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Research Paper

Influence of human activity on gut microbiota and immune responses of Darwin's finches in the Galápagos Islands

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ABSTRACT. Urbanization can influence many environmental factors that can affect the condition, immunity, and gut microbiota of birds. Over the past several decades, the Galápagos Islands of Ecuador have experienced increasing human activity, which has led to recent changes in the morphology, gut microbiota, and immunity of Darwin's finches. However, these traits have not been characterized before the exponential growth of human population size and tourist visitation rates, i.e., before 2009. The goal of this study was to determine the effect of land use on the fecal microbiota, immune response, and body measurements of Darwin's finches in 2008, at a time of rapidly increasing human activity on the islands. Specifically, we compared fecal microbiota (bacterial diversity, community structure and membership, and relative abundance of bacterial taxa), proxies of immunity (lysozyme activity and haptoglobin, complement antibody, and natural antibody levels), and body measurements (body mass and condition, tarsus length) across undeveloped, agricultural, and urban areas for medium ground finches (*Geospiza fortis*) and small ground finches (*G. fuliginosa*). Lysozyme activity was lower and observed bacterial species richness was higher in urban areas compared to non-urban areas across both finch species. In medium ground finches, four genera (*Methylobacterium-Methylorubrum*, *Escherichia-Shigella*, *Brucella*, and *Citrobacter* spp.) were higher in urban areas compared to undeveloped areas. In small ground finches, *Paucibacter*, *Achromobacter*, *Delftia*, *Stenotrophomonas*, and *Brucella* spp. had higher relative abundances in undeveloped and agricultural areas whereas the genus *Cutibacterium* was more abundant in finches from urban and agricultural areas than in finches from undeveloped areas. Medium ground finches were smaller in undeveloped areas compared to the other two areas, but body mass of small ground finches did not differ across areas. Our results suggest that human activity can have an impact on immune measures and gut microbiota of Darwin's finches.

Influence de l'activité humaine sur le microbiote intestinal et la réponse immunitaire des géospizes de Darwin dans les îles Galápagos

RÉSUMÉ. L'aménagement du territoire peut influer sur de nombreux facteurs environnementaux susceptibles d'affecter la condition, l'immunité et le microbiote intestinal des oiseaux. Au cours des dernières décennies, les îles Galápagos, en Équateur, ont subi une hausse de l'activité humaine, ce qui a entraîné des changements récents dans la morphologie, le microbiote intestinal et l'immunité des géospizes de Darwin. Cependant, ces traits n'ont pas été caractérisés avant la croissance exponentielle de la population humaine et du taux de fréquentation touristique, c'est-à-dire avant 2009. L'objectif de cette étude était de déterminer l'effet de l'occupation des terres sur le microbiote fécal, la réponse immunitaire et les mesures corporelles des géospizes de Darwin en 2008, moment où l'activité humaine augmentait rapidement sur les îles. Plus précisément, nous avons comparé le microbiote fécal (diversité bactérienne, structure et composition de la communauté et abondance relative des taxons bactériens), les indicateurs d'immunité (activité du lysozyme et haptoglobine, anticorps du complément et niveaux d'anticorps naturels) et les mesures corporelles (masse et état corporel, longueur du tarse) dans des régions naturelles, agricoles et urbaines chez les Géospizes à bec moyen (*Geospiza fortis*) et les Géospizes fuligineux (*G. fuliginosa*). L'activité du lysozyme était plus faible et la richesse en espèces bactériennes était plus élevée dans les régions urbaines, comparativement aux régions non urbaines, chez les deux espèces de géospizes. Chez les Géospizes à bec moyen, quatre genres (*Methylobacterium-Methylorubrum*, *Escherichia-Shigella*, *Brucella* et *Citrobacter* sp.) étaient plus nombreux dans les régions urbaines que dans les régions naturelles. Chez les Géospizes fuligineux, *Paucibacter*, *Achromobacter*, *Delftia*, *Stenotrophomonas* et *Brucella* sp. avaient une abondance relative plus élevée dans les régions naturelles et agricoles, tandis que le genre *Cutibacterium* était plus abondant chez les géospizes de régions urbaines et agricoles que chez les géospizes de régions naturelles. Les Géospizes à bec moyen étaient plus petits dans les régions naturelles que dans les deux autres régions, et la masse corporelle des Géospizes fuligineux n'était pas différente entre les régions. Nos résultats indiquent que l'activité humaine peut avoir un effet sur les mesures immunitaires et le microbiote intestinal des géospizes de Darwin.

Key Words: *human activity; immunity; microbiome; urbanization*

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INTRODUCTION

Human activity is rapidly changing the natural environment, and can influence avian ecology and evolution. For example, anthropogenic change can influence many avian traits, including morphology, e.g., bill size; behavior, e.g., boldness, predator avoidance, and problem solving; and physiology, e.g., digestion and immunity (Hendry et al. 2006, Møller 2008, Atwell et al. 2012, Samia et al. 2017, Sol et al. 2018, Strandin et al. 2018). Food availability can mediate the effect of human activity on avian traits. Humans can change food-resource availability for birds by increasing the abundance of anthropogenic food through wild bird feeders, the disposal of human food into the environment, and by reducing the abundance of natural food items through environment changes (Murray et al. 2016, Bosse et al. 2017, Start et al. 2018). In turn, birds lacking natural food resources may be forced to invest less in maintaining body mass or physiological processes, such as immune responses, which can be energetically costly (Sheldon and Verhulst 1996, Svensson et al. 1998, Lochmiller and Deerenberg 2000, Demas 2004, Sternberg et al. 2012, Cornet et al. 2014, Howick and Lazzaro 2014).

In addition, human activity can alter avian traits through impacts on the gut microbiota, the community of symbiotic microbial species that live within the gut of their host (Grond et al. 2018). Different environments, including human-altered ones, contain different microbial communities that can colonize the gut of the host. As a result, the gut microbiota of the host can change in response to changes in land use, including urbanization (Phillips et al. 2018, Teyssier et al. 2018, Knutie et al. 2019, Berlow et al. 2021) and pollution of water and soil (Jin et al. 2017). In turn, the gut microbiota can interact with host cells to change host physiological traits, such as the immune system (Round and Mazmanian 2009). Increased bacterial diversity and the presence of specific bacterial taxa can confer increased resistance to parasites and pathogens. As human activity increases, along with the concern over diseases in birds, disentangling these relationships is important to understand how novel stressors can influence birds and help aid in their management and conservation.

The Galápagos Islands of Ecuador are relatively pristine but are facing increasing human activity, which allows for the study of host-associated microbiota and immune systems in response to urbanization. Over the past 20 years, ecotourism and the permanent-resident human population has grown rapidly on the Islands (Walsh and Mena 2016). For example, from 2007 to 2016, the number of permanent human residents increased from approximately 20,000 to 30,000 people, and tourist numbers increased from 140,000 to 225,000 people (Watkins and Cruz 2007, Walsh and Mena 2016). Consequently, in areas with human infrastructure, such as towns and popular tourist sites, the natural habitat and diet of endemic species have been altered with the introduction of agricultural plants and human-processed food (de León et al. 2018). Studies have shown that changes in habitat type can affect gut microbiota and metrics of immunity of endemic Darwin's finch species (Zylberberg et al. 2013, Michel et al. 2018, Knutie et al. 2019, Loo et al. 2019, Solomon et al. 2023). However, all studies on the gut microbiota of Darwin's finches have occurred since 2016 and little is known about how the start of exponential growth in human activity may have influenced microbiota in Darwin's finches. Furthermore, the effect of human-influenced land use on the interaction between gut microbiota and immunity has received little attention. A better understanding of human impacts on avian immune systems could be especially important because introduced parasites, such as avian pox and avian vampire flies (*Philornis downsi)*, have been negatively affecting the survival of birds on the islands (Wikelski et al. 2004, Fessl et al. 2010, Koop et al. 2016).

