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Research Article

Gut microbial taxa are associated with problem-solving success in house sparrows

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Behavioral innovations are important traits that allow individuals to adapt to novel situations and changing environments. These behaviors require cognitive skills and behavioral flexibility but the mechanisms behind them are still poorly understood. The gut microbiome is the collection of microorganisms that reside in the gut, and it can influence the brain of the host organism through the gut–brain axis. We hypothesized that the richness and composition of the gut microbiome would relate to birds' abilities to solve novel problem-solving tasks. For this study, we used wild-caught house sparrows *Passer domesticus*, a species that is invasive in many parts of the world in part due to its flexible behavior and preference for urban habitats. We presented individuals with two food extraction tasks and measured their ability to solve them. We also measured the richness and composition of the birds' microbiomes from fecal samples. While microbial diversity did not significantly differ between solvers and non-solvers, some microbial genera did differ between solvers and non-solvers. We also found that persistence in solving the task was correlated with problem-solving success. These results add to our understanding of the avian gut–brain axis; however, further research will be needed to understand how specific microbes impact the brain and behavior of birds.

Keywords: avian microbiome, bird, cognitive performance, gut–brain axis, *Passer domesticus*

Introduction

Behavioral innovation, or the ability to invent new behaviors or use existing ones in new contexts, is an important aspect of animal cognition (Griffin and Guez 2014). However, innovation can be difficult to study in the wild due to its rarity. In some species (especially birds), innovation and problem-solving are correlated such that problem-solving can be an indirect way to measure innovation (Webster and Lefebvre 2001). Problem-solving is a complex behavior that involves cognition, behavioral



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flexibility, and persistence in solving the task (Lefebvre et al. 2004, Biondi et al. 2022, Overington et al. 2011, van Horik et al. 2016). Innovation and problem-solving are particularly important in the context of foraging and can provide increased access to food, particularly in changing environments. At the species level, birds that are more innovative are more tolerant of habitat change and are less likely to be in danger of extinction (Ducatez et al. 2020). At the individual level, problem-solving in birds is correlated with increased reproductive success (Cauchard et al. 2017, Wetzel 2017). Variation in problem-solving in birds is still being explored but is related to the individual's learning ability, motor skills, and environment (Cole et al. 2011, Griffin and Guez 2014, Griffin et al. 2014, Quinn et al. 2016). Individuals with a wider range of motor skills may be more capable of developing novel behaviors (Diquelou et al. 2015).

One understudied aspect of an animal's biology is the gut microbiome. The gut microbiome is the sum of the microbes and their genomic elements in the gastrointestinal tract of an animal (Ho and Bunyavanich 2018). This community is composed of millions of cells and possesses more genes than the host organism. Because of the large number of cells and functional genes, the microbiome has the potential to have an enormous impact on not only the gut but the rest of the body, including the brain (Cryan et al. 2019).

The gut microbiome can communicate with the host's brain through the gut-brain axis using mechanisms such as circulating metabolites and direct stimulation of nerves connecting the gut and the brain (Davidson et al. 2018, Maniscalco and Rinaman 2018). In laboratory rodents, variation in the microbiome is associated with complex behaviors and learning (Magnusson et al. 2015, Savignac et al. 2015, Oliveros et al. 2016, Buffington et al. 2021), and higher diversity in the microbiome correlates with improved cognition (Gao et al. 2020, Shi et al. 2020). Relatively little is currently known about the gut-brain axis in wild birds. One study found that the composition, but not diversity, of the gut microbiome influences learning and memory of a captive-bred songbird (Slevin et al. 2020). It is therefore possible that the microbiome may influence cognitive abilities or behavioral patterns, such as persistence, that are needed for problem-solving through the gut-brain axis in songbirds.

House sparrows *Passer domesticus* are a highly innovative and adaptive passerine species. They have successfully adapted to habitats across the globe as an invasive species and there are numerous anecdotal accounts of their use of novel foraging methods (Dixon 2010, MacGregor-Fors et al. 2020, Jędro and Jędro 2018). This ability makes them an ideal species to study innovation through problem-solving behavior. House sparrows are frequent subjects in avian studies, which have found that gene expression, hormones, and social learning impact their behavior and cognition (Kelly et al. 2020, 2022, Lattin et al. 2022). Due to house sparrows' behavioral flexibility, several studies have investigated possible correlates of their problem-solving behavior (Liker and Bókony 2009, Bókony et al. 2014, Papp et al. 2015, Wetzel 2017); however, the effect of the microbiome on these behaviors has not

yet been investigated. Previous studies of problem-solving by house sparrows have found that birds with lower amounts of corticosterone and coccidian parasites, and higher antioxidant levels, solved problems faster (Bókony et al. 2014). Furthermore, sparrows that live in more urban landscapes and those living in larger groups were more successful in solving problems (Liker and Bókony 2009, Papp et al. 2015). The ability to solve problems was also positively related to nestling provisioning and offspring survival (Wetzel 2017). These studies support the hypothesis that problem-solving is associated with enhanced fitness, including increased offspring survival and lower stress and infection levels; however, it is unknown if the microbiome plays a role in this relationship.

We investigated the relationship between the diversity and composition of gut microbiome and performance on problem-solving tasks using wild-caught house sparrows. We predicted that individuals that are more persistent in solving the tasks would be more likely to solve the tasks, as has been found in other birds (Davidson et al. 2020). Because our knowledge of avian microbiomes is limited, we are basing our predictions regarding microbiomes and cognition on results from other species; we are doing so as a starting point but acknowledge that species differences may exist. We therefore predicted that individuals that successfully solved the tasks would have higher levels of alpha diversity, or species richness, in their gut microbiome because high microbiome diversity is associated with better cognitive performance in mice and humans (Fröhlich et al. 2016, Canipe et al. 2021). We also predicted there would be differences in beta diversity, or community composition, of the microbiome in individuals that solved and did not solve the tasks and by how persistent they were at solving the tasks. Finally, we predicted that specific microbial taxa would differ in relative abundance between solvers and non-solvers.

