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

Running title: Variable pathogen protection by microbiomes

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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.

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* (MG). 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 MG inoculation doses because the resident microbiome would be less disrupted by invading pathogen. Our five MG dose treatments were fully factorial with an antibiotic treatment to perturb resident microbes just prior to MG inoculation. Unexpectedly, we found no indication of protective effects of the resident microbiome at any pathogen inoculation dose, which was inconsistent with 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.

Introduction

47 Understanding the varied roles of microbial communities in mediating host–pathogen
48 interactions across ecological contexts has become increasingly important. Bacterial community
49 members can interact with invading pathogens along the spectrum from facilitative to
50 antagonistic interactions (Daskin and Alford 2012; Boon *et al.* 2014; Oliver, Smith and Russell
51 2014; Becker *et al.* 2015b). Evidence of antagonistic interactions, whereby bacterial taxa inhibit
52 pathogens in or on a host, has been found across multiple host-pathogen systems. In some cases,
53 the structure of the microbial community at the site of pathogen invasion is predictive of disease
54 severity (Lauer *et al.* 2008; Becker *et al.* 2015a; Holden *et al.* 2015; Harris, Roode and Gerardo
55 2019). Further, studies using antibiotics to perturb resident microbiomes have found increases in
56 host morbidity and mortality when challenged with a pathogen, including higher pathogen loads
57 and greater sickness behaviors (Sekirov *et al.* 2008; Becker and Harris 2010; Weyrich *et al.*
58 2014; Holden *et al.* 2015; Kugadas *et al.* 2016; Thomason *et al.* 2017b). These results indicate
59 that intact microbiomes, i.e. resident microbiomes unmanipulated by antibiotics, often act in a
60 protective role for hosts.
61 Indeed, microbiomes play an important part in a host's innate immune system. Where
62 they interact directly with invading pathogens, microbial communities may act as the first line of
63 defense against invasion. Bacterial communities can inhibit invading pathogens through direct
64 and indirect interactions (Sassone-Corsi and Raffatellu 2015; McLaren and Callahan 2020). For
65 example, some cutaneous bacteria produce metabolites that inhibit growth of an invading fungal
66 pathogen on frogs and salamanders (Brucker *et al.* 2008a, 2008b; Becker *et al.* 2009; Harris *et al.*
67 2009). In other instances, commensal bacteria may outcompete pathogens for space or nutrients
68 (Sassone-Corsi and Raffatellu 2015; Wei *et al.* 2015; McLaren and Callahan 2020). Microbes
69 also help to maintain and trigger immune responses against pathogen invasion and disease, both

70 locally and elsewhere in the body (Ichinohe *et al.* 2011; McDermott and Huffnagle 2014; Thaiss
71 *et al.* 2016; Shukla *et al.* 2017). When resident microbiomes are experimentally disrupted,
72 pathogens can even exhibit distinct virulence phenotypes (Thomason *et al.* 2017b). Thus,
73 microbes can inhibit infection and disease severity in hosts via a range of potential mechanisms.
74 Overall, intact microbiomes appear to provide protection from infection and disease
75 across a variety of hosts and pathogens, but the extent of protection that the microbiome provides
76 is likely to depend on the dose of invading pathogen. Pathogen exposure dose predicts the degree
77 of resulting host morbidity and mortality in diverse disease systems (e.g. Ebert, Zschokke-
78 Rohringer and Carius 2000; Brunner, Richards and Collins 2005; Leon and Hawley 2017);
79 however, potential interactions between the host microbiome and pathogen dose on infection
80 outcomes have rarely been examined. In one study, the presence of an intact microbiome
81 increased the infective dose of *Bordetella pertussis* in mice by three orders of magnitude
82 compared with infective doses in mice given antibiotics to knock down the native microbiome
83 (Weyrich *et al.* 2014), consistent with the hypothesis that microbiome-mediated protection may
84 vary with pathogen dose. In natural systems, pathogen exposures commonly occur at low doses,
85 which may not, in single exposure events, cause disease (Dhondt *et al.* 2007; Regoes 2012;
86 Aiello *et al.* 2016). Heterogeneity of disease and pathogen load in animal populations could
87 result, in part, from the interplay between exposure dose and host protection by the microbiome.
88 In this study, we experimentally assessed the hypothesis that the degree of protection
89 provided by intact microbiomes varies with the dose of invading pathogen. House finches
90 (*Haemorhous mexicanus*) develop mycoplasmal conjunctivitis after infection by the bacterial
91 pathogen *Mycoplasma gallisepticum* (MG) (Kollias *et al.* 2004). Previous experimental work in
92 this system using ocular antibiotics found protective effects of the ocular microbiome against

