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Social Microbial Transmission in a Solitary Mammal

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ABSTRACT

Microbial transmission is hypothesised to be a major benefit of sociality, facilitated by affiliative behaviours such as grooming and communal nesting in group-living animals. Whether microbial transmission is also present in animals that do not form groups because territoriality limits interactions and prevents group formation remains unknown. Here, we investigate relationships among gut microbiota, population density and dynamic behavioural and spatial measures of territoriality in wild North American red squirrels (*Tamiasciurus hudsonicus*). Periods of high population density predicted population-level gut microbial homogeneity but individual-level diversification, alongside changes in obligately anaerobic, non-sporulating taxa indicative of social transmission. Microbial alpha-diversity increased with more frequent territorial intrusions, and pairs with stronger intrusion-based social associations had more similar gut microbiota. As some of the first evidence for social microbial transmission in a solitary system, our findings suggest that fluctuations in density and territorial behaviours can homogenise and diversify host microbiomes among otherwise non-interacting animals.

1 | Introduction

The benefits of sociality are widespread. Prosocial behaviours such as grooming and communal nesting can reduce stress (Bekoff 2015; Keverne et al. 1989), promote social cohesion (Wiszniewski et al. 2010) and improve access to food (Carter and Wilkinson 2013; Cronin et al. 2010; Vucetich et al. 2004) and mates (Duffy et al. 2007; DuVal 2007). Among these benefits is the host-to-host transmission of symbiotic microbiota (i.e., social microbial transmission), which, despite a historical underappreciation, has recently garnered interdisciplinary interest (Lombardo 2008). At the individual level, social microbial transmission can enhance pathogen protection (Koch and Schmid-Hempel 2011; Powell et al. 2014) and support microbial recovery following small and large-scale disruptions (Ng et al. 2019). More broadly, social microbial transmission can buffer against stochastic microbial extinction events caused by bottlenecks over evolutionary time, preserving microbial diversity within a population (Moeller et al. 2016). While deterministic mechanisms (e.g., genetic effects, parent-offspring transmission) undoubtedly shape host microbial communities (Moeller et al. 2018; Bubier et al. 2021), social transmission in highly social species explains as much, and sometimes more, of the microbial similarity among hosts than relatedness (Bensch et al. 2023; Raulo et al. 2021; Wanelik et al. 2023). This points to a role for the social transfer of microbiota in how animals acquire, maintain and potentially fine-tune their microbiomes in response to changing physiological and environmental demands (Sarkar et al. 2024; Alberdi et al. 2016).

Prior work on social microbial transmission has focused almost exclusively on group-living species like nonhuman primates and social rodents (Perofsky et al. 2017; Tung et al. 2015; Sabol et al. 2023), but whether social routes of microbial transmission exist in non-social or solitary systems remains unknown. Tests for microbial transmission in such systems can reveal socio-ecological boundaries of host-to-host microbial transfer and help to disentangle the relative roles of vertical, social and environmental routes of microbial acquisition. For example, transient periods of social isolation can sever transmission pathways in social species, with potentially detrimental effects on host health and fitness (Martignoni et al. 2024). Further, as pathogenic microbiota can also be socially transmitted, animals must balance the costs and benefits of microbial transmission. But with so few opportunities for affiliative microbial transfer, solitary and non-social species may nonetheless maintain pathways for social microbial transmission despite pathogenic risk (Pavez-Fox et al. 2024). As natural disasters (Pavez-Fox et al. 2024), pandemics (Finlay et al. 2021) and habitat fragmentation (Barelli et al. 2015) continue to disrupt social systems and thus the transfer of microbiota, identifying non-affiliative routes of transmission can ultimately shed light on the resilience of social microbial transfer during periods of social disconnect.

In this study, we test the hypothesis that social microbial transmission can be facilitated by periods of high population density and through territorial behaviours in a solitary, non-social small mammal, the North American red squirrel (*Tamiasciurus hudsonicus*). In the southwest Yukon, red squirrel densities fluctuate due to the boom-bust dynamics of masting white spruce (*Picea glauca*), their primary food source. A reliance on this single food item results in relatively low dietary diversity and high intraspecific competition (Fletcher et al. 2013). Food booms (spruce masts) occur episodically every 3–7 years, driving larder hoarding of spruce cones by squirrels into middens located at the centre of stable, non-overlapping, individual territories that are vigorously defended from intruders (Dantzer et al. 2020; Smith 1968; Price et al. 1986). The frequency of intrusions by conspecifics onto territorial spaces also fluctuates: following food booms, squirrel densities peak and territorial intrusions decrease (Webber et al. 2023). By contrast, food scarcity reduces densities and increases the frequency of intrusions (Webber et al. 2023). These fluctuations generate appreciable variation in opportunities for social microbial transmission as a function of dynamic changes in ecology and behaviour, a novel contrast to the more static routes of microbial transmission commonly explored in more social animals (e.g., social bonds, rank, (Rudolph et al. 2022)).

Here, we address these questions by leveraging 10 years of longitudinal data on demographic change, faecal microbiota and territorial intrusions. We expect that when squirrel densities are high, or when territorial intrusions are more frequent, gut microbial communities will homogenise as social microbial transmission weakens microbial individuality. At the same time, we expect that gut microbiota will diversify as hosts acquire conspecific microbiota (Arneberg et al. 1998). We predict corresponding shifts in predominantly beneficial microbial functions, such as those favouring increased investment in key host physiological processes like metabolism and immune function. Finally, recent work in social mice reveals the spread of oxygen-intolerant microbiota to be a primary signal of social microbial transmission. Thus, if transmission routes are direct (i.e., physical contact), we predict these patterns will be driven primarily by obligately anaerobic, non-sporulating bacteria; if they are indirect (i.e., faecal shedding), we expect to identify differential enrichment of anaerobes with at least some capacity for aerotolerance (Raulo et al. 2024).

2 | Materials and Methods

2.1 | Ethics Statement

Approvals were granted by the University of Michigan Institutional Animal Care and Use Committee (PRO00009804, PRO00007805 and PRO00005866) and met the Canadian Council on Animal Care Guidelines and Policies; all relevant guidelines were followed. Fieldwork was permitted under Yukon Territorial Government Wildlife Research Permits and Scientists & Explorer's Permits.

2.2 | Study Population

The Kluane Red Squirrel Project (KRSP) is a long-term field study of North American red squirrels (*Tamiasciurus hudsonicus*) in the southwest Yukon, Canada (Dantzer et al. 2020). Data for this study were collected from 2008 to 2017 (except 2013) from 52 squirrels (41 females, 11 males, age 1–7 years) living on two unmanipulated ~40 ha study areas (‘Kloo’/KL, ‘Sulphur’/SU) experiencing similar environmental conditions (Petrullo, Delaney, et al. 2022). Monitoring included twice yearly censuses of the entire population in May and August/September and measurement of potential confounding variables (e.g., body mass, age, food availability) during routine live-trapping and standardised quantification of spruce cone production (see (Dantzer et al. 2020)).

