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Research Article

Association between movement patterns, microbiome diversity, and potential pathogens of feral pigeons in dairy farms

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Abstract

The feedback between host behavior and disease transmission is well acknowledged, but empirical studies demonstrating the strength and direction of the associations between individuals' pathogens or microbiota composition and their movement are rare. We investigated these associations in feral pigeons (*Columba livia domestica*), a synanthrope species known to host a plethora of zoonotic pathogens, predicting a positive association between individual's movement behavior and microbiota alpha diversity. We captured pigeons in three dairy farms along an urbanization gradient in central Israel and combined GPS-tracking with total RNA-Sequencing to characterize pigeons' movement and microbiota, respectively. We found that pigeons roosted primarily in human settlements, with frequent visits to dairy farms and other agricultural sites. Microbiota diversity and composition varied between sites and the individuals within them, and several pathogens relevant to poultry, cattle, and human-health were frequently detected (e.g. *Escherichia* and *Clostridium*). Pigeons in the urban site covered shorter distances and carried a greater diversity of bacteria compared to those in rural sites. Beyond these among-site differences, exploratory individuals (measured by the number of unique locations they visited) had more diverse microbiota, implying a role for individual behavioral traits in disease exposure and spread. We conclude that pigeons can potentially serve as transmission vectors among wildlife, livestock, and humans. Further, the associations between host behavior and microbiota diversity emphasize the relevance of individually-targeted wildlife movement analyses for disease ecology and One Health.

Key words: Behavioral feedbacks, biotelemetry, disease ecology, individual variation, microbiota, movement ecology, One Health, parasite transmission, spatial analysis

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Introduction

Pathogen and parasite transmissions typically require the movement of hosts or vectors between locations, highlighting the fundamental role of animal movement in understanding dynamics for the diversity of (both pathogenic and non-pathogenic) microbes across large and small scales (Dougherty et al. 2017). Behavioral feedback between host and parasites further stresses the inherent connection

between movement and disease (Ezenwa et al. 2016). Host movement determines the exposure rate to different pathogens (e.g. more mobile hosts may encounter more pathogens), as well as the potential to transmit them into new sites. The pathogens, in turn, are not only affected by the host movement but may affect it either indirectly (e.g. reducing movement due to poor body conditions; Fofana and Hurford 2019), or directly through deliberate behavioral manipulations (Poulin and Maure 2015). Accordingly, the role of host movement ecology in disease transmission, and the variation among individuals in movement, exposure, and transmission traits, attract attention from a broad range of scientists, especially given the potential for zoonotic diseases (Dougherty et al. 2017).

Zoonotic diseases (pathogens that jump across species barriers from animals to humans) are an emerging issue because the interactions among wildlife, livestock, and humans are intensifying due to the population growth of the latter two (Salyer et al. 2017; Craft 2015). A review identified 175 animal-borne pathogens that are associated with emerging infectious diseases (Woolhouse et al. 2001), and Covid19 is a current and urgent reminder of the devastating and lasting effects of zoonotic outbreaks (Mackenzie and Smith 2020). To optimize global health, the One Health approach was created with the goal of using integrated knowledge across animals (both wildlife and livestock), humans, and their environment (Rabinowitz et al. 2013; Davis et al. 2017). Nevertheless, while One Health literature has often focused on broad ecological scales to study the origin, transfer, and potential vectors of zoonotic pathogens in an attempt to predict and prevent outbreaks, mechanistic examples focusing on transmission and feedbacks in specific systems are often left out. For instance, large-scale vector or host movements such as seasonal migration offer important insights into disease transmission (Viana et al. 2016), but routine local movements within populations (e.g. foraging between resource patches), and differences in behavior among conspecifics are also crucial for transmission dynamics (Payne et al. 2025).

Bio-logging techniques, such as GPS telemetry, may provide a detailed picture of individual hosts' movements, bridging these knowledge gaps and identifying where, when, and how individuals move over time for predicting the pathways of potential diseases (Viana et al. 2016; Daversa et al. 2017; Spiegel et al. 2022). Accumulating knowledge in the field demonstrates how movement patterns emerge from complex interactions between individual needs (e.g. to find food or shelter), capacities (e.g. the flight capacity), and external conditions (e.g. resource distribution; Nathan et al. 2008). Indeed, species show a remarkable variation across sites, and among individuals, directly affecting their transmission potential. This variation highlights the contribution of both bio-logging and methods to quantify individual differences as potential vectors (Dougherty et al. 2017; Spiegel et al. 2017).

Similarly to the bio-logging improvements, ongoing technological improvement in high-throughput DNA or RNA sequencing (including Next-Generation Sequencing; NGS) provides important insights into the presence and the potential transmission dynamics of both pathogenic and non-pathogenic microbes between hosts, and the influence of host behavior on the microbiome. Gut microbiome is affected by local environment (Loo et al. 2019) and season (Escallón et al. 2019), as well as host lifestyle, social interactions, and communal behavior (Sarkar et al. 2020). For example, greater natural avian movement

has been linked to increased microbiome diversity (Corl et al. 2020), and food variation between foraging sites has been shown to influence bird microbial composition (Gadau et al. 2019). The microbiome can serve as a pathway that connects an individual's environment to health disparities (Kuthyar and Reese 2021). Accordingly, a study of five different species of ducks found that infection status by the avian influenza virus dramatically affected microbial community variation (Hird et al. 2018), highlighting the potential relevance of studying microbiome diversity and pathogen transmission (Trinh et al. 2018).

Agricultural sites and practices are common environmental contexts in exposing humans and livestock to wildlife populations and the diseases they may carry. Dairy and poultry farming are particularly known to attract a variety of wildlife from peripheral habitats, due to their resource availability including food, shelter, and possible security from natural predators who avoid human habitats. European starlings (*Sturnus vulgaris*), for example, often aggregate in high numbers from surrounding cities and towns to forage at farms, creating an intersection for otherwise distant subpopulations (Cabe 2021). Such use of shared space together with high densities and mobility may facilitate pathogen transmissions among wildlife or into cattle (Jori et al. 2021). Considering contacts with workers (at the farm) and members of the community (at urban roosts) who regularly interact with these wildlife and domestic species highlights the relevance of the One Health approach and the potential harmful epidemiological consequences of these encounters between humans, non-human animals, and the environment.

Indeed, birds have received a lot of attention in the field of zoonotic diseases due to their mobility and potential to spread pathogens over large distances and ecological barriers (Cabe 2021; Nabi et al. 2021). Perhaps a most suitable model system for investigating the association between movement, microbiome diversity and pathogen transmission is the ubiquitous feral pigeon (*Columba livia domestica*; often referred to as rock dove and hereafter simply "pigeon"). Pigeons are common pests found in both urban and agricultural environments worldwide, often sharing their space with humans (Mia et al. 2022). They are social animals, aggregating into large flocks at resource-rich locations, or roosts at human buildings, resulting in spatial overlap between various surrounding subpopulations (Haag-Wackernagel and Moch 2004; Nabi et al. 2021). Pigeons are known reservoirs for pathogens and carry a variety of agricultural and public health-relevant viral, bacterial, fungal, and parasitic pathogens, such as *Salmonella*, *Chlamydia*, *Cryptococcus*, and *Escherichia* (Haag Wackernagel and Moch 2004). They have also been documented to carry a diversity of human pathogens with many documented spillovers and zoonotic transmissions demonstrating their importance in the context of One Health (Haag-Wackernagel and Moch 2004; Osman et al. 2013; Elser et al. 2019).

