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Gut microbiome community structure correlates with different behavioral phenotypes in the Belyaev Farm-Fox Experiment

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Domestication represents one of the largest biological shifts of life on Earth, and for many animal species, behavioral selection is thought to facilitate early stages of the process. The gut microbiome of animals can respond to environmental changes and have diverse and powerful effects on host behavior. As such, we hypothesize that selection for tame behavior during early domestication, may have indirectly selected on certain gut microbiota that contribute to the behavioral plasticity necessary to adapt to the new social environment. Here, we explore the gut microbiome of foxes from the tame and aggressive strains of the "Russian-Farm-Fox-Experiment". Microbiota profiles reveal a significant depletion of bacteria in the tame fox population that have been associated with aggressive and fear-related behaviors in other mammals. Our metagenomic survey allows for the reconstruction of microbial pathways enriched in the gut of tame foxes, such as glutamate degradation, which converge with host genetic and physiological signals, revealing a potential role of functional host-microbiota interactions that could influence behaviors associated with domestication. Overall, by characterizing how compositional and functional potential of the gut microbiota and host behaviors co-vary during early animal domestication, we provide further insight into our mechanistic understanding of this adaptive, eco-evolutionary process.

Behavioral shifts are thought to facilitate the early stages of animal domestication[1,2] with tameness being the single most consistently shared trait across domesticated species[3,4]. Tameness denotes an attenuation of the flight-or-fight response, or in other words, a reduced fear and long-term stress response towards humans, which is often a prerequisite to successful breeding in captivity[5]. Rapid adaptation via behavioral plasticity is traditionally viewed as being primarily encoded by the genome, however, ample evidence shows that the gut microbiome of animals can respond to environmental changes and may play a crucial role on their adaptive capacity[6,7]. Further, mounting evidence has demonstrated how the gut microbiome influences host behavior, cognition, brain development and physiology[8–10], which may have profound implications for host ecology and evolution, including adaptation via behavioral selection[11–13].

The complex bidirectional communication between animals and their gut microbiota, called the microbiota–gut–brain axis, can be mediated through a variety of mechanisms and it is becoming increasingly clear that gut-derived metabolites are important to these interactions[14–18]. The biochemical communication pathways can occur through various direct and indirect mechanisms, which include transport of gut-derived metabolites from circulation into the brain, initiation of immune and vagus nerve

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activation, stimulation of enterochromaffin cells in the gut, and even epigenetic alterations[8,14–16,18,19]. Experimental studies have also implicated microbiome-derived signals in regulating fear and aggressive behavior in animals, as well as, expression of genes in the brain involved in such behaviors[20–25], both of which have interesting implications for domestication. However, the eco-evolutionary relevance of the microbiome—gut–brain axis outside of laboratory animals remains largely unexplored.

The Russian Farm Fox Experiment is one of the most extensive experimental domestication studies. Initiated in 1959 at the Institute of Cytology and Genetics (ICG) (Novosibirsk, Russia), Dimitry K. Belyaev and Lyudmila Trut selectively bred captive populations of the silver fox (*Vulpes vulpes*) that were originally derived from fur farms in eastern Canada[26,27], to understand the evolutionary processes of adapting to a new social environment[1,2,5,28]. After decades of selecting on foxes solely for behavior, specific strains of fox with markedly different behavioral phenotypes were gradually established. Foxes bred for tame phenotypes, herein referred to as the "tame" strain, displayed a reduced fear and positive response towards humans, whereas aggressive phenotypes, herein referred to as the "aggressive" strain, exhibited aggression towards humans along with avoidance behavior[2,5,28,29]. While a genetic basis has been extensively documented for both behavioral phenotypes of these foxes[5,30–33], in several other animals species (e.g., chickens and mice), it has been demonstrated that the host genetic effect can be supplemented by the microbiome[11,14]. We therefore hypothesize that selection for behavioral traits in the fox domestication experiment may have led to selection on specific bacterial gut microbiota community structures that may potentially contribute to shaping fox behaviors - something we have previously demonstrated in a similar red jungle fowl domestication experiment[11].

In this study we characterized the fecal microbiome community (as a proxy for gut) of foxes from the tame and aggressive strains of the Russian Farm Fox Experiment, in order to test the hypothesis that the gut microbial community compositions, and their neuroactive potentials, differ between fox behavioral phenotypes. All foxes were housed in the same or similar facilities and fed identical diets, allowing us to assess the differences between the gut microbiota of the two behavioral phenotypes without introducing the confounding variables of environment, and especially diet, both of which can strongly influence the gut community composition[34].

Results and discussion

In order to profile the gut microbiome of the two fox behavioral strains, we generated both targeted bacterial 16S rRNA amplicon and shotgun metagenomic data from fecal samples collected from 123 foxes that represented two collection years (2015: tame ($n = 10$); aggressive ($n = 10$); 2017: tame ($n = 51$); aggressive ($n = 52$)). Because rare microbial species are typically not well assembled in metagenomic surveys because of low coverage[35], 16S amplicon data was used as a starting point to characterize the microbiota's broad taxonomic composition in order to study links with behavioral phenotypes. Complementary metagenomic data was incorporated to (1) recover and compare novel bacterial species between the behavioral selections lines, through the reconstruction of metagenome assembled genomes (MAGs), and (2) provide insights into the potential microbiome functions relevant to the microbiota–gut–brain axis.

Due to the strong batch effects (namely sample storage treatment known to significantly impact microbial taxonomic profiles[36,37]), between the two collections years (Supplementary Fig. 1), the 2015 dataset was removed from the 16S analysis and additionally removed from the shotgun metagenomic analysis after we generated the MAG reference catalog.

Gut microbial diversity reduced in tame foxes

To create initial community composition profiles of the gut microbiota of the two silver fox behavioral strains, a 16S rRNA gene amplicon survey was performed on the fecal samples collected in 2017 from aggressive ($n = 52$) and tame ($n = 50$) individuals. A total of 1525 amplicon sequence variants (ASVs) were identified after quality filtering. Diversity analysis at the genus level identified a lower Shannon diversity estimate in the microbiota of the tame, compared to aggressive, fox populations (Fig. 1A; $\text{Shannon}_{\text{tame}} = 2.45 \pm 0.006$; $\text{Shannon}_{\text{aggr}} = 2.52 \pm 0.003$; p-value < 0.0001). Marked reductions in the gut microbial diversity of domesticated animals have been identified in a number of species, and are typically associated with anthropogenic factors, such as shifts in environment and diet, during the domestication process[38–41]. Interestingly, increased gut microbial diversity is also associated with aggressive and fearful behavior in mammals[42–47] and chickens[48]. As the foxes from the two selection lines were housed in similar controlled environments, the lower diversity estimates identified in the tame gut microbiota are unlikely due to shifts in environmental factors (Supplementary Fig. 2). It is possible that founder effects influenced the gut microbial structure of the foxes, however, extensive genetic studies showed that the two populations are closely related[32] and inbreeding was strenuously avoided since the beginning of the breeding program to avoid bottlenecks during selection. Gut morphology can influence gut microbiota composition (e.g., refs. 49,50), and changes in gut length have been reported in some domesticated species[51,52]. However, such morphological changes have yet to be explored in the experimentally domesticated foxes. Alternatively, it is possible that the depletion or loss of certain gut microbes during the early stages of the domestication process may initiate the behavioral shifts necessary to adapt to the new social environment, and knowledge of individual microbial species responsible for these phenotypes may be valuable in understanding their biological role during domestication.

