increased over time but did not differ among feeder treatments (electronic supplementary material, figure S2; time $F_{1,170} = 175.30$, $p < 0.001$; treatment $F_{2,170} = 0.61$, $p = 0.54$). Water type influenced CFU density on YM (fungi) and R2A media, at 2 and 4 days of growth (electronic supplementary material, figure S2; $p < 0.05$). Deionized water supported the most fungal growth (electronic supplementary material, figure S2), while tap water or bottled water supported the most bacterial growth for media types where differences were observed.

(d) Microbial communities in feeder solutions

Feeder treatments affected the incidence of live microbial strains found in solutions identified using MALDI-TOF (electronic supplementary material, tables S1 and S2). *Acinetobacter*, *Klebsiella* and *Pseudomonas* were frequently found in bird-visited feeders. Putative pathogens including *Klebsiella* (tentatively identified *Klebsiella oxytoca*) and *Enterobacter* (tentatively identified *Enterobactor kobei*) were identified but uncommon. Culture-independent analysis also showed that visitation treatment influenced bacterial and fungal species composition in feeders (electronic supplementary material, figure S3; PerMANOVA bacteria $p < 0.001$, $R^2 = 0.08$; fungi $p = 0.04$, $R^2 = 0.06$). In bacterial communities (but not fungal communities), feeder age also influenced species composition (bacteria $p < 0.001$, $R^2 = 0.16$; fungi $p = 0.18$, $R^2 = 0.03$).

4. Discussion

Microbial communities in hummingbird feeders are influenced by bird visitation, dynamic over time and distinct from microbial communities in floral nectar. Given the influence of diet on gut microbiome composition across bird species [20,45], the different microbial composition of feeders and flowers may be of consequence to wild birds, as we outline below.

(a) Bird-associated microbial communities

Hummingbird faecal and bill samples contained microbial taxa previously found associated with bird cloaca or gastrointestinal microbial communities, including *Lactobacillus*, *Fructobacillus*, *Corynebacterium* and *Leuconostoc* [46–48]. Other bird-associated taxa including *Rothia*, *Lactococcus*, *Lonsdalea*, *Streptobacillus*, and *Riemerella* were also detected, and despite the association with epizootic infectious disease in poultry and waterfowl [49], no obvious signs of disease were noted in the birds sampled in the current study. Novel taxa, including *Zymobacter* and another ASV from the family Halomonadaceae, were detected in nearly all faecal samples. The related species *Zymobacter palmae* is facultatively anaerobic and ethanol-fermenting, suggesting the potential for this taxon to participate in the hummingbird's unique physiology [26,27]. Previous studies suggest that gut-associated microbial communities may participate in nitrogen recycling through uric acid degradation [25]; our study could not detect if microbes contain genes relating to urease activity, but shotgun metagenomic approaches could examine this potential. Interestingly, we did not detect insect symbionts (e.g. *Wolbachia*, *Buchnera*, *Rickettsia*, etc.) expected based on hummingbird diets (C. Lee 2017, personal observation), suggesting rapid DNA degradation or rapid transit time through the gastrointestinal tract (GI) tract. Although microbes from bill and faecal samples overlap, these regions are distinguished by key taxa, suggesting some differentiation between habitats (figure 2). Nevertheless, further work will be required to determine if microbial communities contribute to the function of the GI tract.

(b) Microbial exchange between birds and feeders

Many microbial ASVs were found on both bird samples (bills or faecal material) and food resources. Notably, the bacteria *Corynebacterium* and fungi *Aureobasidium* (like additional taxa) were highly abundant in faecal samples, but also detected on bills, feeder solutions and in nectar (figure 2), suggesting that transfer of microbes among birds' shared food resources occurs. Despite evidence of microbial transfer, most of the microbial taxa isolated from feeder solutions were not known bird pathogens and instead are taxa frequently isolated from environmental samples. However, a small minority of bacterial and fungal variants identified were identified to genera associated with disease or opportunistic infection in captive birds (e.g. *Aspergillus* and *Candida*) [24] or other animals [50]. Given that 16S sequencing cannot distinguish among strains, our ability to distinguish pathogens is limited. Based on the evidence above and the clear role of bird visitation in the transfer of some microbial taxa, we conclude that microbial pathogens could be transferred via shared food resources, but that neither feeders nor flowers represent abundant sources of bacterial or fungal pathogens. Viral abundance was not assessed, and evidence to demonstrate pathogen transfer would require further experiments or targeted analyses.