Material and methods

Animals and housing

From March through July 2020, we captured 44 adult house sparrows (17 females, 27 males) from five locations (at least 0.5 km apart, 3.2 ± 1.9 km (mean $\pm$ standard deviation)) in College Station and Bryan, Texas. Birds were caught in either baited potter traps or mist nets. We limited sampling to the breeding season to avoid possible changes in birds' behavior based on different times of the year. Immediately after capture, birds were placed in a paper bag with a sterilized weight boat at the bottom to collect a fecal sample (methodology outlined in Knutie and Gotanda 2018). Fecal samples were used as a proxy for the microbial community present in the gut. Although fecal samples represent a distinct microbial community from the gastrointestinal tract, it is similar to the distal gut (Ingala et al. 2018, Berlow et al. 2020) and is commonly used as a non-lethal method to sample the microbiome (Schmidt et al. 2019, Davidson et al. 2020, San Juan et al. 2021). After defecation (within 5 min), the birds were removed from the bag and the feces were placed in an

Eppendorf tube with a sterile spatula. Samples were placed on ice until they were transported to and stored in a -80°C freezer (mean time to freezer 107 ± 63 min). Birds were then transported to the housing facility and individuals were marked with a metal leg band with a unique identification number. Birds were sexed based on their plumage: males have black chests, gray crowns, and chestnut backs while females are duller brown with a light stripe above the eye. Juvenile birds cannot be reliably sexed and were identified based on their paler plumage and bill as well as an enlarged yellow gape (Summers-Smith 1988). Juvenile individuals were released upon capture. At capture, weight and tarsus length was measured from each bird. We used Peig and Green's (2009) method to calculate the scaled mass index with the formula as follows: $M_i = M_i \left[\frac{L_0}{L_i} \right]^b$, where M_i is the weight, L_i is the tarsus length, L_0 is the average tarsus length of all birds measured, and b is the slope of standard major axis regression between mass and tarsus length. Birds were then housed in cages ($0.6 \times 0.33 \times 0.3$ m) at Texas A&M University, College Station Texas, USA ($30^{\circ}36'\text{N}$, $96^{\circ}21'\text{W}$) in an indoor room (5.0×6.3 m). To minimize microbe transfer between individuals, each bird was housed alone in a cage, although they were within visual and auditory contact of other birds in the room. They were kept on a 13 h:11 h light:dark cycle at $24.0 \pm 0.5^{\circ}\text{C}$ (mean $\pm$ standard deviation) provided with food (Royal Wing Wild Bird Food, Tractor Supply Co.; mix of milo, millet, cracked corn, and black sunflower seeds) and water ad libitum.

Problem-solving protocol

Three to eight days after capture (mean $\pm$ standard deviation: 4.54 ± 1.14 days), birds were presented with the first problem-solving task. On the day immediately after being tested on the first task, they were presented with the second problem-solving task. These tasks were based on tasks from Bókonyi et al. (2014, tasks 2 and 3) and are described in detail below. These tasks were chosen based on their previous use with sparrows as well as the level of variation in solving between individuals. The day prior to the first trial, the birds were presented with the first feeder task with no barriers to the food inside, to familiarize them with the apparatus. On the day of the trial, 3 h before the trial began, the birds were moved to an experimental room where they were visually isolated from other birds in the housing room, and all sources of food were removed from the birds' cages. The period of food deprivation was implemented to increase their motivation to solve the task and attempt to standardize the hunger levels of each bird. The birds were then presented with a closed version of the problem-solving task (Fig. 1A–C). They were given 90 min to attempt to solve the task, during which they were recorded with a video camera (VIXIA HF R70; 60 frames/s; Cannon Inc.). For the duration of trials, the experimenter waited in an adjacent room hidden from the birds but monitoring them through video cameras. Following the 90 min, we recorded the mass of the individuals to determine changes in body mass while in captivity and then returned them to

their regular housing conditions. Once returned, the birds were presented with the second feeder with no barriers and the process was repeated with the second task the next day. We found that change in mass was not related to initial scaled mass (Pearson correlation coefficient: $r = -0.1$). All trials took place between 10:00 and 19:00 h (mean start time of $14:45 \text{ h} \pm 192$ min). After completing the two tasks, we collected an additional fecal sample from a subset of the birds ($n = 28$, 19 males and 9 females, using the same methods as above) one to four days after the final task (mean $\pm$ standard deviation: 1.80 ± 1.07 days) and all birds were released to their site of capture. Collection of this additional sample was performed for a separate study (Florkowski et al. 2023) that began after we started collecting data for this study, explaining why not all birds in this study were sampled a second time.

Using the videos of each trial, we assessed which birds solved each task by determining if they were able to open and retrieve seeds from the tasks. If the bird was successful, we measured the latency to solve the task (the latency between the start of the trial and when the bird first picked up a seed). We also measured persistence to solve the task ('persistence') by dividing the number of times they contacted the feeder with their beak by the total time; the total time was the latency to solve the task, or 90 min if they did not solve the task. Birds did contact the feeder with other parts of their body (e.g. standing on top of it) but were only able to solve the tasks by manipulating it with their beak; therefore, contacts with the feeder that did not involve the beak were not included in the persistence measurement.

Problem-solving tasks

The birds were presented with two food-extraction tasks (Fig. 1) with Task 1 always presented first. Task 1 was a clear plastic cup (Fig. 1A; 2.85 cm in height and 3.95 cm in diameter). The cup was filled with the birds' regular seed mixture food and a piece of opaque white paper covered the top, secured with tape. To access the seeds in Task 1, the birds needed to pierce the paper (Fig. 1B). Task 2 was a commercial bird feeder with a plastic reservoir (Fig. 1C; 10 cm high) containing the birds' regular seed mixture food. The reservoir had a slit cut into it (approximately 8 cm high) with a plastic card fitted into the slit holding the seeds up, so they were inaccessible. To access the seeds, the birds needed to remove the plastic card for the seeds to fall to the feeder's opening (Fig. 1D).

Microbiome methods

Using 0.25 g of each frozen fecal sample, we isolated DNA with QIAamp PowerFecal DNA Isolation Kits (Qiagen, Germany) following the manufacturer's protocol (except we increased sample incubation at 65°C from 10 min to at least 8 h in an effort to increase DNA yield). While extended incubation was intended to maximize DNA yield from avian fecal material, it may preferentially lyse thicker-walled Gram-positive bacteria (e.g. Firmicutes versus Gram-negative Proteobacteria; Knudsen et al. 2016). To verify sufficient DNA yield, we used a Qubit fluorometer (dsDNA HS Assay