In light of their zoonotic relevance, several studies investigated pigeon movement (Sol and Senar 1995; Carlson et al. 2011; Santos et al. 2020; Smith et al. 2025) and microbiome diversity (Grond et al. 2019; Oh et al. 2023; Szczuka et al. 2023), but rarely together. In turn, here we address this knowledge gap and study these two components (movement and microbiome) at three dairy farms in central Israel, differing in their level of urbanization. First, we ask whether pigeons differ in their movement, both among and within each site. We expect that individuals at the more urban site will move less than the other two sites

due to the greater proximity to surrounding food availability and roosting sites (Tucker et al. 2018), and that individuals within the site will vary in their movement and transmission potential (Rose et al. 2006). Second, we ask which pathogens do pigeons carry at our focal dairy farms. We expect a diverse list of bacteria (including cattle, and human-relevant ones). This expectation reflects the above-mentioned literature regarding pigeons' diverse pathogenic loads, as well as a couple of local studies reporting *Salmonella* (Yeruham et al. 2006; Osman et al. 2013) and *E. coli* (Vasconcelos et al. 2018). Finally, we ask if individuals' movements are associated with their respective microbial diversity. The microbiome is influenced by a variety of environmental factors such as season, habitat, and diet (Amato et al. 2015; Embriette Hyde et al. 2016). Yet, how movement correlates with microbiome is poorly studied. In a rare example, Corl et al. (2020) reported that alpha diversity in barn owls' (*Tyto alba*) microbiome was higher in individuals that moved greater distances away from the nest each day. Such a positive association can reflect, for instance, exposure to more diverse bacterial environments. Hence, we predict that exploratory individuals who visit more sites throughout their daily foraging activity would display more diverse microbiota. To address these predictions, we combined free-living pigeon GPS tracking data and untargeted next-generation RNA sequencing and taxonomic profiling of a subset of the individuals.

Methods

Study sites

The fieldwork for this project took place at three dairy farms in central Israel representing a gradient of increasing urbanization (Fig. 1A, B): Mevo Horon, Ma'ale Hahamisha, and Mikve Israel. **Mevo Horon** (31.8496°N, 35.0350°E, +250 masl) is a settlement located 13 km northwest of Jerusalem. It is very isolated, with almost no other human settlements within a 5 km vicinity, and no urbanized (built) areas within 2 km area around the settlement (see Methods section in Suppl. material 1 for quantification procedure from aerial imaging). **Kibbutz Ma'ale Hahamisha** is located on a high elevated ridge (31°49.239'N, 35°6.667'E, +800 masl) about 7 km southeast of Mevo Horon. This site is surrounded by approximately 4 small human settlements within ~2 km distance (covering around 13.3% of the area) and several others within a 5 km range. Lastly, **Mikve Israel** (32.0312°N, 34.7833°E, +30 masl) is located approximately 30 km further northwest from Mevo Horon, within the heavily populated coastal plane. It is on the outskirts of the Tel Aviv metropolitan area, surrounded by immediate urban environments and additionally home to poultry houses nearby the dairy farm. Accordingly, 42% of the land surrounding this site is urban, making it our most urban site. For simplicity, we hearafter refer to the three farms as Mevo, Maale and Mikve, respectively.

Pigeon captures, GPS-tagging, and microbiome sampling

We captured a total of 308 pigeons using a combination of mist nets, walk-in ground traps, and falling traps with grains spread nearby to lure individuals towards them. Captures took place between July 2019 to November 2021 throughout all seasons. After capture, morphological measurements were

recorded from each pigeon following standard ornithology procedures for weight, age (juveniles/adults), wing and tail length, color, and chest feathers collection for sexing (by Karnieli Labs, Tivon, Israel; https://www.karnieli-vet.com/). All pigeons were fitted with metal rings containing a unique ID, in addition to an individual-specific combination of colored rings.

Captured individuals that were determined as adults in good body condition (i.e. had healthy plumage condition, showed normal behavior, and were above the 250 g minimal weight threshold) were fitted with an Ornitela OrniTrack 10 g GPS transmitter attached in a backpack configuration (Fig. 1C). These tags employ state-of-the-art technology, including solar power and data download capacity over GSM communication, allowing extended tracking duration. To save energy, tags used a diurnal duty cycle (dawn to dusk) reflecting pigeons' activity. During day-time, pigeon locations were sampled at 10 minute intervals when the battery was properly charged (reducing fix rate to once an hour or once a day if battery was below 50% or 25% power, respectively). Examples of a typical track (after filtering) can be seen in the appendix (Suppl. material 1: fig. S1). In total, 44 individual pigeons were fitted with GPS tags (Maale: 24, Mevo: 11, Mikve: 9).

To determine microbiome composition, we swabbed all captured individuals for the collection of DNA samples. We first swabbed the choanal cleft, followed by the cloaca. This combined swabbing method reflects our interest primarily in the overall microbial composition of each individual, rather than place of occurrence within an individual (and the associated costs of each sample). The swab was then placed into 2 mL of Viral Transport Medium (BHI) containing antibiotics and antimycotics for prevention of bacterial and fungal growth, and the sample was placed immediately onto ice. In total, 237, 23 and 48 samples were

