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RESEARCH ARTICLE

# Experimental test of microbiome protection across pathogen doses reveals importance of resident microbiome composition

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**One sentence summary:** Bacterial communities can be key in protecting hosts against pathogens, but that protection depends on which bacteria make up resident communities during pathogen invasion. Photo credit: Sara Teemer

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## ABSTRACT

The commensal microbes inhabiting a host tissue can interact with invading pathogens and host physiology in ways that alter pathogen growth and disease manifestation. Prior work in house finches (*Haemorhous mexicanus*) found that resident ocular microbiomes were protective against conjunctival infection and disease caused by a relatively high dose of *Mycoplasma gallisepticum*. Here, we used wild-caught house finches to experimentally examine whether protective effects of the resident ocular microbiome vary with the dose of invading pathogen. We hypothesized that commensal protection would be strongest at low *M. gallisepticum* inoculation doses because the resident microbiome would be less disrupted by invading pathogen. Our five *M. gallisepticum* dose treatments were fully factorial with an antibiotic treatment to perturb resident microbes just prior to *M. gallisepticum* inoculation. Unexpectedly, we found no indication of protective effects of the resident microbiome at any pathogen inoculation dose, which was inconsistent with the prior work. The ocular bacterial communities at the beginning of our experiment differed significantly from those previously reported in local wild-caught house finches, likely causing this discrepancy. These variable results underscore that microbiome-based protection in natural systems can be context dependent, and natural variation in community composition may alter the function of resident microbiomes in free-living animals.

**Keywords:** bird; conjunctivitis; *Mycoplasma gallisepticum*; house finch; ocular microbiome; host–microbe interactions

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## INTRODUCTION

Understanding the varied roles of microbial communities in mediating host–pathogen interactions across ecological contexts has become increasingly important. Bacterial community members can interact with invading pathogens along the spectrum from facilitative to antagonistic interactions (Daskin and Alford 2012; Boon *et al.* 2014; Oliver, Smith and Russell 2014; Becker *et al.* 2015b). Evidence of antagonistic interactions, whereby bacterial taxa inhibit pathogens in or on a host, has been found across multiple host–pathogen systems. In some cases, the structure of the microbial community at the site of pathogen invasion is predictive of disease severity (Lauer *et al.* 2008; Becker *et al.* 2015a; Holden *et al.* 2015; Harris, de Roode and Gerardo 2019). Further, studies using antibiotics to perturb resident microbiomes have found increases in host morbidity and mortality when challenged with a pathogen, including higher pathogen loads and greater sickness behaviors (Sekirov *et al.* 2008; Becker and Harris 2010; Weyrich *et al.* 2014; Holden *et al.* 2015; Kugadas *et al.* 2016; Thomason *et al.* 2017b). These results indicate that intact microbiomes, i.e. resident microbiomes unmanipulated by antibiotics, often act in a protective role for hosts.

Indeed, microbiomes play an important part in a host's innate immune system. Where they interact directly with invading pathogens, microbial communities may act as the first line of defense against invasion. Bacterial communities can inhibit invading pathogens through direct and indirect interactions (Sassone-Corsi and Raffatellu 2015; McLaren and Callahan 2020). For example, some cutaneous bacteria produce metabolites that inhibit growth of an invading fungal pathogen on frogs and salamanders (Brucker *et al.* 2008a,b; Becker *et al.* 2009; Harris *et al.* 2009). In other instances, commensal bacteria may outcompete pathogens for space or nutrients (Sassone-Corsi and Raffatellu 2015; Wei *et al.* 2015; McLaren and Callahan 2020). Microbes also help to maintain and trigger immune responses against pathogen invasion and disease, both locally and elsewhere in the body (Ichinohe *et al.* 2011; McDermott and Huffnagle 2014; Thaiss *et al.* 2016; Shukla *et al.* 2017). When resident microbiomes are experimentally disrupted, pathogens can even exhibit distinct virulence phenotypes (Thomason *et al.* 2017b). Thus, microbes can inhibit infection and disease severity in hosts via a range of potential mechanisms.

Overall, intact microbiomes appear to provide protection from infection and disease across a variety of hosts and pathogens, but the extent of protection that the microbiome provides is likely to depend on the dose of invading pathogen. Pathogen exposure dose predicts the degree of resulting host morbidity and mortality in diverse disease systems (e.g. Ebert, Zschokke-Rohringer and Carius 2000; Brunner, Richards and Collins 2005; Leon and Hawley 2017); however, potential interactions between the host microbiome and pathogen dose on infection outcomes have rarely been examined. In one study, the presence of an intact microbiome increased the infective dose of *Bordetella pertussis* in mice by three orders of magnitude compared with infective doses in mice given antibiotics to knock down the native microbiome (Weyrich *et al.* 2014), consistent with the hypothesis that microbiome-mediated protection may vary with pathogen dose. In natural systems, pathogen exposures commonly occur at low doses, which may not, in single exposure events, cause disease (Dhondt *et al.* 2007; Regoes 2012; Aiello *et al.* 2016). Heterogeneity of disease and pathogen load in animal populations could result, in part, from the interplay between exposure dose and host protection by the microbiome.

In this study, we experimentally assessed the hypothesis that the degree of protection provided by intact microbiomes varies with the dose of invading pathogen. House finches (*Haemorhous mexicanus*) develop mycoplasmal conjunctivitis after infection by the bacterial pathogen *Mycoplasma gallisepticum* (Kollias *et al.* 2004). Previous experimental work in this system using ocular antibiotics found protective effects of the ocular microbiome against *M. gallisepticum* conjunctival infection loads and disease severity (Thomason *et al.* 2017b). Treatment with antibiotics prior to inoculation was also associated with increased activity of known virulence-associated phenotypes (sialidase activity and cytadherence) in output *M. gallisepticum* isolates. Thus, while the exact causal mechanisms remain unclear, intact ocular microbiomes appear to provide protection against both *M. gallisepticum* infection and disease when inoculation doses are relatively high, as was the case in Thomason *et al.* (2017b). *Mycoplasma gallisepticum* also interacts with the resident microbiome to cause shifts in the ocular bacterial community composition after *M. gallisepticum* invasion (Thomason *et al.* 2017a).

To assess potential interactions between pathogen invasion dose and the resident ocular microbiome, here we compared infection and disease severity in control and antibiotic-perturbed microbiome treatments for each of five *M. gallisepticum* inoculation dose concentrations. We perturbed the ocular microbiome with cefazolin, a $\beta$-lactam antibiotic to which *M. gallisepticum* is intrinsically resistant due to the absence of a cell wall. After microbiome treatment (antibiotic or control), birds were conjunctivally inoculated with a given *M. gallisepticum* dose. We monitored pathology and *M. gallisepticum* loads in the conjunctiva throughout infection, and measured sialidase activity in output *M. gallisepticum* isolates at peak infection. We predicted that the protective effects of intact ocular microbiomes would be strongest at lower infective pathogen doses, because the microbiome would be less disrupted by invading pathogen.