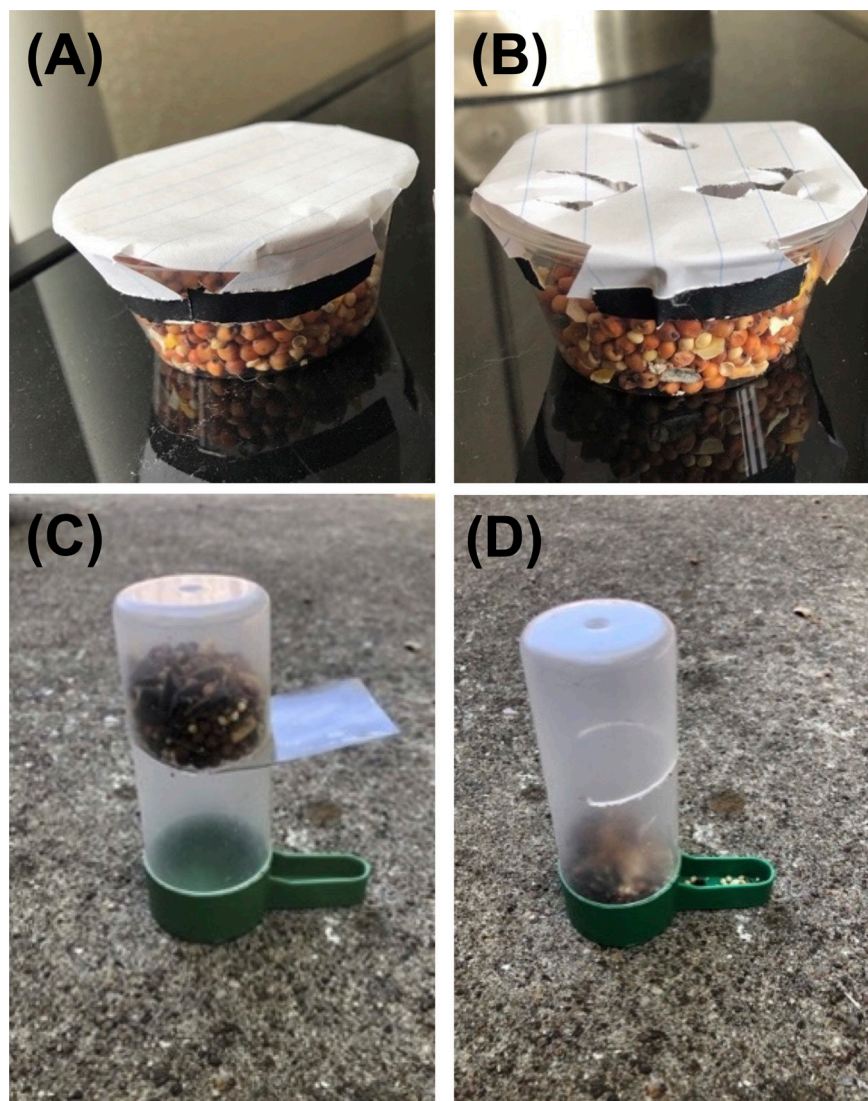


Figure 1. Problem-solving Task 1 closed (A) and open (B); Task 2 closed (C) and open (D).

Kit, Invitrogen, Carlsbad, CA, USA) to measure DNA concentration and then diluted each sample to a concentration of $5 \text{ ng } \mu\text{l}^{-1}$ of DNA. The extracted DNA was then sent to the Michigan State University's genomics core and was processed and sequenced according to Kozich et al. (2013). In brief, at the Michigan State University's genomics core, libraries were constructed by amplifying the V4 region of the 16S rRNA gene using primers 515F and 806R with Illumina adapters and dual indices. The 16S rRNA gene is a prokaryotic gene that contains hypervariable regions, such as the V4 region, and is commonly used to identify bacteria (Caporaso et al. 2011). Samples were amplified using DreamTaq Master Mix (ThermoFisher, Waltham, MA, USA). The PCR reaction was incubated at 95°C for 3 min, followed by 30 cycles of 45 s at 95°C , 60 s at 50°C , and 90 s at 72°C , then a final extension at 72°C for 10 min. Unique dual indices were added to the PCR products, which were then pooled and batch normalized using Invitrogen SequelPrep DNA Normalization plates

(Invitrogen, Carlsbad, CA, USA). The product recovered from the plates were concentrated using an Amicon Ultra centrifugal filter (Sigma-Aldrich, St. Louis, MO, USA) and cleaned using AMPure XP magnetic SPRI beads (Beckman Coulter, Brea, CA, USA). The cleaned pools were sequenced on the Illumina MiSeq platform using v2 2x250 base pair kit (Illumina Inc., San Diego, CA, USA).

We performed initial quality control of raw sequences with Trim Galore (ver. 0.6.6) which was used to remove adaptors and trim reads to remove sequences with base call quality below a Phred score of 20. We then processed trimmed sequences using the Mothur software (ver. 1.45.3; Schloss et al. 2009) using standard operating procedure (accessed May 2021). Briefly, the forward and reverse sequences were assembled into contigs using the *make.contigs* function. The *screen.seqs* function was used to evaluate the resulting sequences, and sequences with incorrect base pair length or those that did not overlap the targeted region were removed. Singletons and

chimeric sequences are common artifacts in DNA sequencing data, which possibly result from PCR errors. Singletons were removed with the *split.abund* function with a cutoff of 1, and chimeric sequences were identified with the *chimeric.uchime* function and removed with the *remove.seq* function. Because singletons were removed, we did not estimate Chao1 richness (which is highly sensitive to singletons) and instead relied strictly on Shannon diversity index and Faith's PD as our primary alpha diversity metrics. Mitochondria and chloroplast sequences were removed using the *remove.lineage* function. Remaining sequences were aligned against the SILVA database (Release 132) with the *align.seqs* function. The sequences were then clustered into operational taxonomic units (OTUs) with 97% similarity, a commonly used threshold including in other studies investigating wild bird microbiomes (Ritter et al. 2019, Garcia-Mazcorro et al. 2021). We evaluated the read count of the 44 initial microbiome samples, and any samples with fewer than 1000 reads ($n = 5$) were dropped from subsequent analysis as the low sequence read count may skew downstream analysis (Momozawa et al. 2011). This resulted in 39 samples used in further analysis. In the absence of blank controls, to minimize contaminant noise, we processed all samples in a single batch using sterile techniques and excluded samples with low read counts (< 1000 reads), which are most vulnerable to contaminant amplification.

