Functional insights into the effect of feralisation on the gut microbiota of cats worldwide

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Abstract

Feralisation, the process by which domesticated organisms revert to a wild state, is a widespread phenomenon across various species. Successfully adapting to a new environment with different access to food, shelter, and other resources requires rapid physiological and behavioural changes, which could potentially be facilitated by gut microbiota plasticity. To investigate whether alterations in gut microbiota support this transition to a feral lifestyle, we analysed the gut microbiomes of domestic and feral cats from six geographically diverse locations using genome-resolved metagenomics. By reconstructing 229 draft genomes from 92 cats, we identified a typical carnivore microbiome structure, with notable diversity and taxonomic differences across regions. While overall diversity metrics did not differ significantly between domestic and feral cats, hierarchical modelling of species communities, accounting for geographic and sex covariates, revealed distinct taxonomic and functional profiles between the two groups. While taxonomic enrichment was balanced, microbial functional capacities were significantly enriched in feral cats. These functional enhancements, particularly in amino acid and lipid degradation, correspond to feral cats' dietary reliance on crude protein and fat. Additionally, functional differences were consistent with behavioural contrasts, such as the more aggressive and elusive behaviour measured in feral cats compared to the docile behaviour of domestic cats. Finally, the observed enrichment in short-chain fatty acid, neurotransmitter, and vitamin B12 production in feral cats aligns with improved cognitive function and potentially contributes to their heightened aggression and elusiveness. Our findings suggest that microbiome shifts may play a significant role in the development of physiological and behavioural traits advantageous for a feral lifestyle, supporting the adaptive success of feral cats in the wild.

Introduction

Feralisation is the process by which a once-domesticated organism detaches from the anthropic environment (Henriksen et al., 2018). While possessing some distinct characteristics, feralisation can generally be perceived as the counterpoint to domestication (Price, 1984). Many species initially domesticated by humans have subsequently given rise to feral populations (Gering et al., 2019), often leading to adverse impacts on both human settlements and biodiversity (Bonacic et al., 2019; Medina et al., 2011; Palmas et al., 2017). Despite the profound implications of this phenomenon, feralisation remains a relatively understudied process compared to its counterpart, domestication.

One of the animal species that is commonly found in feral form is the house cat (*Felis silvestris catus*). The domestication of cats is thought to have taken place in the Near East at the onset of the Neolithic period (Driscoll et al., 2007). This likely occurred concurrently with the rise of agriculture, as cats became valuable assets in controlling rodent populations around grain stores (Clutton-Brock, 1990; Gross, 2020). Since then, cats have spread alongside human populations across most of the planet (Baca et al., 2018; Ottoni and Van Neer, 2020). In every region they have colonised, cats have demonstrated an exceptional ability to thrive outside of domestic settings, forming countless feral cat populations worldwide (Bradshaw et al., 1999). Therefore, while domestication happened in a specific geographic area and time point, feralisation has happened and is continuously happening all over the world. This is partly due to the fact that modern non-pedigree domestic cats still closely resemble their wild ancestors both genetically and morphologically, retaining a significant portion of their wild behavioural repertoire, including proficient hunting skills (Bradshaw, 2006; Cecchetti et al., 2021). Additionally, feral cats typically display behaviours more akin to their wild relatives, such as an aversion to humans (Levy et al., 2003). The feralisation of cats poses a significant threat to the environment, as feral cats have been implicated as the primary cause of at least 14% of global bird, mammal, and reptile extinctions (Dueñas et al., 2021; Medina et al., 2011).