2.3 | Squirrel Densities

We measured squirrel densities by counting the number of squirrels defending individual territories at two scales. First, we calculated ‘grid-wise’ densities based on the number of squirrels enumerated on each study area during the spring census divided by 40 ha (i.e., study area size). We calculated neighbourhood densities as the number of squirrels defending territories within 50, 100 and 150 m of a focal individual's territory centroid, respectively (Dantzer et al. 2012; Siracusa et al. 2021).

2.4 | Territorial Spaces and Intrusions

Each May, we recorded the location of each individual's primary spruce cone hoard (i.e., midden), which is located at/near the territory centre. Because squirrels typically defend the same territory for their entire life and rarely change territories when new ones become available (Lane et al. 2015; Berteaux and Boutin 2000; Price and Boutin 1993), we used this midden location as an anchor for the spatial location of each individual's territorial space in each year (Webber et al. 2023; Larsen and Boutin 1995). We then used trapping, focal and opportunistic behavioural observations (visual, with some radio-collared) to generate estimates of territorial spaces for each squirrel. Spatial locations of behavioural observations were recorded to the nearest 3 m; trapping locations were recorded to the nearest 15 m. We jittered all spatial locations based on the resolution with which we measured the location of each data type to minimise error (behavioural data by an error of ±3 m, trapping data by an error of ±15 m).

We then removed all observations that occurred outside our study area and generated territory estimates for squirrels with > 20 observations/trapping events/year (March 15–September 1). In some cases, observers conducted detailed behavioural observations of squirrels, resulting in a large number of behavioural observations for a given individual throughout the summer (max 132 observations/squirrel/day). For squirrels with > 30 observations in a single day, we randomly selected 30 observations (~95% of squirrels had < 30 observations/day). Finally, we removed all study area-year combinations with < 10 unique squirrels after applying these criteria (Webber et al. 2023). We generated 30% kernels as a conservative measure of the boundaries of an individual's territorial space (Webber et al. 2023), which is likely to be an underestimate of true territory size based on mapping of territorial defence behaviours typically used to estimate territory boundaries (Webber et al. 2023). These estimates are therefore proxies for the full size of an individual's territory, representing an individually variable area around each squirrel's midden (Webber et al. 2023).

We defined intrusions as instances in which a squirrel was observed/trapped within the inferred territorial space of a conspecific (hereafter, 'owner'), excluding juveniles captured on their mothers' territories in the first year (Dantzer et al. 2012). Squirrels occasionally bequeath territories (Berteaux and Boutin 2000), and abandoned territories (e.g., due to death of the owner) are rapidly overtaken by new individuals (Price et al. 1986; Siracusa et al. 2017). We thus used a hierarchical temporal moving window approach to remove potential false positives (Webber et al. 2023). We considered the ownership tenure and the earliest and latest days of observation for each squirrel each year. We removed intrusions where a squirrel was observed on an owner's territorial space before the owner's earliest trapping/observation on territory that year, and after the owner's last trapping/observation day that year if the owner did not own the territory the subsequent year. Therefore, only instances in which a squirrel was observed on the territory of another squirrel during the period of territory ownership were considered 'true' intrusions. When an owner did not own their territory in the previous year, we omitted any instances when a squirrel was observed on a territory before the owner's earliest observed day in that year. Finally, if an owner did not own their territory in the subsequent year, we omitted any instances when a squirrel was observed on a territory after the owner's last observed day in that year. This procedure was designed to discriminate instances where a squirrel was observed in a location that was previously or not yet owned by another squirrel from true intrusions (Webber et al. 2023).

2.5 | Faecal Sampling

We collected 562 faecal samples (mean = 11 samples/individual, range = 1–34) non-invasively during routine live-trapping (Dantzer et al. 2020). We immediately stored samples on wet ice, froze them at −20°C within 5 h, and kept them at −20°C. We then shipped the samples to the University of Michigan, where they were lyophilized and stored at −20°C.

2.6 | Sequencing and Bioinformatics

We used 16S rRNA amplicon sequencing to determine gut microbiota composition from faecal samples. Briefly, microbial DNA was extracted from up to 0.25 g of faecal material using the Qiagen MagAttract PowerMicrobiome kit (Qiagen, USA). Extracted DNA was quantified and the hypervariable V4 region of the 16S rRNA bacterial gene was amplified using a dual-indexing approach (forward primer: GTGCCAGCMGCCGCGGTAA, reverse primer: GGACTACHVGGGTWTCTAAT, (Kozich et al. 2013)). Libraries were pooled, spiked with PhiX to increase complexity and sequenced in two runs on an Illumina Miseq at the University of Michigan. Each PCR plate had 3 positive controls (ZymoBIOMICS Mock Community II) and 3 negative extraction controls (water) in amplification and sequencing.

We processed sequences using Quantitative Insights Into Microbial Ecology 2 (QIIME2, (Bolyen et al. 2019)). We demultiplexed raw reads before running Divisive Amplicon Denoising Algorithm 2 (DADA2) to collapse sequences into amplicon sequence variants (ASVs), which vary by a single nucleotide (Callahan et al. 2016). We denoised sequencing runs separately before merging to account for potential batch effects (Callahan et al. 2016). We removed low-quality end portions of sequences and retained 13,097,568 reads across 562 samples (mean = 23,305 reads/sample, SD = 8595, range = 1243-53,686). Negative controls had 0–15 reads, suggesting minimal contamination. We assigned taxonomy using the q2-feature classifier against SILVA (SILVA-138-SSU-Ref-NR-99) based on 100% similarity to the reference sequence (Quast et al. 2013). Assignment was universal at phylum level (100%), and high at both family (98%) and genus (97%) levels.

2.7 | Statistical Analysis

We imported QIIME2 data into R (version 4.3.1, (R Core team 2015)) using *qiime2R* (Bisanz 2018). All analyses controlled for sample read depth to avoid the inflated false positive rates and removal of meaningful sequence information associated with rarefaction (McMurdie and Holmes 2014), but see (Schloss 2024).

2.7.1 | Among-Individual Microbial Diversity

We calculated Jaccard similarity, an integrated and bounded measure of presence/absence that does not consider relative abundance, in *phyloseq* (McMurdie and Holmes 2013) as our beta-diversity metric because it is more sensitive than others (e.g., Bray-Curtis, UniFrac) to signals of social microbial transmission (Raulo et al. 2021; Raulo et al. 2024). We visualised sample spread using Principal Coordinates Analysis (PCoA) in *vegan* (Oksanen et al. 2007) and used marginal Permutational Multivariate Analysis of Variance (PERMANOVAs, 999 permutations) to test for associations between gut microbiota and variables of interest, controlling for individual ID (blocking factor) using *permute*(). In all models, continuous variables were scaled to permit effect size comparisons.