Statistical analysis

In our analyses, the initial models included the variables sex, scaled mass, weight loss during captivity, capture location, capture date, and days in captivity before the trial. We included these variables to determine if there were differences in behavior based on sex, body size, captivity, or time and location of capture. To account for potential differences among capture sites while avoiding overfitting, we incorporated capture location as a random/frailty effect (frailty(Location)) in all Cox proportional hazards models. For PERMANOVA models, we accounted for capture location by restricting permutations within locations using the *strata* parameter (*strata*=Location), which blocks for site-level differences without treating location as a fixed covariate. To control for potential temporal or seasonal autocorrelation, capture date was included as an initial covariate, though it was removed during stepwise selection if non-significant ($p \geq 0.10$). Non-significant variables were removed stepwise so that the final models reported only contain effects at the $p < 0.10$ level. We used this conservative threshold ($p < 0.10$) to ensure that important variables were not eliminated from the models. We also never eliminated solving success, microbiome diversity, or persistence when they appeared in the models because these were our variables of interest for our predictions.

To investigate the relationship between success in solving the presented tasks and persistence in solving the tasks, we used Cox proportional hazards models in the 'survival' R package (ver. 3.4-0; Therneau 2022). This model relates the time that passes before an event occurs to covariates that may be associated with that quantity of time. This model also

includes censored values, which can account for individuals not solving the task within the time limit. The response variable was the latency to solve Task 1 or 2, and any trials where the task was not solved were coded as a maximum latency of 5400 s (the maximum duration of a trial). The explanatory variables were persistence in solving the task, weight loss in captivity, and capture location for Task 1, and persistence in solving the task and sex for Task 2. We also investigated the effect of weight loss in captivity on persistence in solving the tasks with linear models where persistence in solving either Task 1 or Task 2 was the response variable and weight loss in captivity was the explanatory variable.

We imported microbiome files into RStudio (ver. 4.1.2; www.r-project.org) using the 'phyloseq' R package (ver. 1.36.0, McMurdie and Holmes 2013). We then applied variance stabilization transformation to the OTU counts to account for the differences in library size across samples using the 'DESeq2' R package (ver. 1.32.0; Love et al. 2014). We then used the *estimate_richness* function in the 'phyloseq' package to determine the alpha diversity of each sample using Shannon diversity index (Shannon 1948) and Faith's PD (Faith 1992). The Shannon index reflects how many different microbes are in the sample and how evenly they are distributed within a sample; it looks at the richness of the community, which impacts the stability and function of the community. Faith's PD measures the amount of the phylogenetic tree covered by the community in the sample; it incorporates phylogenetic distance, which also takes into account the potential diversity of genetic function. We used multiple measures of diversity to prevent results from being biased by one diversity index. To determine if alpha diversity was related to the latency to solve the problem-solving tasks, we used Cox proportional hazards with the response variable as the latency to solve Task 1 or 2. The explanatory variables were alpha diversity of each bird's microbiome sample (either Shannon diversity index or Faith's PD), weight loss in captivity, and capture location for Task 1, and alpha diversity and weight loss in captivity for Task 2. We used the Bonferroni correction to account for multiple comparisons with the different measurements of alpha diversity (Bonferroni 1936).

We also tested whether persistence in solving the tasks was related to alpha diversity in the microbiome. We used the linear model function *lm* in the R package 'stats' (ver. 4.1.2). The response variable was the persistence in solving the tasks and the explanatory variable was alpha diversity of the microbiome (either Shannon diversity index or Faith's PD) for Tasks 1 and 2. We used the Bonferroni correction to account for multiple comparisons with the different measurements of alpha diversity (Bonferroni 1936).

To investigate possible differences in microbiome community composition between individuals that solved and did not solve the tasks, we calculated beta diversity measures using Bray–Curtis dissimilarity (Bray and Curtis 1957), as well as weighted and unweighted UniFrac distances (Lozupone et al. 2011). Bray–Curtis dissimilarity measures differences in communities based on taxon abundances. Weighted and unweighted UniFrac distances measure differences in

communities based on taxon abundances or based on taxon presence or absence, respectively, and are weighted by phylogenetic distance. We then used the *adonis2* function to perform PERMANOVAs in the 'vegan' R package (ver. 2.5-7, Oksanen et al. 2013) set at 999 permutations. This function compares the centroids of microbial communities of different groups as the response variable. The explanatory variables of these models were the success of solving Task 1 or 2, scaled mass index, and location in which individuals were captured. We used the Bonferroni correction to account for multiple comparisons with the different measurements of beta diversity (Bonferroni 1936).

We confirmed homogeneity of dispersion in the data with the *betadisper* function. We then plotted these measures on a principle component analysis (PCA) plot to visualize the differences. A PCA plot reduces microbiome community data to two axes and visually represents how similar or different two microbiome communities are based on how close they are on the plot.

Finally, we determined differences in relative abundance of microbial taxa between solvers and non-solvers using analysis of compositions of microbiomes with bias correction (ANCOM-BC) for differential abundance analysis (Lin and Peddada 2020). We also investigated the interaction between persistence in solving the task and beta diversity in the microbiome. We used PERMANOVAs set at 999 permutations. The measures of beta diversity were Bray–Curtis dissimilarity, and weighted and unweighted UniFrac distances. The explanatory variables were persistence, capture location, scaled mass, and days in captivity before the trial for Tasks 1 and 2.

To determine if there was a relationship between the problem solving and the impact of captivity on the microbiome, we repeated the above analyses with the microbiome samples collected from a subset of the birds ($n=28$) just before release.

Results

A total of 14 individuals solved at least one of the tasks. Task 1 was solved by 11 out of 39 individuals (28% success rate) and Task 2 was also solved by 11 out of 39 individuals.

However, the tasks were not solved by the same 11 individuals: only five birds (12% of individuals) successfully solved both tasks. On average, successful individuals solved Task 1 in 2625 ± 1307 s (mean $\pm$ standard deviation; range: 590–5118 s) and Task 2 in 1391 ± 1607 s (mean $\pm$ standard deviation; range: 44–3610 s). We also found that there was a significant positive correlation between persistence in solving Task 1 and Task 2 (Pearson correlation coefficient: $r=0.40$).

In the models with Location frailty, persistence was positively related to solving success in both Task 1 ($p < 0.001$; hazard ratio = 456.81, 95% CI: 15.16, 13768.63; Table 1) and Task 2 ($p=0.011$; hazard ratio = 2.35×10^5 , 95% CI: 17.17, 3.21×10^9 ; Table 1; Fig. 2). We did not find that weight loss in captivity significantly predicted success in solving either task in these models (Task 1: removed during selection; Task 2: $p=0.220$, hazard ratio = 2.07, 95% CI: 0.65, 6.64). Individuals lost, on average, $12.1 \pm 6.1\%$ (mean $\pm$ standard deviation) of their body mass, which is an acceptable reduction based on animal welfare recommendations (Talbot et al. 2020). We found that weight loss did not correlate with persistence in solving either task (Supporting information).