## METHODS

### Bird capture

Hatch-year house finches ($n = 107$) were captured from June 2019 to August 2019 in Montgomery County, Giles County and Radford, VA. Birds were housed singly or in pairs in cages (76 cm × 46 cm × 46 cm) and were provided a constant 12:12 photoperiod and food and water *ad libitum*. We monitored birds for signs of disease every 3–5 days postcapture for 2 weeks and then collected blood samples to assess *M. gallisepticum*-specific antibody concentrations. The combination of age (hatch year) and serological status allowed us to ensure that birds included in our *M. gallisepticum* inoculation treatments did not have prior exposure to *M. gallisepticum* in the wild. No experimental birds had conjunctivitis pathology at any point prior to inoculation, and only birds negative for anti-*M. gallisepticum* antibodies (Hawley *et al.* 2011) were included in treatment groups inoculated with *M. gallisepticum* (see the 'Experimental design' section). Birds were single-housed starting 13 days before experimental *M. gallisepticum* inoculation (i.e. post-inoculation day [PID] –13). Birds were captured under VDGIF (061440) and USFWS (MB158404-0) permits. Experimental procedures were approved by Virginia Tech's Institutional Animal Care and Use Committee.

### Experimental design

Experimental birds were divided among 10 treatment groups in a fully factorial design (Table 1), with the highest *M. gallisepticum* concentration similar to that used by Thomason *et al.* (2017b).

**Table 1.** Sample sizes for house finches in each of 10 treatment groups in a fully factorial design among five *M. gallisepticum* doses and two ocular microbiome treatments ($n = 107$ total).

| | Pathogen dose concentrations (color-changing units/mL) | | | | |
|---|---|---|---|---|---|
| Microbiome treatment | 0 (*M. gallisepticum* control) | $3 \times 10^1$ | $3 \times 10^2$ | $3 \times 10^3$ | $3 \times 10^4$ |
| Catch-only (microbiome control) | 11 | 8 | 11 | 12 | 12 |
| Antibiotic | 11 | 8 | 11 | 12 | 11 |

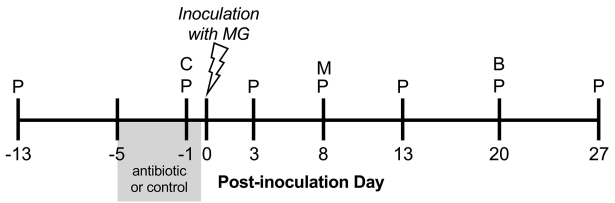


**Figure 1.** Experimental timeline based on PID with respect to inoculation with *M. gallisepticum*. To detect whether the protective effects of an intact ocular microbiome vary with the inoculation dose of *M. gallisepticum* in house finches, we collected: P = pathology scores and nucleic acid swab samples, C = conjunctival culture swab samples from a subset of birds to confirm antibiotic efficacy (Supporting Information), M = *M. gallisepticum* sialidase phenotype swabs and B = blood samples to determine antibody levels (Supporting Information).

Treatment groups had as close to 50:50 sex ratios as possible. For logistical purposes, birds were split between two temporal groups, with all treatments present in each group and a 4-week lag between the first and second group. Because birds given the lowest dose in the first temporal group did not develop any pathology, we reallocated birds in the second temporal group to focus on higher dose treatments.

We used ocular administration of the $\beta$-lactam antibiotic cefazolin (West-Ward Pharmaceuticals, Eatontown, NJ) to disrupt the resident microbiome, as previously described (Thomason *et al.* 2017b). Cefazolin was rehydrated in phosphate buffered saline and diluted to 33 mg/mL in artificial tears (Bausch + Lomb Advanced Eye Relief Dry Eye). We administered the antibiotic by droplet instillation of 15 μL into each conjunctiva three times per day (8:00, 12:30, 17:00) for 5 days (Fig. 1). Because saline administration as a control may have disrupted the resident microbiome, control catch-only birds were caught at the same times as those given the antibiotic, held briefly and released. We used culture techniques to confirm the effectiveness of the antibiotic in reducing resident conjunctival bacterial populations (Supporting Information).

We inoculated house finches with the VA1994 *M. gallisepticum* isolate (7994-1 6 P 9/17/2018) because the ocular microbiome provided protection against this isolate in prior work (Thomason *et al.* 2017b). *Mycoplasma gallisepticum* was diluted in antibiotic-free Frey's broth medium on the day of inoculation. Experimental birds were inoculated by droplet instillation with 70 μL of *M. gallisepticum* diluted to a given concentration (depending on their dose treatment), split between the two conjunctivae (i.e. ~35 μL per eye). Infection controls ('*M. gallisepticum* control', Table 1) were given 70 μL of antibiotic-free Frey's media.

## Pathology, swabbing and *M. gallisepticum* quantification

We collected pathology data and conjunctival swab samples from birds at multiple time points from PID –13 to PID 27 (Fig. 1). Pathology was scored for each conjunctiva on a 0–3 scale, with scores made while blind to a given bird's treatment. Briefly, no clinical signs of conjunctivitis are scored as 0, a score of 1 represents minor swelling around the eye or minor conjunctival eversion, moderate swelling and eversion are scored as 2, and severe swelling, eversion and exudate are scored as 3 (Sydenstricker *et al.* 2005). We summed the scores between the two sides within each time point, resulting in a value from 0 to 6 per bird per time point (Hawley *et al.* 2011). No experimental birds had signs of disease before inoculation. After measuring pathology, we swabbed the conjunctiva with flocked swabs (Copan FLOQSwabs, Copan Diagnostics Inc., Murrieta, CA) lubricated with artificial tears, combining the two swabs from each bird into 300 μL Zymo DNA/RNA Shield (Zymo Research, Irvine, CA). On PID –1, a subset of birds' conjunctival swabs was cultured to confirm the antibiotic effectiveness (Supporting Information). On PID 8, after the DNA/RNA-preserved swab, we swabbed the conjunctivae a second time for *M. gallisepticum* culture and phenotyping of sialidase activity. On the day of collection, *M. gallisepticum* phenotype swabs from *M. gallisepticum* treatment groups were shipped on ice to the University of New England in 3.0 mL Remel M5 media, where they were subjected to a sialidase activity assay after growth as previously described (Thomason *et al.* 2017b) (Supporting Information).

DNA was extracted from conjunctival swab samples from PIDs –1, 3 and 13 with the Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) protocol for Gram-positive bacteria (see the Supporting Information for modifications). To quantify *M. gallisepticum* in swab samples, we conducted quantitative polymerase chain reaction (qPCR) on the MGC2 gene as previously described (Hawley *et al.* 2013), with pathogen load analyzed as $\log_{10}(\text{load} + 1)$. Data from PID –1 was used to verify that the birds did not have *M. gallisepticum* prior to experimental inoculation.

## Statistical analyses

We used R v4.0.2 in RStudio v1.3.1093 to conduct all statistical analyses (R Development Core Team 2015; RStudio Team 2020). First, we tested the effectiveness of the antibiotic in knocking down the resident ocular microbiomes, comparing optical density (OD) values of cultured swab samples between antibiotic and catch-only (microbiome control) birds using analysis of variance (ANOVA). We then analyzed pathology, pathogen load, probability of infection (defined below) and *M. gallisepticum* sialidase phenotype data to detect how the interaction between microbiome treatment and *M. gallisepticum* dose affected infection and disease severity. We used these analyses specifically to test the prediction that ocular microbiomes have stronger protective effects at lower pathogen infective doses. We ran analyses on data from all experimental birds, as well as a data set including only birds inoculated with *M. gallisepticum*. These two data sets provided similar results, so below we present analyses of just *M. gallisepticum*-inoculated birds (see the Supporting Information for full data set results).