Feralisation success is often linked to phenotypic plasticity (Gering et al., 2019), as feral animals must adapt to significantly more variable and unpredictable environments compared to their domestic counterparts. In this regard, there is growing interest in the potential role that the gut microbiota may play in assisting such transitions. In general, exposure to new environments can lead to the acquisition of novel intestinal microorganisms, and these can interact with the host in diverse ways (Candela et al., 2012). While traditional microbiological and veterinary research has largely concentrated on pathogens, there is an increasing recognition of the beneficial effects of gut microorganisms (Lee and Hase, 2014; Ma et al., 2023). Researchers have hypothesised that these microorganisms can influence the phenotypic plasticity of animals, thereby enhancing their ability to thrive in diverse environments (Alberdi et al., 2016; Henry et al., 2021; Moeller and Sanders, 2020; Rosenberg and Zilber-Rosenberg, 2022; Voolstra and Ziegler, 2020). For example, variability in gut microbiota is acknowledged as one of the most effective mechanisms for adapting

to dietary changes (Teullet et al., 2023). Moreover, changes in gut microbiota have been linked to animal behaviour, as gut microorganisms are known to produce and metabolise neurotransmitters and hormones that can influence neurological processes (Forsythe et al., 2014; Johnson and Foster, 2018; Mayer, 2011). In fact, studies on domestic chickens and foxes kept in controlled conditions have demonstrated how certain microbiome features are associated with different behavioural responses to humans (Puetz et al., 2024, 2021). These observations raise the hypothesis that the differentiation in behaviour often observed when comparing feral and domestic individuals, may be partially influenced by differences in their gut microbial communities.

In this study, we elected to explore how the gut microbiota of cats changes in association with feralisation. Specifically, to explore their potential implications in facilitating the process and shaping behavioural changes in hosts, we used genome-resolved metagenomics to compare the taxonomic and functional features of gut microbiomes of 92 domestic and feral cats, sampled in six different locations across the world. With this data, we tested whether feral cats derived from domestic populations recovered an extended repertoire of microbial functions compared to domestic cats in the same region, and whether these changes were in line with the observed behavioural phenotypes.

Results

A global catalogue of cat-associated microbial genomes

We generated 298.1 GB of shotgun sequencing data (3.3±1.4 GB/sample) from faecal samples of 96 cats collected across six countries (Figure 1a). Metagenomic assembly and binning of this dataset yielded a total of 229 non-redundant metagenome-assembled genomes (MAGs) (Figure 1b). The average CheckM completeness of these MAGs was 90.02 ± 7.79%, with an average CheckM contamination of 1.32 ± 1.18% (Supplementary Fig. S1).

The MAGs were taxonomically assigned to 10 bacterial phyla (Figure 1b), with nearly half of them belonging to Bacillota A. Despite their phylogenetic relatedness, MAGs belonging to Bacillota A displayed extensive functional variability, covering over half of the functional space in the functional ordination (Supplementary Fig. S2). Bacterial communities were dominated by Bacteroidota (30.62±17.35%), Bacillota_A (21.47±10.53%), and Actinomycetota (17.85±14.45%), with the remaining bacterial phyla with average value with less than 9% (Figure 1c). At the family level, Bacteroidaceae (28.71±16.84%), Lachnospiraceae (12.18±8.03%), Coriobacteriaceae (9.96±9.09%), Helicobacteraceae (4.59±7.93%), and Megasphaeraceae (4.37±7.38%) were the five most abundant in the gut microbiome of cats (Supplementary Fig. S3). Furthermore, *Prevotella* (13.11±13.07%), *Collinsella* (9.96±9.08%), *Phoecaeicola* (8.98±8.41%),

Bacteroides (4.85±4.99%) and *Megasphera* (4.33±7.35%) the five most abundant bacterial genera (Supplementary Fig. S4).

Differences across localities

While 66 MAGs (29%) were present across all locations, the rest were specific to one or a few locations (Supplementary Fig. S5). Brazilian samples exhibited the highest number of MAGs, including 20 unique MAGs, with Cabo Verde showing both the fewest total and unique number of MAGs. The spread of bacteria was not affected by the lifestyle of the cats (MANN-WHITNEY; W = 25706, p-value = 0.713), with MAGs from domestic and feral cats being present in 3.12±1.93 and 3.19±2.03 locations, respectively. Diversity metrics also varied across geographic locations, with Aruba and Cabo Verde exhibiting the lowest neutral diversities, yet similar phylogenetic and functional metrics as the rest of the locations (Figure 1d).