We found that alpha diversity of the microbiome did not predict the success in solving either task (Shannon diversity index: Task 1: $p=0.35$; Task 2: $p=0.61$; Faith's PD: Task 1: $p=0.86$; Task 2: $p=0.088$; Table 2; Fig. 3). We also found no relationship between the microbiome and persistence in solving either task (Shannon diversity index: Task 1: $p=0.069$; Task 2: $p=0.116$; Faith's PD: Task 1: $p=0.489$; Task 2: $p=0.852$; Supporting information).

We found no significant difference in beta diversity between individuals that solved and did not solve Task 1 or 2 (Bray–Curtis dissimilarity Task 1: $p=0.997$; Task 2: $p=0.789$; weighted UniFrac Task 1: $p=0.944$; Task 2: $p=0.512$; unweighted UniFrac Task 1: $p=0.789$; Task 2: $p=0.918$; Table 3; Fig. 4). There is also no significant difference between persistence and beta diversity in individuals that solved and did not solve Task 1 or 2 (Bray–Curtis dissimilarity Task 1: $p=0.554$; Task 2: $p=0.808$; weighted UniFrac Task 1: $p=0.859$; Task 2: $p=0.363$; unweighted UniFrac Task 1: $p=0.810$; Task 2: $p=0.844$; Supporting

Table 1. Results of Cox proportional hazards models investigating the relationship between success in solving Task 1 or 2 and persistence, weight loss during captivity, sex, or capture location. Significance is indicated with an asterisk. *In Cox proportional hazards models, capture location was modeled as a random/frailty grouping factor. Because random effects do not have standard coefficient estimates, hazard ratios, or confidence intervals, we report the estimated random effect variance.

Task	Variables	df	Estimate (coef)	SE	z value	p-value	Hazard ratio (HR)	95% CI for HR	Variance
1	Persistence	1	6.124	1.738	3.52	< 0.001*	456.81	[15.16, 13768.63]	–
	Sex (male)	1	1.430	0.927	1.54	0.120	4.18	[0.68, 25.73]	–
	Location (frailty)†	–	–	–	–	0.970	–	–	5.0e-7
2	Persistence	1	12.366	4.859	2.54	0.011*	2.35×10^5	[17.17, 3.21×10^9]	–
	Weight loss	1	0.728	0.595	1.22	0.220	2.07	[0.65, 6.64]	–
	Sex (male)	1	0.491	1.354	0.36	0.720	1.63	[0.12, 23.21]	–
	Location (frailty)†	–	–	–	–	0.970	–	–	5.0e-7

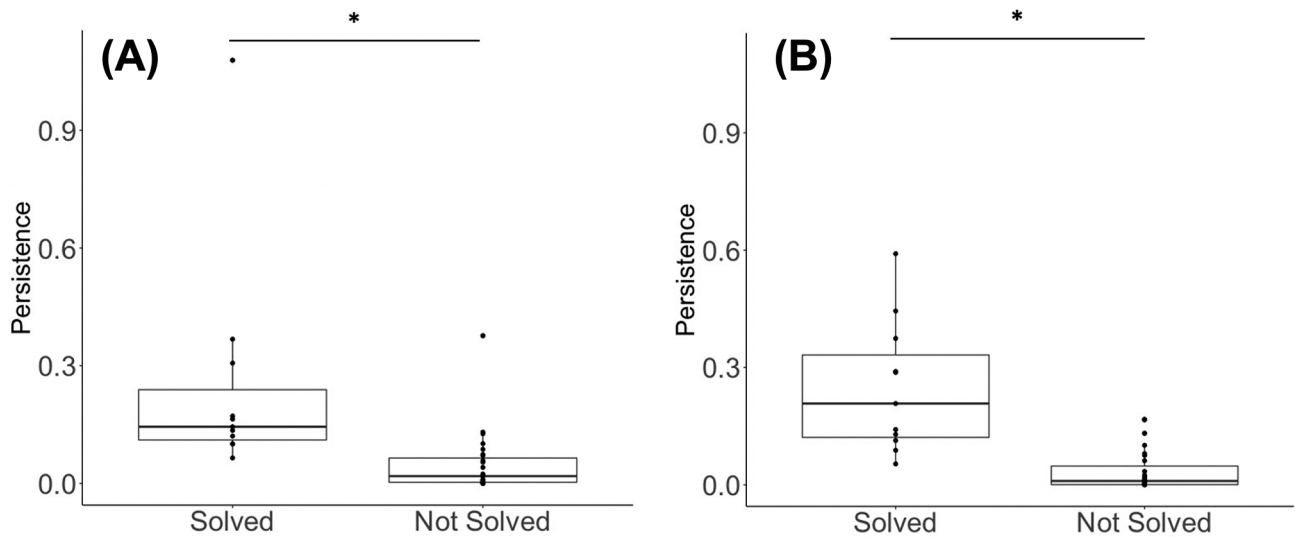


Figure 2. The relationship between persistence and problem-solving success in (A) Task 1 and (B) Task 2. Asterisks indicate significant differences.

information). We found no significant difference in microbiome community composition related to differences in scaled mass index (all $p > 0.05$; Table 3).

We found taxa with significantly different relative abundances between solvers and non-solvers of both tasks using ANCOM-BC2. In Task 1, we found two taxa with higher abundance in solvers and no taxa with higher abundance in non-solvers (Table 4, Supporting information). In Task 2, we found that nine taxa had higher abundance in solvers and two taxa with higher abundance in non-solvers (Table 4, Supporting information). The average abundance of microbial phyla found in the samples was as expected, based on other studies of songbird microbiomes (Skeen et al. 2021). The abundance of these phyla are shown in Fig. 5.

Results from the analyses run on the second microbiome sample are reported in the Supporting information. These

results were generally similar to the analyses run on the first microbiome sample. The microbiomes of the birds after captivity generally were less diverse than those sampled at capture, and they also had a shift in composition. Specifically, there was no significant relationship between diversity (alpha and beta) and problem-solving success using the post-captivity samples (all $p > 0.05$; Supporting information); there was also no significant relationship between persistence and diversity (alpha and beta) using the post-captivity samples (all $p > 0.05$; Supporting information). Alpha diversity decreased significantly during captivity (observed richness, Shannon index, and Faith's PD all showed reductions; Supporting information), and ANCOM-BC2 identified 13 genera that shifted significantly in abundance; Supporting information). There were different, and fewer, taxa that differed in abundance between solvers and non-solvers (Supporting information).