To determine the effects of *M. gallisepticum* dose and microbiome treatments on disease severity over time, we modelled pathology data after inoculation using negative binomial generalized linear mixed effects models (GLMM) in the glmmTMB package (Brooks *et al.* 2017), with bird ID as a random variable. We used model simplification to arrive at a minimal model addressing our question, sequentially removing interaction terms and covariates with $P < 0.1$ from Wald's chi-squared tests using the car package (Fox and Weisberg 2019). The full model included interactions between *M. gallisepticum* dose ($\log_{10}$[*M. gallisepticum* concentration + 1]) and PID, *M. gallisepticum* dose and microbiome treatment, and microbiome treatment and PID, along with sex and temporal group as covariates. Regardless of P-value, *M. gallisepticum* dose and microbiome treatment were kept in the final model, though we removed their interaction from the model where applicable. We used Akaike information criterion (AIC) to determine if a better model fit was provided if *M. gallisepticum* dose or PID were analyzed as quadratic variables ($\log_{10}$[*M. gallisepticum* concentration + 1]$^2$ and PID$^2$, respectively).

To determine if pathogen load (our metric of infection severity) differed among the treatment groups, we analyzed load data from PIDs 3 and 13 using linear mixed effects models in the lme4 package (Bates *et al.* 2015). We conducted model simplification as above, except PID was only included as an ordinal variable because we had fewer time points available.

Pathogen load data were also used to assess successful infection in experimental birds, conservatively defined as any post-inoculation *M. gallisepticum* load ($\log_{10}$[load + 1]) greater than 3.1 $\log_{10}$ copies as per prior work (Adelman *et al.* 2015; Leon and Hawley 2017). We analyzed whether microbiome treatment affected the probability of infection using binomial GLM with a probit link. The main predictor variables of interest included the interaction between microbiome and *M. gallisepticum* dose treatments, with host sex and temporal group as covariates.

When sialidase activity was present in the cultured *M. gallisepticum* isolates from PID 8, sialidase activity (mU/mg total protein) was analyzed using ANOVA, with the same predictor variables as in the probability of infection analyses.

### Describing starting ocular microbiomes

A subset of ocular swab samples were used to compare the resident ocular microbiomes in birds at the beginning of this experiment with those in a previous study assessing the effect of antibiotic on responses to *M. gallisepticum* (Thomason *et al.* 2017b). In order to describe the resident microbial community without confounding effects of experimental perturbation, samples used here were collected either prior to ocular antibiotic treatment at PID –13 ($n = 7$) or on PID –1 from catch-only birds ($n = 4$ microbiome controls). We used the Zymo Quick DNA/RNA Microprep extraction kit (Zymo Research, Irvine, CA) to extract samples, eluting in 15 μL DNase/RNase-free water. We conducted library prep for Illumina MiSeq sequencing as previously described, amplifying a portion of the V4 region of 16S bacterial rRNA using 515F and barcoded 806R primers (Caporaso *et al.* 2012; Thomason *et al.* 2017a). Single-end sequence reads were demultiplexed using QIIME2, and reads were trimmed and quality-filtered with a maximum of two expected errors using the DADA2 package (Callahan *et al.* 2016; Bolyen *et al.* 2019). We similarly filtered and trimmed reads from 14 microbiome control PID –1 samples from Thomason *et al.* (2017b), and combined those reads from 2016 with our 2019 data set to denoise reads to detect amplicon sequence variants (ASVs) with DADA2. Sample collection protocols differed for the 2016 data set (which used sterile cotton swabs, storage in tryptose phosphate broth, and DNA extraction with Qiagen DNeasy Blood and Tissue Kit), but PCR and sequencing protocols were identical; thus, the data sets should be broadly comparable (Fouhy *et al.* 2016; Panek *et al.* 2018; Bjerre *et al.* 2019). We assigned taxonomy to our combined read file with the Silva v132 database and filtered out nonbacterial, chloroplast and mitochondrial reads. Sequences are openly available on figshare (DOI: 10.6084/m9.figshare.14541390).

We used analysis of composition of microbiomes (ANCOM) on unrarefied data in QIIME2 to assess differential abundance of bacterial genera between the two study years. After inspecting rarefaction curves, we rarefied the data to 7500 reads per sample, removing three samples from the 2016 data. We used QIIME2 to calculate ASV richness, alpha diversity metrics (Pielou's evenness, Shannon's diversity metric and Faith's phylogenetic diversity) and beta diversity (weighted and unweighted UniFrac distances). Analyses of these metrics focused on detecting differences between the study years in starting microbial communities in the absence of antibiotic perturbation and prior to pathogen inoculation. We compared ASV richness and alpha diversity metrics between the sampling years with Kruskal–Wallis tests. With the vegan package (Oksanen *et al.* 2018), we compared beta diversities between sampling years with permutational ANOVA (PERMANOVA) and further tested for differences in multivariate dispersion with permutational multivariate analysis of beta-dispersion.

## RESULTS

Culture-based assays indicated that the topical ocular antibiotic significantly suppressed the overall abundance of viable resident bacteria (Fig. S1, Supporting Information), consistent with prior work (Thomason *et al.* 2017b) and indicating that our microbiome antibiotic treatment successfully perturbed the resident ocular microbiome. Following antibiotic treatment, but just prior to *M. gallisepticum* inoculation (PID –1), none of the experimental birds had pathology or detectable *M. gallisepticum* by qPCR.

### Disease severity

Overall, we found no support for our primary hypothesis that *M. gallisepticum* dose and microbiome antibiotic treatment would interact to influence disease severity. Both *M. gallisepticum* dose and PID were significant predictors of disease severity in our simplified model (Table 2), with pathology score increasing with *M. gallisepticum* dose, consistent with prior work (Leon and Hawley 2017). However, microbiome perturbation with antibiotics was not significantly predictive of disease severity (Table 2), either alone or in interaction with *M. gallisepticum* dose, such that the interaction was removed during model simplification. Antibiotic treated birds generally had lower pathology scores, in the opposite direction of prior work (Thomason *et al.* 2017b), although this was not statistically significant ($P = 0.055$; Fig. 2A; Fig. S2, Supporting Information). Because a single house finch in the catch-only (microbiome control) $3 \times 10^3$ color-changing units/mL (CCU/mL) *M. gallisepticum* dose treatment had abnormally high pathology scores from PID 8 onward, we also evaluated the simplified model without this individual. This analysis suggested that this one individual was important in driv-

**Table 2.** Final models of pathology, pathogen load, probability of infection and sialidase activity for experimental house finches that either had microbiomes left intact or perturbed with antibiotic prior to inoculation with *M. gallisepticum* ($n = 85$ inoculated birds). Bold denotes significant *P*-values (<0.05). PID = post-inoculation day. Italicized *P*-values indicate where trends were greatly influenced by an outlier. Outlier in pathology data was a bird with severe pathology in the microbiome control, $3 \times 10^3$ CCU/mL *M. gallisepticum* dose group.