Tame foxes are depleted in bacteria associated with fearful and aggressive behavior

To investigate whether certain microbes were associated with the different behavioral phenotypes, we modeled relative abundance of 16S sequence data at different taxonomic levels. At the phylum level, we observed that the gut microbiota of tame foxes were significantly depleted of Tenericutes in comparison to aggressive foxes (Wald test, t-value = −4.785, p-value = 1.53e-05), mainly due to the reduction of the bacterial family *Anaeroplasmataceae* (Wald test, t-value = −4.785, p-value = 7.88e-05). This finding is interesting for several reasons. Firstly, in other mammals such as hamsters and mice, Tenericutes, and more specifically *Anaeroplasmataceae*, have been reported to play a role in social behavior, and consistently and positively correlate with aggression[42,43,47,53]. Secondly, not only do some members of Tenericutes show heritability in multiple human populations[54], but some Tenericutes are also less capable of recovering after perturbation in the gut[47]. Although taken at a broad taxonomic level, together, these findings suggest that it may be possible to select against at least some bacteria during early domestication, that remain depleted or lost throughout the process.

When considering the data at the order level, we noted a depletion of bacteria from the order *Desulfovibrionales* in tame foxes (Wald test, t-value = −2.938, p-value = 0.04). Maternal stress and perturbations in the gut microbiota of Siberian hamsters produced offspring that were not only enriched in *Desulfovibrionales* but also displayed increased levels of aggression when treated with stress[55]. Cusick and co-workers[55] went on to suggest that both maternal microbiome and response to stress interact in ways that impact the behavior and gut microbiota of their offspring, both of which would have interesting implications in the eco-evolutionary processes of domestication.

Modeled relative abundance at the genus level revealed three additional taxa depleted in the tame fox strain, specifically *Ruminococcaceae (UCG-014)*, *Anaeroplasma* and *Lachnospiraceae (UCG-010)* (Fig. 1B, C), all three of which have intriguing links to behavior in other mammals. In particular, *Ruminococcaceae* and *Lachnospiraceae* not only positively associate with aggressive behaviors in mammals, including dogs, mice and hamsters[24,43,45,46,53], but also exhibit lower abundance in some captive and domestic animal populations of gaur and yaks[39,41]. Further, *Lachnospiraceae* are positively associated with brain reactivity to fear in humans, particularly in the prefrontal cortex[56], which is a brain region involved in memory, learning and regulating fear, and shown to be modulated by changes in gut microbiota[10,57,58].

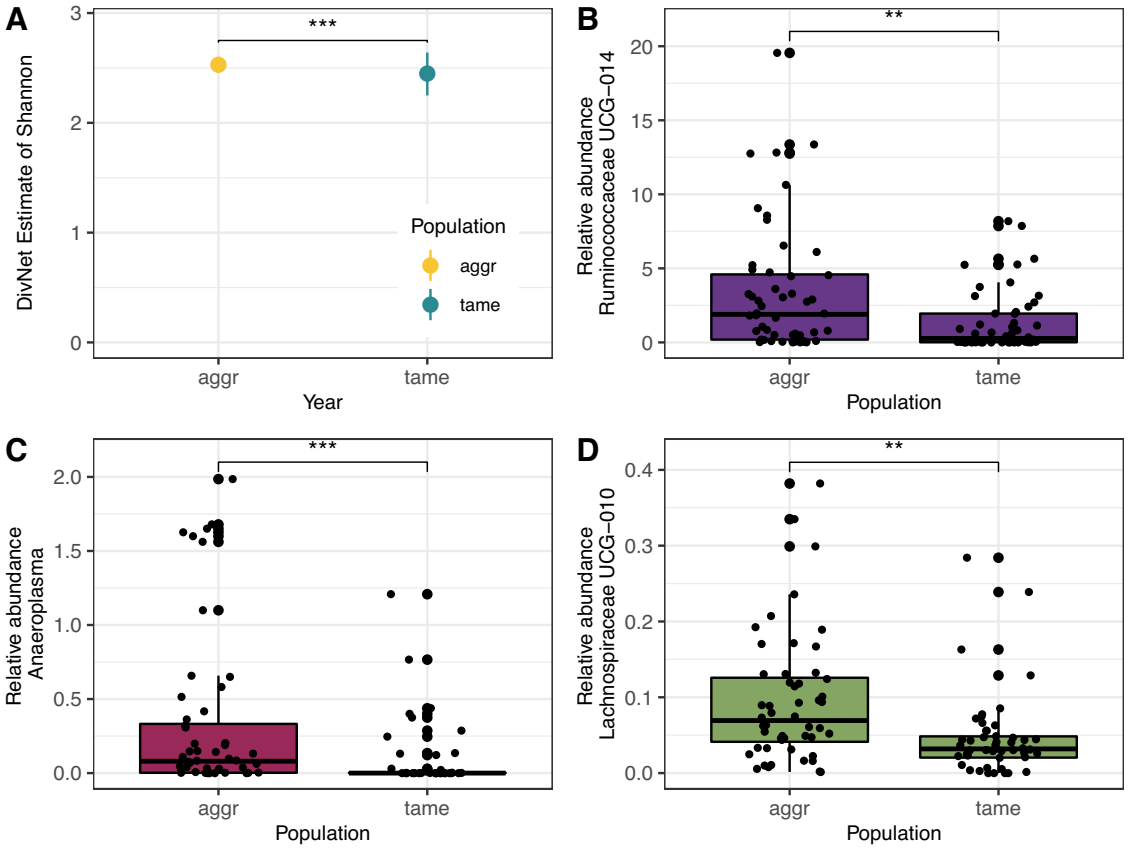

Fig. 1 | Diversity differences of fecal microbiota between aggressive ($n = 52$) and tame ($n = 50$) fox strains from the bacterial 16S rRNA gene amplicon survey. **A** Diversity estimates with uncertainties of Shannon diversity in fox fecal samples collected in 2017. The dots represent the mean Shannon diversity estimate and the error bars (whiskers) represent the 95% confidence intervals. Differential relative abundance of the genera (**B**) *Ruminococcaceae (UCG-014)*, (**C**) *Anaeroplasma* and (**D**) *Lachnospiraceae (UCG-010)*. Microbial community composition was modeled at the genus level for the 16S dataset by fitting the beta-binomial regression model implemented in the 'corncob' package in R. Differentially abundant taxa were considered significant using the parametric Wald test with a controlled false discovery rate (p-value cut-off <0.05) $^{**}p \le 0.01$, $^{***}p \le 0.001$.