Table 2. Cox proportional hazards models investigating the relationship between success in solving Task 1 or 2 and alpha diversity (Shannon diversity index or Faith's PD) and weight loss during captivity. Location-restricted permutations (adonis|strata=Location) were used to control for capture site. †In Cox proportional hazards models, capture location was modeled as a random/frailty grouping factor. Because random effects do not have standard coefficient estimates, hazard ratios, or confidence intervals, we report the estimated random effect variance.

Task	Microbiome metric	Variables	df	Estimate (coef)	SE	z value	p-value	HR [95% CI]	Variance
1	Shannon	Diversity	1	-0.511	0.548	-0.93	0.350	0.60 [0.21, 1.76]	–
		Weight loss	1	0.268	0.231	1.16	0.250	1.31 [0.83, 2.06]	–
		Location (frailty)†	–	–	–	–	0.960	–	5e-7
	Faith's PD	Diversity	1	0.029	0.165	0.17	0.860	1.03 [0.75, 1.42]	–
		Weight loss	1	0.194	0.218	0.89	0.370	1.22 [0.79, 1.86]	–
		Location (frailty)†	–	–	–	–	0.960	–	5e-7
2	Shannon	Diversity	1	-0.340	0.670	-0.51	0.610	0.71 [0.19, 2.65]	–
		Weight loss	1	0.654	0.377	1.73	0.083	1.92 [0.92, 4.03]	–
		Location (Frailty)†	–	–	–	–	0.970	–	5e-7
	Faith's PD	Diversity	1	0.250	0.147	1.70	0.088	1.28 [0.96, 1.71]	–
		Weight loss	1	0.504	0.376	1.34	0.180	1.66 [0.79, 3.45]	–
		Location (frailty)†	–	–	–	–	0.970	–	5e-7

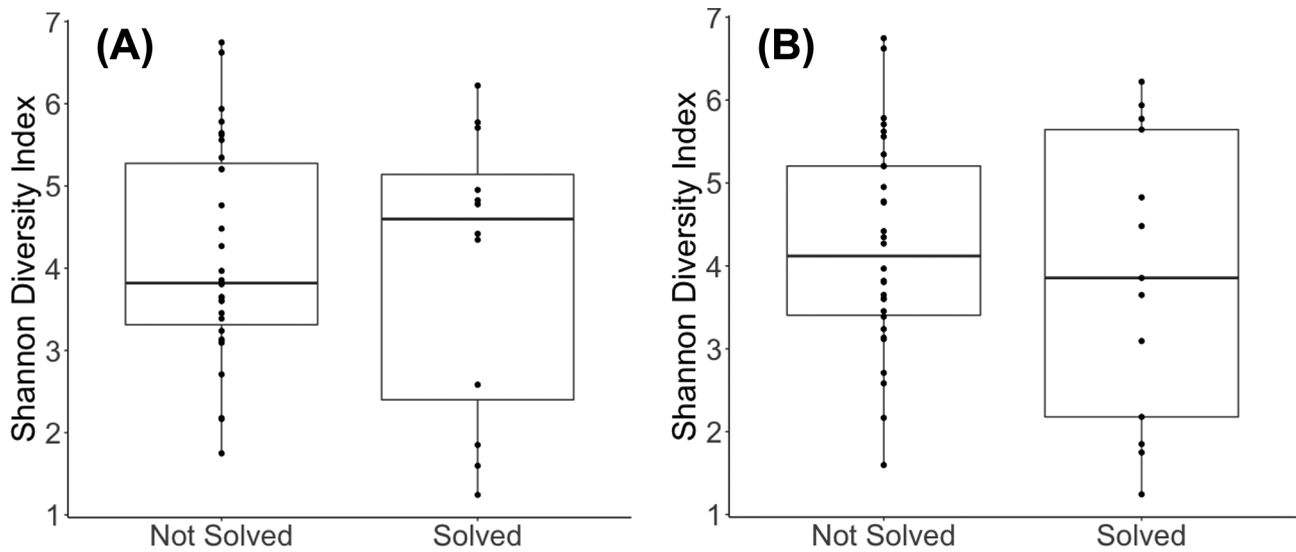


Figure 3. The relationship between alpha diversity (Shannon's diversity index) and problem-solving success in (A) Task 1 and (B) Task 2.

Discussion

We did not find support for our predictions that alpha diversity and beta diversity would be correlated with problem-solving success in food extraction tasks. However, we did find that the relative abundances of several microbial taxa were correlated with success in solving the tasks. We also found that persistence in attempting to solve the tasks predicted success in solving both tasks (but was not correlated with diversity or composition of the microbiome). These results suggest that overall gut microbiome composition is not a significant predictor of behavioral innovation in wild birds, although some taxa may play a role.

Although we did not find significant differences in alpha or beta diversity between solvers and non-solvers, we found

several microbial taxa that had different abundance between these groups. Because the functions of many microbes in the gut are unknown, especially in terms of how they may impact the brain, we are unable to make definitive conclusions on the impact these taxa had on the birds' performance. We found that birds that were successful in solving the tasks have a gut microbiome enriched in fiber-fermenting taxa (Bacteroidetes, Ruminococcaceae). Fiber-fermenting bacteria produce short-chain fatty acids (SCFAs), which can cross the blood–brain barrier and influence neurological function through the gut–brain axis (O'Riordan et al. 2022). The enrichment of Muribaculaceae and Prevotellaceae, both associated with complex carbohydrate metabolism, suggests these birds may have enhanced nutrient extraction capabilities (Betancur-Murillo et al. 2023; Zhu et al. 2024). The reduction of

Table 3. PERMANOVA models investigating the influence of success in solving and scaled mass index on beta diversity (Bray–Curtis dissimilarity, weighted, or unweighted UniFrac distances). Location-restricted permutations (adonis|strata=Location) were used to control for capture site.