| Response | Predictors | Estimate ± SEM | $\chi^2$ | *P* | *P* (no outlier) |
|---|---|---|---|---|---|
| Pathology | | | | | |
| | *M. gallisepticum* dose | 1.311 ± 0.179 | 53.43 | **<0.0001** | **<0.0001** |
| | Microbiome treatment | –0.523 ± 0.272 | 3.69 | 0.055 | 0.11 |
| | PID$^2$ | –0.002 ± 0.0004 | 29.05 | **<0.0001** | **<0.0001** |
| Pathogen load | | | | | |
| | *M. gallisepticum* dose × PID | –0.605 ± 0.136 | 19.93 | **<0.0001** | |
| | *M. gallisepticum* dose | 1.962 ± 0.130 | 227.53 | **<0.0001** | |
| | Microbiome treatment | –0.360 ± 0.278 | 1.68 | 0.19 | |
| | PID | 0.975 ± 0.446 | 4.78 | **0.029** | |
| Probability of infection | | | | | |
| | *M. gallisepticum* dose | 2.993 ± 0.629 | 91.17 | **<0.0001** | |
| | Microbiome treatment | –0.652 ± 0.596 | 1.29 | 0.26 | |
| *M. gallisepticum* sialidase phenotype ($n = 48$) | | | *F*-value | | |
| | *M. gallisepticum* dose | –13.48 ± 12.32 | 0.014 | 0.91 | |
| | Microbiome treatment | –5.62 ± 17.83 | 0.048 | 0.83 | |
| | Temporal group | –41.59 ± 17.47 | 8.30 | **0.006** | |

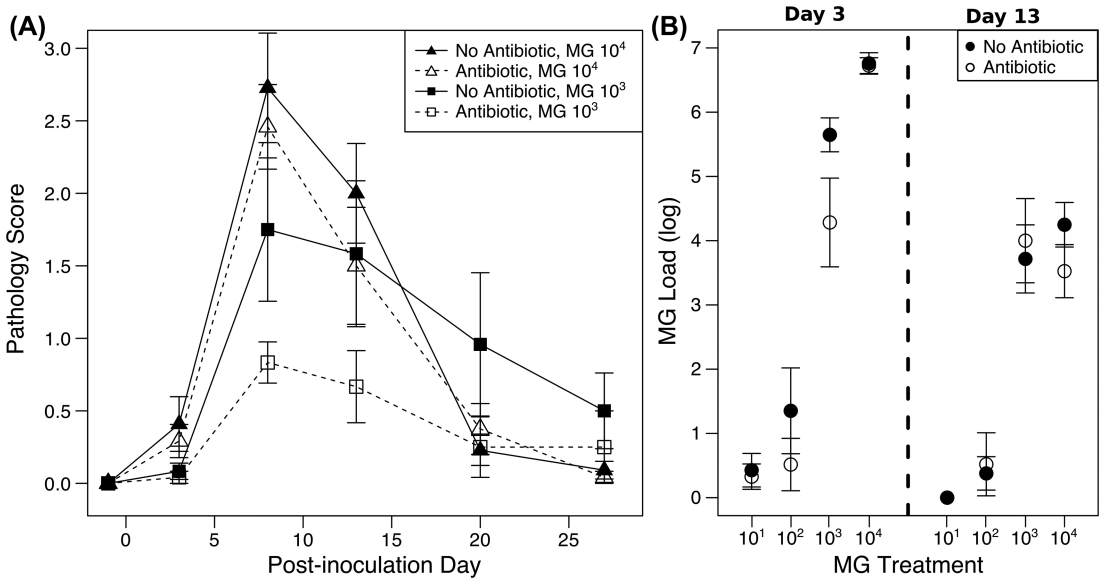


**Figure 2.** Intact ocular microbiomes were not protective against *M. gallisepticum* (MG) disease or infection severity in house finches. **(A)** Pathology scores for the two highest *M. gallisepticum* dose concentrations (only two doses shown for ease of visualization, but see Fig. S2, Supporting Information, for lower dose results) across PIDs. Squares indicate $3 \times 10^3$ CCU/mL *M. gallisepticum* dose. Triangles indicate $3 \times 10^4$ CCU/mL *M. gallisepticum* dose. **(B)** *Mycoplasma gallisepticum* loads on days 3 and 13 post-inoculation were predicted by *M. gallisepticum* dose, PID and their interaction, but not antibiotic treatment. Only birds in the four treatment groups with *M. gallisepticum* dose > 0 are shown for visual clarity. In both panels, open symbols signify birds given antibiotic to perturb the resident microbiome, and closed symbols signify control birds not given antibiotic. Points and bars signify mean and standard error.

ing the marginal effects of microbiome treatment on pathology (Table 2).

## Pathogen load and probability of infection

We used linear mixed effects models to detect effects of microbiome treatment and *M. gallisepticum* dose on infection loads quantified on PIDs 3 and 13. Similar to the results for disease severity, we found no support for interactions between *M. gallisepticum* dose and microbiome treatment on infection load. In the simplified model, *M. gallisepticum* load was significantly predicted by *M. gallisepticum* dose, PID and their interaction, but not microbiome treatment (Table 2; Fig. 2B).

We further used pathogen load to determine if the birds were successfully infected and whether probability of infection differed with microbiome and *M. gallisepticum* dose treatments. Consistent with *M. gallisepticum* load results, probability of infection differed based on *M. gallisepticum* dose, but not microbiome antibiotic treatment, with birds given higher *M. gallisepticum* doses more likely to become infected (Table 2).

### *Mycoplasma gallisepticum* sialidase phenotype

Of the 85 birds inoculated with *M. gallisepticum*, 48 cultured swab samples from PID 8 grew and exhibited sialidase activity (*M. gallisepticum* doses: $3 \times 10^1$, $n = 1$; $3 \times 10^2$, $n = 4$; $3 \times 10^3$, $n = 20$; $3 \times 10^4$, $n = 23$). From linear models, only temporal group was a significant predictor of sialidase activity (Table 2; Fig. S3, Supporting Information), with *M. gallisepticum* isolated from birds in temporal group 2 exhibiting lower sialidase activity. Neither microbiome treatment, nor *M. gallisepticum* dose, was significant.

### Resident ocular microbiomes

Prior to *M. gallisepticum* inoculation, resident ocular microbiomes from the present experiment were dominated by *Proteobacteria* and *Actinobacteria*, with *Sphingomonas*, *Pseudomonas*, *Comamonas*, *Mycobacterium* and other genera in greater abundance in the 2019 samples based on ANCOM compared with house finch conjunctival samples collected for a previous experiment from birds captured and housed at the same localities and conditions (Thomason *et al.* 2017b) (Fig. 3; Table S4, Supporting Information). In contrast, resident ocular microbiomes sampled in 2016 were dominated by *Firmicutes*, with greater abundance of *Lactococcus* and *Enterococcus* in 2016 compared with our 2019 samples (Fig. 3; Table S4, Supporting Information). Though the genus *Lactococcus* was the dominant taxon in finch ocular microbiomes in 2016, this genus accounted for <0.5% of the reads in the three (of 11) 2019 samples where it was detected. All samples were collected from ocular microbiomes that were not perturbed with antibiotic.