In addition to *Lachnospiraceae*, *Bacteroides* similarly associate with brain reactivity to fear[56]. In this regard, when we explored our data at the ASVs level, we see many *Bacteroides* ASVs depleted in the tame strain (Fig. 2A). Interestingly, *Bacteroides* are observed in high abundance in the gut microbiota of wild red foxes from the grasslands in China[59]. Additional analysis at the ASV level revealed 53 ASVs to be differentially abundant between the aggressive and tame behavioral strains, and an additional 23 ASVs were discriminant to either selection line (Fig. 2; Supplementary Data 1). Most of these differences occurred in abundant and highly prevalent taxa (Fig. 2B). In addition to the depletion of *Bacteriodes* ASVs, we detected a significant depletion of *Alloprevotella, Prevotellaceae and Blautia ASVs* (Fig. 2A; Supplementary Data 1), all of which have previously been associated with aggression in dogs, hamsters, mice and voles[43,45,46,53,60]. Taken together, these data suggest that the gut microbiota in tame foxes are depleted of bacteria not only associated with aggressive and fearful behaviors, but also in taxa found in abundance in their wild counterparts. Furthermore, similar patterns in the gut microbiome of other domesticated animals have been observed, suggesting a role of the gut microbiome in domestic behaviors.

Genome-resolved metagenomics characterize the neuroactive potential of the fox gut microbiota

To identify novel gut bacterial species, and assess the functional potential of the gut microbiota found in the aggressive and tame fox strains, we next carried out shotgun metagenomic sequencing. Initially, we generated a reference catalog of metagenome-assembled genomes (MAGs) using a dataset of 123 samples that represented two collection years (2015 + 2017) (tame ($n = 61$); aggressive ($n = 62$)). Briefly, the metagenomic data yielded 15.8 billion high-quality short-reads, of which ca. 30–70% (per sample) mapped to the fox genome (Supplementary Data 2a). After removing reads corresponding to the fox genome, we performed a large metagenomic co-assembly (7.09 billion reads), which produced 105,106 contigs >2500 nt. Subsequent manual binning within the anvi'o[61] framework using differential coverage across all samples resulted in 237 non-redundant MAGs of which 50% of the reads mapped back to (Fig. 3A; Supplementary Fig. 3; Supplementary Data 2b). Four samples had a sequencing depth of less than 10 million single-end reads (<1 Gb) after quality control, and were removed from downstream analyses (as per recent recommendations[35]). At the phylum level, MAGs were affiliated to *Firmicutes* ($n = 145$), followed by *Bacteroidetes* ($n = 27$), *Proteobacteria* ($n = 25$), *Actinobacteria* ($n = 17$), *Tenericutes* ($n = 17$), *Spirochaetes* ($n = 3$), *Cyanobacteria* ($n = 1$), *Fusobacteria* ($n = 1$) and *Deferribacteres* ($n = 1$) (Fig. 3A). In addition, all MAGs were affiliated to known bacterial orders, and 43% of them could also be assigned to a known species (average nucleotide identity > 95%). Complete taxonomic assignments using the Genome Taxonomy Database Toolkit (GTDB-Tk)[62] are found in Supplementary Data 2b.

Modeled microbial abundance revealed 22 MAGs to be significantly differentially abundant between the aggressive and tame fox strains, and an additional 3 MAGs were discriminant to either strain (Fig. 3). Although overlap between shotgun metagenomic and 16S data exist at broad taxonomic levels[63,64], comparison of community structures across the two sequencing strategies do not necessarily merge perfectly as a consequence of differences in taxonomic databases[65–67], detection limits[68], sequencing depths[67,69] and PCR biases[63,70]. Nonetheless, corroborating 16S sequence