Task	Metric	Variables	df	Sum of squares	R ²	F value	p-value
1	Bray–Curtis	Solving success	1	0.1179	0.0103	0.3751	0.997
		Scaled mass index	1	0.2986	0.0261	0.9505	0.546
		Residual	35	10.9967	0.9628	–	–
	Weighted UniFrac	Solving success	1	0.1607	0.0141	0.5227	0.944
		Scaled mass index	1	0.4767	0.0418	1.5505	0.092
		Residual	35	10.7604	0.9424	–	–
	Unweighted UniFrac	Solving success	1	0.2954	0.0252	0.9341	0.789
		Scaled mass index	1	0.3565	0.0304	1.1272	0.074
		Residual	35	11.0687	0.9448	–	–
2	Bray–Curtis	Solving success	1	0.2037	0.0178	0.6533	0.789
		Scaled mass index	1	0.3188	0.0279	1.0225	0.451
		Residual	35	10.9109	0.9552	–	–
	Weighted UniFrac	Solving success	1	0.2827	0.0248	0.9300	0.512
		Scaled mass index	1	0.5165	0.0452	1.6992	0.073
		Residual	35	10.6384	0.9317	–	–
	Unweighted UniFrac	Solving success	1	0.2820	0.0241	0.8905	0.918
		Scaled mass index	1	0.3502	0.0299	1.1059	0.091
		Residual	35	11.0821	0.9460	–	–

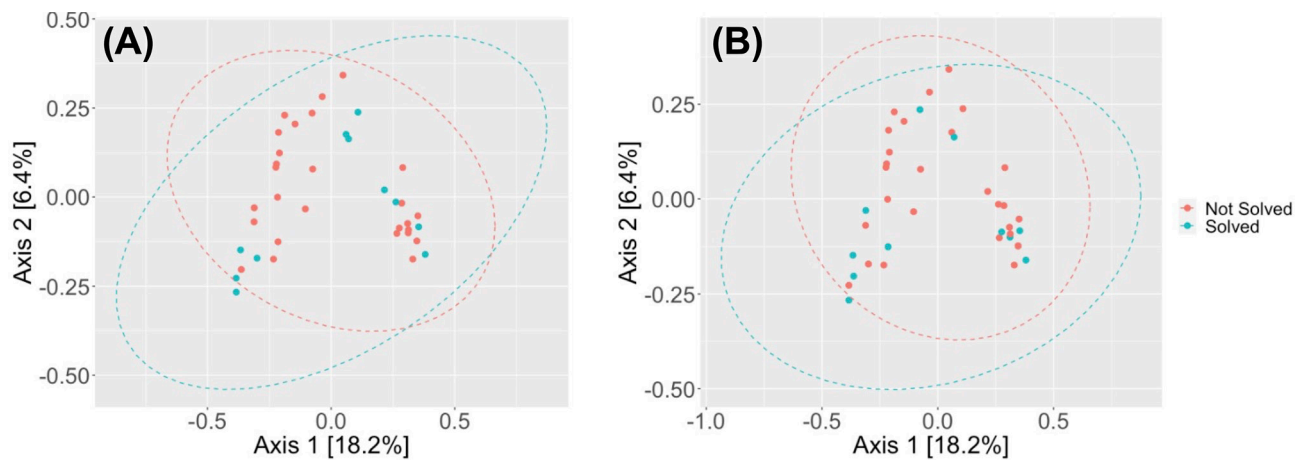


Figure 4. Principal component analysis of beta diversity using Bray–Curtis dissimilarity between individuals who did solve (teal) and did not solve (red) Task 1 (A) and Task 2 (B). Percentages indicate the percentage of total variation explained by each axis.

Pseudomonas and *Shimwellia* (both Proteobacteria) in individuals that solved the tasks may indicate a reduction in potentially pathogenic bacterial load, which may reflect better overall gut health (Shin et al. 2015, Song et al. 2022). Although we did not find significant patterns in microbiome diversity, single species can have significant impact on the gut–brain axis (Zhu et al. 2020, Zhong et al. 2023) and additional investigation into this topic is needed to understand how individual microbial taxa may drive behavior in birds. The microbiome can house hundreds of taxa, and although these observations were corrected for multiple comparisons, replication with a larger sample size would be valuable to confirm which taxa are consistently different between birds with different problem-solving behavior. The microbiomes of birds have also not been studied as extensively as humans or model laboratory organisms, leading to many of our sequences remaining unclassified. These unclassified bacteria could play an important role, but we are not yet able to investigate them. The microbiome of solvers and non-solvers differed by fewer taxa when using microbiome data collected after the birds were living in captivity, which

indicates that differences between the microbiomes of solvers and non-solvers decreased while in captivity.

One methodological limitation of our study was the lack of blank negative controls. This prevented the computational subtraction of potential kit and laboratory contaminants (Salter et al. 2014). To minimize contamination risk, we processed all samples in a single batch using sterile techniques and excluded low-read samples (< 1000 reads), which are most vulnerable to contaminant amplification. Furthermore, we acknowledge that our extended DNA extraction incubation (8 h at 65°C) may have biased lysis toward thick-walled Gram-positive bacteria, potentially altering the perceived relative abundances of specific taxa (Knudsen et al. 2016). Future avian microbiome studies should incorporate negative controls and standardize extraction parameters to ensure reproducibility.

The effect of the microbiome on cognition and behavior in humans has been well supported (Verdi et al. 2018, Johnson 2020, Canipe et al. 2021, Carlson et al. 2021, Park et al. 2022); however, evidence in birds has been mixed.

Table 4. Microbial taxa that are statistically more abundant in either solvers or non-solvers of Task 1 and Task 2. Log fold change is displayed.

Task	Group	Taxon (genus)	Log fold change (LFC) [q-value]
1	Solvers (enriched)	<i>Escherichia–Shigella</i>	3.37 [0.0003]
		Ruminococcaceae_unclassified	1.63 [0.0034]
2	Non-solvers (enriched)	None	–
	Solvers (enriched)	Muribaculaceae_ge	2.00 [0.0126]
		Prevotellaceae_uncultured	1.90 [0.0376]
		Bacteroidia_unclassified	1.65 [< 0.0001]
		Ruminococcaceae_unclassified	1.64 [0.0038]
		Acidaminococcaceae_unclassified	1.60 [0.0005]
		Ruminococcaceae_NK4A214_group	1.53 [0.0155]
		Bacteroidales_unclassified	1.49 [0.0117]
		Ruminococcaceae_UCG-005	1.46 [0.0057]
		Rikenellaceae_RC9_gut_group	1.42 [0.0174]
	Non-solvers (enriched)	<i>Pseudomonas</i>	1.49 [0.0230]
		<i>Shimwellia</i>	1.62 [< 0.0001]