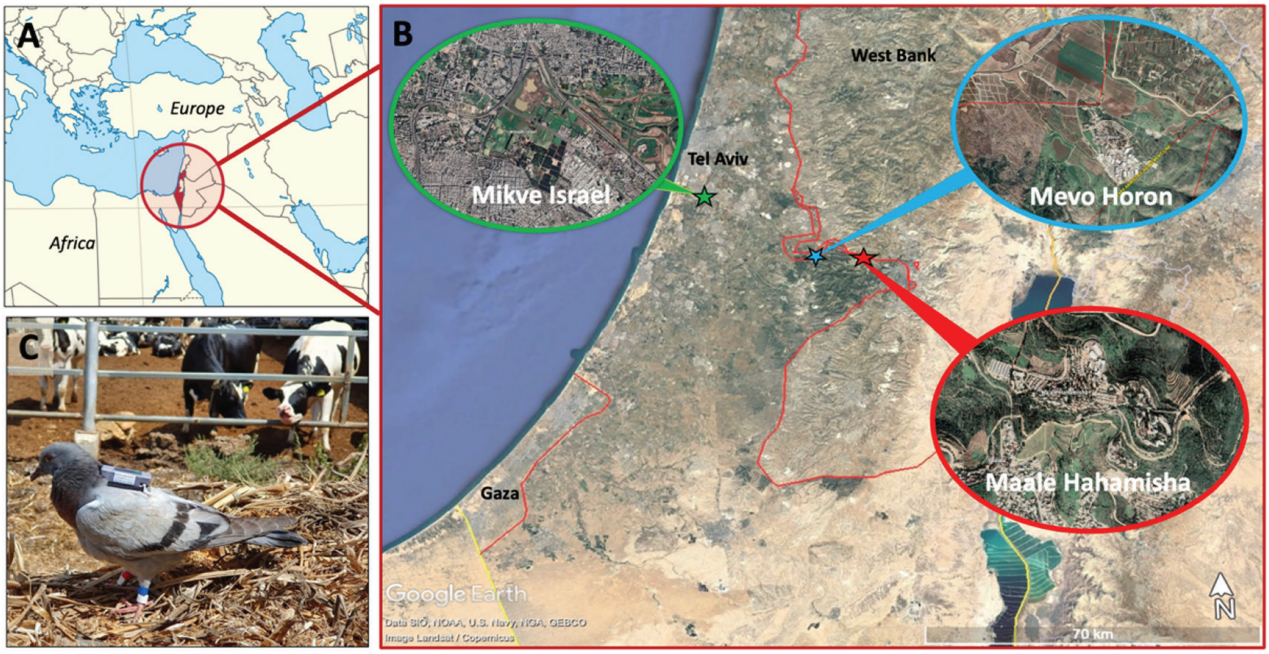


Figure 1. The dairy farm – feral pigeon (*Columba livia domestica*) study system. **A.** A map of the Mediterranean and Israel's position; **B.** A focal map of the three study sites within Israel; Mevo Horon being the most rural, Ma'ale Hahamisha is intermediately rural, and Mikve Israel is urban (hereafter Mevo, Maale and Mikve, respectively). **C.** Pigeon tagging and banding. Color-bands enable individual identification in the field. A subset of individuals were equipped with GPS-GSM transmitters in a backpack configuration. Photo credit: Orr Spiegel.

available for analysis from Maale, Mevo, and Mikve, respectively. From this total captured population, we chose a subset of 29 samples (of the GPS-tagged individuals) to send for NGS. These samples were spotted onto AniCards (a paper card by AniCon Labor GmbH, Höltinghausen, Germany, https://www.anicon.eu) for RNA preservation. While NGS is more expensive than classical species-specific tests (e.g. swabbing and growing *Campylobacter* colonies) – allows us to link these observed loads across many species with relevant ecological predictors. Validating NGS results with *Campylobacter* and *Salmonella* colony growth showed a strong agreement between methods, with both approaches revealing relatively high prevalence of the former and negligible prevalence of the latter (Crafton et al. in preparation). All capture and sampling procedures were authorized by research permit 2019/ 04-19-059 provided by the Tel Aviv University research animal ethics committee.

Next-Generation Sequencing (NGS)

To present a more in-depth comparison between movement and the microbiome, a total of 29 samples (Maale: 19, Mevo: 5, Mikve: 5) were analyzed using NGS by AniCon. AniCon is a private company that discloses their full protocols, thus here only we briefly overview these analytical protocols of total RNA sequencing. Readers are also referred to the Suppl. material 1 for additional details and to previous studies applying a similar approach with highly-specialized protocols of NGS swab preparations (Ayala et al. 2016; Dimitrov et al. 2017; Ferreira et al. 2019).

In brief, the AniCards were first applied with the appropriate volume of sample media, properly dried, individually packaged in zip-lock bags with desiccants, and later sent for NGS.

In the lab, DNA was digested from the AniCard samples, and total RNA was extracted. After reverse transcription and inclusion of barcodes and sequencing adapters, the samples were analyzed for library preparation in a total RNA-seq workflow. Random primers were used for RT after DNase digestion on a MiSeq machine in paired read mode, with a read length of 150 base pairs. This RNA sequencing approach was chosen because it outperforms 16S amplicon sequencing and shotgun metagenomics, by facilitating detection of viruses and bacterial sequences outside of the 16S locus (and therefore in most cases it has higher discriminatory power than other sequencing methods). These methodologies represent current cutting edge molecular and bioinformatics techniques capable of identifying microbiome composition at a higher resolution compared to traditional methodologies (Hempel et al. 2022). Accordingly, these protocols require more resources, thus limiting the sample size.

Bioinformatics analysis

Taxonomic profiling of microbial agents (pathogens and others): organisms in the data obtained from the MiSeq sequencing of the KAPA libraries were profiled at BASE2BIO LLC (Madison, WI, USA) by untargeted metagenomics discovery (avian) workflow. The pre-processed reads were searched against a custom database composed of known microbial sequences, host sequences and common contaminants (AniCon). Data were filtered to reduce false

positive hits based on cutoffs determined by bootstrapped simulations. Final classifications were filtered based on unique k-mer count (number of k-mers that can be assigned uniquely to that taxon or below) and adjusted read count or a-score, to which an adjustment factor was applied accounting for highly abundant species in a closely related clade. Each of the assembled contigs was annotated with the lowest common ancestor, which can be assigned to the sequence based on BLAST searches (GenBank). This produced datasets with two different stringency levels: "relaxed" and "strict". For this particular study, we chose to analyze only reports of bacterial agents (i.e. excluding viral and fungal data from subsequent analysis) produced with "strict" cutoffs for k-mer count of 200 in order to limit the potential of relatively high numbers of false positives. Generally, the higher the taxonomic level examined, the more accurate both classification and abundance become, therefore we chose to examine bacteria primarily at the genus level. The aim of this array of cutting-edge analytical and bioinformatics methods is to identify the list of microorganisms (and pathogens) associated with each individual pigeon. Full details of bioinformatics protocols can be found in Suppl. material 1.

Data analysis of movement

First, to appropriately represent individual's movements, we filtered GPS data by removing erroneous localizations, and excluding fixes with the following criteria: Horizontal dilution of precision (HDOP) >2.1, Altitude >2000, Number of Satellites <4. Additionally, for ensuring comparable movement indices, we unified sampling rates across all individuals by sub-sampling data to fixes taken every hour. After subsampling, we excluded tracking days with fewer than 6 points per day and individuals with fewer than 7 days of tracking, leaving a total of 32 pigeons for the analysis of movement data.