There were also quantifiable differences between study years with respect to alpha and beta diversity of resident bacterial communities. Using Kruskal–Wallis tests, the 2016 and 2019 samples significantly differed in ASV richness and the three alpha diversity metrics ($P \leq 0.0001$ each; Table S2 and Fig. S4, Supporting Information). Similarly, samples from the two study years significantly differed in beta diversity (weighted and unweighted UniFrac $P = 0.001$; Table S3 and Fig. S5, Supporting Information). Analysis of dispersion detected significant differences in dispersion between the years ($P \leq 0.01$ each). Thus, we found strong support for differences in microbiome composition and structure between studies.

## DISCUSSION

This experiment assessed dose-dependent protective effects of the house finch ocular microbiome against the conjunctival pathogen *M. gallisepticum*. Although both disease and infection severity increased with *M. gallisepticum* inoculation dose as expected based on previous work in this and other systems (Timms *et al.* 2001; Regoes, Ebert and Bonhoeffer 2002; Spekreijse *et al.* 2011; Leon and Hawley 2017), we did not detect the presence of protective effects of the intact microbiome at any inoculation dose, including the dose used in prior work that found significant protective effects of intact ocular microbiomes (Thomason *et al.* 2017b). Thus, we were unable to adequately test whether protective effects of the ocular microbiome are dose dependent. Instead, we show that variation in the resident microbiome present in the conjunctiva at the time of pathogen inoculation likely explains the discrepancies between ours and the previous study. This and other studies finding that microbiome variation at the time of pathogen invasion predicts variation in disease (e.g. Becker *et al.* 2015a; Walke *et al.* 2015; Nava-González *et al.* 2021) underscore the potential for microbiome function to be highly variable and context dependent in natural systems.

We found that the ocular microbiomes at the start of this experiment differed notably from those in previous experiments (Thomason *et al.* 2017a,b), such that the bacteria contributing to the microbiome's protective effects in Thomason *et al.* (2017b) were likely not present or were very rare in our study. Specifically, the ocular communities in birds in 2016, where protective effects of the ocular microbiome were detected, were largely composed of the bacterial genus *Lactococcus*, with over 70% of the relative abundance representing this genus. A high relative abundance of *Lactococcus* was also documented in another prior study describing ocular bacterial microbiomes and their shifts after *M. gallisepticum* inoculation (Thomason *et al.* 2017a). *Lactococcus* can inhibit growth of other bacteria through lactic acid production and other antimicrobial metabolites, and lactic acid-producing bacteria can provide important protection when they dominate a microbiome (Røssland *et al.* 2005; Vásquez *et al.* 2012; O'Hanlon, Moench and Cone 2013). In birds from the present experiment, which were captured from the same areas of Virginia as finches in past studies, we found that *Lactococcus* comprised <1% of the ocular communities. Importantly, the two groups of microbiome samples were collected and processed under slightly different protocols, which may explain some of the variation between sampling years, given that microbiome studies often find differences in community composition and diversity across extraction protocols (Fouhy *et al.* 2016; Bjerre *et al.* 2019). However, distinct extraction protocols are unlikely to cause the degree of divergence between predominant taxa detected between the two studies here (Fig. 3). Overall, the low abundance of *Lactococcus* and large shift in the resident microbiome compared with previous descriptions could have resulted in its lack of protective function against mycoplasmal conjunctivitis in this study. Further, the dominant ocular community members in this experiment may not have responded to experimental antibiotic treatment in the same way as the prior *Lactococcus*-dominated communities, potentially limiting our ability to adequately disrupt the ocular microbial community and thus test its protective function. Though we used culture-based methods to verify that administering cefazolin reduced ocular bacterial growth in this study, we also detected abundant genera in the sequenced ocular samples that may not be susceptible to this antibiotic, such as *Pseudomonas* and *Sphingomonas* (Reller *et al.* 1973; Mustafa, Maulud and Hamad 2018). Thus, it is challenging to compare the role of these distinct ocular communities in ocular health and disease.

Past work also found that perturbing the ocular microbiome increased activity of sialidase enzymes associated with *M. gallisepticum* virulence (Thomason *et al.* 2017b), and that activity was correlated with variation in disease severity among individuals. Here, we did not detect any influence of ocular microbiome disruption on the sialidase activity of output *M. gallisepticum* isolates, consistent with the absence of significant effects of antibiotic on disease severity. Modulation of sialidase activity, and often its functional balance with host cell cytadherence, is an important mechanism for fine-scale rheostasis (i.e. regulation in a changing environment) during infection for many pathogens. Overall, the differences between these results and those of past work suggest that prior protective effects, including the potential modulation of sialidase activity in *M. gallisepticum*, were likely driven by *Lactococcus* and other resident community members that were largely absent in the resident ocular microbiomes in this experiment.

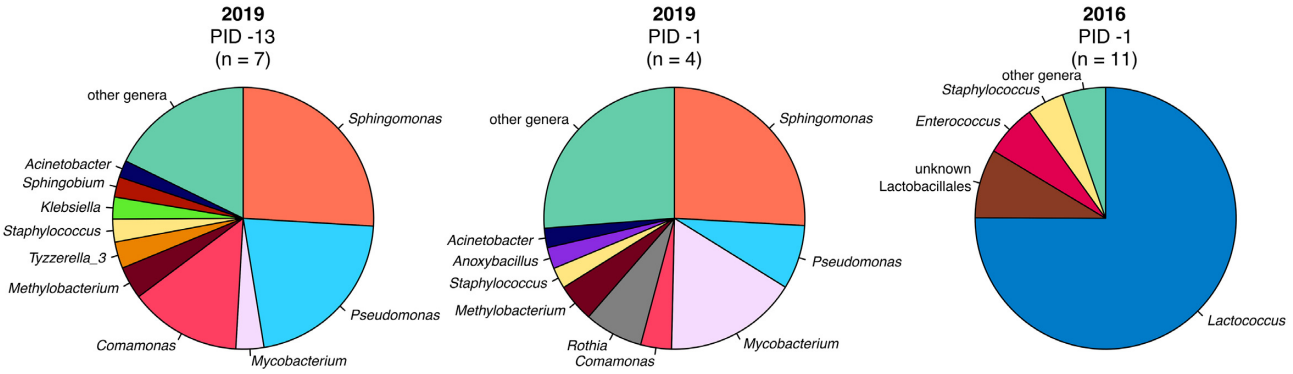


**Figure 3.** Abundant genera in the resident ocular microbiomes sampled in 2019 (this experiment) compared with those sampled in 2016 (far right; Thomason *et al.* 2017b). All microbiomes were sampled either 13 days or 1 day prior to inoculation with *M. gallisepticum* and thus represent resident microbiome communities (birds given ocular antibiotic were excluded for clarity). Proportions based on relative abundance of rarefied reads. Samples grouped by PID and treatment. Included genera account for at least 2% of the reads in each sample type.