The goal of this study was to determine the effect of land use on the gut microbiota, immune response, and body measurements of Darwin's finches in 2008. Specifically, we characterized the following traits in small ground finches (*Geospiza fuliginosa*) and medium ground finches (*G. fortis*) across three different habitat types in 2008 on Santa Cruz Island in the Galápagos: gut microbiota, i.e., bacterial diversity, community structure and membership, and relative abundance of bacterial taxa; immune response, i.e., lysozyme activity and haptoglobin, complement antibody, and natural antibody levels; and body measurements, i.e., body mass, tarsus length, and scaled mass index. Finches were caught in three different areas that varied in human activity and presence, including an area with (1) little human activity and no permanent human population (hereon, undeveloped); (2) developed land for agricultural activities but only a small permanent human population (hereon, agricultural); and (3) developed land for a substantial, permanent human population (hereon, urban).

Previous studies have shown that the immune response of Darwin's finches can vary temporally across land-use types. For example, Zylberberg et al. (2013) found that finches living in agricultural and undeveloped areas had different immune responses across years, whereas finches in urban areas showed no change across years. One explanation is that food availability changed across years but not in all habitats. Variation in food availability could either directly affect the immune response or the gut microbiota, which in turn could affect the immune response. However, Zylberberg et al. (2013) did not examine the effect of land use on the immune response within each year. Thus, for the present study, we examined the impact of land use on three metrics of constitutive immunity, including lysozyme activity, complement antibody levels, natural antibody levels, and one metric of inducible immunity, including haptoglobin levels, using PIT54 acute phase proteins. These four aspects of the innate immune system were chosen because each plays an integral and complementary role in the first line of defense against novel pathogens (Zylberberg et al. 2013). In short, the complement system of proteins activates the lysis of foreign cells, enhances antibody activity, and directly destroys viruses (Hirsch 1982, Murphy et al. 2017). Natural antibodies, i.e., constitutive, bind novel pathogens, facilitate phagocytosis, and promote cell lysis (Casali and Schettino 1996, Caroll and Prodeus 1998). The PIT54 protein minimizes self-damage during inflammation and stimulates the white blood cell response upon pathogen exposure (Wicher and Fries 2006, Quaye 2008). Lysozymes lyse gram positive bacteria (Millet et al. 2007). Together, these measures afford a broad view of the innate immune system by providing information on both inducible and constitutive components of innate immunity.

Because land use can affect the host diet, which consequently can affect the gut microbiota and immune responses, we hypothesized that variation in land use would influence the immune response and gut microbiota of Darwin's finches. Specifically, we predicted that finches in areas with lower human activity, e.g., agricultural and undeveloped areas, would have more similar immune responses and gut microbiota than finches living in areas with higher human activity, e.g., urban areas. Furthermore, because studies have shown that the metrics of the immune system can relate to changes in the gut microbiota community (Round and Mazmanian 2009), we hypothesized that bacterial diversity would relate to immune measures in the finches. Finally, because food availability and thus diet differ across sites, we hypothesized that body size, i.e., mass and tarsus length, and condition, i.e., scaled mass index (Peig and Green 2009), would differ across land-use type, as found in Knutie et al (2019). Overall, our study aimed to provide insight into the effect of human activity on the gut microbiota and immune proxies of island birds, which could have conservation implications for Darwin's finches (Ohmer et al. 2021).

METHODS

Study system

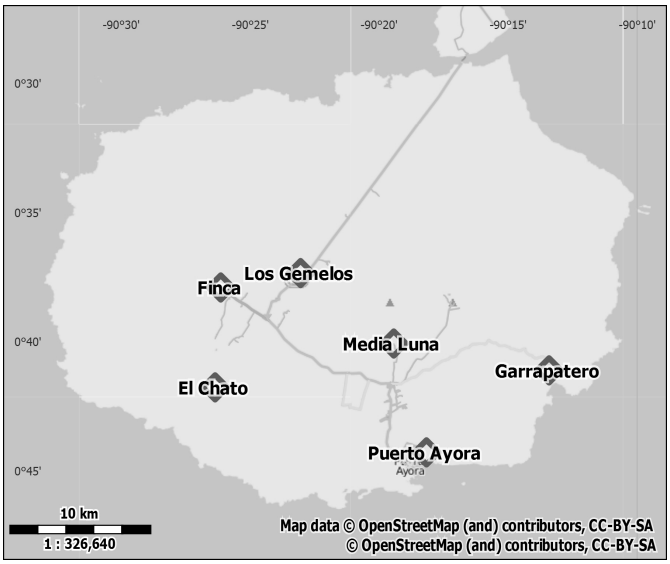
Small and medium ground finches are residents of the island of Santa Cruz (Dvorak et al. 2012). The small ground finch has a smaller body and bill size, and eats smaller sized food, e.g., seeds, than the medium ground finch, who has a larger body and bill size, and eats larger sized food (Schluter 1982).

We conducted this study on Santa Cruz Island, Galápagos, Ecuador, during the breeding season from 1 to 9 February 2008 (Fig. 1). To examine the effect of land-use patterns on microbiota, immune response, and body size of finches, we sampled small and medium ground finches at six sites, spanning three different land-use types: urban, agricultural, and undeveloped (Table S1, Appendix 1). We selected the study sites to represent the range of vegetation types and precipitation present in each land-use and elevation category. The urban site was in Puerto Ayora in the lowland arid zone and the human population size was approximately 11,000 in 2010 (Guerrero et al. 2017). Three sites were located within the agricultural zone of the island, which contains a combination of small farms, fruit plantations, and cattle ranches. Two sites were in undeveloped areas, representing both arid lowland and moist highland zones. Both undeveloped sites have few human residents. The Galápagos is well known for extreme fluctuations in precipitation because of the El Niño weather pattern; however, 2008 was neither particularly wet nor particularly dry, on average (Zylberberg et al. 2013).

Sample collection

To obtain immune function and microbial data, we caught birds in mist nets that were actively watched. Birds were removed from the mist nets and processed as quickly as possible. In the event that multiple birds entered the mist net, they were removed and kept in cloth bags until they were banded and a blood sample collected from the brachial vein, with the area not swabbed with alcohol prior to sample collection. Body mass (g) and tarsus length (mm) were measured, and a scaled mass index, i.e., a metric of body condition, was calculated based on the methods from

Fig. 1. The location of the six study sites on Santa Cruz Island, Galápagos. Puerto Ayora (-0.743101, -90.310493) is the single urban site. Los Gemelos (-0.625779, -90.385080) and Garrapatero (-0.689600, -90.222585) are undeveloped park land, whereas El Chato (-0.682552, -90.432501), Finca (-0.633890, -90.433617), and Media Luna (-0.672168, -90.323153) are agricultural sites. Lowland sites are 0–200 m in elevation and highland sites are 300–500 m in elevation. Lines on the map indicate roads, whereas triangles indicate elevational peaks.



Peig and Green (2009). We banded each bird and collected blood samples using heparinized microcapillary tubes. Blood samples were kept on ice for four to six hours until they were centrifuged to separate the red blood cells from the plasma. Plasma was then frozen at -20 °C for four to eight months prior to conducting immunoassays. For birds that defecated during handling, the fecal sample was immediately collected and placed in formalin, which was 37% formaldehyde by weight, until used for the fecal bacterial DNA extraction at the University of Connecticut in 2019. Because fecal samples were collected opportunistically, they were not collected using sterile technique and no field blanks were taken. Samples were collected by the same researcher (M. Z.) and the collection method was similar across individuals and locations. Therefore, any issues with contamination should be relatively consistent across species and locations. Additionally, studies show that the bacterial community in avian feces does not always represent the entire digesta of the host, e.g., in the cecum (Wilkinson et al. 2017), but fecal samples are generally representative of the bacterial community in the large intestines (Wilkinson et al. 2017, Videvall et al. 2018, Yan et al. 2019, Berlow et al. 2020) and are used when hosts cannot be euthanized.