(c) Microbial growth in and modification of feeder solutions

All feeder solutions (including bird-excluded feeders) accumulated dense microbial communities (electronic supplementary material, figure S1) associated with changes in solution optical density, and acidification (figure 3). This demonstrates that diverse microbial taxa can survive, grow and alter feeder solutions, and substantial microbial modification of feeder solutions can occur even without

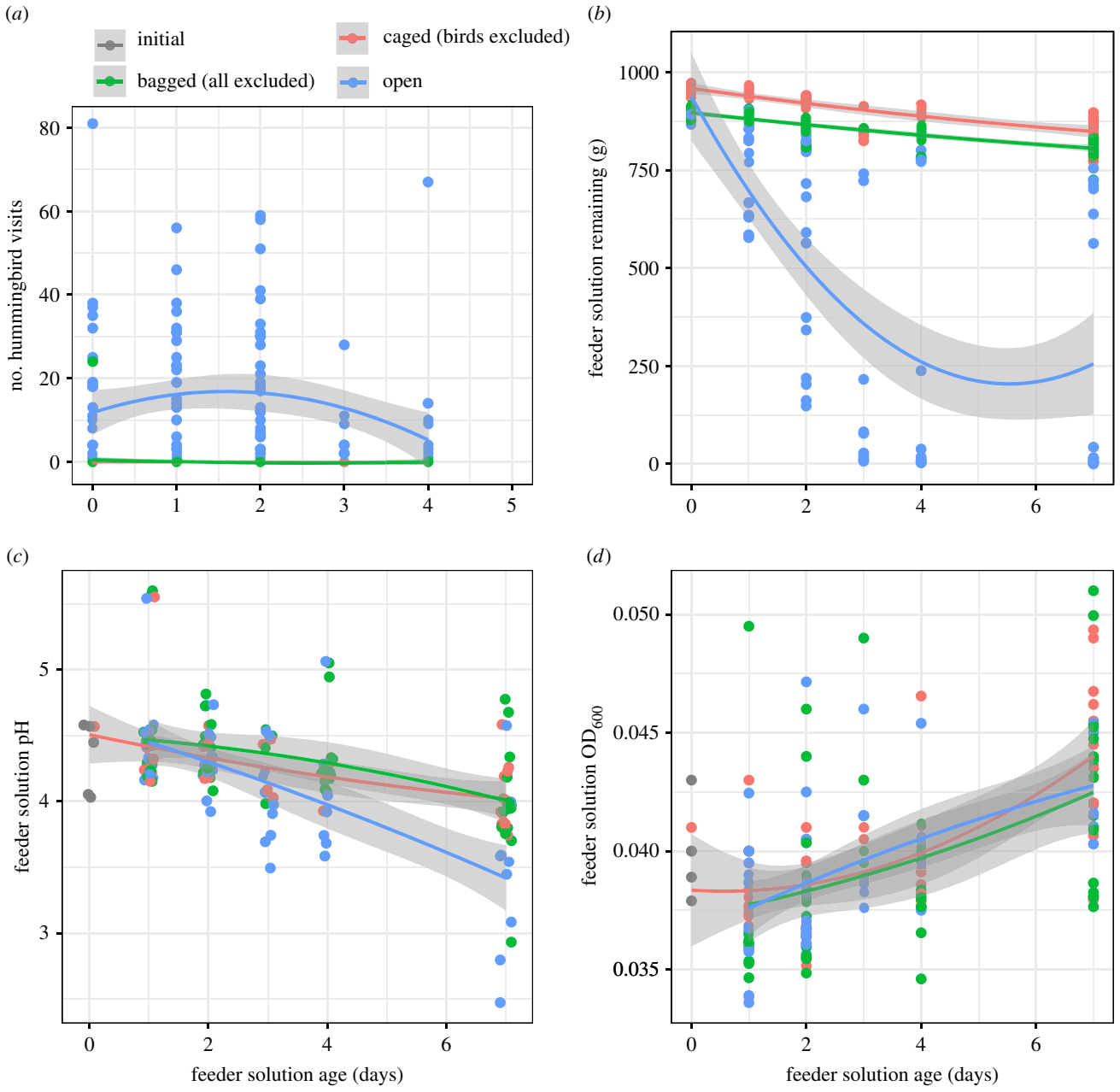

Figure 3. Hummingbird feeder visitation frequency influences hummingbird visits and most characteristics of feeder sucrose solutions. Feeders that were bagged, caged or open to birds vary in (*a*) hummingbird visits in 15 min (*b*) mass of sucrose solution remaining in the feeder (*c*) pH and (*d*) optical density (OD_{600}) of feeder sucrose solutions.