The location of the cats explained most of the compositional variability of the gut microbiota (Figure 1e). Over 20% of the variability of the neutral (PERMANOVA; R^2=0.221, p-value=0.001) and phylogenetic (PERMANOVA; R^2=0.268, p-value=0.001) diversity was explained by the location and 58% of the functional variability (PERMANOVA; R^2=0.584, p-value=0.001). Spain and Denmark exhibit more similar microbial communities than the rest of the localities (pairwise-Permanova: R^2=0.0972, p-adjusted=0.075). Despite geographical differences, cats from Brazil had more similar gut microbiota to cats from Spain (pairwise-Permanova: R^2=0.0630, p-adjusted=0.555) than from the nearby Aruba (pairwise-Permanova: R^2=0.1377, p-adjusted=0.015). Cats from Aruba, Cabo Verde and Malaysia exhibited a more distinct microbial community than those from Denmark, Brazil, and Spain, which clustered closely together (Supplementary Fig. S6).

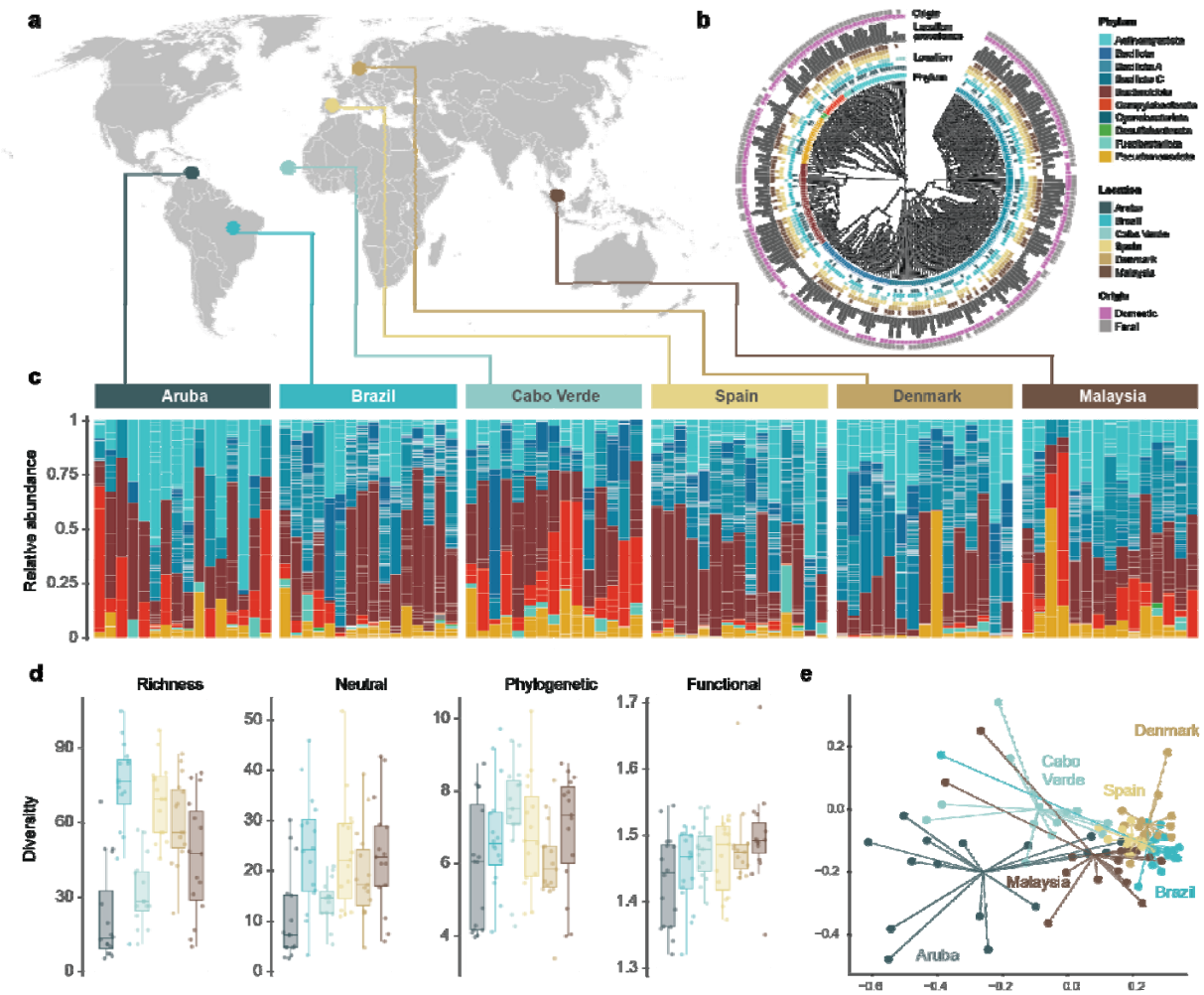

Figure 1. Overview of the geographic locations and genome catalogue characteristics. a) World map with the geographic locations of the samples. **b)** Reference metagenome-assembled genome (MAG) catalogue generated from the cat faecal samples, with outer rings indicating detection of MAGs in different geographic regions and origin (domestic or feral). **c)** Taxonomic composition of the faecal samples, each tile representing a MAG coloured by taxonomic phyla. **d)** Alpha diversity differences across geographic regions, based on four different Hill numbers diversity metrics. **e)** Ordination of richness-based compositional differences across samples, coloured and grouped by location.

Functional microbiome differences between domestic and feral cats