Immune measures

We used a hemolysis-hemagglutination assay to measure levels of natural antibodies, i.e., lysis activity, and complement antibodies, i.e., agglutination activity (Matson et al. 2005). We used a commercial kit from Tri-delta Diagnostics Inc., Morris Plains, New

Jersey, USA, to determine the plasma concentration of PIT54 acute phase protein, i.e., haptoglobin levels, following Millet et al. (2007). We used the lyso-plate assay described in Millet et al. (2007) to measure levels of lysozyme activity in plasma samples. Each of these assays was carried out with previously described protocols (Zylberberg et al. 2014).

Bacterial DNA extraction and sequencing

Before starting the extraction from the finch feces, samples were centrifuged for 10 minutes at 10,000 rpm at 4 °C and the supernatant, i.e., the 37% formaldehyde, was then removed. Nanopure water (500 µL) was added, and the sample was vortexed and centrifuged again for 5 minutes at 4 °C at 10,000 rpm. The supernatant was removed and the step was repeated. Nanopure water (200 µL) was then added to the sample and the sample was vortexed. Total DNA was extracted from finch feces using a Qiagen PowerFecal DNA Isolation Kit. The protocol listed in the kit was followed except for the heating step; samples were heated for 30 minutes at 65 °C. Samples were extracted in five batches and then the DNA extracts were sent to the University of Connecticut Microbial Analysis Resources and Services (MARS) for library preparation. Bacterial inventories were conducted by amplifying the V4 region of the 16S rRNA gene using primers 515F and 806R (Caporaso et al. 2012) and with Illumina adapters and dual indices (Kozich et al. 2013). Samples were amplified in triplicate 15 µl reactions using Go-Taq DNA polymerase (Promega) with the addition of 3.3 µg BSA (New England BioLabs). To overcome inhibition from host DNA, 0.1 pmol primer without the indexes or adapters was added to the mastermix. The PCR reaction was incubated at 95 °C for 3.5 minutes, the 30 cycles of 30 s at 95 °C, 30 s at 50 °C, and 90 s at 72 °C, followed by final extension at 72 °C for 10 minutes. MARS also amplified a laboratory blank to control for kit contamination and had no detectable product. PCR products were sequenced with an Illumina MiSeq platform and v2 2x250 base pair kit (Illumina, Inc.).

We used the DADA2 Version 1.22.0 pipeline (Callahan et al. 2016) in R Version 4.3.1 (R Core Team 2023) to process sequence data. Sequences were trimmed to remove low quality read areas and chimeric reads. We classified amplicon sequence variant (ASV) taxonomies using RDP's Naive Bayesian Classifier (Wang et al. 2007) with the Silva Reference Database Version 138.1 (Quast et al. 2012). After classification, we removed sequences identified as chloroplast and mitochondria from the dataset. Additionally, we identified and removed four likely bacterial contaminants with the package decontam (Davis et al. 2018) in R using PCR blanks that were processed in parallel with the samples as controls. Sequences were aligned using the DECIPHER package Version 2.22.0 in R (Wright 2015), and a generalized time-reversible maximum likelihood tree of the remaining ASVs was constructed with the phangorn package Version 2.9.0 (Schliep 2011). The ASV table, taxonomic information, phylogeny, and sample metadata were joined for further analyses using the package *phyloseq* (McMurdie and Holmes 2013). We filtered the feature table to retain samples with at least 3000 total reads. The resulting dataset contained 929 unique ASVs across 71 samples. Samples had an average of 28,079 ± 3,000 reads per sample (min: 3214; max: 124,843).

Statistical analyses

Generalized linear mixed models (GLMMs) were used to determine the effect of land use on immune metrics, i.e., lysozyme activity and haptoglobin, complement antibody, and natural antibody levels; and on body size and condition, i.e., body mass, tarsus length, and scaled mass index. We ran models using the glmmTMB package (Brooks et al. 2017) with field-site location included as a random effect, with a poisson error structure for complement antibodies, and with a gaussian structure for lysozyme activity and haptoglobin, natural antibody levels, body mass, tarsus length, and scaled mass index. A dispersion parameter to account for heteroscedasticity related to land-use type was included in the model for lysozyme activity and to account for heteroscedasticity related to species in the model for body mass. Additionally, a zero-inflation parameter (ziformula = ~1) was included in the model for natural antibodies. Land-use type, i.e., undeveloped, agricultural, and urban; species, i.e., small or medium ground finch; and the interaction between species and land-use type were considered in all models. Undeveloped land use was set as the reference for comparison in all models, and we used the DHARMa package (Hartig 2019) to plot residuals and confirm the suitability of each model. Probability values were calculated using log-likelihood ratio tests using the Anova function in the car package (Fox and Weisberg 2018).

We analyzed alpha diversity metrics using two methods. For the first method, we rarefied samples to the sample with the lowest read count (3214). The rarefied dataset contained 881 ASVs after random subsampling. From this rarefied dataset, we calculated observed richness, Shannon diversity index, and Simpson diversity index. Observed richness describes the number of observed ASVs, i.e., the number of bacterial taxa as specified by amplicon sequence variants. The Shannon and Simpson diversity indices are both estimators of species richness and species evenness, which describes the distribution of abundance across the species. Species richness is weighted more for Shannon, and species evenness more for Simpson. Because there is still some debate over the appropriateness of rarefying microbiome data (McMurdie and Holmes 2014), we also quantified the Shannon and Simpson diversity indices utilizing a compositional approach on the unrarefied dataset (Gloor et al. 2016). For this method, the feature table was first filtered using the codaSeq.filter function from the R package CoDaSeq (Gloor et al. 2016), where sequences found in less than 0.2% of reads and fewer than 2% of samples were removed. This filtering step reduced the number of ASVs from 929 to 117. We then used Bayesian-multiplicative replacement to impute zero-count sequences following the methods in Grieves et al. (2023) using the R package zCompositions (Palarea-Albaladejo and Martin-Fernandez 2015), and calculated the Shannon and Simpson diversity indices from this zero-replaced dataset using the diversity function in vegan (Oksanen et al. 2022). For all alpha diversity metrics, we ran generalized linear mixed models using the glmmTMB package (Brooks et al. 2017) with field-site location included as a random effect and with a negative binomial (nbinom1) error structure for observed richness, a Gaussian structure for Shannon diversity, and a beta regression (beta_family) structure for Simpson. Additionally, a dispersion parameter to account for heteroscedasticity related to land-use type was included in the

richness model. Land-use type, i.e., undeveloped, agricultural, and urban; species, i.e., small and medium ground finch; and the interaction between species and land-use type were considered in all models. Undeveloped land use was set as the reference for comparison in all models, and we used the DHARMa package (Hartig 2019) to plot residuals and confirm the suitability of each model. The Anova function in the car package (Fox and Weisberg 2018) was used to determine significance. The emmeans package (Lenth et al. 2023) was used for pairwise comparisons where appropriate. Correlation function in the performanceanalytics package (Peterson et al. 2018) was used to test for and visualize correlations between alpha diversity metrics and immune endpoints, i.e., lysozyme activity and haptoglobin, complement antibody, and natural antibody levels, using the Pearson correlation method.

For beta diversity, we examined Euclidean (Aitchison) distances by applying a centered log-ratio (CLR) transformation to the filtered and zero-replaced dataset, following methods in Grieves et al. (2023). We conducted a PCA on the CLR-transformed data using the prcomp function in R and retained the first two PCs, which accounted for 16.56% of the variance. To test for differences between samples, we ran two GLMMs with a Gaussian error structure in glmmTMB (Brooks et al. 2017) with PC1 and PC2 as dependent variables and land-use type, species, and the interaction between land-use type and species as predictor variables. Field-site location was included as a random effect in both models. In the model for PC1, we included a dispersion parameter to account for heteroscedasticity related to land-use type. Undeveloped land use was set as a reference for comparison in all models, and we used the DHARMa package (Hartig 2019) to plot residuals and confirm the suitability of each model. The Anova function in the car package (Fox and Weisberg 2018) was used to determine significance.