To discover among-site and among-individual differences in movement or in the associated bacterial abundance, we extracted a few relevant movement indices that represent the spatial extent of the individual activity, and exploration that can affect potential exposure to different locations. First, we calculated 'Daily Max Displacement' as the straight-line distance between the pigeon's first location of the day (typically at the roost at dawn) and most distant point of the day. 'Daily Travel Distance' is the sum of the straight-line segments between all consecutive GPS points of the day. To determine exploration level, we quantified the daily number of spatially different stops each pigeon made. Each localization was considered a unique "stop" if it stayed at least one hour in the same location (within 200 m), and if this was at least 200-m away from the last stop. This 200-meter threshold was chosen based on the typical size of the dairy farms and ensured that two stops inside a given farm were not counted as a separate location, whereas other nearby sites (e.g. other facilities within the same settlement) were not grouped together, and that the scale is appropriate given the pigeons space-use patterns. A Kruskal-Wallis test was performed to determine if any of our movement metrics (average across all days for each individual) were significantly different between capture sites. Dunn's post-hoc comparison with Benjamini-Hochberg correction was used for pairwise comparison. In addition, we also used linear mixed models with pigeon ID as a random factor to determine the combined influence of capture location, sex, and weight. Since the latter two

predictors had only very weak influence on the movement indices in our dataset, these results are presented in the appendix (Suppl. material 1: table S1). Because tracking duration was very variable, we also explored the possible effect of this factor on the movement indices (Suppl. material 1: table S2), and tested whether it influenced any of the three core daily movement metrics (travel distance, max displacement, and exploration) using linear mixed models with pigeon ID as a random effect and tracking duration and capture location as fixed effects. Because we found no effect of this predictor it was not included in the final models.

Microbiome alpha diversity and its association with movement

To identify core bacteria in our NGS sample population at all three sites (n = 29), we filtered the top 20 taxa present in at least 90% of the pigeons, with a relative abundance higher than 0.1% and visualized them in a Venn diagram, grouped by site, using the Eulerr package in R (Larsson 2024). In the Suppl. material 1, we also provide a heat map of this subset (Suppl. material 1: fig. S2).

We investigated how pigeons and sites differed in their microbial diversity. We performed several exploratory analyses and differential diversity analyses of pigeon microbial profiles in R using the Phyloseq package (McMurdie and Holmes 2013) with a $\log_{10}$ transformation to normalize the data. There are three available indices of richness (Observed, Chao1, ACE) and four indices of diversity (also measuring evenness: Shannon, Simpson, Inverse Simpson, Fisher's α). Our results of all indices largely agreed with each other (Suppl. material 1: fig. S3); for simplicity, we present here results for one index of each currency: **Chao1** for richness and **Fisher's α** for diversity. The Chao1 metric for richness estimates the number of different bacterial genera in a sample and is particularly useful for data sets skewed toward the low-abundance classes, as is likely to be the case with microbes (Chao et al. 2006). Fisher's α index is recommended as a reliable measure due to its ability to compensate for potentially incomplete sampling (Hayek 1997); it is relatively unaffected by variation in sample size and aims to measure diversity by accounting for "evenness" or homogeneity. For instance, communities dominated by one species will exhibit low evenness, as opposed to communities where most species are relatively well represented (high evenness).

Focusing on the association between movement and bacterial abundance (while accounting for differences among sites, and individual traits) is possible for the subset of pigeons with both sufficient GPS movement data and NGS data. We investigated whether variation in microbiome diversity (Chao1 and Fisher's α indices) was associated with capture location, sex, body weight, and movement behavior, using a subset of 19 individuals with both high-quality GPS tracking data and available NGS microbiome profiles. Because diversity was measured once per pigeon, for this analysis all predictors were calculated as per-individual averages across the GPS tracking period. We decided to use the full available tracking duration (and not a limited time window of the tracking period immediately after the microbiome sampling events) because alpha diversity was unrelated to sampling date and because movement metrics (e.g. exploration) were temporally stable (Suppl. material 1: fig. S4, table S2). Linear models were used to fit all diversity models, and model selection was based on AICc (Akaike information criterion with a correction for small sample sizes). The top model for each response variable was retained for interpretation, and

competing models with lower support (ΔAICc > 2) are reported in the Suppl. material 1. Plots showing the effect of Julian day and season on diversity indices are included in Suppl. material 1: fig. S5.

Microbiome Beta diversity and its association with movement

Beyond the effect on alpha diversity, we also explored the effects of capture site, individual sex and movement indices (daily max displacement, travel distance and exploration) on Beta diversity. Here we implemented a redundancy analysis (RDA) using the vegan package (Oksanen et al. 2024). Broadly speaking, redundancy analysis combines methods of linear regression analysis with methods of principal component analysis to predict how specific variables (here the capture site and movement indices of each individual) affect a response variable (here diversity). Because most linear methods (including RDA) are sensitive to the distribution of the data — and specifically to the many zeros that characterize microbiome count data — the Hellinger transformation of relative abundance is recommended as a preliminary step (Legendre and Gallagher 2001). The significance of separation in RDA analysis was estimated with a permutation test using 5,000 permutations. Distance-based comparisons among individuals were made using the Bray-Curtis dissimilarity coefficient calculated between each and every sample pair and visualized with RDA. Type II ANOVA was used to test for the significance of movement indices on Beta diversity using a 5% alpha level.

Results

Pigeon movement

Out of the 308 color-ringed individuals, 102 were re-sighted during our routine fieldwork. A total of 32 pigeons with adequate GPS data (out of 44) accumulating to 8,473 days (265 ± 198; range: 8–832 days per individual) of tracking were analyzed for travel distance, max displacement, and exploration (Table 1). Tracking duration did not differ significantly across capture sites (Kruskal-Wallis test: $\chi^2 = 2.51$, df = 2, $P = 0.29$), suggesting that site-level comparisons were not confounded by variation in this predictor, nor did it significantly affect any outcome (all p > 0.5), and was, thus, excluded from final models.

Travel distance differed significantly between capture sites ($\chi^2 = 7.52$, df = 2, $P = 0.023$), with pigeons from Mikve (n = 8) traveling significantly shorter distances than those from Maale (n = 18, Z = 2.62 $P = 0.026$), and compared to Mevo (n = 6, Z = 2.11 $P = 0.052$). Mevo and Maale did not differ significantly ($P = 0.94$). A similar pattern was observed for max displacement ($\chi^2 = 10.39$, df = 2, $P = 0.005$; Fig. 2A), where pigeons in urban Mikve had more restricted ranges compared to rural and semi-rural sites (Maale: Z = 3.12 $P = 0.005$; Mevo: Z = 2.35 $P = 0.037$). In contrast, for exploration (number of daily stops; $\chi^2 = 6.51$, df = 2, $P = 0.038$), differences were much weaker due to high variability within each location, with only Maale and Mikve differing significantly (Z = 2.46 $P = 0.041$), but not other pairs (Fig. 2B). Model comparison and effect sizes showed similar results. Neither sex nor body weight significantly predicted movement behavior (Suppl. material 1: table S1), and the effect sizes from the leading models are reported in Suppl. material 1: table S3.

Table 1. Tracking data and basic movement characteristics in the three study sites (Mean ± SD).