2.7.2 | Density-Dependent Shifts in Individuality

To test for homogeneity of dispersion, we binned samples into low, medium, or high-density categories using a three-way split. We used *betadisper*() on the Jaccard matrix to calculate the distance of samples from their group centroid, extracted these distances and built a generalised linear mixed-effects model (family = beta regression) to test whether squirrel density (low, medium and high) predicted sample distance to centroid (dependent variable), controlling for grid, season, prior year food availability, age, body mass, sex, sample read depth, year and individual ID.

2.7.3 | Within-Individual Microbial Diversity

To quantify sample alpha-diversity, we calculated observed richness (number of unique ASVs per sample), Shannon Index (ASV richness and evenness) and Faith's phylogenetic diversity (diversity of phylogenetic relationships among ASVs) in *phyloseq* (McMurdie and Holmes 2013) and *picante* (Kembel et al. 2010).

2.7.4 | Predicted Microbial Functional Capacity

We used Phylogenetic Investigation of Communities by Reconstruction of Observed States version 2 (PICRUSt2) to estimate microbial gene content and infer microbial function (Douglas et al. 2020). We assigned ASVs to reference sequences using HMMER (Finn et al. 2011) and placed them into a reference tree using EPA-NG (Barbera et al. 2019) and gappa (Czech et al. 2020). Data were normalised with castor (Louca and Doebeli 2018) and mapped onto gene pathways using MinPath (Ye and Doak 2009). We investigated functional profiles based on Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs (KOs) mapped onto functional categories at Level 2 of the BRITE map (Kanehisa et al. 2012). One ASV was poorly aligned and excluded from analysis (2ce86713ae7cbabc639eb411191e6128). We assessed functional accuracy by calculating sample-wise weighted Nearest Sequence Taxon Indices (NSTI), which index similarity between ASVs and reference genomes [82]. The mean NSTI across our dataset was 0.11 ± 0.02, falling within the range of NSTI values obtained for mammalian gut microbiota in a validation of PICRUSt2 against metagenomic data (mean NSTI 0.14 ± 0.06, (Langille et al. 2013; Muegge et al. 2011)).

2.7.5 | Differential Abundance of Microbial Taxa and Functional Pathways

We agglomerated taxa at the genus level and transformed to relative abundance. We used a sample-wise abundance threshold, retaining microbial taxa with an average relative abundance of at least 0.05%. For taxonomic data, we constructed zero-inflated negative binomial mixed-effects models (dependent variable = taxa). Models included season, study area, sequencing run, individual ID, year and sample read depth as covariates. For functional data, we built linear mixed-effects models including the same covariates and all functional pathways. For all models, we adjusted for multiple hypothesis testing via Benjamini-Hochberg P-value correction (Benjamini and Hochberg 2000).

2.7.6 | Assignment of Aerotolerance and Sporulation Capacity to Microbial Taxa

We assigned phenotypes of facultative and obligate aerotolerance and capacity for sporulation using Bergey's Manual of Systematics of Archaea and Bacteria (Whitman et al. 2015); where taxa were not available in Bergey's Manual (e.g., more recently named and/or classified taxa), we referred to Raulo et al. (2024) for assignment of microbial phenotype (Raulo et al. 2024).

2.7.7 | Investigating Microbial Similarity With Social Network Analysis and Dyadic Bayesian Regression

We first generated an intrusion-based social network for pairs of squirrels using a lifespan-overlap-corrected simple ratio index (Firth and Sheldon 2016). This index defined social association strength as the total number of unique intrusions between individuals A and B (number of times individual A invaded B, B invaded A) divided by the total number of intrusions either A or B were part of (total times A invaded/was invaded, times B invaded/was invaded). We used our entire dataset of intrusions to generate this measure accurately, including squirrels not faecal sampled for the current study (total $N = 555$ squirrels, (Webber et al. 2023)).

We then used dyadic Bayesian modelling to predict microbiota sharing (Jaccard similarity) by intrusion-based social association strength with a multi-membership beta regression model that allows random effect structures to account for the inherent interdependence of pairwise data and the non-independence of repeated sampling in *brms* (Raulo et al. 2021; Raulo et al. 2024; Bürkner 2017). Models used pairwise