Finally, to investigate whether bacterial genera differ in abundance across land-use types, we used an analysis of composition of microbes with bias correction (ANCOM-BC; Mandal et al. 2015, Lin and Das Peddada 2020). ANCOM-BC uses the structure of the microbiota data to identify differentially abundant bacterial taxa between treatment groups and controls for false discovery rates. Prior to ANCOM-BC analyses, we used the filter_taxa function in phyloseq (McMurdie and Holmes 2013) to remove taxa not seen in at least 5% of the samples. This filtering step limited analyses to 17 bacterial genera in medium ground finches (undeveloped: n = 5; agricultural: n = 18; urban: n = 8) and 21 bacterial genera in small ground finches (undeveloped: n = 13; agricultural: n = 19; urban: n = 4). We then used the ANCOMBC package in R to test for differences in bacterial genera with a significance of $P < 0.5$ after Bonferroni correction (Lin and Das Peddada 2020). ANCOM-BC analyses were run separately for each species to determine the effect of land-use type on the differential abundance of bacterial genera.

RESULTS

Effect of land use and finch species on the gut microbiota

Bacterial diversity, as measured by observed richness, differed among land-use types for finches ($P = 0.0003$; Table S2, Appendix 1; A, Fig. 2). Pairwise comparisons revealed that birds in the urban area had higher richness values than birds in agricultural ($P = 0.003$) and undeveloped ($P = 0.0009$) areas, whereas richness in birds from agricultural areas did not differ significantly from birds in undeveloped areas ($P = 0.93$). There was no effect of species or an interaction between species and land-use type on richness values (Table S2, Appendix 1). Land use, bird species, and the interaction between land-use type and bird species did not affect the Shannon or Simpson indices (Tables S2, S3, and Fig. S1, Appendix 1) regardless of the method in which these indices were calculated, i.e., from rarefied dataset or from compositional zero-replaced dataset. For beta diversity, the principal components (PC1, PC2) associated with the CLR-transformed feature table did not vary by land use, species, or the interaction between land use and species (Table S4 and Fig. S2, Appendix 1).

ANCOM-BC analyses identified four genera in medium ground finches and five genera in small ground finches that were differentially abundant across land-use types (Table S5, Appendix 1; Fig. 3). In medium ground finches, three genera (*Methylobacterium-Methylorubrum*, *Escherichia-Shigella*, and *Brucella*) were higher in urban areas and agricultural areas when compared with samples from finches in undeveloped areas. Abundance of the genus *Citrobacter* was highest in medium ground finches from urban areas when compared with any other land-use type. In small ground finches, *Paucibacter*, *Achromobacter*, *Delftia*, *Stenotrophomonas*, and *Brucella* had higher abundances in undeveloped and agricultural areas (Table S6, Appendix 1; Fig. 4). Conversely, the genus *Cutibacterium* was more abundant in finches from urban and agricultural areas than in finches from undeveloped areas.

Effect of land use and finch species on the immune response

Lysozyme activity differed among land-use types for both finch species (B, Fig. 2; Table S7, Appendix 1). Overall, urban finches had statistically lower lysozyme levels than finches in the agricultural and undeveloped areas, but this effect was more pronounced in medium ground finches (Table S8, Appendix 1). Complement antibodies also varied among land-use types and were lowest in finches from undeveloped areas (C, Fig. 2; Tables S7 and S8, Appendix 1). Haptoglobin and natural antibody levels did not differ significantly among land-use types in either small or medium ground finches (Tables S7 and S8, Appendix 1). Simpson index was negatively correlated with lysozyme activity (Table S9 and Fig. S3, Appendix 1: correlation coefficient = -0.26, $P = 0.04$). However, upon visualization, this relationship appeared to be driven by one individual with high lysozyme activity and when this bird was excluded from analyses, this relationship was no longer significant (Fig. S3, Appendix 1: correlation coefficient = -0.11, $P = 0.39$). No other immune metrics correlated significantly with the bacterial diversity metrics (Table S9, Appendix 1: $P > 0.15$ for all pairs).

Effect of land use and finch species on body size and mass

Body mass differed between small and medium ground finches (A and B, Fig. 5; Tables S10 and S11, Appendix 1). For small ground finches, body mass did not differ significantly among land-use types (B, Fig. 5), but for medium ground finches, individuals in the undeveloped area had lower body mass than individuals in agricultural and urban areas (A, Fig. 5; Table S11, Appendix 1). Tarsus length and scaled mass index differed significantly between species but not among land-use types (C and D, Fig. 5; Tables S10 and S11, Appendix 1).

Fig. 2. The effect of land-use type (undeveloped, agricultural, and urban) on the mean (± SE) observed bacterial richness (A), lysozyme activity (B), and complement antibodies (C) of medium ground finches (undeveloped: n = 5; agricultural: n = 18; urban: n = 8–10) and small ground finches (undeveloped: n = 13–14; agricultural: n = 19–21; urban: n = 4–5) on Santa Cruz Island, Galápagos in 2008. Each point represents an individual bird.

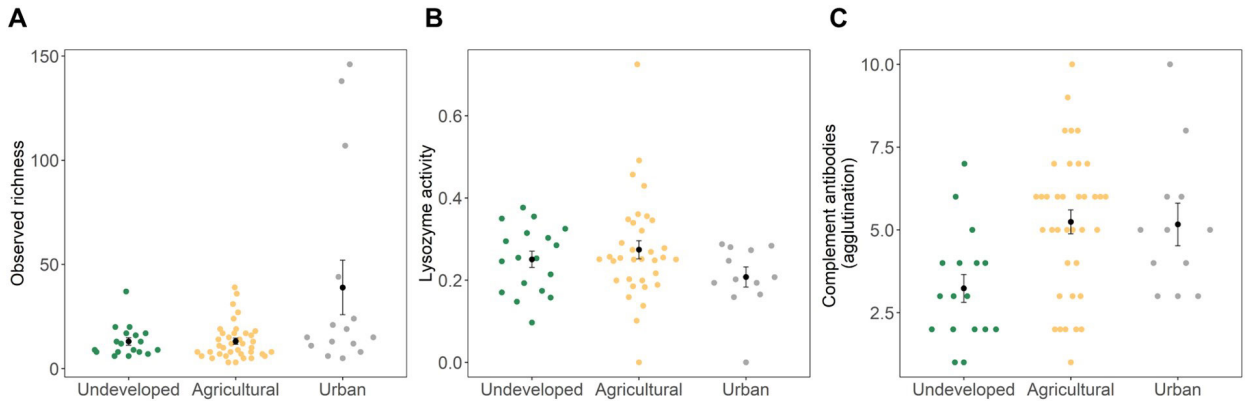
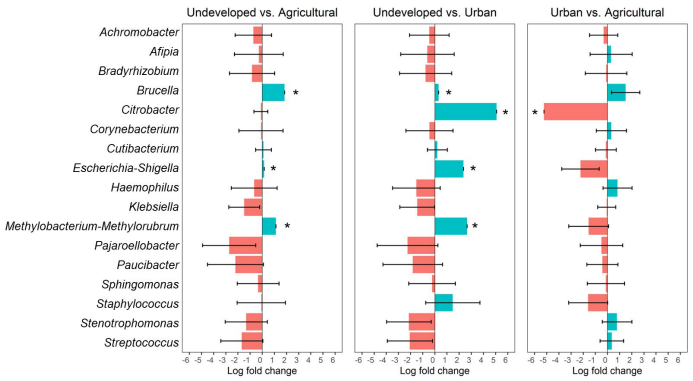


Fig. 3. Analysis of composition of microbiomes with bias-correction (ANCOM-BC) of bacterial genera that were differentially abundant across land-use types (undeveloped, agricultural, and urban) in medium ground finches. Genera with significantly different abundances across land-use types are indicated with an asterisk. Within each comparison, negative log-fold change values indicate an increased abundance in the land-use type that is listed first in the comparison and positive log-fold change values signify an increase in abundance land-use type that is listed second. For example, the genus *Citrobacter* is significantly more abundant in medium ground finches from urban areas when compared with finches from undeveloped and agricultural areas.