Capture Site	n pigeons	Tracking duration (days)	Daily Travel Distance (m)	Daily Max Displacement (m)	Exploration (number of stops)
Mevo Horon (rural)	6	313.8 ± 192.4	2,096 ± 2,946	3,163 ± 3,304	4.40 ± 1.70
Ma'ale Hahamisha (semi-rural)	18	401.5 ± 218.2	2,481 ± 3,019	3,031 ± 3,251	5.29 ± 2.60
Mikve Israel (urban)	8	163.2 ± 96.1	1,004 ± 1,039	1,297 ± 1,133	3.83 ± 1.38
Total	**32**	351.6 ± 216.5	2,206 ± 2,865	2,833 ± 3,127	4.91 ± 2.36

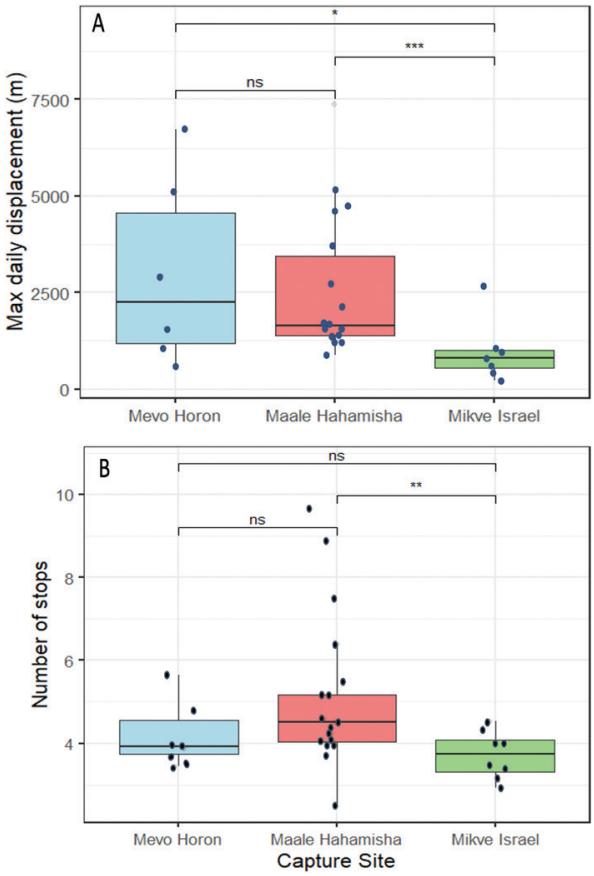
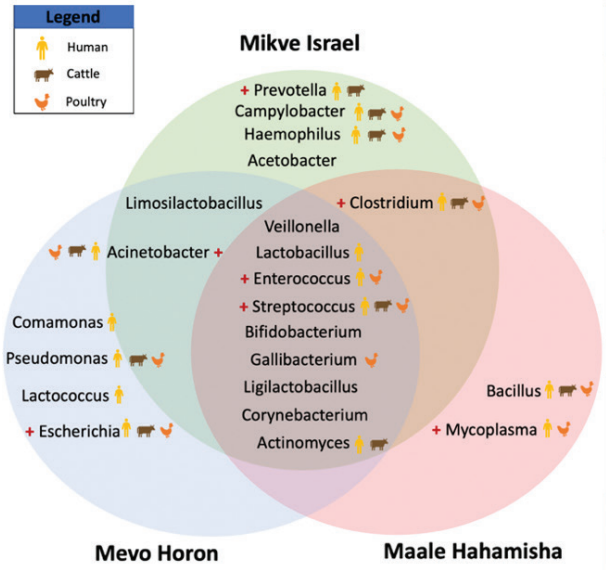


Figure 2. Pigeon movement within the three study sites. Movement behavior of GPS-tracked pigeons across the three capture sites. **A.** Maximal daily displacement and **B.** Exploration (average number of daily stops), shown by site and ordered by urbanization: Mevo (rural), Maale (semi-rural), and Mikve (urban). Black bars indicate statistically significant pairwise differences with Dunn's test ($p < 0.05$). Pigeons captured in Mikve traveled significantly shorter distances compared to pigeons from the more rural sites. Exploration was less variable among sites, differing only between Maale and Mikve. Travel distance comparison are shown in Suppl. material 1: fig. S6.

Pathogen presence in pigeon microbiome

To compare the core taxa shared between the three study sites, we identified the top 20 bacterial genera (Fig. 3A; see Suppl. material 1: table S5 for a full list of identified genera). The majority of these genera (identified by our NGS reports found at samples from all sites) such as *Clostridium*, *Escherichia*, *Campylobacter*, and *Streptococcus* are known to contain many species of disease-causing pathogens. Thus, to establish the presence of pathogenic species

A



B

+ Genera	Pathogenic species detected	n individuals	Associated infections
Prevotella	*P. intermedia*	3	Human, bovine periodontis
Clostridium	*C. perfringens*	6	Food poisoning
	C. difficile	17	
Mycoplasma	*M. penetrans*	6	Infections in immunocompromised humans
Streptococcus	*S. equis*	3	Equine "strangles disease"
	S. dysgalactae	2	Bovine mastitis
	S. mitis	5	
Escherichia	*E. Coli*	22	Food poisoning
Enterococcus	*E. cecorum*	26	Emerging infectious diseases in poultry, humans
	E. faecalis	15	
	E. hirae	5	
Acinetobacter	*A. baumanni*	2	Infections in immunocompromised humans; cattle, horses

Figure 3. A. Top 20 taxa at the genus level (filtered by highest abundance and identified for at least 90% of samples at each site), by their occurrence in the different study sites. Genera with known pathogenic species (one or more) within them to either humans, cattle or poultry are labeled. **B.** List of individual species with known pathogenic potential identified from NGS results (when species was available) within top 20 genera.

within these candidate genera, we further examined the data at the species level, where possible. We report those species which have known cases of associated illnesses in humans, cattle, and poultry (Fig. 3B), and thus demonstrating the pigeons' potential to carry relevant pathogens at our population, and not only pathogen-relevant genera.

Effect of site on alpha and Beta microbiome diversity

First, we tested alpha diversity between capture locations. The Chao1 index for richness showed a significant variation among capture locations (Kruskal-Wallis $\chi^2 = 8.81$, df = 2, $P = 0.012$; Fig. 4A), with pigeons from Mikve showing significantly higher richness than pigeons from both Mevo (posthoc Dunn's test Z=-2.42, $P = 0.023$) and Maale (Z=-2.27, $P = 0.017$). This is congruent with other richness metrics (Observed, ACE) suggesting that pigeons in Mikve (highly urban) contain a higher number of lower abundance species as opposed to pigeons from Mevo (highly rural) and Maale (semi rural) that did not substantially differ from each other. Despite the apparent differences among locations in the Fisher's α diversity index (Fig. 4B), this result was not statistically significant ($\chi^2 = 4.77$, df = 2, $P = 0.12$), and here different diversity indices varied in their predicted effects, with ACE, for instance, showing a similar trend, but with significant differences ($\chi^2 = 8.82$, df = 2, $P = 0.013$), reflecting differences in the calculation method of the different indices.