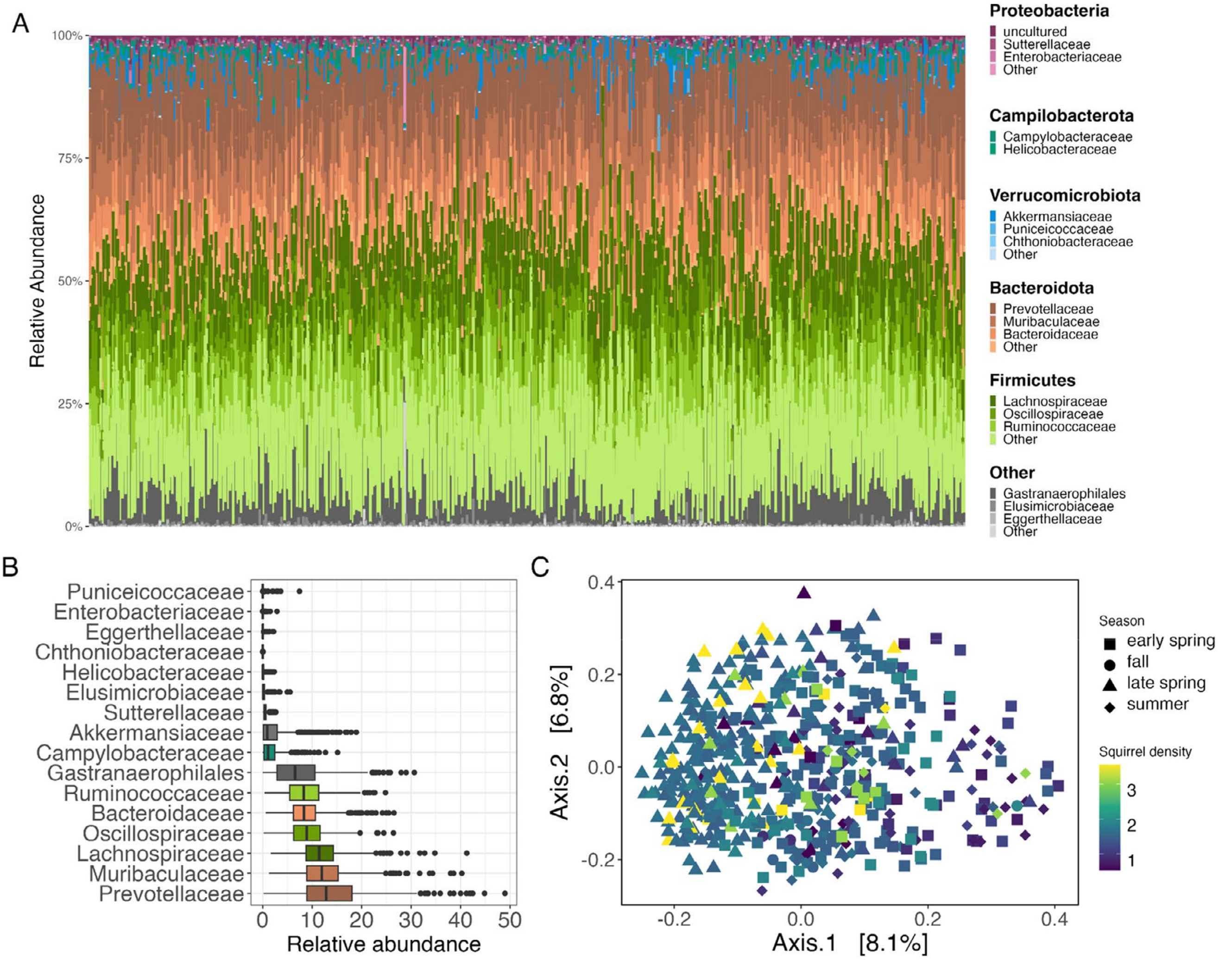

FIGURE 1 | The red squirrel gut microbiota is shaped by temporal, spatial, and ecological factors. (A) Taxonomic composition of all gut microbiota samples, showing sample-wise variation in the 5 most abundant phyla and the 3 most abundant families within each of those phyla. (B) Variation in the relative abundance of the top microbial families in the red squirrel gut (median relative abundance between 1% and 13%). (C) Spatial and temporal clustering by season and squirrel density (average number of squirrels per hectare) based on Jaccard similarity indices (presence/absence of microbial taxa).

Jaccard values as the response (excluding self-comparisons) and a Markov chain Monte Carlo sampler implemented with *rstan* (Raulo et al. 2021; Raulo et al. 2024). We first used the entire Jaccard matrix ($N = 153{,}581$ comparisons, $N = 1326$ unique pairs) to determine how similarity in intrinsic (host age, sex), temporal (sampling season, year), and spatial (study area) variables predicted microbial similarity. We controlled for differences in read depth between paired samples and included a multi-membership random effect for ID (Individual A, B) and sample (Sample A, B). We then tested intrusion-based social association as a predictor of microbial similarity for pairs at relevant spatio-temporal scales, including only comparisons among pairs inhabiting the same study area sampled in the same year ($N = 25{,}381$ comparisons, $N = 436$ pairs). We controlled for season (same/different), sex (same/different), age difference and sample read depth difference. We also controlled for spatial autocorrelation and shared environment by including spatial distance between a pair's territory centroids. Models were run with 4 parallel chains threaded across 32 cores, each with 3000 warm-up samples preceding 12,000 actual iterations. We used posterior checks to assess reliable performance and chain convergence, and ensured Rhat values < 1.10.

3 | Results

3.1 | Socio-Environmental Structuring of the Red Squirrel Gut Microbiota

We detected 4547 amplicon sequence variants (ASVs) across all samples, with the majority of ASVs belonging to *Firmicutes* (mean relative abundance = 47.4%) and *Bacteroidota* (38.3%), with fewer taxa assigned to *Campilobacterota*, *Proteobacteria*, *and Actinobacterota* (Figure 1A). Within these phyla, 16 families were highly abundant (Figure 1B). The most abundant bacterial families were *Prevotellaceae*, *Muribaculaceae*, *Lachnospiraceae*, *Oscillospiraceae*, *Ruminococcaceae* and *Bacteroidaceae*, each