DISCUSSION

Our study examined the effect of land use on the gut microbiota, immune metrics, and body measurements of two Darwin's finch species in 2008, at the start of a rapid increase in human activity in the Galápagos Islands (Watkins and Cruz 2007, Walsh and Mena 2016). Studies of samples collected in 2016 have found significant impacts of human activity on metrics of the gut microbiota and body measurements of Darwin's finches (Knutie et al. 2019). Based on data collected eight years earlier, urbanization affected the observed richness across bird species but did not affect the Shannon or Simpson diversity metrics, suggesting that land-use type affects the number of bacterial taxa that are present in the gut microbiota, i.e., richness, but not the evenness of those taxa, i.e., diversity. However, when analyzing the abundance of the most common bacterial taxa, i.e., genera found in at least 5% of samples, land-use type altered the abundance of a few bacterial genera in both small and medium ground finches. In medium ground finches, the genus *Citrobacter* was more abundant in urbanized areas, and *Brucella, Escherichia-Shigella*, and *Methylobacterium-Methylorubrum* were more abundant in both urban and agricultural areas when compared with undeveloped areas. In small ground finches, five genera, i.e., *Paucibacter*, *Achromobacter*, *Delftia*, *Stenotrophomonas*, and *Brucella*, were less abundant in urban areas when compared with undeveloped and agricultural areas. Conversely, the genus *Cutibacterium* was more abundant in small ground finches from urban and agricultural areas. Similarly, land use had few significant effects on immune metrics and body measurements, with urbanization affecting lysozyme and complement antibody levels across bird species and body mass in medium ground finches. Simpson index was negatively correlated with lysozyme activity; however, this appeared to be driven by a single bird with high lysozyme activity. Bacterial diversity did not correlate with any other immune measures. Although humans have had a permanent presence in the Galápagos for decades, our results suggest that, initially, increased human activity starting ~2007 had marginal effects on the finches (Walsh and Mena 2016).

Lysozyme activity was influenced by land-use type, with urban birds having lower lysozyme activity than non-urban birds. Timing of breeding could be responsible for differences in lysozyme activity in urban versus non-urban birds. Lysozymes are transferred from mothers to egg whites and, during the breeding season, lysozyme activity declines in females during pre-laying and egg laying (Saino et al. 2002). Urban Darwin's finches lay eggs earlier than non-urban finches (Harvey et al. 2021) and we collected samples in February when urban finches were preparing to breed or were laying eggs, but non-urban finches were not yet breeding. Furthermore, although lysozymes are

Fig. 4. Analysis of composition of microbiomes with bias-correction (ANCOM-BC) of bacterial genera that were differentially abundant across land-use types (undeveloped, agricultural, and urban) in small ground finches. Genera with significantly different abundances across land-use types are indicated with an asterisk. Within each comparison, negative log fold change values indicate an increased abundance in the land-use type that is listed first in the comparison and positive log fold change values signify an increase in abundance in the land-use type that is listed second.

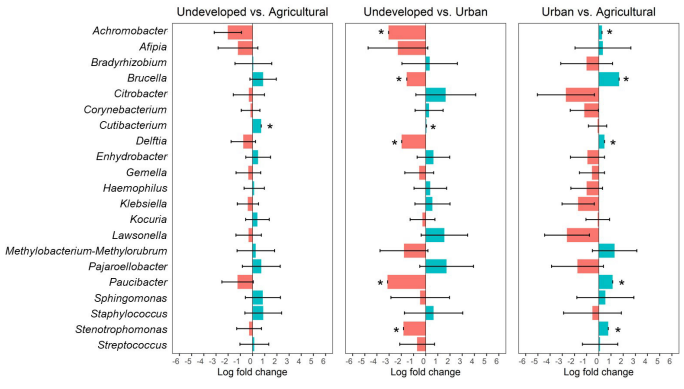
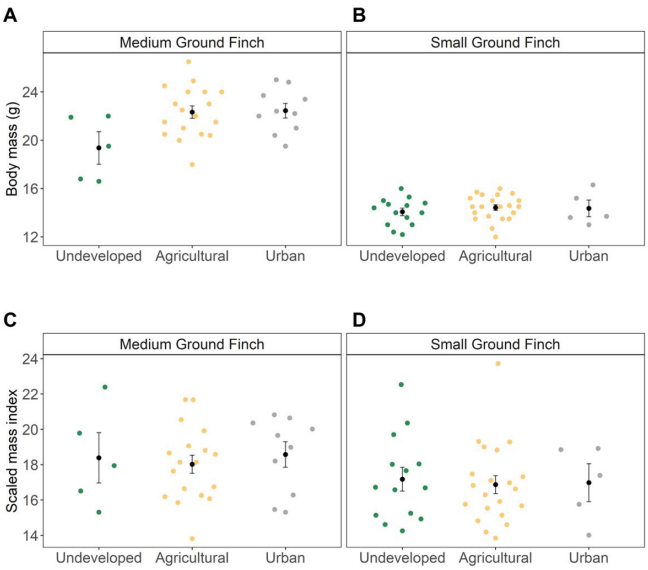


Fig. 5. The effect of land-use type (undeveloped, agricultural, and urban) on body-mass (grams) and scaled-mass index of medium ground finches (A: body mass, C: scaled mass; undeveloped: n = 5, agricultural: n = 18, urban: n = 10) and small ground finches (body mass: B, scaled mass: D; undeveloped: n = 14, agricultural: n = 21, urban: n = 5) on Santa Cruz Island, Galápagos in 2008. Black circles denote the mean values (± SE) of birds from each treatment.



antimicrobial enzymes, lysozyme activity and bacterial diversity did not correlate significantly. This lack of relationship is likely because the lysozymes quantified in our study were in the plasma, rather than in the gut mucosal tissue. Overall, because lysozyme activity differed across land-use categories in 2008, at the onset of rapid human activity change, future studies could focus on how lysozyme activity has changed in finches since then, especially in response to emerging diseases (Wikelski et al. 2004). Complement antibodies also varied by land-use type. Specifically, finches caught in agricultural and urban areas had higher complement antibody levels than finches from undeveloped areas. Higher complement activity in agricultural and urban areas could be because of increased or altered parasite communities, such as the avian pox virus, in areas associated with higher rates of human disturbance (Minias 2023). For example, in the Common Myna (*Acridotheres tristis*), higher levels of urbanization are associated with stronger immune responses and higher ectoparasite loads (Peneaux et al. 2021).

Observed species richness was higher in urban areas compared to non-urban areas. However, this result was driven by a small number of individuals with very high bacterial richness. The other alpha diversity metrics that consider species evenness did not differ across habitats. Other studies that have found higher observed species richness and Shannon index in urban birds indicate that this could be caused by man-made landscapes and a higher grass and tree cover (Phillips et al. 2018, Berlow et al. 2021). However, there have also been studies demonstrating less bacterial diversity in species living in an urban habitat when compared to species living in a non-urban habitat (Barelli et al. 2015, Teyssier et al. 2018, 2020, Knutie et al. 2019). A less diverse gut community in urban areas could be caused by habitat disturbances or differences in diets between urban and non-urban populations (Sonnenburg et al. 2016, Teyssier et al. 2018, 2020, Knutie 2020). The time of sampling should also be considered, because Tessyier et al. (2018) found that birds in a non-urban habitat exhibited less microbiome diversity during the winter months, whereas microbiome diversity in birds in an urban habitat did not differ across seasons. Because the birds included in this study were sampled at the start of the rainy, i.e., breeding, season, it is possible that a seasonal effect contributed to the difference seen between birds from non-urban and urban habitats. Overall, our results contribute to the growing body of literature demonstrating that there are many factors to consider when studying the relationship between urbanization and gut microbiome diversity.