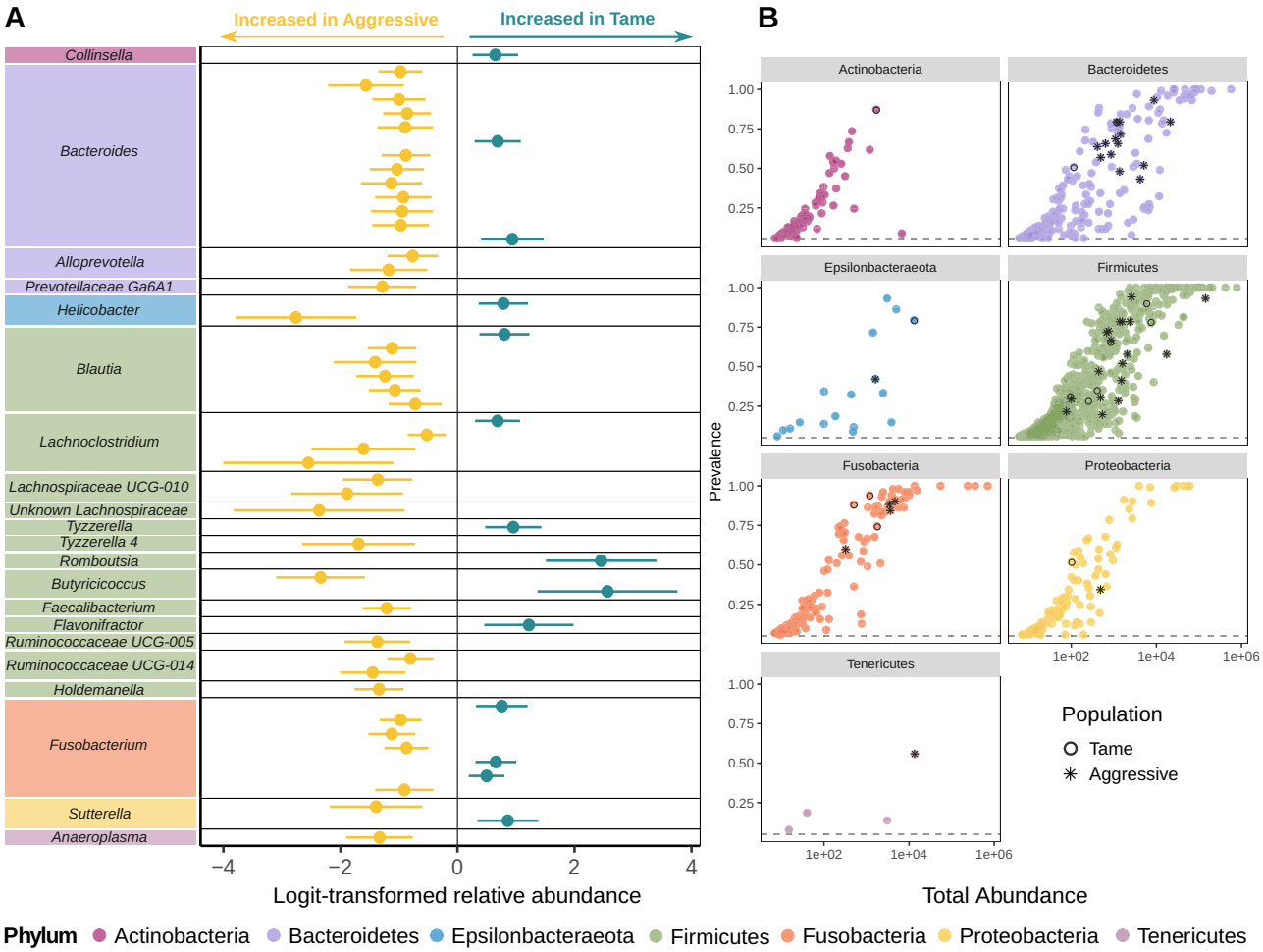

Fig. 2 | Differential abundance of bacterial 16S ASVs between aggressive and tame foxes ($n = 102$). A Differences in the modeled abundance of ASVs, grouped by genus and phylum level taxonomic classifications, between aggressive and tame populations estimated with corncob using the Wald test with a controlled false discovery rate using the Benjamini–Hochberg procedure. The dots represent the estimated model coefficients for abundance (effect sizes) and the errors bars (whiskers) represent the 95% confidence intervals. **B** Prevalence plots of 1525 bacterial 16S rRNA amplicon sequence variants (ASVs) found in fox fecal samples at the phylum level. Each point represents the total counts of a unique ASV corresponding to the fraction of individuals it was detected in. ASVs that were differentially abundant ($n = 53$) or discriminant ($n = 23$) in either the aggressive (*) or tame (O) population are highlighted.

data, there was an enrichment of *Anaeroplasmataceae*, *Helicobacter C sp.* and several *Bacteroidaceae* MAGs, namely *Paraprevotella sp.*, *Prevotellamassilia sp.*, and *Prevotellamassilia sp.000437675*, in the aggressive fox strain, among six additional enriched MAGs (Figs. 2A and 3B). Conversely, the tame fox strain was enriched in the MAGs *Collinsella sp.*, *Fusobacterium sp.900015295* and *Helicobacter bilis*, similarly identified at higher taxonomic resolution in the 16S data (Fig. 2A) in addition to eight other MAGs (Fig. 3B).

In order to describe the neuroactive potential of gut microbiota in relation to gut–brain interactions in the foxes, we applied a previously described module-based framework[71]. This framework identifies microbial pathways that metabolize molecules with the potential to interact with the host nervous system. We found 35 out of the 56 annotated gut–brain modules (GBMs) known to produce or degrade neuroactive compounds, spread widely across the phylogenic range of MAGs (Fig. 3A). We subsequently compared the microbial neuroactive potential of the gut microbiota between tame and aggressive fox strains by assessing the detection of GBMs in the 22 MAGs that were significantly enriched in one of the behavioral phenotypic groups (Fig. 3B). We detected six GBMs associated with the tame fox strain, three of which were identified in less than 5% of all MAGs (Fig. 3B; Supplementary Fig. 4). Two of the GBMs enriched in the tame population were associated with short chain fatty acids (SCFA), namely butyrate synthesis II and isovaleric acid synthesis I, and an additional three GBMs were from the glutamate-derived pathway, glutamate degradation II, GABA synthesis III and g-Hydroxybutyric acid (GHB) degradation (Fig. 3B). The final GBM was associated with the estrogen hormone signaling pathway, 17-beta-estradiol degradation, and was present in five out of the eleven MAGs enriched in the tame selection line (Fig. 3B). Interestingly, circulating levels of estradiol have been linked to variation in social behaviors, including a positive correlation with aggression in multiple animal species, namely sparrows, cichlids and mice[72–76]. It is therefore possible that the gut bacteria in the tame fox strain have the potential to reduce estradiol in circulation and in turn decrease aggressive behaviors important in the early domestication process.

We further applied the GBM framework to the cleaned shotgun data (7.09 billion reads), prior to assembly, for all fox samples, in order to increase the potential detection of GBMs associated to behavioral phenotypes. Most GBMs ($n = 31$) were present in over 75% of all fox samples and one was rare, namely nitric oxide degradation II and exclusively found in the tame fox selection line (Supplementary Fig. 5; Fig. 4). Modeled differential abundance of GBMs per metagenomic sample revealed three significant enrichments associated with the aggressive behavioral group; S-adenosylmethionine (SAM) synthesis (Wald test, t-value = −4.23, p-value = 3.9e-04), acetate synthesis I (Wald test, t-value = −4.34, p-value = 3.8e-04) and acetate synthesis III (Wald test, t-value = −3.15, p-value = 0.01) (Fig. 4).