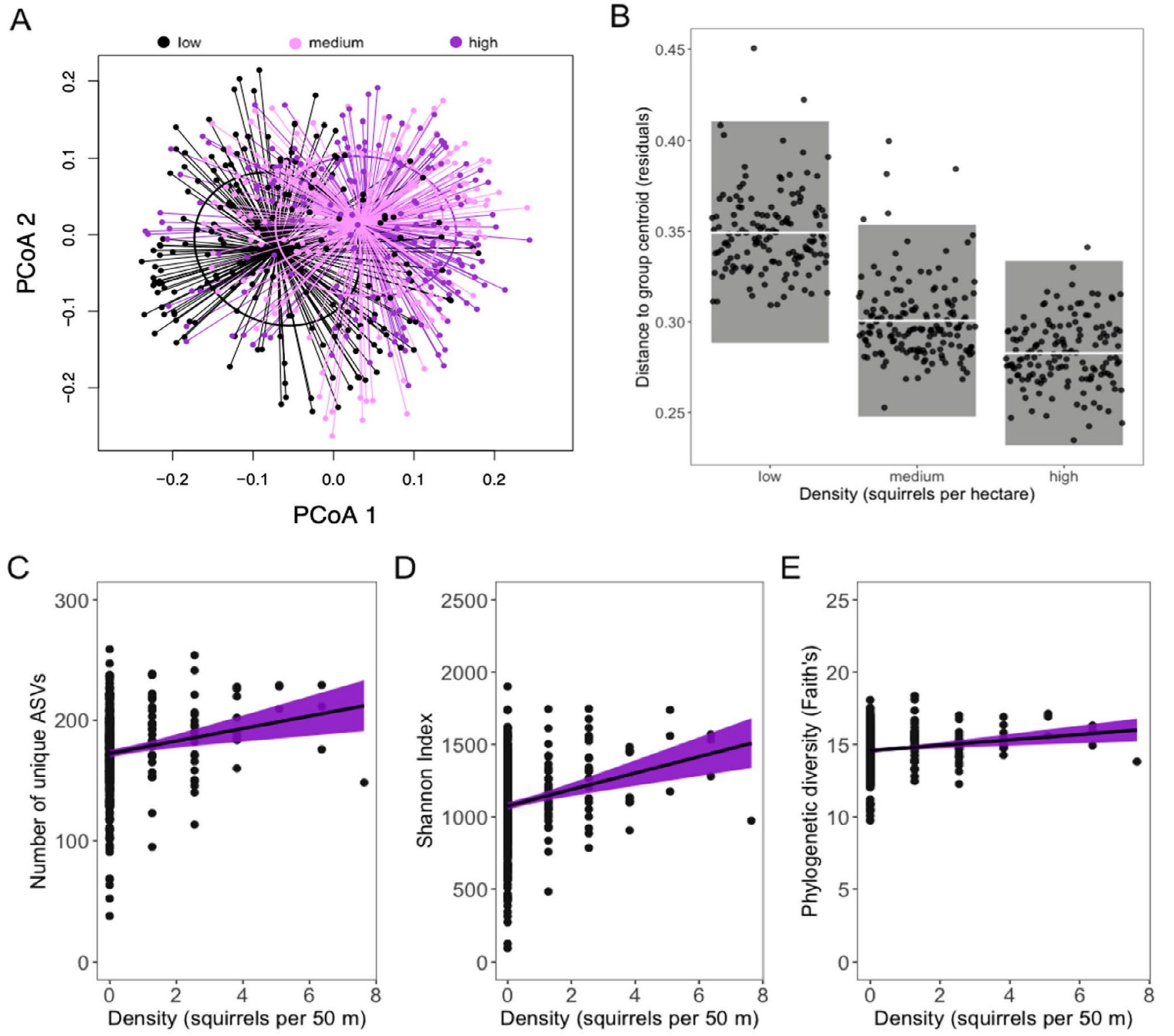

FIGURE 2 | High density weakens gut microbial individuality but increases microbial alpha-diversity. Homogenisation at higher densities indicated by significant differences in (A) beta dispersion at various squirrel densities (permutest, $p < 0.009$; confidence ellipses reflect 1 standard deviation from the median) and (B) individual variability of the gut microbiota, measured as distance to the group centroid in Jaccard compositional space at different squirrel densities (y axis), controlling for individual identity in a generalised linear mixed-effects model (family = beta). Individual microbial communities also exhibit greater (C) microbial richness (number of unique ASVs), (D) abundance weighted diversity (Shannon Index), and (E) phylogenetic diversity (Faith's) at higher neighbourhood densities (within 50 m of an individual's territorial space). Scatterplots depict results from linear mixed-effects models; points reflect samples (partial residuals); line and bands show fitted regression with 95% CIs.

comprising on average between 9% and 15% of the total gut microbiota (Figure 1B).

Compositional variation (Jaccard similarity) in the gut microbiota was explained by social, environmental, temporal and host factors to different degrees. Among all factors, host identity explained most (16%) of the variance, indicating highly individualised microbial communities. Temporal and ecological factors related to food availability also significantly predicted variation, but to lesser extents (year = 3%, season = 2%, and prior year food availability = 0.02%, Table S1). Squirrel density, which fluctuated dramatically at both fine and coarse scales across this study (Figure S1), explained some compositional variation (0.04%), remaining significant after controlling for individual identity (Table S2). Host factors predicted less variance than density did (only sex was significant, 0.03%, with no effect of host age or body mass, Table S2).

3.2 | Gut Microbiota Homogenise and Diversify at High Densities

Tests for homogeneity of dispersion revealed changes in microbial variability rather than changes in average composition as a function of squirrel density ($p = 0.009$, Figure 2A). At the population level, inter-individual variation in gut microbial communities (measured as the mean distance of samples to group

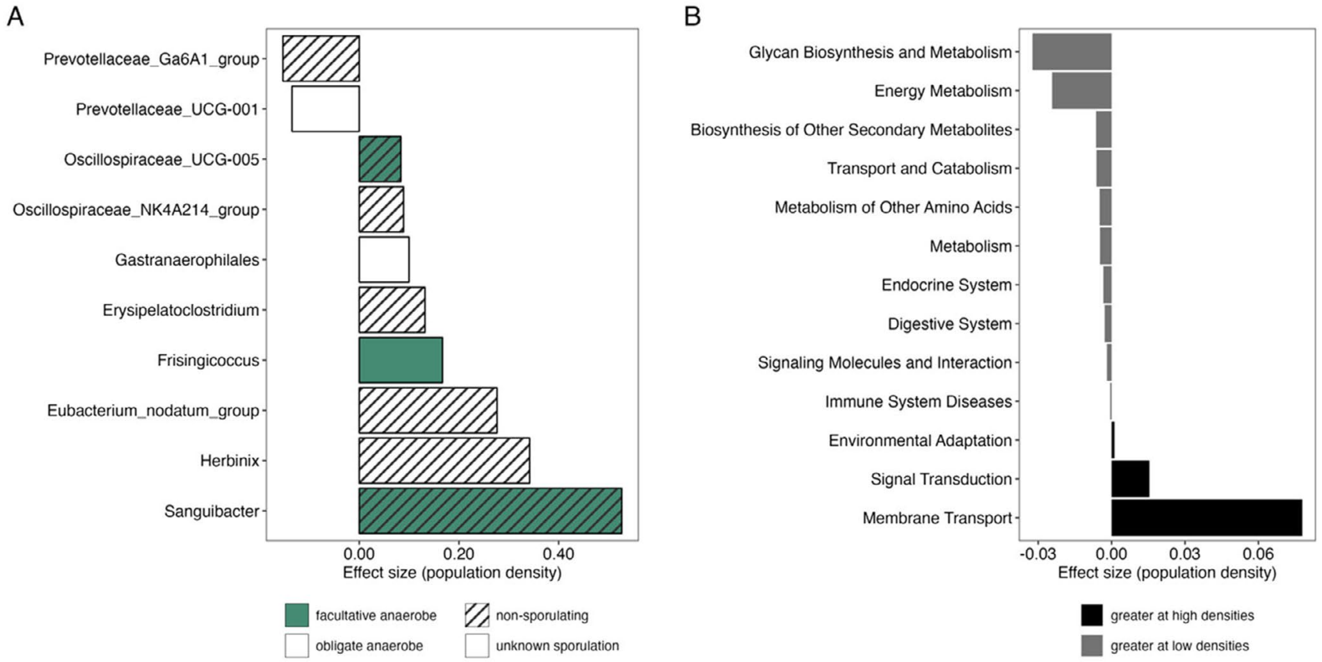

FIGURE 3 | Density predicts shifts in anaerobic, non-sporulating bacteria and changes in microbial functional capacity. Differential enrichment and depletion of microbiota as a function of density among (A) microbial genera with variable levels of aerotolerance, and (B) microbial functional pathways. Plots depict results of mixed-effects models (A: zero-inflated negative binomial; B: linear distribution) testing microbial genera ($N = 84$ bacterial genera) and predicted functional pathways ($N = 41$ KOs) that significantly ($p_{\mathrm{FDR}} > 0.05$) differ in relative abundance as a function of changing squirrel densities.

centroids in Jaccard compositional space) was greatest at low densities, intermediate at medium densities and weakest at high densities ($\beta = -0.07$, $z = -3.32$, $p = 0.002$; Figure 2B, Table S3). This breakdown of microbial individuality predicted by elevated densities was stronger than population-level shifts in individuality predicted by seasonal change ($\beta = 0.03$, Table S3).