Prior to comparing the microbiomes of the feral vs domestic cats, we validated that cat origin did correlate with behavioral traits. Specifically, feral cats exhibited less acceptance (X^2 = 24.525, df = 1, *p*-value = 7.337e-07) and more hisses (X^2 = 11.221, df = 1, *p*-value = 0.0008), bites (X^2 = 11.808, df = 1, *p*-value = 0.0006) and retreats (X^2 = 5.4412, df = 1, *p*-value = 0.0197) towards humans compared to domestic cats.

When comparing the gut microbiota of these behaviourally different groups we observed that overall alpha (Mixed models, $p>0.05$) and beta diversity (PERMANOVA, $p>0.05$) analyses did not yield any significant differences in microbiome diversity and composition between feral and domestic cats (Figure 2a-b). Accordingly, the only bacteria unique to either of the two origins were rare species found in just one location, with no widespread MAGs specific to either domestic or feral origins. However, when considering relative abundances, hierarchical modelling of species communities (HMSC) revealed that 61 MAGs were associated with domestic cats while 34 MAGs were associated with feral cats (Figure 2c-d). MAGs belonging to a range of bacterial phyla were enriched in domestic cats, including Pseudomonadota (11 MAGs from CAG-239, Burkholderiaceae, and Succinivibrionaceae), Campylobacterota (3 MAGs from Helicobacteraceae), Bacteroidota (9 MAGs from Bacteroidaceae), Actinomycetota (18 MAGs from Actinomycetaceae, Atopobiaceae, Bifidobacteriaceae, Coriobacteriaceae, Mycobacteriaceae), Bacillota A (11 MAGs from Butyricicoccaceae, Oscillospiraceae, Peptoniphilaceae and Ruminococcaceae) and Bacillota C (8 MAGs from Acidaminococcaceae, Dialisteraceae, Megasphaeraceae), Desulfobacterota (1 MAG from Desulfovibrionaceae). In feral cats, MAGs belong to Bacillota A (14 MAGs from Acutalibacteraceae, Anaerotignaceae, Clostridiaceae, Lachnospiraceae, Peptostreptococcaceae, and UBA1381), Bacillota (17 MAGs from Enterococcaceae, Lactobacillaceae, and Streptococcaceae) and Fusobacteriota (3 MAGs from Fusobacteriaceae).