MG 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 MG isolates. Thus, while the exact causal mechanisms remain unclear, intact ocular microbiomes appear to provide protection against both MG infection and disease when inoculation doses are relatively high, as was the case in Thomason *et al.* (2017b). MG also interacts with the resident microbiome to cause shifts in the ocular bacterial community composition after MG 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 MG inoculation dose concentrations. We perturbed the ocular microbiome with cefazolin, a β-lactam antibiotic to which MG is intrinsically resistant due to absence of a cell wall. After microbiome treatment (antibiotics or control), birds were conjunctivally inoculated with a given MG dose. We monitored pathology and MG loads in the conjunctiva throughout infection, and measured sialidase activity in output MG 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–August 2019 in Montgomery County, Giles County, and Radford, Virginia. Birds were housed singly or in pairs

in cages (76 x 46 x 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 post-capture for two weeks and then collected blood samples to assess MG-specific antibody concentrations. The combination of age (hatch-year) and serological status allowed us to ensure that birds included in our MG inoculation treatments did not have prior exposure to MG in the wild. No experimental birds had conjunctivitis pathology at any point prior to inoculation, and only birds negative for anti-MG antibodies (Hawley *et al.* 2011) were included in treatment groups inoculated with MG (see *Experimental Design*). Birds were single-housed starting 13 days before experimental MG 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 ten treatment groups in a fully factorial design (Table 1), with the highest MG concentration similar to that used by Thomason *et al.* (2017b). 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 four-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 β-lactam antibiotic cefazolin to disrupt the resident microbiome, as previously described (Thomason *et al.* 2017b). Cefazolin was rehydrated in PBS 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 five days (Figure 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 (Supplemental Materials).

We inoculated house finches with the VA1994 MG isolate (7994-1 6 P 9/17/2018) because the ocular microbiome provided protection against this isolate in prior work (Thomason *et al.* 2017b). MG 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 MG diluted to a given concentration (depending on their dose treatment), split between the two conjunctiva (i.e. approximately 35 µL per eye). Infection controls ("MG controls", Table 1) were given 70 µL of antibiotic-free Frey's media.

Pathology, Swabbing, and MG Quantification

We collected pathology data and conjunctival swab samples from birds at multiple timepoints from PID -13 to PID 27 (Figure 1). Pathology was scored for each conjunctivia on a 0–3 scale, with scores made while blind to a given bird's treatment. Briefly, no clinical signs of conjunctivitis is scored as 0, a score of 1 represents minor swelling around the eye or minor conjunctival eversion, moderate swelling and eversion is scored as 2, and severe swelling, eversion, and exudate is scored as 3 (Sydenstricker *et al.* 2005). We summed the scores between the two sides within each time point, resulting in a value from 0–6 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 were cultured to confirm the antibiotic effectiveness (Supplemental Materials). On PID 8, after the DNA/RNA-preserved swab, we swabbed the conjunctivae a second time for MG culture and phenotyping of sialidase activity. On the day of collection, MG phenotype swabs from MG 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) (Supplemental Materials).

DNA was extracted from conjunctival swab samples from PID -1, 3, and 13 with the Qiagen DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) protocol for Gram-positive bacteria (see Supplemental Materials for modifications). To quantify MG in swab samples, we conducted quantitative PCR (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 MG 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 ANOVA. We then analyzed pathology, pathogen load, probability of infection (defined

below), and MG sialidase phenotype data to detect how the interaction between microbiome treatment and MG 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 dataset including only birds inoculated with MG. These two datasets provided similar results, so below we present analyses of just MG-inoculated birds (see Supplemental Materials for full data set results).

To determine the effects of MG 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 MG dose ($\log_{10}$(MG concentration + 1)) and PID, MG dose and microbiome treatment, and microbiome treatment and PID, along with sex and temporal group as covariates. Regardless of p-value, MG 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 MG dose or PID were analyzed as quadratic variables ($\log_{10}$(MG 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 PID 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.