The limited data on avian ocular microbiomes restrict our ability to discern the microbiome composition that should be considered 'normal' or expected in house finches. Though no other studies beyond those discussed above have described ocular communities in birds with high-throughput sequencing, samples from nonhuman mammals have found *Proteobacteria*, *Actinobacteria* and *Firmicutes* on ocular surfaces, with the predominant phylum differing among and within host species (Alfano *et al.* 2015; Leis and Costa 2019; Leis, Madruga and Costa 2021). The abundant genera within those mammals, however, do not overlap with the principal genera in house finches. As with other microbiomes with such high exposure to the environment (e.g. skin and respiratory tract), the bacteria in ocular samples are likely affected by transient taxa that are detected in the eye, but are not true commensals (Lauer *et al.* 2007; Kong and Segre 2012; Hammer, Sanders and Fierer 2019). Further, the house finch ocular microbiomes that have been described have all derived from captive birds, and studies from other systems show that captivity can strongly affect microbiomes (e.g. Cheng *et al.* 2015; Kohl *et al.* 2017). The birds in this experiment and in the first study characterizing house finch ocular microbiomes (Thomason *et al.* 2017a), which found *Lactococcus* predominating in the microbiome, were housed in captivity for ~2–4 months prior to *M. gallisepticum* inoculation. Thus, time in captivity is unlikely to explain the large differences in abundance of *Lactococcus* between studies. The two studies directly compared here (Fig. 3) both included hatch-year males and females captured from the same population in Southwest Virginia during summer and housed under identical captive conditions in the same laboratory space, though other unrecognized differences in housing and care among the years could have affected the ocular communities at the beginning of the experiments. Overall, the microbial differences between study years represent, at least in some capacity, differences across years between the microbiomes at the time of capture. Further study should quantify temporal variation in the ocular microbiome of free-living house finches, as well as effects of captivity on such variation, to begin to unravel the mechanisms that determine ocular microbial composition in house finches and potentially other songbird taxa.

Large differences in microbial communities within a host species are common (e.g. Tung *et al.* 2015; Escallón *et al.* 2017; Springer *et al.* 2017; Kueneman *et al.* 2019; Hernandez *et al.* 2020) and likely play a large role in the variation of other aspects of host ecology. Much of what we know about patterns of microbiomes in and on wild organisms, particularly outside of the gut, and their interactions with pathogens comes from research on amphibian skin microbiomes, which can vary significantly with respect to spatial, temporal and environmental factors (Walke and Belden 2016; Familiar López *et al.* 2017; Christian *et al.* 2018; Kueneman *et al.* 2019; Loudon *et al.* 2020; Douglas, Hug and Katzenback 2021). Although studies of amphibian skin microbiomes focus on interactions with invading fungal and viral pathogens, studies in other natural systems are increasingly focusing on bacterial microbiome patterns and their functional roles in and on wild animals (e.g. Kohl 2012; Ainsworth *et al.* 2015; Colston and Jackson 2016; Thomason *et al.* 2017a; Allender *et al.* 2018; Weitzman, Sandmeier and Tracy 2018). While most studies seek patterns among individuals, microbial communities are labile even within single hosts, with microbial community shifts affected by factors such as horizontal transmission from social interactions, season and food availability (particularly for the gut microbiome), and infection (Jani and Briggs 2014; Bradley *et al.* 2016; Springer *et al.* 2017; Thomason *et al.* 2017a; Zhu *et al.* 2020). Much like the skin, ocular surfaces are constantly exposed to the outside world. In humans, ocular microbiomes not only vary among individuals (Ozkan *et al.* 2018; Delbeke *et al.* 2021), but also fluctuate across time within healthy individuals (Ozkan *et al.* 2017). Considering the many factors that could lead to temporal changes in microbial communities, it is reasonable to expect that house finch ocular microbiomes differ among generations and years. In this and other systems, changes in community composition across time could affect the microbiome's protective role, because members of the community influence the microbiome's effectiveness as protective. Thus, understanding the factors that drive changes in microbial community composition and function in this and other natural systems is critical.

This experiment attempted to conceptually expand upon results generated 4 years ago, by asking whether microbiome-mediated protection is dose dependent. Instead, we found that intact microbiomes that provided significant protection from infection and disease in an earlier experiment provided no detectable protection here, despite use of the same pathogen and free-living host. While this lack of protection meant that we were unable to adequately test our motivating hypothesis with respect to dose, the broader pattern of variable microbiome-mediated protection against infection is likely a common occurrence in ecological studies, as many abiotic and biotic changes

occur across spatial and temporal scales that could affect ecological patterns. The publication bias in ecological literature implies that many fields may be lacking the true range of results needed to fully assess ecological hypotheses and their generalizability (Jennions and Møller 2002; Fidler *et al.* 2017). Importantly, our study contributes to our understanding of how and when the host microbiome is protective, and the context dependence of interactions between complex microbial communities and invading pathogens. With these considerations, investigations should examine microbial variation, and associated potential inconsistencies of results, when exploring functions of resident microbiomes in free-living animals.

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## SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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## REFERENCES

Adelman JS, Moyers SC, Farine DR *et al.* Feeder use predicts both acquisition and transmission of a contagious pathogen in a North American songbird. *Proc Biol Sci* 2015;**282**:20151429.

Aiello CM, Nussear KE, Esque TC *et al.* Host contact and shedding patterns clarify variation in pathogen exposure and transmission in threatened tortoise *Gopherus agassizii*: implications for disease modelling and management. *J Anim Ecol* 2016;**85**:829–42.

Ainsworth TD, Krause L, Bridge T *et al.* The coral core microbiome identifies rare bacterial taxa as ubiquitous endosymbionts. *ISME J* 2015;**9**:2261–74.

Alfano N, Courtiol A, Vielgrader H *et al.* Variation in koala microbiomes within and between individuals: effect of body region and captivity status. *Sci Rep* 2015;**5**:1–12.

Allender MC, Baker S, Britton M *et al.* Snake fungal disease alters skin bacterial and fungal diversity in an endangered rattlesnake. *Sci Rep* 2018;**8**:12147.

Bates D, Mächler M, Bolker B *et al.* Fitting linear mixed-effects models using lme4. *J Stat Softw* 2015;**67**:1–48.

Becker MH, Brucker RM, Schwantes CR *et al.* The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. *Appl Environ Microbiol* 2009;**75**:6635–8.

Becker MH, Harris RN. Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. *PLoS One* 2010;**5**:e10957.

Becker MH, Walke JB, Cikanek S *et al.* Composition of symbiotic bacteria predicts survival in Panamanian golden frogs infected with a lethal fungus. *Proc Biol Sci* 2015a;**282**:20142881.

Becker MH, Walke JB, Murrill L *et al.* Phylogenetic distribution of symbiotic bacteria from Panamanian amphibians that inhibit growth of the lethal fungal pathogen *Batrachochytrium dendrobatidis*. *Mol Ecol* 2015b;**24**:1628–41.

Bjerre RD, Hugerth LW, Boulund F *et al.* Effects of sampling strategy and DNA extraction on human skin microbiome investigations. *Sci Rep* 2019;**9**:17287.

Bolyen E, Rideout JR, Dillon MR *et al.* Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 2019;**37**:852–7.