The abundances of several bacterial genera were higher in urban areas than non-urban areas in medium ground finches (*Citrobacter*, *Brucella*, *Escherichia-Shigella*, and *Methylobacterium-Methylorubrum*) and small ground finches (*Cutibacterium*) in 2008. Conversely, in small ground finches, five genera (*Paucibacter*, *Achromobacter*, *Delftia*, *Stenotrophomonas*, and *Brucella*) were less abundant in urban areas when compared with undeveloped and agricultural areas. Increases in some bacterial genera in urban environments might be because of stress-induced or immune-induced shifts in gut microbiota (Minias 2023, Watson et al. 2017). Urban environments are associated with physiological stress in some studies (Minias 2023) and stress hormones, such as glucocorticoids, can alter gut microbiome composition in birds (Noguera et al. 2018). Stress can increase *Citrobacter* spp. abundances in mice (Bailey et al. 2010), which we found at higher levels in medium ground finches from urban areas. We also found higher levels of *Escherichia-Shigella* in urban medium ground

finches, which has been associated with increased urbanicity in humans (Bowyer et al. 2022). Similarly, the phylum Proteobacteria, which includes *Escherichia-Shigella* spp. and *Brucella* spp., was higher in urban House Sparrows (*Passer domesticus*) than their rural counterparts (Gadau et al. 2019). Interestingly, urbanization was associated with higher *Brucella* abundances in medium ground finches, whereas it was less abundant in small ground finches from urban areas, possibly because of species differences in dietary preferences across land-use types. The lower abundances of several genera detected in small ground finches in urban areas could be associated with variable diets between land-use types. One such genera, *Stenotrophomonas*, has been linked to chitin digestion (Huang and Liao 2021), and could be lower in small ground finches from urban environments because of lower insect abundance in urban areas or feeding preferences for other food items associated with urban environments. Small and medium ground finches do eat different natural diets (Abbott et al. 1977) and differences in feeding preferences between the two species across land-use types could possibly explain the variable effects of urbanization on the abundances of gut microbiota between species. Interestingly, small ground finches in 2016 only showed increases in the relative abundance of *Steroidobacter* spp. in the presence of humans (Knutie et al. 2019). Although we found no effect of human activity on bacterial phyla in 2008 finches, 2016 finches in areas with higher human activity had higher relative abundances of phyla *Chlamydiae* and Cyanobacteria than in other areas.

One explanation for differences in bacterial taxa between 2008 and 2016 finches is that the gut microbiota of finches has changed over eight years, coinciding with a change in the finches' environment. Davidson et al. (2020) found that relative abundances of the phyla Proteobacteria, Bacteroidetes, and Actinobacteria in Great Tits (*Parus major*) were higher in urban areas compared to rural areas and/or when Tits were fed an insect-based diet compared to a seed-based diet. Recent studies have found that urban small and medium ground finches tend to prefer human food diets, e.g., chips and cooked rice, rather than their natural diet, e.g., seeds, which could explain our results (de León et al. 2018). Alternatively, urban contaminants could be responsible for changes in the gut bacterial taxa observed in the finches. For example, human-associated toxicants like per- and polyfluoroalkyl substances (PFASs) alter the abundances of genera *Delftia* and *Methylobacterium-Methylorubrum* in the gut of frogs, albeit in opposite directions from what we observed in finches in the present study (Lin et al. 2022). Although human activity in the Islands did not rapidly increase until approximately 2007, sources of human-driven pollution were present in towns, such as Puerto Ayora. For example, the major presence of vehicles began in the 2000s with the completion of major paved roads. One final possibility is that the difference between 2008 and 2016 results is because of sampling technique. Samples from 2016 were systematically collected using Knutie et al. (2018) for a microbiome study, compared to 2008 samples, which were opportunistically collected, i.e., without a specific study in mind.

Body mass differed across land-use types for medium ground finches, with non-urban birds having lower body mass than those in urban areas. These results were also found by McNew et al. (2017) and can potentially be explained by differences in food availability across sites. Human activity can affect food availability and preference for wildlife. For example, the diet of non-urban birds includes mostly natural foods, such as seeds, fruits, and insects, whereas urban birds can prefer human-processed food (Murray et al. 2016, de León et al. 2018, Phillips et al. 2018). Consumption of human-processed food can affect body morphometrics of animals, including increased body mass (Banks and Dickman 2000, Bayol et al. 2007, Wilcoxen et al. 2015). In contrast, land use did not influence the body mass of small ground finches. Small ground finches have smaller bill sizes (length, width, and depth) and therefore different diets than medium ground finches (Abbott et al. 1977). One explanation is that perhaps medium ground finches are better able to exploit the urban diet than small ground finches. Interestingly, small ground finches in areas with more human activity in 2016 were larger than in areas with no human activity, which might be related to the increase in human activity over time. Since museum specimens and long-term field data exist, a future study could determine the effect of human activity on different finch species in urban and non-urban areas across islands.

Our study suggests that immunity, gut microbiota, and body mass of two species of Darwin's finches vary across land use at the start of the rapid increase in human activity in Galápagos Islands. These results suggest that the onset of rapid human change affects the ecology of birds. Over the past several decades, Darwin's finches have faced increasing challenges from invasive parasites (Wikelski et al. 2004, Fessl et al. 2010, Koop et al. 2016, Knutie 2018) and predators (Gotanda 2020), anthropogenic debris (Theodosopoulos and Gotanda 2018, Harvey et al. 2021), and dynamic annual changes in natural and novel food availability (Grant and Grant 1995, de León et al. 2018), which can all affect the physiology and gut microbiota of animals. Given our results and the novel challenges facing the Galápagos Islands, the iconic Darwin's finch system has exciting potential for future ecoimmunology and microbiome research in a changing world (Ohmer et al. 2021).

Author Contributions:

M. Z. and S. A. K. conceived the study; M. Z. collected the samples and field data; M. Z. and A. A. conducted the laboratory work; A. C. L. and A. A. conducted the bioinformatics; and A. C. L. did the data analyses. All authors wrote, revised, and approved the manuscript.

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Data Availability:

Data are available at FigShare (https://doi.org/10.6084/m9.figshare.25134524.v1) and sequences have been uploaded to GenBank (BioProject accession number: PRJNA1070320).

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Appendix 1

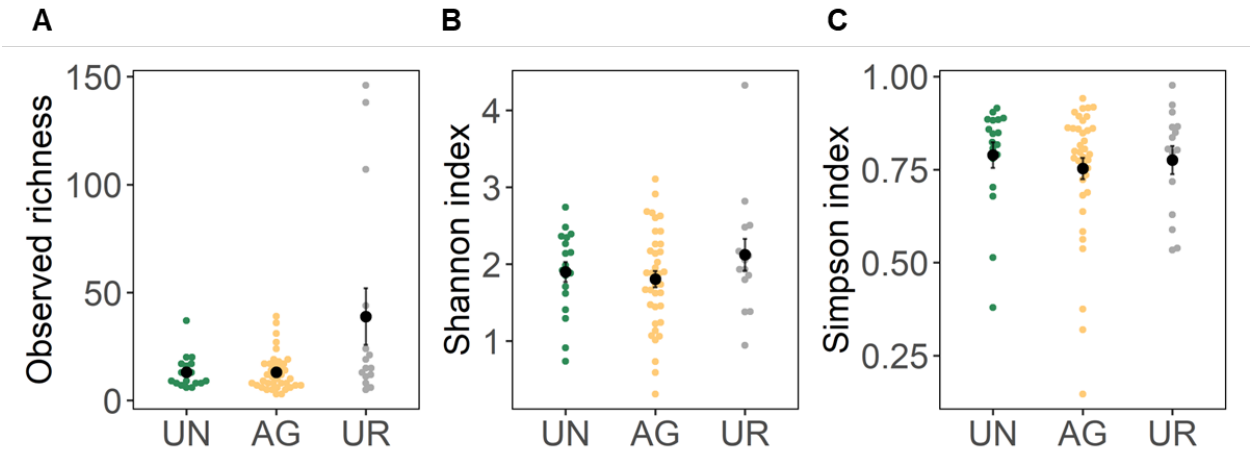

Fig. S1. Effect of land use type (green = undeveloped (UN), yellow = agricultural (AG), gray = urban (UR)) on alpha diversity of small and medium ground finch fecal microbiotas. Panels depict (A) Observed ASV richness, (B) Shannon index, and (C) Simpson index. Points represent individual birds. Black circles denote the mean values (± SE) of birds from each treatment.