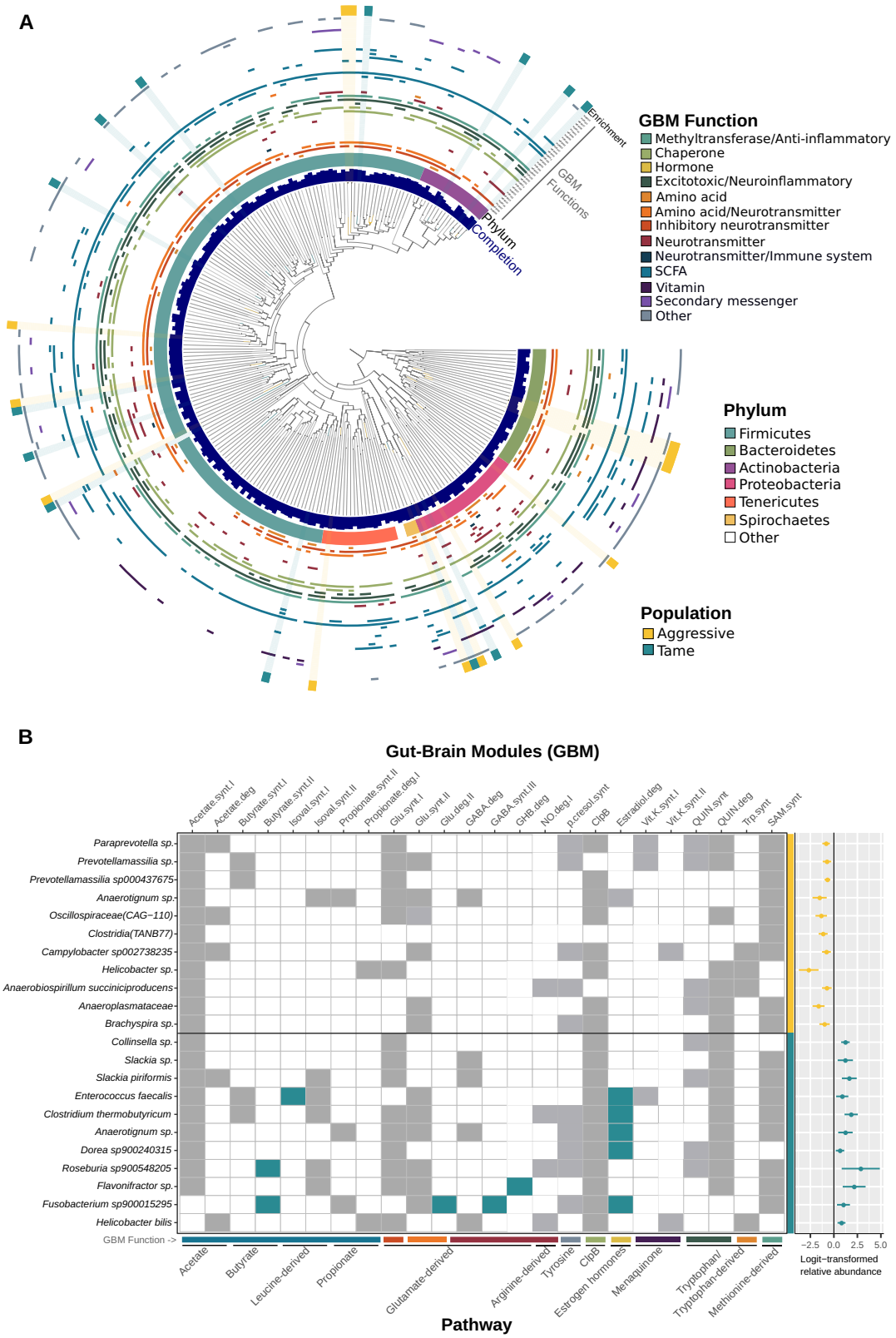

Fig. 3 | Gut–brain module (GBM) distribution in fox MAGs. A Maximum-likelihood phylogenetic tree comprising 237 gut-associated MAGs identified in 123 silver fox fecal samples, rooted at the level of Bacteroidetes. The innermost circular layer represents the percent completion of each genome followed by the associated phylum in the second layer. The following middle layers represent the 35 gut–brain modules (GBMs) detected in the collections of MAGs and categorized by functional association. Twenty-five MAGs were differentially abundant or exclusive to a behavioral phenotypic group and highlighted on the tree based on the enrichment in either the aggressive (yellow) or the tame (teal) populations. **B** Detection of GBMs (minimum 66% coverage) in the 22 differentially occurring fox MAGs highlighted in (**A**). Pathways and functions were annotated for each GBM along the bottom x-axis. GBMs exclusively present in MAGs enriched in the tame population are highlighted in teal whereas none were exclusively present in the aggressive population.

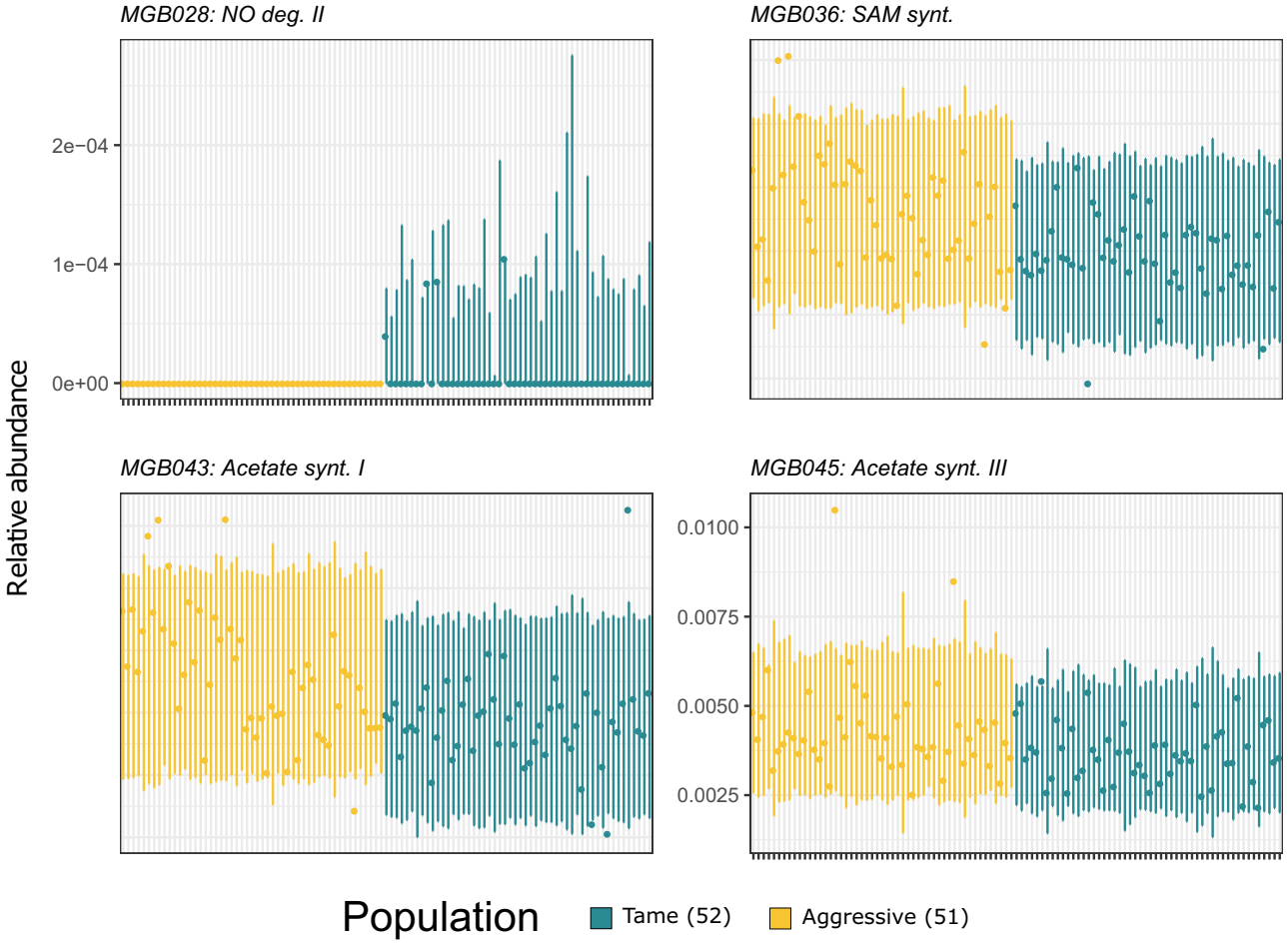

Fig. 4 | Differential abundance of gut–brain modules (GBMs) between aggressive and tame foxes ($n = 102$). Differences in the modeled relative abundance of GBMs detected in fecal metagenomes of silver foxes selected for tame and aggressive behaviors towards humans were estimated with corncob using the Wald test with a controlled false discovery rate using the Benjamini–Hochberg procedure. The dots represent the estimated relative abundance of the GBM detected in the metagenomes of the foxes and the bars (whiskers) represent the 95% prediction intervals for the observed relative abundance for each individual fox with behavioral phenotype as a covariate.