At the individual level, squirrels with more neighbours defending territories within 50 m of their own territory exhibited greater gut microbial richness ($\beta = 5.43$, $t = 2.46$, $p = 0.01$), abundance-weighted diversity (Shannon, $\beta = 59.85$, $t = 3.34$, $p = 0.001$) and phylogenetic diversity (Faith's, $\beta = 0.19$ $t = 2.09$, $p = 0.04$) compared to squirrels with fewer neighbours (Figure 2C–E, Table S4). As the neighbourhood radius increased (to 100 and 150 m, respectively), the relationship between density and microbial richness disappeared (the relationship between density and abundance-weighted and phylogenetic diversity remained, Table S5). At the coarsest scale (i.e., within an individual's entire ~40 ha study area), squirrel density did not predict alpha-diversity by any measure tested (Table S6).

As neighbourhood densities and microbial alpha-diversity increased, microbial communities exhibited lower relative abundances of *Prevotellaceae* (UCG-001 and Ga6A1-group, Figure 3A, Table S7), but became enriched in several genera, including *Sanguibacter* ($\beta = 0.527$, $p_{FDR} = 0.047$), *Herbinix* ($\beta = 0.342$, $p_{FDR} = 0.026$), *Eubacterium_nodatum_group* ($\beta = 0.276$, $p_{FDR} < 0.0001$), *Frisingicoccus* ($\beta = 0.167$, $p_{FDR} = 0.047$), *Erysipelatoclostridium* ($\beta = 0.132$, $p_{FDR} = 0.004$), *Gastranaerophilales* ($\beta = 0.100$, $p_{FDR} = 0.035$) and two genera in the family *Oscillospira* (NK4A215_group [$\beta = 0.089$, $p_{FDR} = 0.047$] and UCG-005 [$\beta = 0.084$, $p_{FDR} = 0.035$], Figure 3A, Table S7). Of the taxa enriched at high densities, five were obligate anaerobes and three had some aerotolerance (Figure 3A). At lower densities, obligately anaerobic *Prevotella* (Ga6A1_group: $\beta = -0.153$, $p_{FDR} = 0.040$ and UCG-001: $\beta = -0.135$, $p_{FDR} = 0.011$) exhibited lower relative abundances. All taxa that varied with fluctuating densities were non-sporulating, except two with unknown or unresolved sporulation capacity.

The functional capacity of gut microbial communities also changed appreciably with changing density. Overall, the red squirrel gut microbiota was dominated by microbial functions related to membrane transport (median relative abundance = 10.4%, range = 8.2%–12.7%), replication and repair (10.3%, 9.6%–11.0%) and carbohydrate (9.7%, 9.3%–10.8%) and amino acid (9.7%, 9.3%–10.4%) metabolism (Figure S2). At higher densities, communities shifted toward greater capacity for genes involved in environmental adaptation ($\beta = 0.001$, $p_{FDR} = 0.046$), signal transduction ($\beta = 0.015$, $p_{FDR} = 0.008$), and membrane transport ($\beta = 0.078$, $p_{FDR} = 0.046$, Figure 3B, Table S8). By contrast, at lower densities, functional pathways associated with major host organ systems and physiological processes like energy metabolism ($\beta = -0.024$, $p_{FDR} = 0.008$), immunity ($\beta = -0.001$, $p_{FDR} = 0.008$), digestion ($\beta = -0.003$, $p_{FDR} = 0.003$) and endocrine function ($\beta = -0.003$, $p_{FDR} = 0.006$) dominated (Figure 3B, Table S8).

3.3 | Territorial Intrusions Predict a More Diverse Gut Microbiota

In general, males had larger territorial spaces than females ($\beta = 0.26$, $t = 2.40$, $p = 0.02$), and higher neighbourhood densities within 50 m predicted smaller territorial spaces in both sexes

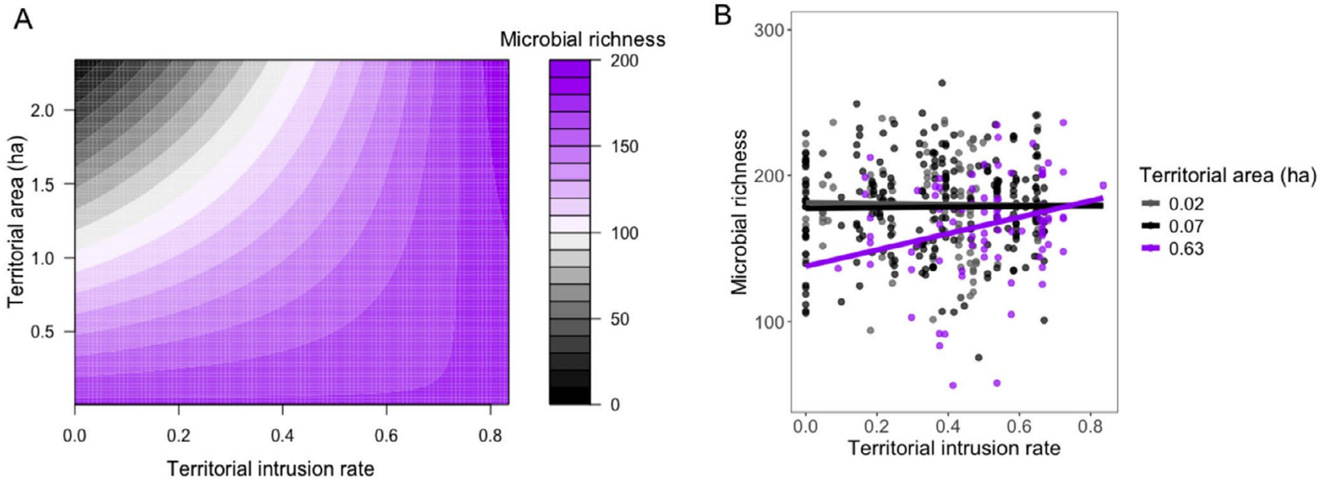

FIGURE 4 | Frequent intrusions diversify gut microbial communities. (A) Surface plot showing slope and direction of the interaction between territorial space size and territorial intrusions on gut microbial richness. As territorial area (*y*-axis) and the rate of territorial intrusions (*x*-axis) increase, so does gut microbial richness (*z*). (B) Individuals with larger territorial spaces that experience more territorial intrusions exhibit greater gut microbial richness. Plot depicts partial residuals from a linear mixed-effects model controlling for density and other covariates (see Table S11), and three overlaid slopes at different territorial space sizes. Note one outlier removed from plot for visualisation purposes, but retained in the model.