Boon E, Meehan CJ, Whidden C *et al.* Interactions in the microbiome: communities of organisms and communities of genes. *FEMS Microbiol Rev* 2014;**38**:90–118.

Bradley CW, Morris DO, Rankin SC *et al.* Longitudinal evaluation of the skin microbiome and association with microenvironment and treatment in canine atopic dermatitis. *J Invest Dermatol* 2016;**136**:1182–90.

Brooks ME, Kristensen K, van Benthem KJ *et al.* glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *R J* 2017;**9**:378–400.

Brucker RM, Baylor CM, Walters RL *et al.* The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. *J Chem Ecol* 2008a;**34**:39–43.

Brucker RM, Harris RN, Schwantes CR *et al.* Amphibian chemical defense: antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. *J Chem Ecol* 2008b;**34**:1422–9.

Brunner JL, Richards K, Collins JP. Dose and host characteristics influence virulence of ranavirus infections. *Oecologia* 2005;**144**:399–406.

Callahan BJ, McMurdie PJ, Rosen MJ *et al.* DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods* 2016;**13**:581–3.

Caporaso JG, Lauber CL, Walters WA *et al.* Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* 2012;**6**:1621–4.

Cheng Y, Fox S, Pemberton D *et al.* The Tasmanian devil microbiome: implications for conservation and management. *Microbiome* 2015;**3**:76.

Christian K, Weitzman C, Rose A *et al.* Ecological patterns in the skin microbiota of frogs from tropical Australia. *Ecol Evol* 2018;**8**:10510–9.

Colston TJ, Jackson CR. Microbiome evolution along divergent branches of the vertebrate tree of life: what is known and unknown. *Mol Ecol* 2016;**25**:3776–800.

Daskin JH, Alford RA. Context-dependent symbioses and their potential roles in wildlife diseases. *Proc Biol Sci* 2012;**279**:1457–65.

Delbeke H, Younas S, Casteels I *et al.* Current knowledge on the human eye microbiome: a systematic review of available amplicon and metagenomic sequencing data. *Acta Ophthalmol (Copenh)* 2021;**99**:16–25.

Dhondt AA, Dhondt KV, Hawley DM *et al.* Experimental evidence for transmission of *Mycoplasma gallisepticum* in house finches by fomites. *Avian Pathol* 2007;**36**:205–8.

Douglas AJ, Hug LA, Katzenback BA. Composition of the North American wood frog (*Rana sylvatica*) bacterial skin microbiome and seasonal variation in community structure. *Microb Ecol* 2021;**81**:78–92.

Ebert D, Zschokke-Rohringer CD, Carius HJ. Dose effects and density-dependent regulation of two microparasites of *Daphnia magna*. *Oecologia* 2000;**122**:200–9.

Escallón C, Becker MH, Walke JB *et al.* Testosterone levels are positively correlated with cloacal bacterial diversity and the relative abundance of Chlamydiae in breeding male rufous-collared sparrows. *Funct Ecol* 2017;**31**:192–203.

Familiar López M, Rebollar EA, Harris RN *et al.* Temporal variation of the skin bacterial community and *Batrachochytrium dendrobatidis* infection in the terrestrial cryptic frog *Philoria loveridgei*. *Front Microbiol* 2017;**8**:2535.

Fidler F, Chee YE, Wintle BC *et al.* Metaresearch for evaluating reproducibility in ecology and evolution. *Bioscience* 2017;**67**:282–9.

Fouhy F, Clooney AG, Stanton C *et al.* 16S rRNA gene sequencing of mock microbial populations: impact of DNA extraction method, primer choice and sequencing platform. *BMC Microbiol* 2016;**16**:123.

Fox J, Weisberg S. *An R Companion to Applied Regression*. 3rd edn. Thousand Oaks, CA: Sage, 2019.

Hammer TJ, Sanders JG, Fierer N. Not all animals need a microbiome. *FEMS Microbiol Lett* 2019;**366**:fnz117.

Harris EV, de Roode JC, Gerardo NM. Diet–microbiome–disease: investigating diet's influence on infectious disease resistance through alteration of the gut microbiome. *PLoS Pathog* 2019;**15**:e1007891.

Harris RN, Brucker RM, Walke JB *et al.* Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *ISME J* 2009;**3**:818–24.

Hawley DM, Grodio J, Frasca S *et al.* Experimental infection of domestic canaries (*Serinus canaria domestica*) with *Mycoplasma gallisepticum*: a new model system for a wildlife disease. *Avian Pathol* 2011;**40**:321–7.

Hawley DM, Osnas EE, Dobson AP *et al.* Parallel patterns of increased virulence in a recently emerged wildlife pathogen. *PLoS Biol* 2013;**11**:e1001570.

Hernandez J, Escallón C, Medina D *et al.* Cloacal bacterial communities of tree swallows (*Tachycineta bicolor*): similarity within a population, but not between pair-bonded social partners. *PLoS One* 2020;**15**:e0228982.

Holden WM, Hanlon SM, Woodhams DC *et al.* Skin bacteria provide early protection for newly metamorphosed southern leopard frogs (*Rana sphenocephala*) against the frog-killing fungus, *Batrachochytrium dendrobatidis*. *Biol Conserv* 2015;**187**:91–102.

Ichinohe T, Pang IK, Kumamoto Y *et al.* Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proc Natl Acad Sci USA* 2011;**108**:5354–9.

Jani AJ, Briggs CJ. The pathogen *Batrachochytrium dendrobatidis* disturbs the frog skin microbiome during a natural epidemic and experimental infection. *Proc Natl Acad Sci USA* 2014;**111**:E5049–58.

Jennions MD, Møller AP. Publication bias in ecology and evolution: an empirical assessment using the 'trim and fill' method. *Biol Rev Camb Philos Soc* 2002;**77**:211–22.

Kohl K. Diversity and function of the avian gut microbiota. *J Comp Physiol B* 2012;**182**:591–602.

Kohl KD, Brun A, Magallanes M *et al.* Gut microbial ecology of lizards: insights into diversity in the wild, effects of captivity, variation across gut regions and transmission. *Mol Ecol* 2017;**26**:1175–89.

Kollias GV, Sydenstricker KV, Kollias HW *et al.* Experimental infection of house finches with *Mycoplasma gallisepticum*. *J Wildl Dis* 2004;**40**:79–86.

Kong HH, Segre JA. Skin microbiome: looking back to move forward. *J Invest Dermatol* 2012;**132**:933–9.

Kueneman JG, Bletz MC, McKenzie VJ *et al.* Community richness of amphibian skin bacteria correlates with bioclimate at the global scale. *Nat Ecol Evol* 2019;**3**:381–9.

Kugadas A, Christiansen SH, Sankaranarayanan S *et al.* Impact of microbiota on resistance to ocular *Pseudomonas aeruginosa*-induced keratitis. *PLoS Pathog* 2016;**12**:e1005855.

Lauer A, Simon MA, Banning JL *et al.* Common cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. *Copeia* 2007;**2007**:630–40.

Lauer A, Simon MA, Banning JL *et al.* Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. *ISME J* 2008;**2**:145–57.

Leis ML, Costa MO. Initial description of the core ocular surface microbiome in dogs: bacterial community diversity and composition in a defined canine population. *Vet Ophthalmol* 2019;**22**:337–44.