Using our HMSC model, we predicted the typical microbial community compositions for feral and domestic cats, while controlling for the rest of the variables. This enabled us to estimate the community-level functional differences of the microbiota associated with both conditions (Figure 2c-e). The microbial community of domestic cats exhibited a higher capacity to degrade polysaccharides such as mixed-linkage glucans. The serine and tyrosine amino acid biosynthetic capacity was also higher in domestic cats, and the capacity to biosynthesise the aromatic compound indole-3-acetate, a major plant growth hormone. The overall repertoire of enriched functions was considerably wider in the case of feral cats (Figure 2e). These included an enhanced capacity for biosynthesising the amino acids glutamate, cysteine and proline, and the amino acid derivative spermidine. The capacities to produce the SCFA acetate, propionate and butyrate, and vitamin E (Tocopherol/tocotorienol) and B12 were also increased. The catabolic repertoire of enriched functions listed capacities for degrading the amino acids histidine, tryptophan, proline, and threonine, the lipids triglyceride, oleate, fatty acid, and dicarboxylic acids, the antibiotics streptogramin, lincosamide and macrolide, the sugar sucrose, D-mannose and lactose, various nitrogen compounds such as methylamine, and allantoin, the alcohol 2,3,-Butanediol and two xenobiotics, such as toluene, and xylene.