($\beta = -0.03$, $t = -2.14$, $p = 0.03$, Table S9; (Webber et al. 2023)). Individuals using larger territorial spaces experienced more intrusions ($\beta = 0.06$, $t = 7.41$, $p < 0.0001$; Table S10, (Webber et al. 2023)), but had lower gut microbial richness, abundance-weighted and phylogenetic diversity (Table S11). The frequency of intrusions by conspecifics onto these spaces significantly predicted gut microbial alpha-diversity as a function of territorial space size and independent of density. For individuals with larger territorial spaces, more intrusions predicted greater microbial richness ($\beta = 6.90$, $t = 2.62$, $p = 0.009$, Figure 4) and phylogenetic diversity with an effect size double that of density ($\beta = 0.38$, $t = 3.53$, $p < 0.0001$), with no effect on abundance-weighted alpha-diversity (Table S11).

3.4 | Intrusions Homogenise the Gut Microbiota of Intruder Pairs

The mean Jaccard similarity across our entire dataset was 0.18 (se = 0.06), indicating that on average, pairs of squirrels shared approximately 18% of their gut microbiota. Using Bayesian dyadic multi-membership models (Raulo et al. 2021; Raulo et al. 2024), we investigated drivers of variation in similarity across pairs. Pairs of squirrels ($N = 1326$ unique pairs) had more similar microbiota if they were sampled in the same year (posterior mean = 0.1, CI = 0.01–0.01) and season (0.14, CI = 0.14–0.14), if they inhabited the same study area (0.02, CI = 0.01–0.02) and if they were similar in age (−0.01, CI = −0.02 to 0.0, Table S12). There was a methodological effect of sample read depth difference (−0.86, CI = −0.88 to 0.85), but no effect of similarity in host sex (Table S12).

We then built an intrusion-based social association network to test whether the frequency of intrusions between pairs predicted pairwise microbial sharing (Figure 5A). Limiting comparisons to pairs inhabiting the same study area and sampled in the same year ($N = 436$ unique pairs), pairs with higher intrusion-based social association indices (more pairwise intrusions relative to total intrusions) had more similar gut microbiota. This was the strongest effect in the model (after sample read depth; social association strength posterior mean = 0.22, CI = 0.14–0.29, Table S13, Figure 5B,C) and was independent of, and twice as large as, the effect of spatial distance between a pair's territory centroids (pairs in closer proximity had more similar gut microbiota, −0.1, CI = 0.14–0.29, Table S13).

4 | Discussion

Applications of island biogeography theory to host microbial ecology typically view animal hosts as microbial islands (Armstrong et al. 2022; Ramos Sarmiento et al. 2024). In territorial animals, non-overlapping, individual territories can reinforce these islands as territorial boundaries and behaviours constrain the social spread of microbiota. This can curtail transmission of pathogens, beneficial ‘core’ microbiota and host-specific strains generated by rapid microbial evolution within the gastrointestinal tract that increase microbial community diversity (Barreto and Gordo 2023; Sarkar et al. 2020). If and when population densities swell, however, routes of microbial transmission can emerge (Arneberg et al. 1998). Here, we show that population density positively predicts greater gut microbial alpha-diversity in non-social red squirrels, echoing work on density-driven microbial transmission in highly social pikas (*Ochotona curzoniae*, (Li et al. 2016)). Immediate neighbours within 50 m had the strongest effect on microbial diversification, and this effect largely weakened at more distant spatial scales, suggestive of local microbial transmission.

If microbial diversification were unrelated to social transmission, elevated densities should exacerbate inter-individual compositional differences and reduce homogeneity because there would be more individuals harbouring their own unique microbiota (Arneberg et al. 1998). However, population-level microbial similarity increased with increasing density, indicating

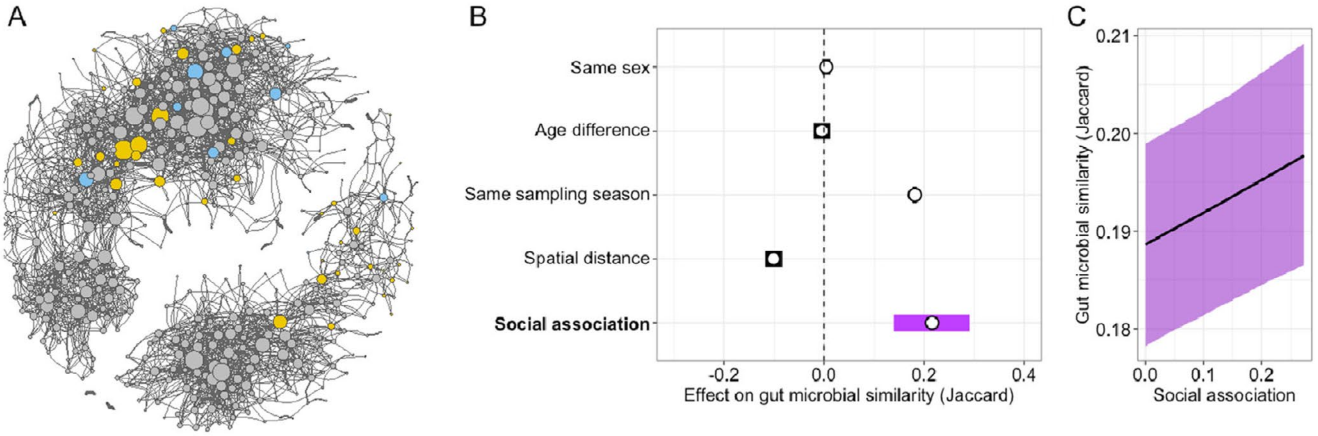

FIGURE 5 | Intrusion-based social association strongly predicts pairwise microbial similarity. (A) Social network constructed from intrusion-based social association index ($N = 555$ squirrels). Node size corresponds to intrusion frequency, with blue nodes indicating males, yellow nodes indicating females, and grey nodes indicating non-focal squirrels (i.e., those without microbiota samples); edge thickness is intrusion-based social association index, corrected for lifespan overlap. (B) Social association strength predicts gut microbial similarity, controlling for spatial distance between pairs. Forest plot shows posterior means (points) and their 95% credible intervals from Bayesian regression model using gut microbiome similarity (Jaccard similarity) between pairs as the response variable. Credible intervals (CIs) that do not overlap zero indicate factors that significantly predicted gut microbial similarity. The effect of read depth difference was removed from the plot for better visualisation of CIs but retained in the model (Table S13). (C) Slope of the effect of pairwise social association on gut microbial similarity (conditional effects with 95% CIs).

homogenisation and a loss of microbial individuality (Arneberg et al. 1998). The homogenising effect of density was three times greater than that of seasonal change, which indexes dietary differences (though squirrels have relatively low dietary diversity that is lowest during periods of high density, see (Fletcher et al. 2013; Dantzer et al. 2020; Ren et al. 2017)). Early spring—which coincides with the mating season and thus increased opportunities for direct and indirect transmission—was the season of greatest microbial homogeneity and individual-level diversification. As we found no consistent effects of host condition (age, body mass) on microbial composition, and because earlier work showed hormonal responses to density decrease, rather than increase, microbial diversity (Petrullo, Ren, et al. 2022), social transmission remains a compelling explanation for density-related microbial homogenisation and diversification (Li et al. 2016).