Leis ML, Madruga GM, Costa MO. The porcine corneal surface bacterial microbiome: a distinctive niche within the ocular surface. *PLoS One* 2021;**16**:e0247392.

Leon AE, Hawley DM. Host responses to pathogen priming in a natural songbird host. *Ecohealth* 2017;**14**:793–804.

Loudon AH, Kurtz A, Esposito E *et al.* Columbia spotted frogs (*Rana luteiventris*) have characteristic skin microbiota that may be shaped by cutaneous skin peptides and the environment. *FEMS Microbiol Ecol* 2020;**96**:fiaa168.

McDermott AJ, Huffnagle GB. The microbiome and regulation of mucosal immunity. *Immunology* 2014;**142**:24–31.

McLaren MR, Callahan BJ. Pathogen resistance may be the principal evolutionary advantage provided by the microbiome. *Philos Trans R Soc B Biol Sci* 2020;**375**:20190592.

Mustafa K, Maulud SQ, Hamad PA. Detection of *Sphingomonas paucimobilis* and antibacterial activity of *Prosopis farcta* extracts on it. *Karbala Int J Mod Sci* 2018;**4**:100–6.

Nava-González B, Suazo-Ortuño I, López PB *et al.* Inhibition of *Batrachochytrium dendrobatidis* infection by skin bacterial communities in wild amphibian populations. *Microb Ecol* 2021:**82**:666–76.

O'Hanlon DE, Moench TR, Cone RA. Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. *PLoS One* 2013;**8**:e80074.

Oksanen J, Blanchet FG, Friendly M *et al.* vegan: community ecology package. R package version 2.5-2. 2018. https://CRAN.R-project.org/package=vegan (13 August 2018, date last accessed).

Oliver KM, Smith AH, Russell JA. Defensive symbiosis in the real world: advancing ecological studies of heritable, protective bacteria in aphids and beyond. *Funct Ecol* 2014;**28**:341–55.

Ozkan J, Coroneo M, Willcox M *et al.* Identification and visualization of a distinct microbiome in ocular surface conjunctival tissue. *Invest Opthalmol Vis Sci* 2018;**59**:4268–76.

Ozkan J, Nielsen S, Diez-Vives C *et al.* Temporal stability and composition of the ocular surface microbiome. *Sci Rep* 2017;**7**:1–11.

Panek M, Čipčić Paljetak H, Barešić A *et al.* Methodology challenges in studying human gut microbiota: effects of collection, storage, DNA extraction and next generation sequencing technologies. *Sci Rep* 2018;**8**:5143.

R Development Core Team. R: a language and environment for statistical computing. *GBIFORG*. 2015. http://www.gbif.org/resource/81287 (17 November 2016, date last accessed).

Regoes RR, Ebert D, Bonhoeffer S. Dose–dependent infection rates of parasites produce the Allee effect in epidemiology. *Proc R Soc Lond B Biol Sci* 2002;**269**:271–9.

Regoes RR. The role of exposure history on HIV acquisition: insights from repeated low-dose challenge studies. *PLoS Comput Biol* 2012;**8**:e1002767.

Reller LB, Karney WW, Beaty HN *et al*. Evaluation of cefazolin, a new cephalosporin antibiotic. *Antimicrob Agents Chemother* 1973;**3**:488–97.

Røssland E, Langsrud T, Granum PE *et al*. Production of antimicrobial metabolites by strains of *Lactobacillus* or *Lactococcus* co-cultured with *Bacillus cereus* in milk. *Int J Food Microbiol* 2005;**98**:193–200.

RStudio Team. *RStudio: Integrated Development Environment for R*. Boston, MA: RStudio, 2020.

Sassone-Corsi M, Raffatellu M. No vacancy: how beneficial microbes cooperate with immunity to provide colonization resistance to pathogens. *J Immunol* 2015;**194**: 4081–7.

Sekirov I, Tam NM, Jogova M *et al*. Antibiotic-induced perturbations of the intestinal microbiota alter host susceptibility to enteric infection. *Infect Immun* 2008;**76**:4726–36.

Shukla SD, Budden KF, Neal R *et al*. Microbiome effects on immunity, health and disease in the lung. *Clin Transl Immunol* 2017;**6**:e133.

Spekreijse D, Bouma A, Stegeman JA *et al*. The effect of inoculation dose of a highly pathogenic avian influenza virus strain H5N1 on the infectiousness of chickens. *Vet Microbiol* 2011;**147**:59–66.

Springer A, Fichtel C, Al-Ghalith GA *et al*. Patterns of seasonality and group membership characterize the gut microbiota in a longitudinal study of wild Verreaux's sifakas (*Propithecus verreauxi*). *Ecol Evol* 2017;**7**:5732–45.

Sydenstricker KV, Dhondt AA, Ley DH *et al*. Re-exposure of captive house finches that recovered from *Mycoplasma gallisepticum* infection. *J Wildl Dis* 2005;**41**:326–33.

Thaiss CA, Zmora N, Levy M *et al*. The microbiome and innate immunity. *Nature* 2016;**535**:65–74.

Thomason CA, Leon A, Kirkpatrick LT *et al*. Eye of the Finch: characterization of the ocular microbiome of house finches in relation to mycoplasmal conjunctivitis. *Environ Microbiol* 2017a;**19**:1439–49.

Thomason CA, Mullen N, Belden LK *et al*. Resident microbiome disruption with antibiotics enhances virulence of a colonizing pathogen. *Sci Rep* 2017b;**7**:1–8.

Timms R, Colegrave N, Chan BHK *et al*. The effect of parasite dose on disease severity in the rodent malaria *Plasmodium chabaudi*. *Parasitology* 2001;**123**:1–11.

Tung J, Barreiro LB, Burns MB *et al*. Social networks predict gut microbiome composition in wild baboons. *eLife* 2015;**4**:e05224.

Vásquez A, Forsgren E, Fries I *et al*. Symbionts as major modulators of insect health: lactic acid bacteria and honeybees. *PLoS One* 2012;**7**:e33188.

Walke JB, Becker MH, Loftus SC *et al*. Community structure and function of amphibian skin microbes: an experiment with bullfrogs exposed to a chytrid fungus. *PLoS One* 2015;**10**:e0139848.

Walke JB, Belden LK. Harnessing the microbiome to prevent fungal infections: lessons from amphibians. *PLoS Pathog* 2016;**12**:e1005796.

Wei Z, Yang T, Friman V-P *et al*. Trophic network architecture of root-associated bacterial communities determines pathogen invasion and plant health. *Nat Commun* 2015;**6**:8413.

Weitzman CL, Sandmeier FC, Tracy CR. Host species, pathogens and disease associated with divergent nasal microbial communities in tortoises. *R Soc Open Sci* 2018;**5**:181068.

Weyrich LS, Feaga HA, Park J *et al*. Resident microbiota affect *Bordetella pertussis* infectious dose and host specificity. *J Infect Dis* 2014;**209**:913–21.

Zhu L, Clayton JB, Van Haute MJS *et al*. Sex bias in gut microbiome transmission in newly paired marmosets (*Callithrix jacchus*). *mSystems* 2020;**5**:e00910–9.