Gut microbiota have the functional potential to produce neuroactive metabolites that influence the serotonergic system relevant to domestic behaviors

Serotonin (5-HT) has been implicated as one of the main neurotransmitters involved in animal aggression and plays an inhibitory role across a wide range of species[77–81]. Similarly, some domesticated animals have higher levels of brain 5-HT, including the Belyaev foxes as previously demonstrated, or peripheral 5-HT, that correlates with reduced aggressive and fear-related phenotypes[82–87]. Although the majority of 5-HT is produced in the gut (~90%), it is generally believed it cannot directly affect levels in the brain, because 5-HT cannot pass the blood–brain barrier (BBB)[88]. However, peripheral levels of 5-HT can alter brain functionality and behavior[89]. Furthermore, germ-free rodents have altered 5-HT concentrations and turnover in the brain, altered levels of circulating 5-HT and its precursor L-tryptophan, and decreased cecal and fecal 5-HT[90–94]. Gut bacteria can also modulate serotoninergic gene expression profiles in the brain[95,96]. Together, this suggests a role for the gut microbiota in modulating 5-HT signaling pathways in the central nervous systems, however, the mechanistic link between the gut microbiota and 5-HT production in the brain is not yet fully defined.

Here, we identified that foxes from the tame strain were significantly enriched in *Enterococcus faecalis*, *Roseburia sp900548205* and *Clostridiales* MAGs, all taxa known to increase peripheral host serotonin levels by either directly producing it, promoting host serotonin biosynthesis, or upregulating the expression of serotonin transport genes in the gut[71,88,97–101] (Fig. 3B; Wald test, t-value = 0.9, p-value = 0.007, Wald test, t-value = 2.86, p-value = 0.008 and order-level *Clostridiales*: t-value = 3.7, p-value = 0.001). Interestingly, *Clostridiales* were also enriched in red jungle fowl gut microbiota selected for low fear of humans[11], and positively associated with impaired fear memory in mice[102], which further suggest a role for this taxa in early domestication. The SCFAs butyrate and acetate can also influence the host serotonin system. Experimental studies have revealed that butyrate induces 5-HT colonic secretion from the gut, whereas acetate has been shown to do the opposite[88,103], and the potential for butyrate and acetate synthesis were enriched in either the tame or aggressive fox strain gut microbiota, respectively (Figs. 3B and 4). Our 16S taxonomic data further support this pattern, where acetate producing bacteria[104] were significantly enriched in the aggressive fox strain, namely *Bacteroides spp.*, *Prevotella spp.*, *Ruminococcus spp.* and *Blautia spp.* (Fig. 2). Intriguingly, butyrate is implicated in neuronal plasticity, fear memory formation, and increased fear extinction[19,105–107], whereas acetate has been proposed to induce impairments in learning and coping with stress[108]. Further, SCFAs, such as butyrate and acetate, can cross the BBB and are known to be strong epigenetic modulators[15] and epigenetic mechanisms may play an integral role in both the microbiota–gut–brain axis[20–25,109] and domestication[110–116].

We detected the nitric oxide (NO) degradation II GBM exclusively in the tame fox metagenomic data, although in low abundance (Fig. 4). Nitric oxide appears to play an important role in normal brain 5-HT functioning

and has been implicated in both fear-related and aggressive behavior in mammals[117–121]. Together these data suggest that fox gut microbiota have the potential to influence the host serotonin system, however, how this translates into behavior remains to be defined. Nonetheless, the taxonomic and functional potential of the gut microbiota enriched in tame foxes indicate the capacity to influence fear memory formation and promote fear extinction learning, both of which would be relevant to overcoming a fear response toward humans during domestication.

Convergence of host and microbial selection signals on glutamate signaling pathway

Extensive studies have demonstrated that genes coding for different types of glutamate receptors in the host are associated with domestication in not only dogs, ducks, rabbits and chickens[122–126], but also on the Belyaev foxes, where genomic regions, gene expression and allele frequencies involved in glutamatergic signaling are different between the tame and aggressive strains[32,33]. Mounting evidence has now shown that the gut microbiota can influence the genetic signatures (e.g., gene expression, epigenetics, dysregulate miRNAs) and functional connectivity of certain regions in the brain of the host[10,57,58,127] and, further, alter gene expression of glutamatergic receptors in the brain[128,129]. In light of these findings, it is interesting that GBMs associated with glutamate degradation, GABA synthesis and g-Hydroxybutyric acid (GHB) degradation, all from the glutamate-derived pathway, were enriched in the MAGs from the gut of the tame fox strains (Fig. 3B). We additionally detected the potential for acetate synthesis in the aggressive population and gut-derived acetate can cross the blood–brain barrier and influence GABAergic and glutamatergic neurotransmission in the brain[130].

Glutamate is the main excitatory neurotransmitter in the brain and plays an important role in fear conditioning, synaptic plasticity, learning, and memory[131,132]. GABA, on the other hand, is an inhibitory neurotransmitter that counteracts glutamate, and GABA signaling has been implicated in fear extinction learning[133,134]. Further, increased levels of glutamate in the brain can trigger aggression in mice[135,136] whereas GABA is mainly associated with an inhibitory role in aggression[137–143]. GHB has also been implicated in aggressive behavior in animals and can increase levels of glutamate in the brain[144–146]. Moreover, the potential for glutamate synthesis has been identified in the gut microbiota of aggressive mice and red jungle fowl selected for high fear towards humans[11,43]. As with 5-HT, glutamate and GABA cannot pass the blood–brain barrier[147], however, certain gut bacteria have been shown to increase GABA and glutamate in the brain[148] and additionally promote consistent changes in GABA receptors in the brain accompanied by behavioral shifts in the host[20]. Further, GABA-producing bacteria in the gut can alter mood and fear-related behavior in studies modeling depression[149]. As such, these findings suggest a role for glutamate and GABA signaling in the behavioral shifts shared among animals during domestication, with both a host genomic and gut microbial component to its regulation.