Further, we assessed the overall joint effect of capture location and movement on microbiome diversity using Type II ANOVA (Suppl. material 1: table S6.1). Here, capture site had a significant overall effect on Fisher's α ($F_{2,15} = 4.68$, $p = 0.026$), while the exploration (number of daily stops) approached significance ($F_{1,15} = 4.28$, $p = 0.056$). A post-hoc pairwise comparison with Tukey

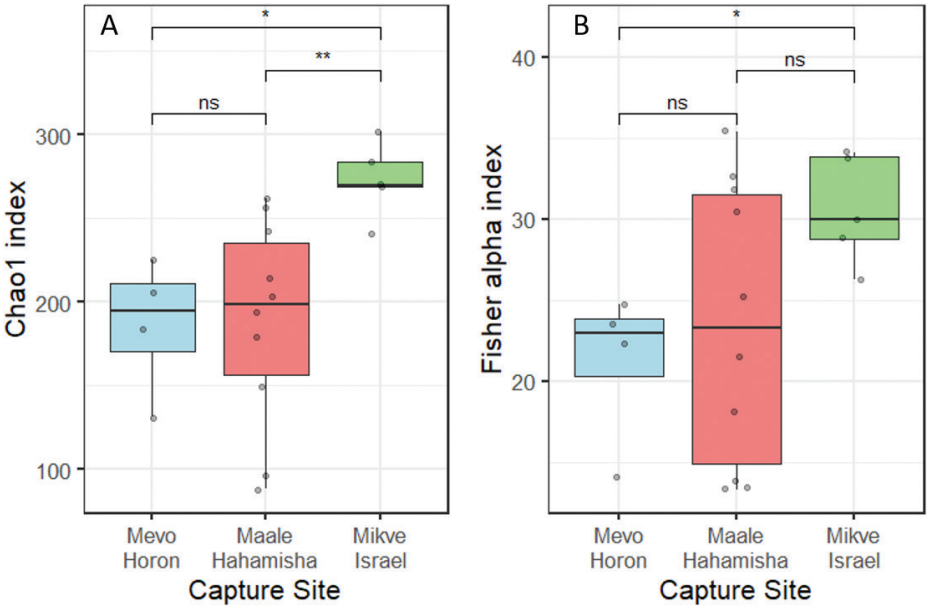


Figure 4. A. Microbiome diversity across the three study sites. The comparison of microbiome alpha diversity between sites, including **A.** Genera richness (represented by Chao1) and **B.** Evenness (represented by Fisher's *α* index). The microbial communities show that pigeons in Mikve (the most urban site) had higher richness than the two rural sites at Mevo and Maale, with fewer differences in the evenness.

correction showed the Fisher's *α* diversity was significantly lower in pigeons from Mikve compared to both Maale (estimate = −10.8, p = 0.039) and Mevo (estimate = −12.6, p = 0.037), while the two rural sites did not differ from each other (estimate = 1.8, p = 0.885; Suppl. material 1: table S6.2). These findings reinforce both a site-level influence on diversity and highlight the urban site's distinct microbial community.

Effects of movement and capture site on microbiome alpha diversity

Arguably, our most relevant hypothesis to individual-based ecology refers to the association between each subject's movement and its respective microbiome. After filtering for pigeons with both NGS data and sufficient GPS movement data, we were left with a total of 19 individuals for an analysis of this relationship between movement and bacterial abundance. Interestingly, when modeling jointly the effect of site and movement on alpha diversity/richness, we found that exploration ($F_{2,18}$ = 6.02, P = 0.02) and capture site ($F_{2,18}$ = 4.02, P = 0.02), but not their interaction ($F_{2,18}$ = 1.9, P = 0.47) were significantly associated with alpha diversity (Table 2). Beyond the clear differences among sites (~10 more genera in Mikve compared to the other two), within each site pigeons that regularly made more stops per day (i.e. explored more unique locations) showed higher alpha diversity. This finding implies that the higher environmental exposure by individuals that visit more diverse locations indeed is associated with a more diverse microbiome in both richness and diversity. Effects of daily travel distance, max daily displacement, and the interaction between number of stops × capture site were performed in each model, but were not significant and therefore not included in the table. The lack of interaction implies that this positive association was similar at all three sites (Fig. 5A). The

Table 2. Effects of site and movement patterns on alpha diversity and bacterial species richness indices. Generalized Linear Models for Chao1 and Fisher's *α* showing that feral pigeons in central Israel varied in their microbiome alpha diversity. Significant *P* values are in boldface.

Diversity index	Factor	Estimate	Std. error	p. value
Chao 1	***Movement***			
	Number of stops	37.75	13.44	**0.012**
	Capture Site			
	Mevo Horon	13.46	24.94	0.596
	Ma'ale Hahamisha	60.15	52.38	0.261
	Mikve Israel	102.12	23.25	**0.0004**
Fisher's *α*	***Movement***			
	Number of Stops	5.59	1.90	**0.023**
	Capture Site			
	Mevo Horon	0.278	3.28	0.938
	Ma'ale Hahamisha	2.91	7.89	0.718
	Mikve Israel	9.98	3.52	**0.007**

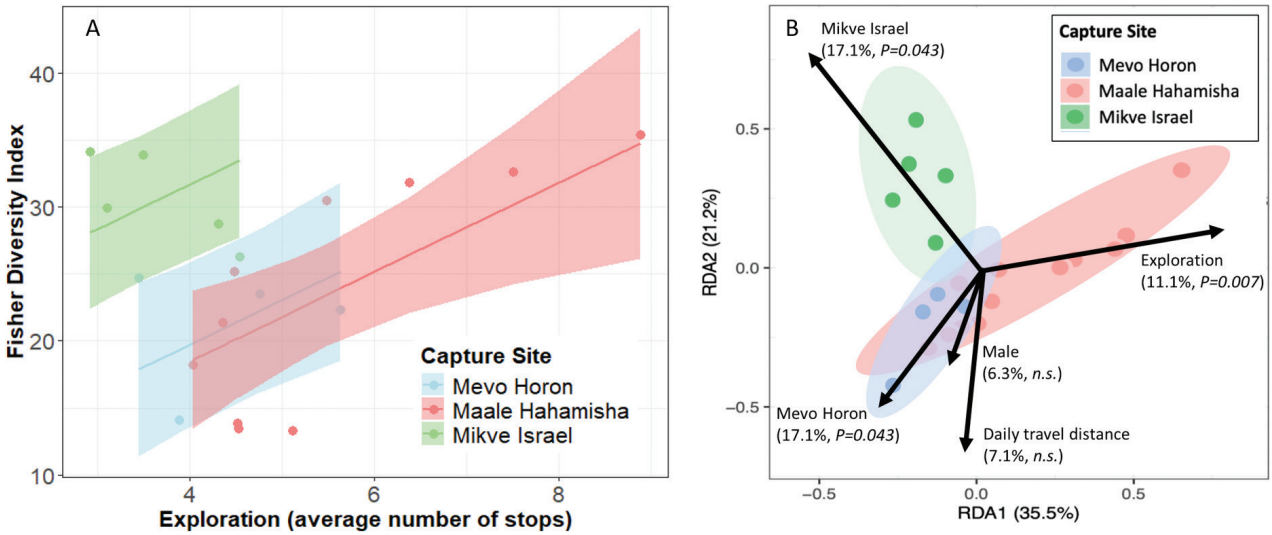


Figure 5. The effect of pigeon movement on microbiome diversity. Relationship between exploration average (number of spatially-different daily stops) and microbiome diversity (Fisher's *α*) across GPS-tracked pigeons (N = 19). Individuals are colored by capture site: Mevo (blue), Maale (red), and Mikve (green). The lines indicate the linear regression fit, with the shaded areas representing the 95% confidence interval. The positive association between exploration and Fisher's *α* diversity suggests that individuals engaging in more exploratory movement harbor more diverse gut microbiota. **B.** Redundancy analysis (RDA) of Beta diversity of 20 pigeon samples at the genus level. RDA plot shows the result of pigeons grouped by capture location. Comparison against the permutation test shows a significant effects of capture sites and exploration.