hummingbird visitation to feeders. This contrasts with microbial modification of floral nectar, where pollinator visitation is often required for dispersal of nectar-specialized microbial taxa that influence nectar characteristics (e.g. [30]). However, open feeders had a lower pH than other feeders and were also characterized by the presence of the acetic acid bacteria *Neokomagataea* and *Asaia* (figure 2; electronic supplementary material, figure S3) which produce acidic short chain fatty acids, suggesting that specialized microbes dispersed by birds can strongly modify feeder solutions.

Interestingly, bird visitation to feeders increased as feeder solutions aged, until day 2, then decreased until feeders were depleted (figure 3*a*). It is possible that microbial-mediated changes to feeder solutions could influence attractiveness to birds. Alternatively, birds may require a few days to explore new feeders and engage in territorial or dominance behaviours at the feeders, affecting visitation rate [51]. Further experiments are required to identify the cause of this relationship. In addition, water type affected composition and growth of microbial populations in sucrose solutions (electronic supplementary material, figure S2), but further study is required to inform which water type best supports hummingbird preference or health.

(d) Nectar microbiome composition and bird health

Like floral nectar, sucrose solutions in hummingbird feeders harbour abundant microbial populations, but the microbial species found in feeders differ from those in nectar. Superficially, these microbial habitats appear similar: both nectar and feeder solutions are approximately 10–20% sugar (w/v), low in nitrogen and probably receive similar microbial inputs via birds, sugar-feeding insects and wind dispersal. Nevertheless, our results reveal that the dominant microbial taxa differ substantially between the two habitats. The observed difference may be owing to a difference in solution volume, sugar composition or other physical or chemical characteristics of floral nectar, including antimicrobial proteins [52]. The consequences

of this difference in the microbial community in bird diets remain to be determined; however, the effects of diet on avian microbiome composition have been documented previously [21].

Bird microbiomes vary with a myriad of factors [21,48,53–55]. Hummingbirds' unique physiology as tiny, hovering, nectar-eating (high water, high-sugar, low nitrogen) birds that lack caeca [25,56] may also contribute to microbiome structure, as well as their diet, including feeder use. Although feeders do harbour microbes, at least some hummingbird species seem to benefit from feeders [2]. Moreover, individuals can practice responsible wildlife stewardship while provisioning food resources for local populations. Unlike floral nectar, which is renewed regularly, sucrose solutions in feeders can remain stagnant for extended periods of time and provide a rich substrate for microbial proliferation and a surface for biofilm formation. Because the quality of feeder sugar water solution is shaped by many factors, sugar water should be replenished on a regular basis (ideally at least every 12–24 h during warm temperatures and every 48–72 h during cooler temperatures). In addition, routine feeder hygiene is essential to prevent excessive microbial growth and solution spoilage.

(e) Conclusions

Anthropogenic provisioning can positively influence population size and health of diverse bird species but also has the potential for negative influence on birds. Our study provides a unique comparison of the microbial composition of sugar water from hummingbird feeders versus floral nectar sources and hummingbird bill and faecal material, providing further insight into how the introduction of microbes to the sucrose solution shapes the microbial community over time. Although our study does not directly inform hummingbird health outcomes, shifts in microbial composition in bird diets may influence bird microbiomes as a consequence. Our results highlight the need to understand the effects of consumed microbes on the health of free-ranging hummingbirds, particularly with regard to anthropogenic effects on wildlife.

Ethics. All animal procedures related to this study were approved by the UC Davis Institutional Animal Care and Use Committee (Protocol number 20355) and California State and United States Federal Agencies (LAT; USGS BBL Permit no. 23947, US Fish and Wildlife Permit MB55944B-2 and California Department of Fish and Wildlife SC-013066).

Data accessibility. Sequence data are available at NCBI SRA under BioProject no. PRJNA512536, and datasets, bioinformatics scripts and metadata used in the current study are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.hr5t61c [57].

Competing interests. We declare we have no competing interests.

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