Conclusion

Here we identified shifts in the fecal (as a proxy for gut) microbiota between behavioral phenotypes of the Belyaev foxes that have been linked to aggressive and fear-related behaviors, behaviors that are consistently reduced in domestic animals. Although our approach does not allow for interpretation of causality nor directionally of the microbiota–gut–brain axis interactions, and with this dataset we are unable to identify whether the gut microbiota is directly under selection, or alternatively responding to selection on host behavior[150–155], such correlative findings set the stage for generating mechanistic hypotheses for further exploration. While the depletion of certain gut microbiota, such as *Ruminococcaceae*, *Anaeroplasmataceae*, and *Lachnospiraceae* during the early stages of the domestication process may initiate the behavioral shifts necessary to adapt to the new social environment, the enrichment of others, such as *Enterococcus faecalis* and *Clostridiales* may be equally important. Our metagenomic survey also allowed for the reconstruction of several microbial pathways enriched in the gut of tame foxes, such as glutamate degradation and GABA synthesis, which converged with host genetic and physiological signals, revealing a potential role of functional host-microbiota interactions that could influence behaviors associated with domestication. In future studies, the coupling of metagenomics, metatranscriptomics and metabolomics would provide opportunities to validate which bioactive metabolites are being produced, and together with antibiotic treatment and/or fecal transplant experiments, may establish a causative role of the functional pathways by which bacteria affect behavior and to what extent. Further, longitudinal studies of individuals across generations could provide further insights as to (i) when, and how quickly compositional changes occur within the microbiota of animals during early domestication, (ii) how these changes may reflect causal links to the behavioral shifts detected throughout the process, and even (iii) which host genetic mechanisms are driving the gut microbiome community trends. With regards to this latter point, we propose two hypotheses that may warrant future exploration. Firstly, previous characterization of the genomic differences found between the Belyaev fox behavioral strains reported not only significant enrichment for GO terms linked to the nervous system, but also immune responses, specifically "cytokine activity" and "interleukin-1 receptor binding"[32]. Given that differentiation in the host immune system can lead to differentiation in the gut microbiome[156], we hypothesize that this intriguing observation may suggest that selection for behavioral phenotypic differences in the earliest stages of domestication may include, or even predominantly focus on, immune system differences, and that these in turn could shape the gut microbiome, and hence behavior. However, this relationship is bi-directional: the gut microbiota can also modulate signals for immune system development and function[157], and this dynamic cross-talk may play a central role in the early stages of domestication. Our second hypothesis draws both on the recent observations from fish[158] and humans[159], that epigenetic changes in host genomes can directly shape the gut microbiome, and that rapid epigenetic divergence has been reported in the early stages of chickens subjected to behavioral selection[110–112,114,115]. As such, an alternate (or complementary) hypothesis, is that epigenetic changes in the fox genomes may be involved in shaping their gut microbiomes. Although this is yet to be tested, the fact that gut microbiomes are also well known to shape their host epigenomes[160], this process could even involve some degree of reciprocal feedback.

Ultimately, however, although intriguing, we of course acknowledge that any role for the microbiota in the evolution of domestic behaviors in animals does not displace other contributing factors, but rather adds an additional layer to our understanding of how such behaviors arise. Understanding the eco-evolutionary mechanisms involved in the process of domestication provides crucial insights into how wild animals may adapt to human encounters over time. In this process, there is likely a bidirectional relationship between microbiota and host factors, including behavior, that further interact with the environment.

Methods

Animals and sample collection

Fecal samples were obtained from 61 tame and 62 aggressive silver foxes (*Vulpes vulpes*) maintained at the experimental farm of the Institute of Cytology and Genetics (ICG) (Novosibirsk, Russia). Foxes were approximately 5 months old at the time of collection. Each fox was housed individually in a cage with a wire net floor. The ground under the fox cage was covered with a piece of tissue at ~7 am and collected in the morning before feeding (~7–9 am) to ensure that only fresh fecal samples were obtained. No contact with urine was allowed. All foxes were raised in standard conditions and underwent identical treatment with minimally necessary human interaction until behavioral testing was performed[161]. Fox behavior was systematically scored based on their response to humans using a categorical scoring system that was well established early in the breeding program[161].

Selection for the tame strain began in 1959 at the ICG, and was developed through selection of conventional farm-bred foxes from across the former Soviet Union due to their less aggressive and fearful behavior towards humans. The aggressive strain was developed by selecting conventional farm-bred foxes for an aggressive response towards humans,

beginning in the late 1960s at the ICG. Both the tame and aggressive foxes ultimately originated from a conventional farm-bred fox population of Canadian descent that was established in the second part of the nineteenth century[27]. A description of the selective breeding program was previously described[5,28,161]. Fecal samples were collected and either frozen at −20 °C in the summer of 2015 (tame: $n = 10$, aggressive: $n = 10$) or preserved in RNAlater stabilization solution in the summer of 2017 (tame: $n = 51$, aggressive: $n = 52$) and stored at −20 °C.

DNA extraction

Prior to DNA extraction, RNAlater was removed with centrifugation (13,000 × *g* for 10 min) and the pellet was washed twice with 1 mL of PBS. DNA was extracted from approximately 100 mg of fecal sample using the DNeasy PowerSoil Kit DNA (Qiagen, Venlo, NL) following the manufacturer's protocol with several modifications. Samples were incubated for 10 min at 65 °C after adding Solution C1 and bead-beaten for 10 min at 30 Hz using a TissueLyser II (Qiagen, Hilden, Germany). Purified DNA was incubated in Solution C6 for 15 min at 37 °C before the final elution spin. Four negative controls (i.e., all reagents except sample continued in the workflow from extraction to sequencing as any other extracts) were included in order to check for potential reagent contamination.

Bacterial community composition from 16S rRNA amplicon sequencing

A dual-indexed PCR approach was used to target the V3-V4 variable region of the bacterial 16S rRNA gene (~465 bp) for all fecal samples ($n = 103$) using the primer pair Bact-341F (5'-CCTAYGGGRBGCASCAG-3') and Bact-806R (5'-GGACTACNNGGGTATCTAAT-3') with Illumina Nextera overhang adapters (Illumina Inc., San Diego, CA, USA)[162–165]. PCR was performed in triplicates and pooled prior to indexing PCR for each individual in order to reduce PCR bias. Pooled libraries were sequenced on an Illumina MiSeq platform using 250PE. Full methodological details can be found in Supplementary information.

Illumina adapters and primer sequences were removed from the 16S metabarcoding sequence data using cutadapt v.2.6[166] and subsequently analyzed using the program DADA2 v.1.12.1[167] and R v.3.6.1[168] to infer amplicon sequence variants (ASVs). Complete code was modified from ref. 169. Briefly, forward and reverse reads were trimmed to 230 bp. The entire dataset was used to define an error rate at each base pair, and all sequences were denoised using the pooled approach to increase the likelihood of resolving rare sequence variants. Forward and reverse reads were merged, and any pair without perfect overlap and <400 bp was removed prior to chimeric sequence filtering. Each ASV was annotated with the RDP Bayesian classifier[170] against the SILVA database[171] to produce a 16S amplicon taxa table. All subsequent analyses were done in R v.3.6.3 unless otherwise stated[172]. ASV data was pre-processed with the phyloseq package v.1.30.0[173], and potential contaminants were assessed with the decontam package v 1.6.0[174]. Twelve putative contaminants were removed from the ASV table. Only samples with >10,000 reads and ASVs present in a minimum of 5% of all samples were included in downstream 16S data analysis.