differences between site in travel distance (Mikve vs. Mevo) was not correlated with variation in alpha diversity among pigeons within each site, nor did capture site and travel distance interact in their effect on diversity. Similarly, max displacement did not affect alpha diversity beyond the site effect. Sex and body weight did not have a significant effect on movement or microbiome diversity and are therefore not presented in the main text but can be found in the Suppl. material 1 section (Suppl. material 1: table S4).

Effects of movement and site on microbiome Beta diversity

Focusing on Beta diversity, the RDAs models (Fig. 5B) resulted with 35.5% and 21.2% of the variance explained by the first and second ordination axes. The results suggest that individuals from different capture locations tended to be colonized by different bacterial taxa ($F_{2,18}$ = 1.357, P = 0.043). In addition, comparison against the permutation test shows that the average number of stops had a significant effect on community composition (P = 0.007), while sex and daily travel distance were not significant (P = 0.758, P = 0.400). These results agree with the results mentioned above for alpha diversity.

Discussion

In this study, we explored the role of feral pigeons as potential pathogen vectors at dairy farms and the association between movement and microbiome. First, GPS tracking showed that pigeons on the urban farm (Mikve Israel, shortened to Mikve) were largely less mobile and more local than those in our two rural sites, and that individuals (within sites) differed in their movement patterns, namely in their exploration (Fig. 2). Second, NGS results were consistent with previous literature, showing that the pigeons in our population have a high prevalence of pathogens relevant to human, cattle, and poultry. The top 20 most abundant taxa include *Escherichia*, *Campylobacter*, *Clostridium* and *Streptococcus*, and several other genera that contain important pathogenic species of bacteria which were identified in our NGS reports (Fig. 3). Third, pigeons' microbiomes differed among sites with higher alpha diversity and richness found in the urban site (Fig. 4). Further, pigeons' movement patterns (within each site), but not their sex or weight, affected their microbiome diversity – explorer individuals who stop at more diverse places show a corresponding increase in microbiome diversity beyond the effect of sites (Fig. 5). To the best of our knowledge, these links between spatial behavior studied *in situ* and bacterial diversity in wildlife were only rarely investigated (e.g. in Fuirst et al. 2018; Corl et al. 2020), and are especially missing from studies in the context of an agricultural One Health approach. The finding that pigeons potentially transmit pathogens among dairy farms, human settlements (where they almost exclusively roost), and wildlife (encountered in open habitats where they often forage) highlights the potential importance of their relationship with surrounding habitats. The association between exploratory behavior and epidemiology in our analyses of movement and microbiome diversity directly offers an example of the link that pigeons create between human and animal populations (and their pathogens), in urban and agricultural sites. Below, we discuss differences in pigeon movement between sites, how each site differs in microbial diversity, the effect of pigeon movement on diversity, and finally the broader implications of our findings.

Variation in movement patterns and shorter distances in urban pigeons

First, we studied whether there were differences in movement between individuals and sites, addressing the spatial extent of pigeon transmission potential and its variation with respect to urbanization. Pigeons captured at each

site were active in its vicinity and crossing among sites was very rare. Mevo and Maale are more rural than Mikve, with Mevo being slightly more isolated. Accordingly, pigeons' daily travel distances and displacement were longer at these two sites compared to Mikve, likely reflecting different resource distributions among sites. All pigeons roosted in buildings within human settlements and routinely visited the respective dairy farms and similar anthropogenic food sources (e.g. parks, poultry farms, and commercial areas). Yet, rural pigeons spent summer months foraging for grains at harvested fields, while the urban ones rarely did so (and only in a few nearby fields). Instead, they foraged year-round on human waste. Thus, pigeons foraging at Mevo must travel further from their urban roosting locations when searching for surrounding food, offering a likely explanation for higher displacement when compared to other sites. These patterns agree with general findings that urban animals move less thanks to the availability of stable and rich food sources (Tucker et al. 2018).

Identifying heterogeneities in host space use – here among sites as discussed above – offers important insights toward understanding pathogen transmission dynamics (Dougherty et al. 2017). Pigeons can spread pathogens over a range of agricultural and urban habitats, affecting livestock, wildlife, and humans. For instance, non-resident pigeons in cattle farms in Colorado are responsible for pathogen transmission among farms, and the resident ones for amplification within farms (Carlson et al. 2011). The significantly longer travel distance and displacement of the pigeons foraging at Mevo and Maale, as well as the composition of habitats used, might suggest a more widespread range of transmission potential when compared to pigeons foraging at Mikve (Fig. 2). The pigeons at Mikve, on the other hand, might pose a greater risk of zoonotic diseases due to their high reliance on urban centers and dairy farms and the high rate of alternation between the two, sometimes located within hundreds of meters. Further research is needed to directly address this 'urbanization-dependent transmission potential', but nevertheless this hypothesis is supported by a growing consensus that variation in host home range has the ability to influence disease spread (McClure et al. 2020).

Pigeons from different sites vary in their microbiome diversity

Our results show that pigeons captured at Mikve had significantly higher microbial alpha diversity compared to both Mevo and Maale (Table 2, Fig. 5). Each pigeon in Mikve had around 160 genera of bacteria (Chao1) and diversity of ~13 (Fisher's α) compared to those in the latter two sites with a similar diversity of around 70 and 3, for the two indices respectively. Admittedly, working with merely three sites that may differ in various aspects (e.g. local ground elevation, resource composition, local density, and others) does not permit confident identification of the main cause for this result. Nevertheless, the urbanization gradient is an apparently obvious possible explanation for this finding. Indeed a substantial positive effect of urbanization on microbial diversity and the microbial communities of wildlife was attributed to acquiring parasites, natural infections, and other physiological stressors at urban habitats (Rouffaer et al. 2017).

However, the literature is inconsistent with respect to the effect of urbanization on microbial diversity, and the opposite (negative) trend was also found in

synanthropic birds (Jatzlauk et al. 2017). For instance, Fuirst et al. (2018) studied the effects of urbanization on foraging and microbiota in three gull colonies (*Larus argentatus*) and found that bacterial diversity was highest in the colony that had the lowest urban exposure. Such disagreement can either reflect either a methodological difference or a genuine variation between systems in their response patterns to urbanization. Indices used for urbanization and diversity, the sampling methods, study season and the spatiotemporal scale of investigation can all act as confounding factors.