208 Pathogen load data were also used to assess successful infection in experimental birds,
209 conservatively defined as any post-inoculation MG load ($\log_{10}$(load + 1)) greater than 3.1 $\log_{10}$
210 copies as per prior work (Adelman *et al.* 2015; Leon and Hawley 2017). We analyzed whether
211 microbiome treatment affected the probability of infection using binomial GLM with a probit
212 link. The main predictor variables of interest included the interaction between microbiome and
213 MG dose treatments, with host sex and temporal group as covariates.
214 When sialidase activity was present in the cultured MG isolates from PID 8, sialidase
215 activity (mU/mg total protein) was analyzed using ANOVA, with the same predictor variables as
216 in the probability of infection analyses.
217
218 *Describing Starting Ocular Microbiomes*
219 A subset of ocular swab samples were used to compare the resident ocular microbiomes
220 in birds at the beginning of this experiment with those in a previous study assessing the effect of
221 antibiotics on responses to MG (Thomason *et al.* 2017b). In order to describe the resident
222 microbial community without confounding effects of experimental perturbation, samples used
223 here were collected either prior to ocular antibiotic treatment at PID -13 (n = 7) or on PID -1
224 from catch-only birds (n = 4 microbiome controls). We used the Zymo Quick DNA/RNA
225 Microprep extraction kit (Zymo Research, Irvine, CA) to extract samples, eluting in 15 µL
226 DNase/RNase-free water. We conducted library prep for Illumina MiSeq sequencing as
227 previously described, amplifying a portion of the V4 region of 16S bacterial rRNA using 515F
228 and barcoded 806R primers (Caporaso *et al.* 2012; Thomason *et al.* 2017a). Single-end sequence
229 reads were demultiplexed using QIIME2, and reads were trimmed and quality-filtered with a
230 maximum of two expected errors using the DADA2 package (Callahan *et al.* 2016; Bolyen *et al.*

231 2019). We similarly filtered and trimmed reads from 14 microbiome control PID -1 samples
232 from Thomason *et al.* (2017b), and combined those reads from 2016 with our 2019 data set to
233 denoise reads to detect amplicon sequence variants (ASVs) with DADA2. Sample collection
234 protocols differed for the 2016 dataset (which used sterile cotton swabs, storage in tryptose
235 phosphate broth, and DNA extraction with Qiagen DNeasy Blood and Tissue Kit), but PCR and
236 sequencing protocols were identical; thus, the datasets should be broadly comparable (Fouhy *et*
237 *al.* 2016; Panek *et al.* 2018; Bjerre *et al.* 2019). We assigned taxonomy to our combined read file
238 with the Silva v132 database and filtered out non-bacterial, chloroplast, and mitochondrial reads.
239 Sequences are openly available on figshare (DOI: 10.6084/m9.figshare.14541390; temporary
240 link: https://figshare.com/s/ce3aea67c701bb061b48).
241 We used analysis of composition of microbiomes (ANCOM) on unrarefied data in
242 QIIME2 to assess differential abundance of bacterial genera between the two study years. After
243 inspecting rarefaction curves, we rarefied the data to 7,500 reads per sample, removing three
244 samples from the 2016 data. We used QIIME2 to calculate ASV richness, alpha diversity metrics
245 (Pielou's evenness, Shannon's diversity metric, and Faith's phylogenetic diversity) and beta
246 diversity (weighted and unweighted UniFrac distances). Analyses of these metrics focused on
247 detecting differences between the study years in starting microbial communities in absence of
248 antibiotic perturbation and prior to pathogen inoculation. We compared ASV richness and alpha
249 diversity metrics between the sampling years with Kruskal-Wallis tests. With the vegan package
250 (Oksanen *et al.* 2018), we compared beta diversities between sampling years with
251 PERMANOVA and further tested for differences in multivariate dispersion with permutational
252 multivariate analysis of beta-dispersion.
253

Results

Culture-based assays indicated that the topical ocular antibiotic significantly suppressed the overall abundance of viable resident bacteria (Figure S1), 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 MG inoculation (PID -1), none of the experimental birds had pathology or detectable MG by qPCR.

Disease Severity

Overall, we found no support for our primary hypothesis that MG dose and microbiome antibiotic treatment would interact to influence disease severity. Both MG dose and PID were significant predictors of disease severity in our simplified model (Table 2), with pathology score increasing with MG 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 MG 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 ($p = 0.055$; Figure 2a, Figure S2). Because a single house finch in the catch-only (microbiome control), $3x10^3$ CCU/mL MG 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 driving 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 MG dose on infection loads quantified on post-inoculation days 3 and 13. Similar to the results for disease severity, we found no support for interactions between MG dose and microbiome treatment on infection load. In the simplified model, MG load was significantly predicted by MG dose, PID, and their interaction, but not microbiome treatment (Table 2, Figure 2b).