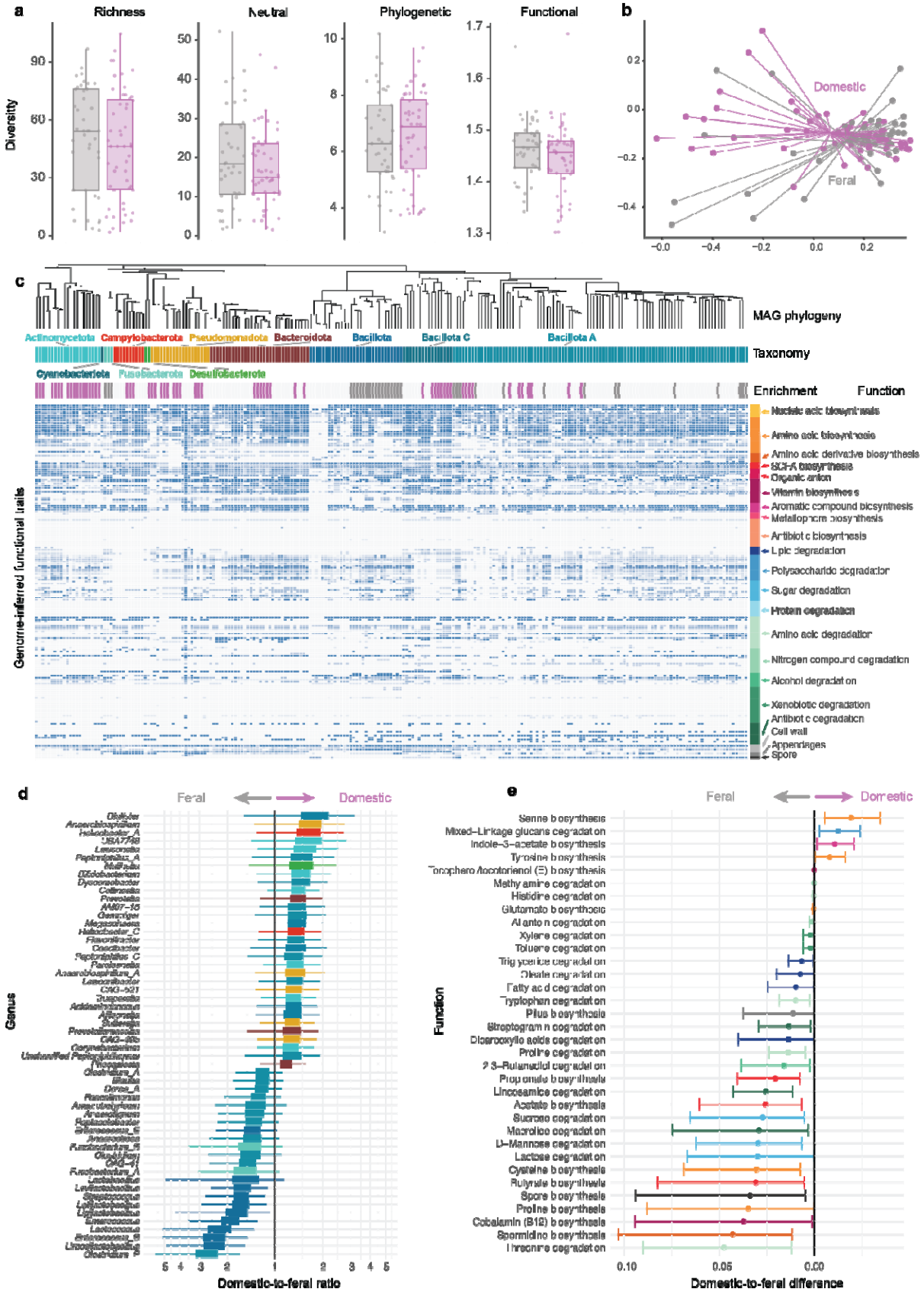
a
Richness
Neutral
Phylogenetic
Functional
Diversity
b
Domestic
Feral
c
MAG phylogeny
Actinomycetota
Campylobacterota
Pseudomonadota
Bacteroidota
Bacillota
Bacillota C
Bacillota A
Taxonomy
Cyanobacteriota
Fusobacteriota
Desulfobacterota
Enrichment
Function
Genome-inferred functional traits
Nucleic acid biosynthesis
Amino acid biosynthesis
Amino acid derivative biosynthesis
SCFA biosynthesis
Organic anion
Vitamin biosynthesis
Aromatic compound biosynthesis
Metallophore biosynthesis
Antibiotic biosynthesis
Lipid degradation
Polysaccharide degradation
Sugar degradation
Protein degradation
Amino acid degradation
Nitrogen compound degradation
Alcohol degradation
Xenobiotic degradation
Antibiotic degradation
Cell wall
Appendages
Spore
d
Feral
Domestic
Genus
Domestic-to-feral ratio
e
Feral
Domestic
Function
Serine biosynthesis
Mixed-linkage glucans degradation
Indole-3-acetate biosynthesis
Tyrosine biosynthesis
Tocopherol/tocotrienol (E) biosynthesis
Methylamine degradation
Histidine degradation
Glutamate biosynthesis
Allantoin degradation
Xylene degradation
Toluene degradation
Triglyceride degradation
Oleate degradation
Fatty acid degradation
Tryptophan degradation
Pilus biosynthesis
Streptogramin degradation
Dicarboxylic acids degradation
Proline degradation
2,3-Butanediol degradation
Propionate biosynthesis
Lincosamide degradation
Acetate biosynthesis
Sucrose degradation
Maltose degradation
D-Mannose degradation
Lactose degradation
Cysteine biosynthesis
Butyrate biosynthesis
Spore biosynthesis
Proline biosynthesis
Cobalamin (B12) biosynthesis
Spermidine biosynthesis
Threonine degradation
Domestic-to-feral difference

Figure 2. Microbiota differences between domestic and feral cats. a) Alpha diversity differences between domestic and feral cats, based on four different Hill numbers diversity metrics. **b)** Compositional differences between domestic and feral cats, samples coloured by domestic or feral origin. **c)** Heatmap of 170 genome-inferred functional traits, with blue colour indicating higher capacity to perform a given metabolic function. Metagenome-assembled genomes (MAGs) are phylogenetically sorted, with their respective enrichment levels annotated above the heatmap. GIFTs are sorted according to their functional group. **d)** Enrichment of bacterial genera towards domestic or feral cats, sorted by effect size and coloured by phyla. **e)** Enrichment of functions towards domestic or feral cats, sorted by effect size and coloured by functional group (see legend in panel c).