Individual host movement is correlated with their microbiome alpha diversity

Beyond the variation among sites, more exploratory pigeons from all sites that averaged more stops in unique locations (i.e., excluding repeated stops within a day at the same location) had higher richness and diversity than individuals that typically spent their day foraging in fewer places (e.g. a dairy farm; Fig. 5). These results agree with previous findings indicating the association of microbiota with foraging patterns in other birds (e.g. Corl et al. 2019; Pekarsky et al. 2021). In agreement with our result, also the gulls in the above-mentioned study by Fuirst et al. (2018) had the maximal bacterial diversity in the colony that used a wider variety of foraging sites (i.e. lower foraging site fidelity). Nevertheless, it is noteworthy that this specific colony also had the lowest urban exposure among the three colonies included in their study. This coupling between urbanization and foraging behavior impairs the ability to distinguish between the independent effects (if any) of foraging and urbanization in their system and can explain the opposite trend here. In our case, the number of stops was relatively similarly spread in all three sites (Fig. 2B), allowing us to distinguish between the effect of (site) urban level and (individual) foraging behavior.

Why should an individual behavior be correlated to their microbiome diversity? There are at least two broad explanations for this finding. First, it may indicate that individuals that utilize an increased diversity of spaces encounter a higher diversity of bacteria on route (i.e. sample their region more thoroughly). Fuirst et al. (2018) for instance, suggested this explanation – the diverse foraging locations resulted in a more diverse microbial community. An alternative mechanism suggests that the microbiome composition itself affects the pigeons' behavior (and not vice versa), through sickness or host manipulation done by one or a few of the agents within the microbiome (Poulin and Maure 2015). It is possible that individuals with higher microbiota diversity may need to visit more locations in order to meet their nutritional requirements. For instance, a negative relationship was found between gut diversity and home range size of white-tailed deer (Webb et al. 2010). The authors suggested that deer with higher gut microbiota evenness are able to use more diet sources overall (i.e. the gut bacteria allowed for eating different plant species) and thus can meet their nutritional requirements within a smaller area relative to individuals with less diverse gut microbiomes (Webb et al. 2010). Importantly, these two explanations can be non-mutually exclusive or vary in their relative contributions across different systems. Future and ongoing studies will further illuminate these pathways (Couch and Epps 2022).

Concluding remarks

The combined effects of high mobility (daily ranges of a few kilometers), high densities (hundreds of individuals in each site), high proximity to humans and cattle (at roost and foraging sites, respectively), as well as opportunistic visits to open areas, point to the very strong potential of feral pigeons to directly transfer pathogens between livestock, humans, and wildlife. Understanding host microbial diversity allows critical insights and identification of important viral and bacterial pathogens that may have the potential to cause devastating human pandemics; for example, three global plagues caused by *Yersinia pestis*, and a Cholera pandemic caused by *Vibrio cholerae* (Sacchi et al. 2002).

Other synanthropes are likely to play a similar role in a variety of anthropogenic systems. While the scope of this study is human-centered with the concern of zoonotic diseases, these patterns should be considered also for conservation and spillover effects since pigeons are similarly likely to transmit pathogens in the opposite direction, risking wildlife populations (e.g. while foraging at mixed flocks in open fields). The massive outbreak of Avian Flu in 2022 (H5N1), likely originating from poultry farms and infecting many endangered species, is a sad reminder of this scenario.

In addition to the obvious agreement with the main premise of the One Health approach (e.g. Hassell et al. 2017), the remarkable differences among and within sites (in both local diversity and movement patterns), emphasize the need to adapt response strategies tailored to local conditions and investigate focal systems towards a mechanistic understanding of these processes. For instance, preventive pigeon culling, if applied in rural areas, should be done in concert across much larger areas than their more urban counterparts. Similarly, within sites, more exploratory pigeons should be targeted when possible (e.g. by trapping at larger distances from the center of activity or prioritizing individuals on the move). Amending practical culling protocols with such insights regarding wildlife movement can indicate how movement ecology studies can facilitate applied disease management, and emphasize the importance of individual variation in shaping disease dynamics (Dougherty et al. 2017; Spiegel et al. 2017; Lazebnik and Spiegel 2025).

Our study is simplistic in several aspects. First sample size limitations imposed by the high costs of both tracking devices and NGS processing prevent investigating the question across a larger set of sites (e.g. better representing a gradient of urbanization) and accounting for additional factors such environmental conditions (e.g. seasonality) and demographics (e.g. age-related differences, density, social contexts) and others. Similarly, the study suffers from an unbalanced design with better representation of Maale Hahamisha, but poorer coverage of the other two sites. This bias reflects both historical reasons in the study development as well as methodological challenges with the ability and permits to work in the latter two sites. While we acknowledge this as an important consideration to the study, we do not believe it invalidates our results, and reported effect size and certainty levels reflect these variable sample sizes. Second, while our exploratory analyses allow us to determine the co-variation between simple indices of movement and bacterial diversity (higher in more exploratory pigeons), they are short from targeting specific mechanisms or

identifying how specific pathogens are affected by differences among sites and behaviors. Future studies can build on these general patterns and proceed beyond our observational approach to identify the causality of this positive correlation. Experiments like inoculation, translocations, movement restrictions, and other *in situ* manipulation can reveal the relative contributions of higher exposure (i.e. movement drives pathogens prevalence) vs. pathogenic impact on movement (e.g., different needs or host manipulation). Assimilation of tools and concepts from movement ecology into studies of disease ecology is a promising direction toward this end (Dougherty et al. 2017,Ezenwa et al. 2022).

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

All capture and sampling procedures were authorized by research permit 2019/ 04-19-059 provided by the Tel Aviv University research animal ethics committee.

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Author contributions

MMC, SC, and OS initiated the study; OS, AL, and SC secured the funds; MMC, SC, and OS did the fieldwork, MMC and AL did labwork; MMC and OS analyzed the data with inputs from SC, AL, LR, CA; MMC and OS wrote the manuscript with a substantial contribution from all other authors who approved the final version.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Additional methodological details figures, tables and R code

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Data type: docx

Explanation note: **figure S1.** Variation in the travel distance among sites. **figure S2.** Heatmap of top 20 (most common) bacterial taxa at the genus level. **figure S3.** A comparison of microbiome richness diversity among sites. **figure S4.** Fisher's *α* diversity across seasons. **figure S5.** Temporal distribution of captures by site. **figure S6.** Max Daily Displacement among sites. **figure S7.** Redundancy analysis (RDA) of 20 pigeon samples at the genus level. **table S1.** Model ranking tables for linear mixed models explaining movement metrices. **table S2.** The effect of tracking duration on daily movement metrics. **table S3.** Effect size of capture location on movement indices. **table S4.** Predictors of microbiome species richness. **table S5.** Factors affecting alpha diversity. **table S6.** Full Pathogen List. **table S6.1.** Type II ANOVA for Fisher's *α* diversity. **table S6.2.** Pairwise comparisons of capture sites on Fisher's *α* diversity.

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