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

MG Sialidase Phenotype

Of the 85 birds inoculated with MG, 48 cultured swab samples from PID 8 grew and exhibited sialidase activity (MG doses: $3x10^1$ n = 1; $3x10^2$ n = 4; $3x10^3$ n = 20; $3x10^4$ n = 23). From linear models, only temporal group was a significant predictor of sialidase activity (Table 2, Figure S3), with MG isolated from birds in temporal group 2 exhibiting lower sialidase activity. Neither microbiome treatment, nor MG dose, was significant.

Resident Ocular Microbiomes

Prior to MG 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) (Figure 3, Table S4). 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 (Figure 3, Table S4). Though the genus *Lactococcus* was the dominant taxon in finch ocular microbiomes in 2016, this genus accounted for less than 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 antibiotics.

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, Figure S4). Similarly, samples from the two study years significantly differed in beta diversity (weighted and unweighted UniFrac $p = 0.001$; Table S3, Figure S5). 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 *Mycoplasma gallisepticum*. Although disease and infection severity both increased with MG 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 predict 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, 2017b), 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 comprised of the bacterial genus *Lactococcus*, with over 70% of the relative abundance representing this genus. A high relative abundance of *Lactoccocus* was also documented in another prior study describing ocular bacterial microbiomes and their shifts after MG 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 less than 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

346 community composition and diversity across extraction protocols (Fouhy *et al.* 2016; Bjerre *et*
347 *al.* 2019). However, distinct extraction protocols are unlikely to cause the degree of divergence
348 between predominant taxa detected between the two studies here (Figure 3). Overall, the low
349 abundance of *Lactococcus* and large shift in the resident microbiome compared to previous
350 descriptions could have resulted in its lack of protective function against mycoplasmal
351 conjunctivitis in this study. Further, the dominant ocular community members in this experiment
352 may not have responded to experimental antibiotic treatment in the same way as the prior
353 *Lactococcus*-dominated communities, potentially limiting our ability to adequately disrupt the
354 ocular microbial community and thus test its protective function. Though we used culture-based
355 methods to verify that administering cefazolin reduced ocular bacterial growth in this study, we
356 also detected abundant genera in the sequenced ocular samples that may not be susceptible to
357 this antibiotic, such as *Pseudomonas* and *Sphingomonas* (Reller *et al.* 1973; Mustafa, Maulud
358 and Hamad 2018). Thus, it is challenging to compare the role of these distinct ocular
359 communities in ocular health and disease.
360 Past work also found that perturbing the ocular microbiome increased activity of sialidase
361 enzymes associated with MG virulence (Thomason *et al.* 2017b), and that activity was correlated
362 with variation in disease severity among individuals. Here, we did not detect any influence of
363 ocular microbiome disruption on the sialidase activity of output MG isolates, consistent with the
364 absence of significant effects of antibiotics on disease severity. Modulation of sialidase activity,
365 and often its functional balance with host cell cytadherence, are important mechanisms for fine-
366 scale rheostasis (i.e. regulation in a changing environment) during infection for many pathogens.
367 Overall, the differences between these results and those of past work suggest that prior protective
368 effects, including the potential modulation of sialidase activity in MG, were likely driven by

Lactoccocus and other resident community members that were largely absent in the resident ocular microbiomes in this experiment.

The limited data on avian ocular microbiomes restricts 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 non-human 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, 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 approximately two to four months prior to MG inoculation. Thus, time in captivity is unlikely to explain the large differences in abundance of *Lactococcus* between studies. The two studies directly compared here (Figure 3) both included hatch-year males and females captured from the same population in SW 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

415 world. In humans, ocular microbiomes not only vary among individuals (Ozkan *et al.* 2018;
416 Delbeke *et al.* 2021), but also fluctuate across time within healthy individuals (Ozkan *et al.*
417 2017). Considering the many factors that could lead to temporal changes in microbial
418 communities, it is reasonable to expect that house finch ocular microbiomes differ among
419 generations and years. In this and other systems, changes in community composition across time
420 could affect the microbiome's protective role, because members of the community influence the
421 microbiome's effectiveness as protective. Thus, understanding the factors that drive changes in
422 microbial community composition and function in this and other natural systems is critical.
423 This experiment attempted to conceptually expand upon results generated four years ago,
424 by asking whether microbiome-mediated protection is dose dependent. Instead, we found that
425 intact microbiomes that provided significant protection from infection and disease in an earlier
426 experiment provided no detectable protection here, despite use of the same pathogen and free-
427 living host. While this lack of protection meant that we were unable to adequately test our
428 motivating hypothesis with respect to dose, the broader pattern of variable microbiome-mediated
429 protection against infection is likely a common occurrence in ecological studies, as many abiotic
430 and biotic changes occur across spatial and temporal scales that could affect ecological patterns.
431 The publication bias in ecological literature implies that many fields may be lacking the true
432 range of results needed to fully assess ecological hypotheses and their generalizability (Jennions
433 and Møller 2002; Fidler *et al.* 2017). Importantly, our study contributes to our understanding of
434 how and when the host microbiome is protective, and the context-dependency of interactions
435 between complex microbial communities and invading pathogens. With these considerations,
436 investigations should examine microbial variation, and associated potential inconsistencies of
437 results, when exploring functions of resident microbiomes in free-living animals.