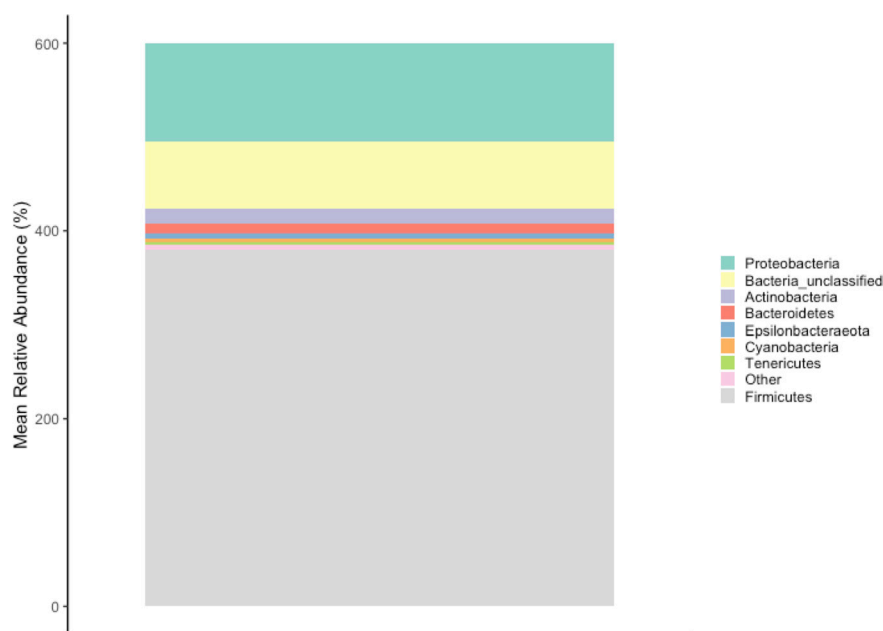


Figure 5. The aggregate relative abundance of bacterial phyla found in the microbiome samples.

For example, microbiome diversity positively correlates with exploratory behavior (Florkowski and Yorzinski 2023) and learning and memory tasks (Slevin et al. 2020). Antibiotic treatment, which can restructure the gut microbiome, impacts social behavior (Rouse and Kaji 2021). However, another study found a correlation between diversity and cognition only after dietary manipulation (Davidson et al. 2020). Other studies have found no relationship between microbiome diversity and behavior in birds, such as neophobia (Kelly et al. 2022), and no association between antibiotic treatment and aggressive behavior (Rouse and Kaji 2021). Birds have not been studied as extensively as mammals in terms of their microbiome, and to date there has not been an experimental manipulation investigating the relationship between the microbiome and behavior in birds. Therefore, continued research will be needed to understand the gut-brain axis in birds.

The two tasks presented to the birds in this study had similar success rates; however, Task 1 was solved, on average, faster than Task 2 (Task 1 in 2625 s versus Task 2 in 1391 s). These differences in latencies may be due to the different tasks requiring cognitive or motor skills that differ between individuals. This may explain the low success rate and why only 12% of individuals in this study were able to solve both tasks. A study using similar tasks in house sparrows had similar success rates in the task analogous to our Task 1 (28 versus 22%) but higher success in the task analogous to our Task 2 (28 versus 72%; Bókony et al. 2014). These results may reflect differences in populations, as Bókony and colleagues studied house sparrows in Europe and our study used birds in North America. Bókony and colleagues did not record the persistence in attempting to solve the tasks, so we are unable to compare that metric between these studies.

We found the most significant predictor of solving the tasks was the persistence in attempting to solve the task, which is consistent with previous studies (Benson-Amram and Holekamp 2012, Thornton and Samson 2012, Chow et al. 2016). For both tasks, the hazard ratio was high (> 100), indicating a strong effect size of this relationship. We found that persistence was correlated between the two tasks, suggesting consistent differences in motivation between individuals. Individuals with higher persistence may have solved the tasks through trial-and-error by attempting to open the task in multiple ways. Previous studies in birds and mammals have found that persistent individuals with higher motor diversity were more successful in food extraction tasks (Griffin et al. 2014, Daniels et al. 2019). Using multiple methods to solve the tasks may not require insight into how to solve the task, but instead involve a larger behavioral repertoire. However, problem-solving tasks likely involve some level of cognition, as birds given tasks repeatedly were found to solve tasks faster on subsequent attempts, which implies they are learning the solution (Morand-Ferron et al. 2011).

We also noted that the microbiome of the birds changed during their time in captivity. Fecal samples collected just prior to release showed a significant decrease in alpha diversity (observed richness, Shannon index, and Faith's PD all decreased) and a marked shift in community composition, with ANCOM-BC2 identifying 13 genera that changed significantly in relative abundance (such as decreases in *Enterococcus* and *Weissella*, and increases in *Streptococcus*). Captivity-induced shifts are well documented in wild birds, driven by standardized diets, reduced exposure to natural environmental microbes, and chronic stress (Levin et al. 2021, Kelly et al. 2022). The differences in microbial taxa between solvers and non-solvers became less pronounced after captivity, suggesting that a standardized laboratory environment

can homogenize individual variation. This highlights the importance of sampling wild animals immediately at capture to detect ecologically relevant behavior–microbiome correlations. When repeating the primary analyses (relationships between problem-solving success and alpha/beta diversity) with the post-captivity samples, we similarly found no significant associations. However, the specific microbial taxa associated with problem-solving success differed, with fewer and different genera showing differences in abundance between solvers and non-solvers after captivity, likely due to the massive composition shifts and diversity loss that occurred during captivity. The changes that occurred in the microbiome during captivity are discussed in more depth in another study (Florkowski et al. 2023).

Overall, we found partial support for the hypothesis that the gut microbiome is correlated with problem-solving in wild-caught house sparrows. We did find that persistence in attempting to solve the problem correlated with problem-solving success. This may indicate that motivation or chance is important for problem-solving. We did not find that microbiome diversity measures were related to solving the presented tasks, but relative abundances of several microbial taxa were correlated with success in solving the tasks. We still know relatively little about the gut–brain axis in birds and continued research on this topic will be necessary to understand the connection between the microbiome and behavior in this taxon.

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Author contributions

Melanie R. Florkowski: Conceptualization (lead); Formal analysis (equal); Funding acquisition (supporting); Methodology (lead); Writing – original draft (lead); Writing – review and editing (equal). **Giridhar Athrey:** Formal analysis (equal); Writing – review and editing (equal). **Jessica L. Yorzinski:** Conceptualization (supporting); Funding acquisition (lead); Methodology (supporting); Resources (lead); Supervision (lead); Writing – review and editing (supporting).

Data availability statement

Data are available from the Harvard Dataverse, <https://doi.org/10.7910/DVN/RDMYOW> (Florkowski 2026).

Supporting information

The Supporting information associated with this article is available with the online version.

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