Metagenomic shotgun sequencing

Shotgun metagenome sequence data were prepared on all DNA extracts ($n = 103$) using the BEST single-tube library preparation protocol[175] as optimized to be BGISEQ-500 compatible[176]. Briefly, genomic DNA was fragmented to 350 bp using a M220 Focused Ultrasonicator (Covaris, Woburn, MA). Sheared DNA was converted into BGISEQ-500 libraries following four steps: blunt end-repair, adapter ligation (20 µM BGI 2.0 adapters), fill-in reaction and SPRI magnetic bead purification (Sigma-Aldrich). Indexing PCR cycle number for all metagenomic libraries (7–11 cycles) were determined through qPCR library quantification. Libraries were pooled equimolar over 6 lanes in 100 bp or 150 bp paired-end mode on the BGISeq-500 platform aiming for a minimum of 50 million reads per sample.

Assembly and genome-resolved metagenomics

Prior to sequence assembly, all paired-end reads were demultiplexed and quality filtered. AdapterRemoval v.2.3.1[177] was used to trim unidentified bases and adapter sequences from the ends of the read and PCR duplicates were removed with seqkit v.0.8.0[178]. Host and human reads were removed using bwa-mem algorithm v.0.7.15[179] against the human (RefSeq: GCF_000001405.26) and fox (RefSeq: GCF_000002315.4) reference genomes. Quality filtered metagenomic reads were then co-assembled using MEGAHIT v.1.1.1 with k-mer sizes[77,87,97,107,127,137,147,157,167]: and default parameters[180]. Contigs less than 2500 nt were removed from the resulting assembly output and corresponding header names were simplifying using anvi'o v.6.2[61]. Metagenomic reads were mapped to the assembled contigs using bwa-mem algorithm v.0.7.15 with default parameters[179] and Samtools v.1.9[181] was used to sort and index the output SAM files into BAM files.

BAM files were used to generate a contig depth of coverage table with jgi_summarize_bam_contig_depths (MetaBAT2 v.2.12.1)[182]. We then applied the automatic binning algorithm in CONCOCT[183] on this coverage table to generate 10 large contig clusters to maximize explained patterns while minimizing fragmentation error, as performed elsewhere[184,185]. Subsequently, a manual binning and curation was performed for each CONCOCT cluster following the genome-resolved metagenomic workflow implemented in anvi'o v.6.2[61]. Briefly, anvi'o was used to generate a contigs database that identified open reading frames using Prodigal v.2.6.3[186] and single-copy core genes using HMMER v.3.2.1[187] against the collection of built-in HMM profiles for Bacteria and Archaea. Gene-level taxonomy was classified using Kaiju v.1.5.0[188], with NCBI's non-redundant protein database, including fungi and microbial eukaryotes, and genes were further annotated with functions using the NCBI's Clusters of Orthologous Groups (COG)[189]. Anvi'o was then used to profile each metagenomic BAM file to estimate the coverage and detection statistics of contigs in the contigs database, and combined mapping profiles into a merged profile database for all individuals. In addition, we imported an anvi'o collection corresponding to the 10 CONCOCT clusters. Finally, each CONCOCT cluster was manually binned and further refined using the anvi'o interactive interface[190] taking into account sequence composition, differential coverage, GC-content, and taxonomic signal of the considered contigs. MAGs with completeness >50% and redundancy <10% were retained for downstream analyses[191] (Genomic features of the MAGs can be found in Supplementary Data 2b).

The taxonomy of MAGs was inferred using the Genome Taxonomy Database Toolkit (GTDB-Tk)[62] version 95. However, we used NCBI taxonomy from the GTDB output to describe the phylum of MAGs in the results and discussion sections, in order to be in line with the literature. A phylogenetic tree of the MAGs was generated with FastTree 2.1.10[192] and visualized in anvi'o.

MAGs were considered to be detected in a given sample when >50% of their length was covered by reads to minimize non-specific read recruitments[184]. The number of recruited reads below this cut-off was set to 0 before determining vertical coverage, the number of bases covering each genome divided by its length.

Gut–brain module (GBM) detection

The fox shotgun metagenomic data was translated into neuroactive potential using a previously described module-based reconstruction framework[71]. Briefly, we searched for the presence of 56 gut–brain modules (GBMs), each corresponding to a process of synthesis or degradation of a neuroactive compound by the gut microbiota, in each of the fox MAGs ($n = 204$). As module structure follows the Kyoto Encyclopedia of Genes and Genomes (KEGG) database syntax, gene calls for each MAG were exported from the contig database within anvi'o and functionally annotated with KEGG identifiers using GhostKoala[193]. GBM coverage was calculated as the number of pathway steps for which at least one of the orthologous groups is found in a genome, divided by the total number of steps constituting the GBM using Omixer-RPM v.0.3.2 (https://github.com/raeslab/omixer-rpm). GBM presence in microbial MAGs was defined with a

detection threshold of at least 66% coverage, to provide tolerance to miss-annotations and missing data in incomplete genomes[71]. GBM detection was visualized with corrplot v.0.84[194] in the 28 differentially abundant fox MAGs to identify over/under-represented metabolic GBMs between the two behavioral selection lines.

Differential abundance estimates

Diversity estimates and hypothesis testing of Shannon diversity were performed with the breakaway package v.4.6.16[195,196] and DivNet package v.0.3.5[197] for the 16S dataset. These packages use sophisticated models to account for sequencing depth and rare taxa in high-dimensional data and incorporate taxon interactions when estimating α-diversity. Diversity estimates with uncertainties were used to support hypothesis testing between selection lines[198]. Expected relative abundance of microbial taxa was modeled directly from read counts for 16S and shotgun sequence data at different taxonomic levels (phylum, class, order, family, genus, and ASVs) using a beta-binomial model. The model was fit using corncob v.0.1.0[199], an r-based package designed specifically for marker gene compositional data, which uses sophisticated models to account for sequencing depth and rare taxa in high-dimensional data and estimates abundance with uncertainties to support hypothesis testing between selection lines. The Wald test was used to test for differential taxon abundances between selection lines with a controlled false discovery rate using the Benjamini–Hochberg procedure (p-value cutoff <0.05)[200]. Graphical representations were performed in R using the package ggplot2 v.3.2.1.9000[201].

Statistics and reproducibility

Statistics were carried using R/v.3.6.1 and R/v.3.6.3 to generate results that are reported throughout the paper and at https://github.com/lcpuetz/Fox_microbiome.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The sequence data that support the findings of this study are available in European Nucleotide Archive (ENA) at https://www.ebi.ac.uk/ena/, study accession number PRJEB29232.

Code availability

The scripts for analysis are available at https://github.com/lcpuetz/Fox_microbiome.

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Author contributions

M.T.P.G. and L.C.P. conceived the study with input from A.V.Kuk. and L.N.T. Sampling was organized and performed by D.V.S., A.V.K., A.V.Kuk., L.N.T., M.T.P.G., and L.C.P. Laboratory work was performed by L.C.P. and G.Z. L.C.P. and T.O.D. performed the computational analysis with input from A.L.M. and R.D.F. L.C.P. wrote the paper with input from all authors.

Competing interests

The authors declare no competing interests.

Additional information

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