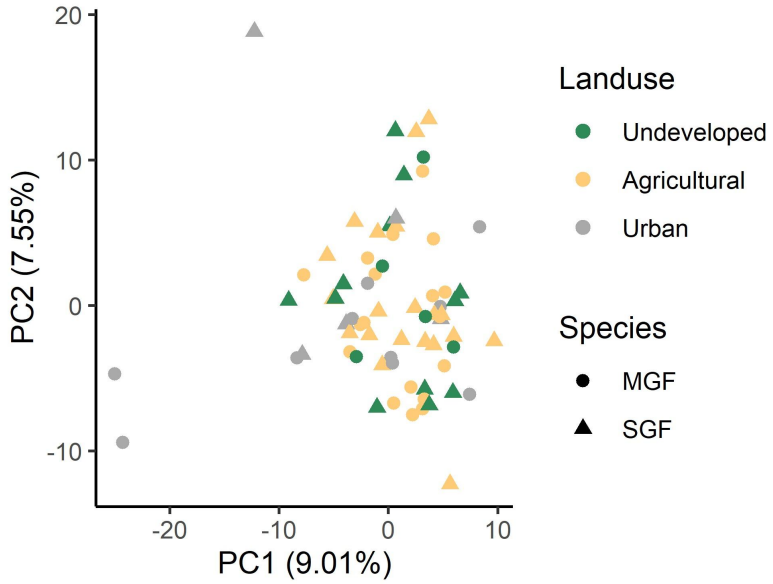

Fig S2. PC1 and PC2 scores derived from relative abundances of bacterial amplicon sequence variants (ASVs) from fecal samples of medium and small ground finches. Colors indicate land use type (green = undeveloped, yellow = agricultural, gray = urban) and shape denotes species (circles = medium ground finches, triangles = small ground finches).

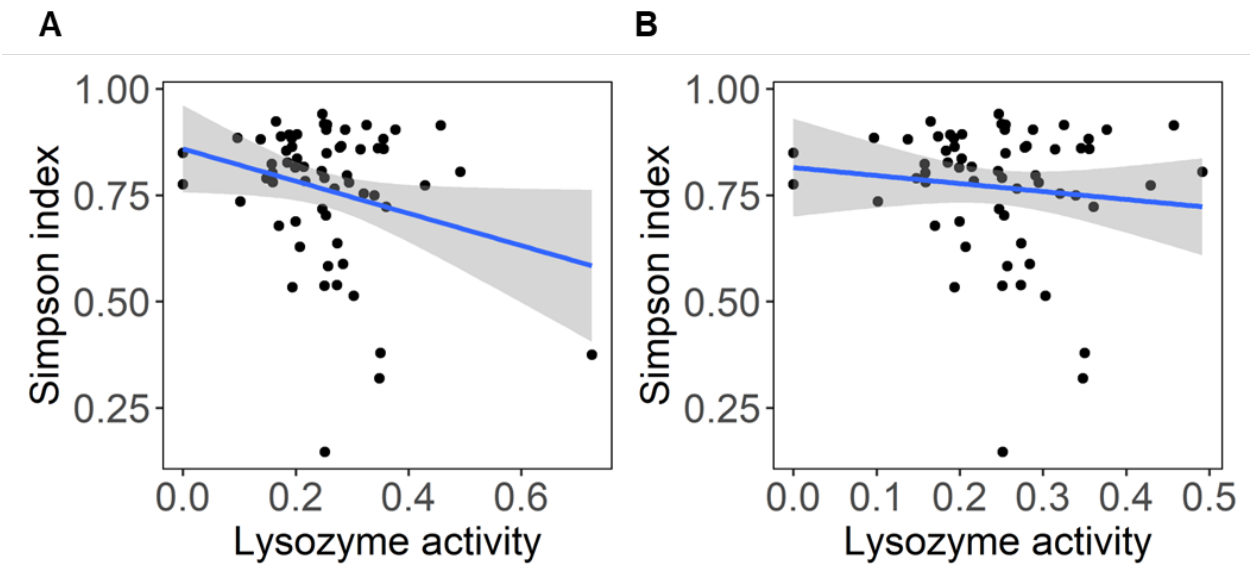

Fig. S3. Correlation between Simpson index and lysozyme activity in medium and small ground finches (A) with all data points included and (B) when the individual with the highest lysozyme activity (outlier) is removed. Each point represents an individual bird.

Table S1. GPS locations for each field site and the number of individuals caught for each field site.

Land use type	**Field site name**	**Latitude**	**Longitude**	**Elevation (m)**	**# individuals *G. fortis***	**# individuals *G. fuliginosa***
Urban	Puerto Ayora	-0.743101	-90.310493	7	10	5
Agricultural	El Chato	-0.682552	-90.432501	202	6	7
	Finca	-0.633890	-90.433617	376	12	4
	Media Luna	-0.672168	-90.323153	405	0	10
Undeveloped	El Garrapatero	-0.689600	-90.222585	21	5	8
	Los Gemelos	-0.625779	-90.385080	605	0	6
Total					**33**	**40**

Table S2. ANOVA results from the generalized linear models examining the effect of land use type, finch species, and their interaction on bacterial diversity metrics calculated from the rarefied dataset.

	χ^2	df	P-value
Observed richness			
Land use type	15.92	2	0.0003
Species	0.23	1	0.63
Interaction	2.87	2	0.24
Shannon index			
Land use type	2.55	2	0.28
Species	1.67	1	0.20
Interaction	2.89	2	0.24
Simpson index			
Land use type	0.52	2	0.77
Species	1.48	1	0.22
Interaction	2.46	2	0.29

Table S3. ANOVA results from the generalized linear models examining the effect of land use type, finch species, and their interaction on Shannon index and Simpson index calculated from the zero-replaced dataset.

	χ^2	df	P-value
Shannon index			
Land use type	0.56	2	0.75
Species	0.95	1	0.33
Interaction	4.04	2	0.13
Simpson index			
Land use type	0.43	2	0.81
Species	1.62	1	0.20
Interaction	3.36	2	0.19

Table S4. ANOVA results from the generalized linear models examining the effect of land use type, finch species, and their interaction on the first two principal components of a PCA calculated from the centered log-ratio (CLR) transformed and zero-replaced feature table.

	χ^2	df	P-value
PC1			
Species	0.01	1	0.94
Land use type	4.01	2	0.13
Interaction	0.48	2	0.79
PC2			
Species	2.23	1	0.14
Land use type	0.07	2	0.97
Interaction	3.28	2	0.19

Table S5. Results of ANCOM-BC analysis, including log fold change (LFC), standard errors (SE), and adjusted P-values (Padj). Differential abundance in bacterial genera between undeveloped, agricultural (Ag.), and urban land use types in medium ground finches.

	Ag. - Undeveloped			Urban - Undeveloped			Ag. - Urban		
Genus	LFC	SE	P_{adj}	LFC	SE	P_{adj}	LFC	SE	P_{adj}
Citrobacter	-0.11	0.57	1	5.09	0.00	0	-5.22	0.00	0
Afipia	-0.28	2.01	1	-0.61	2.20	1	0.30	1.73	1
Pajaroellobacter	-2.72	2.19	1	-2.25	2.50	1	-0.49	1.75	1
Bradyrhizobium	-0.84	1.86	1	-0.76	2.15	1	-0.11	1.71	1
Staphylococcus	-0.08	1.99	1	1.48	2.23	1	-1.59	1.61	1
Brucella	1.84	0.00	0	0.31	0.00	0	1.50	1.16	1
Sphingomonas	-0.34	1.72	1	-0.23	1.92	1	-0.13	1.54	1
Methylobacterium-Methylorubrum	1.13	0.00	0	2.66	0.00	0	-1.55	1.64	1
Paucibacter	-2.21	2.30	1	-1.82	2.45	1	-0.42	1.28	1
Haemophilus	-0.66	1.89	1	-1.52	1.96	1	0.83	1.19	1
Stenotrophomonas	-1.32	1.73	1	-2.14	1.83	1	0.79	1.22	1
Achromobacter	-0.73	1.50	1	-0.45	1.63	1	-0.31	1.16	1
Streptococcus	-1.68	1.73	1	-2.06	1.86	1	0.36	0.97	1
Corynebacterium	-0.10	1.82	1	-0.44	1.94	1	0.32	1.24	1
Escherichia-Shigella	0.16	0.00	0	2.36	0.00	0	-2.22	1.54	1
Klebsiella	-1.48	1.27	1	-1.45	1.43	1	-0.06	0.74	1
Cutibacterium	0.11	0.65	1	0.21	0.82	1	-0.13	0.85	1

Table S6. Results of ANCOM-BC analysis, including log fold change (LFC), standard errors (SE), and adjusted P-values (P_{adj}). Differential abundance in bacterial genera between undeveloped, agricultural (Ag.), and urban land use types in small ground finches.