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Conflicts of interest

The authors declare no conflicts of interest.

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Tables

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

Microbiome treatment	**Pathogen Dose Concentrations** (color-changing units/mL)				
	0 (MG control)	*3 x 10^1*	*3 x 10^2*	*3 x 10^3*	*3 x 10^4*
Catch-only (microbiome control)	11	8	11	12	12
Antibiotics	11	8	11	12	11

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 antibiotics prior to inoculation with *Mycoplasma gallisepticum* (MG; 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, $3x10^3$ CCU/mL MG dose group.

Response	Predictors	Estimate ± SEM	ChiSq	*P*	*P* no outlier
Pathology					
	MG 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					
	MG dose x PID	–0.605 ± 0.136	19.93	**<0.0001**	
	MG 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					
	MG dose	2.993 ± 0.629	91.17	**<0.0001**	
	Microbiome treatment	–0.652 ± 0.596	1.29	0.26	
MG Sialidase Phenotype			*F*-value		
(n = 48)	MG 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 Legends

Figure 1. Experimental timeline based on post-inoculation day (PID) with respect to inoculation with *Mycoplasma gallisepticum* (MG). To detect whether the protective effects of an intact ocular microbiome vary with the inoculation dose of MG 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 (Supplemental Materials), M = MG sialidase phenotype swabs, and B = blood samples to determine antibody levels (Supplemental Materials).

Figure 2. Intact ocular microbiomes were not protective against *Mycoplasma gallisepticum* (MG) disease or infection severity in house finches. (a) Pathology scores for the two highest MG dose concentrations (only two doses shown for ease of visualization, but see Figure S2 for lower dose results) across post-inoculation days (PID). Squares indicate $3x10^3$ CCU/mL MG dose. Triangles indicate $3x10^4$ CCU/mL MG dose. (b) MG load on days 3 and 13 post-inoculation were predicted by MG dose, PID, and their interaction, but not antibiotic treatment. Only birds in the four treatment groups with MG 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.

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 antibiotics were excluded for clarity). Proportions based on relative abundance of rarefied reads. Samples

grouped by post-inoculation day and treatment. Included genera account for at least 2% of the reads in each sample type.