Most microbial taxa enriched at high densities were obligately anaerobic and non-sporulating, unlikely to survive outside of the anoxic gut environment. This includes *Gastranaerophilales*, the only order of Melainabacteria lacking genes for aerobic respiration (Hu and Rzymski 2022; Soo et al. 2019), commonly found in the gastrointestinal tracts of humans and other animals (Almeida et al. 2019). Other enriched obligate anaerobes were *Herbinix*, non-sporulating cellulose degraders (Koeck et al. 2016), and butyrate-producing *Frisingicoccus*, involved in host metabolism and ketogenesis (Luo et al. 2024). The social transfer of such obligately anaerobic bacteria is thought to require direct host-to-host interactions (Raulo et al. 2024; Sarkar et al. 2020), but physical contact between red squirrels is rarely observed (Dantzer et al. 2012). Social transmission may instead occur indirectly through faecal-oral routes common across rodents (e.g., coprophagy, (Browne et al. 2017; Falkow 1997)) or through faecal shedding and host-environment-host linkages (Raulo et al. 2021). In line with this interpretation, high density most strongly predicted an increase in *Sanguibacter*, facultative anaerobes with some capacity for aerotolerance (Pikuta et al. 2017). Microbes with at least some ability to tolerate oxygen may thus be most successful at transmitting in species with limited social interactions. Overall, the lack of aerobic and spore-forming microbiota in the red squirrel gut could reflect limited evolutionary selection for bacterial traits associated with host-to-host dispersal in solitary, non-social animal systems (Sarkar et al. 2024).

Intimate host-microbe symbioses largely govern microbial community structure such that microbial functions couple with changing host physiologic and energetic demands (Kundu et al. 2017). At lower densities, microbiota expressed greater capacity for host-centric functions related to fundamental physiological systems like metabolism, immunity and digestion. At higher densities, microbiota shifted toward greater capacity for sensing and processing environmental information: pathways related to membrane transport, signal transduction and environmental adaptation (including thermogenesis and circadian rhythm entrainment) increased. This shift from 'inward' (involved in major host physiological systems) to "outward" (involved in environmental sensing) functions was opposite to our predictions. If social transmission shifts microbial function away from host physiologic regulation, this may indicate a cost of density-driven changes in gut microbiota. For example, the stochastic incorporation of socially-acquired microbiota may displace core taxa (e.g., *Prevotella* spp., (Ren et al. 2017)) central to the regulation of host organ systems (Moeller et al. 2018). Alternatively, functional shifts may reflect host prioritisation of microbiota with the capacity to detect and integrate environmental cues when intraspecific competition is high (Dantzer et al. 2012; Petrullo et al. 2025). Research investigating the adaptive value of social microbial transmission should consider that socially-mediated shifts in microbial composition may dynamically reshape host physiological priorities.

Finally, in highly social animals, strong social bonds and affiliative interactions like grooming can diversify host microbiota (Dill-McFarland et al. 2019). Our data suggest that territorial intrusions have a similar effect: intrusions positively predicted microbial richness and phylogenetic diversity, but not abundance-weighted diversity, among the owners of large territorial spaces, independent of density. This is in line with data from social species in which social microbial transmission shapes the type and number of unique microbes present in the gut, while within-host selective processes (e.g., diet, physiology) shape the subsequent distribution and abundance of those taxa (Raulo et al. 2024). Moreover, because territory space size and intrusion frequency are positively correlated (Webber et al. 2023), a threshold effect among owners of large (and thus frequently intruded upon) territorial spaces may modify detectable signatures of intrusion-mediated changes in gut microbiota.

Intrusions also facilitated microbial homogenisation. In social and semi-social animals, strong social relationships can maintain microbial similarity among pairs (Raulo et al. 2021; Tung et al. 2015). Here, we find a parallel effect of agonistic, rather than affiliative, interactions. Pairs with stronger intrusion-based social association had greater microbial similarity, independent of the effect of spatial proximity and surpassing the effects of seasonality. Given the risks of resource pilferage and energetic stress associated with territorial intrusions, microbial transmission may be a cryptic benefit that reduces the cost of intrusions for both owners and intruders. For example, intrusions weaken the stability of territorial systems, making resources less valuable and defence more costly (Siracusa et al. 2017); these costs may be balanced by the spread of beneficial microbial symbionts for intruders (who may gain food and microbes), and owners (who may lose food but gain microbes). If intrusions further spread bacteria and/or symbiotrophic fungi that enrich soil or plant communities, such benefits may extend into the broader ecosystem. At the same time, intrusions could facilitate opportunistic pathogen spread across hosts, augmenting the cost of intruding and offsetting potential benefits.

Nonetheless, homogenisation and diversification of host microbiota when densities are high or intrusions are frequent may still enhance host fitness and contribute to population persistence. At the individual level, a diverse microbiota can block invading pathogens from colonising via competitive exclusion (Spragge et al. 2023). At the population level, diversification of host microbiomes may buffer against random microbial extinction events associated with drift (Moeller et al. 2016). If extinctions do occur, a diverse set of microbes could confer functional redundancy, preserving adaptive host-microbe symbioses (Louca et al. 2018; Yang 2021). Indeed, our findings demonstrate that social microbial transmission can be maintained via demographic change and agonistic behaviour in species where physical contact is rare. Despite potential costs, the host-to-host transfer of microbial symbionts in solitary animals may ultimately be an inconspicuous benefit of relaxed territoriality in otherwise territorial systems.

Author Contributions

Lauren Petrullo and Quinn Webber conceived the study. Lauren Petrullo generated microbiome data and performed all analyses. Quinn Webber generated territoriality data. Aura Raulo performed social network analyses. Lauren Petrullo wrote the first draft of the manuscript. All authors contributed edits and revisions to all versions of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data and code associated with the analyses presented in this manuscript are available at http://doi.org/10.6084/m9.figshare.27307227. All raw microbiome sequence data are available through the NCBI Sequence Read Archive (SRA) under PRJNA1237724.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** ele70186-sup-0001-supinfo.docx.