Discussion

Our global genome-resolved metagenomic data set provides unique insights into the taxonomic and functional diversity of cat-associated microbiomes across different geographies and lifestyles. We reconstructed 229 bacterial genomes, with 42 (18%) displaying ANI values below 95% compared to the closest representatives in the GTDB database, indicating potential new species (Rodriguez-R et al., 2024). The gut microbiome of cats was predominantly composed of Bacteroidota, Bacillota A, and Actinomycetota, with notable presences of Campylobacterota and Pseudomonadota, reflecting a typical microbiome profile of a carnivorous mammal (Milani et al., 2020; Zoelzer et al., 2021).

Cat microbiome variation across geographic space responds to anthropisation rather than climatic variables

Nearly one-third of the bacteria detected in the faecal samples were present in all six sampled locations, indicating a globally distributed core microbiota spanning eight phyla. The remaining bacteria were found in only one or a few locations, resulting in substantial compositional variation across different geographies, consistent with previous observations in other domestic animals, such as horses (Ang et al., 2022) and dogs (Yarlagadda et al., 2022).

The fact that cats from Brazil, Spain, and Denmark formed a cluster, while those from Aruba, Cabo Verde, and Malaysia showed greater geographic and interindividual variation suggests that geographical microbiome variation is influenced more by the level of urbanisation or anthropisation than by climatic variables or geographical proximity. Similar to patterns observed in other species (Dillard et al., 2022), including humans, animals from industrialised areas had more similar and uniform microbial compositions than cats from less urbanised environments. This could be due to the standardisation of dietary resources and the buffering of environmental fluctuations, such as droughts or extreme temperature variations, provided by

anthropic environments (Suzuki and Ley, 2020). Nevertheless, these changes were not reflected in alpha diversity metrics, with Aruba and Cabo Verde exhibiting the most and least diverse microbial communities, respectively, across most of the analysed metrics.

Domestic and feral cats exhibit a taxonomic footprint of lifestyle origin

Despite the significant differences observed across geographic regions, hierarchical modelling of microbial communities revealed taxonomic differences between domestic and feral cats once these regional variations were accounted for. The number of bacterial taxa enriched in domestic and feral cats was similar, corresponding to the fairly balanced alpha diversities in microbiomes from both origins. However, the taxonomic profiles of these enriched taxa differed. Bacteria belonging to Pseudomonadota, Bacteroidota, Campylobacterota, and Desulfovibriota were exclusively enriched in domestic cats. The genera that exhibited the largest effect sizes were *Dialister*, *Anaerobiospirillium* and *Helicobacter_A*. In contrast, the microbiome of feral cats was characterised by an enrichment of many Bacillota and Fusobacteriota species. While members of phylum Bacillota such as *Limosilactobacillus*, *Lactobacillus* and *Ligilactobacillus* are often employed as probiotics due to their capacity to produce lactic acid, many Fusobacteria are pathogenic to humans (Roberts, 2000; Robinson et al., 2020). The enrichment of *Megaspheaera* and *Prevotella* in domestic cats, along with increased *Clostridium* and *Fusobacterium* and decreased *Bifidobacterium* in feral cats, aligns largely with previous studies evaluating the impact of dietary transitions from a kibble diet high in carbohydrates to a more high-protein raw diet in cats and dogs (Bermingham et al., 2017; Butowski et al., 2019; Hooda et al., 2013; Lubbs et al., 2009). Moreover, *Bifidobacterium* and *Dialister*, enriched in domestic cats in our study, were identified as the most featured genera in obese cats distinguishing them from normal cat microbiota (Ma Xiaolei et al., 2022). These shifts in the microbial community are probably the reflection of the dietary differences between domestic and feral cats.