Figure 1

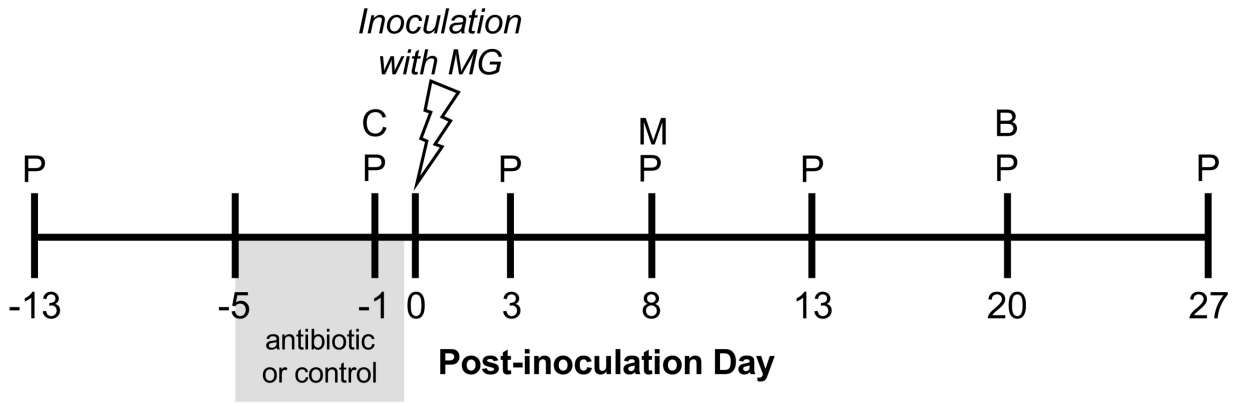
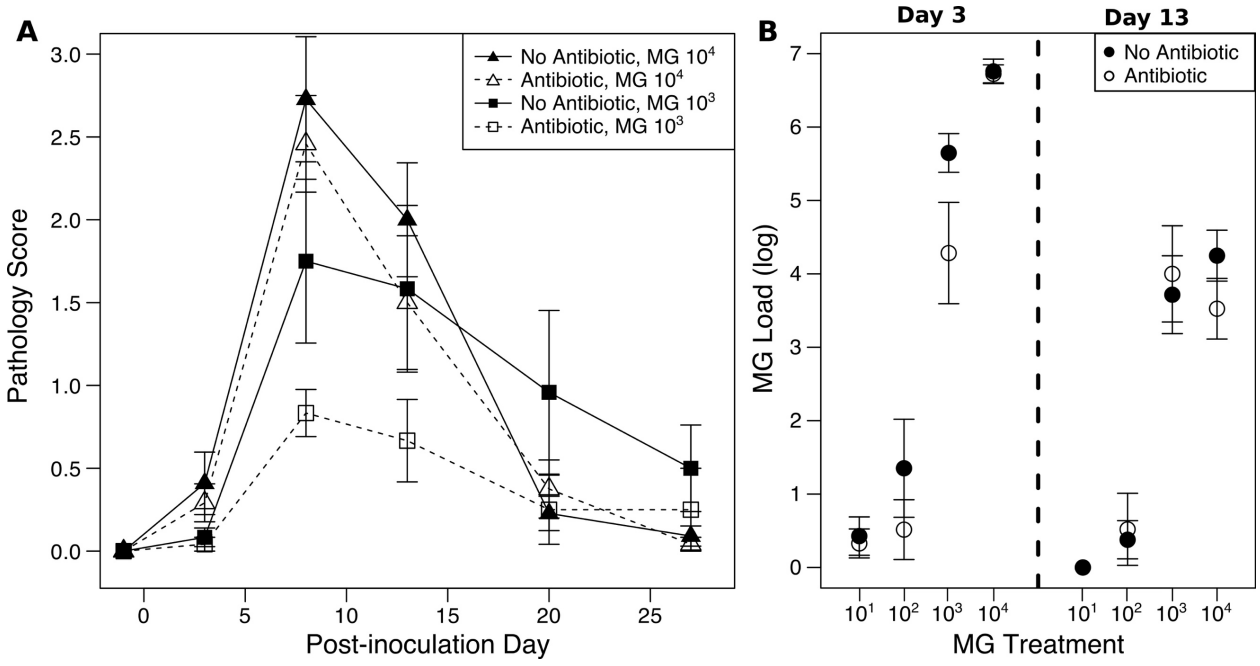
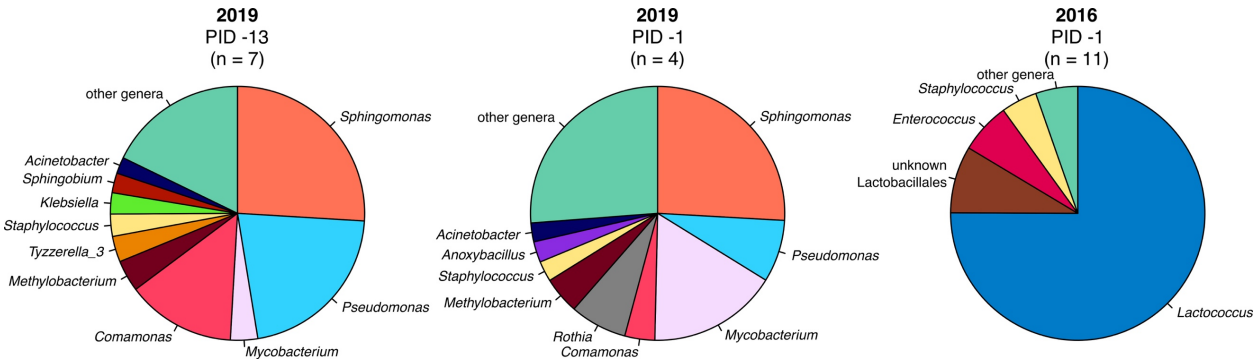


Figure 2



67 Figure 3



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