	Ag. - Undeveloped			Urban - Undeveloped			Ag. - Urban		
Genus	LFC	SE	P_{adj}	LFC	SE	P_{adj}	LFC	SE	P_{adj}
Citrobacter	-0.30	1.28	1	1.65	2.46	1	-2.70	2.34	1
Afipia	-1.19	1.63	1	-2.28	2.45	1	0.34	2.29	1
Pajaroellobacter	0.72	1.55	1	1.72	2.21	1	-1.75	2.13	1
Bradyrhizobium	0.07	1.51	1	0.33	2.28	1	-1.00	2.13	1
Staphylococcus	0.88	1.51	1	0.65	2.39	1	-0.52	2.37	1
Brucella	0.87	1.08	1	-1.55	0.00	0	1.67	0.00	0
Sphingomonas	0.85	1.44	1	-0.45	2.40	1	0.54	2.34	1
Methylobacterium-Methylorubrum	0.27	1.54	1	-1.78	1.97	1	1.30	1.82	1
Paucibacter	-1.23	1.29	1	-3.13	0.00	0	1.14	0.00	0
Haemophilus	0.14	0.83	1	0.38	1.34	1	-0.99	1.30	1
Stenotrophomonas	-0.28	1.02	1	-1.82	0.00	0	0.78	0.00	0
Achromobacter	-2.03	1.12	1	-3.04	0.00	0	0.26	0.00	0
Enhydrobacter	0.46	1.02	1	0.64	1.34	1	-0.93	1.40	1
Streptococcus	0.15	1.18	1	-0.70	1.43	1	0.10	1.45	1
Lawsonella	-0.32	1.04	1	1.54	1.91	1	-2.61	1.84	1
Corynebacterium	-0.16	0.77	1	0.28	1.15	1	-1.19	1.16	1
Delftia	-0.76	1.00	1	-1.97	0.00	0	0.47	0.00	0
Gemella	-0.34	1.01	1	-0.53	1.18	1	-0.56	1.03	1
Kocuria	0.40	0.97	1	-0.26	1.02	1	-0.09	0.97	1
Klebsiella	-0.38	0.87	1	0.56	1.44	1	-1.70	1.32	1
Cutibacterium	0.71	0.00	0	0.07	0.00	0	-0.10	0.75	1

Table S7. The results of GLMMs on the effect of land use type, finch species, and their interaction on immune metrics.

	Land use type	Species	Interaction
Haptoglobin	$\chi^2 = 3.27$, df = 2, $P = 0.19$	$\chi^2 = 0.15$, df = 1, $P = 0.70$	$\chi^2 = 1.45$, df = 2, $P = 0.48$
Lysozyme	$\chi^2 = 7.64$, df = 2, ***P* = 0.02**	$\chi^2 = 3.63$, df = 1, $P = 0.06$	$\chi^2 = 4.53$, df = 2, $P = 0.10$
Complement antibodies	$\chi^2 = 9.41$, df = 2, ***P* = 0.01**	$\chi^2 = 0.37$, df = 1, $P = 0.55$	$\chi^2 = 0.24$, df = 2, $P = 0.89$
Natural antibodies	$\chi^2 = 1.72$, df = 2, $P = 0.42$	$\chi^2 = 0.06$, df = 1, $P = 0.80$	$\chi^2 = 3.03$, df = 2, $P = 0.22$

Table S8. Mean ± SE of each immune metric across land use types and finch species. Sample size in parenthesis.

	Undeveloped	Agricultural	Urban
Small ground finches			
Haptoglobin	0.97 ± 0.16 (13)	0.94 ± 0.08 (20)	0.52 ± 0.14 (3)
Lysozyme	0.23 ± 0.02 (13)	0.25 ± 0.02 (21)	0.24 ± 0.03 (3)
Complement antibodies	3.17 ± 0.55 (12)	5.00 ± 0.48 (20)	5.50 ± 0.50 (2)
Natural antibodies	1.21 ± 0.28 (12)	1.00 ± 0.18 (20)	1.50 ±1.0 (2)
Medium ground finches			
Haptoglobin	0.81 ± 0.18 (5)	0.89 ± 0.09 (18)	0.74 ± 0.07 (10)
Lysozyme	0.31 ± 0.02 (5)	0.31 ± 0.05 (14)	0.20 ± 0.03 (9)
Complement antibodies	3.40 ± 0.51 (5)	5.53 ± 0.53 (17)	5.10 ± 0.74 (10)
Natural antibodies	0.60 ± 0.29 (5)	1.32 ± 0.19 (17)	1.50 ± 0.37 (10)

Table S9. Correlation coefficients for bacterial diversity metrics (observed richness, Shannon index, Simpson index) and immune metrics (lysozyme activity, and haptoglobin, complement antibody, and natural antibody levels).

Dataset	Alpha diversity metric	Haptoglobin levels	Lysozyme activity	Complement antibodies (agglutination)	Natural antibodies (lysis)
Rarefied	Observed richness	-0.09	-0.04	0.09	0.07
Rarefied	Shannon index	-0.17	-0.21	0.11	0.04
Rarefied	Simpson index	-0.10	-0.26	0.09	0.04
Zero-replaced	Shannon index	-0.10	-0.19	0.07	-0.05
Zero-replaced	Simpson index	-0.03	-0.22	0.05	-0.01

Table S10. The results of GLMMs on the effect of land use type, finch species, and their interaction on body mass and size.

	Land use type	Species	Interaction
Body mass	$\chi^2 = 3.88$, df = 2, $P = 0.14$	$\chi^2 = 353.03$, df = 1, ***P* < 0.0001**	$\chi^2 = 6.60$, df = 2, ***P* = 0.04**
Tarsus length	$\chi^2 = 3.14$, df = 2, $P = 0.21$	$\chi^2 = 92.54$, df = 1, ***P* < 0.0001**	$\chi^2 = 2.26$, df = 2, $P = 0.32$
Scaled mass index	$\chi^2 = 0.51$, df = 2, $P = 0.78$	$\chi^2 = 5.57$, df = 1, ***P* = 0.02**	$\chi^2 = 0.10$, df = 2, $P = 0.95$

Table S11. Mean (± SE) of each body mass (g) and tarsus length (mm) across land use types and finch species. Sample size in parenthesis.

	Undeveloped	Agricultural	Urban
Small ground finches			
Body mass	14.07 ± 0.30 (14)	14.43 ± 0.23 (21)	14.36 ± 0.61 (5)
Tarsus length	21.12 ± 0.31 (14)	21.41 ± 0.22 (21)	21.30 ± 0.34 (5)
Scaled mass index	14.27 ± 0.26 (14)	14.33 ± 0.23 (21)	14.37 ± 0.62 (5)
Medium ground finches			
Body mass	19.36 ± 1.17 (5)	22.32 ± 0.50 (18)	22.44 ± 0.58 (10)
Tarsus length	22.88 ± 0.56 (5)	24.12 ± 0.27 (18)	23.93 ± 0.31 (10)
Scaled mass index	21.33 ± 1.23 (5)	21.86 ± 0.47 (18)	22.39 ± 0.65 (10)