Feral cats are enriched for microbial functions compared to domestic cats

Beyond taxonomic differences, our bioinformatic and statistical analyses enabled us to capture differences in the functional repertoires of bacteria associated with domestic and feral cats, shedding light on the pathways potentially affecting the domestication and feralisation processes. While taxonomic enrichment was relatively balanced between domestic and feral cats, functional differences were more pronounced. Of the 170 genome-inferred functional traits (GIFTs) analysed, only four showed consistent patterns of enrichment in domestic cats. The reduced number of enriched functional capacities in domestic animals may be associated with their consumption of commercial cat food, which is highly digestible and supplemented with rapidly absorbed nutrients. This likely leads to less substrate available for bacterial activity, depleting the functional repertoire of microbial communities and potentially diminishing their contribution to host biology.

In contrast, we found 30 GIFTs enriched in feral cats. These functional traits encompassed many biosynthetic and degradation pathways, suggesting a more significant role of gut microbiota in feral cats compared to domestic ones. These findings are consistent with earlier observations that domestic animals tend to harbour simplified microbial communities, exhibiting reduced functional potential compared to their wild counterparts (Alessandri et al., 2019). A meta-analysis of the dietary habits of feral cats confirmed that they are obligate carnivores (Plantinga et al., 2011), consuming a diverse range of prey, including rodents, birds, fish, and insects, along with a significant intake of human garbage (Doherty et al., 2015). It has been estimated that 98% of the daily energy intake derives from crude protein and fat (Plantinga et al., 2011). Accordingly, we observed enrichment of many microbial pathways associated with the degradation of such dietary components. Specifically, the microbiome of feral cats showed an enhanced capacity to break down several amino acids and derivatives, as well as lipids including dicarboxylic acids, fatty acids, oleate, and triglycerides. Although only 2% of the daily energy intake has been estimated to derive from nitrogen-free nutrients (Plantinga et al., 2011), we also found enriched capacities for carbohydrate breakdown, indicated by saccharolytic traits for lactose, D-mannose, and sucrose degradation. We hypothesise that this could be linked to consumption of mixed foods found in human waste (Doherty et al., 2015), which may also explain the enrichment of antibiotic degradation pathways, including those for macrolides, lincosamides, and streptogramins.

Enriched microbial functions align with cat behaviour

The taxonomic and functional differences observed between microbial communities of domestic and feral cats not only reflect dietary changes but also align with the behavioural distinctions seen between these two groups, particularly the fearful and defensive behaviour feral cats exhibited when in contact with humans, in contrast to the docile behaviour observed in domestic cats. This is consistent with studies showing that microbiota can indeed influence animal behaviour (Kamimura et al., 2024; Sudo et al., 2004; Warda et al., 2019; Watanabe et al., 2021; Zhang et al., 2022). As feralisation and domestication are rapid processes involving drastic changes in an animal's environment and diet, they may trigger microbiota shifts that could alter the microbiota-gut-brain interaction.

Taxonomic shifts observed in feral cats have previously been associated with similar behavioural differences in animals. For instance, *Lactobacillus* and *Streptococcus*, both enriched in feral cats, have been associated with increased fear and anxiety in dogs, chickens, and horses (Bulmer et al., 2019; Mondo et al., 2020; Puetz et al., 2021). Additionally, *Lactobacillus*, *Dorea* and *Blautia* are also more abundant in the gut microbiomes of aggressive dogs (Kirchoff et al., 2019). While these behavioural traits are generally selected against in domestic animals, they may provide an anti-predatory advantage for feral cats. Conversely, *Bifidobacterium*, which was enriched in domestic cats, is commonly used as a probiotic in animals and has been shown to significantly reduce anxiety (Bercik et al., 2011; Desbonnet et al., 2010).

While most of these examples relied on taxonomic profiling derived from 16S rRNA amplicon sequencing, a major advantage of the genome-resolved metagenomics approach we employed lies in its ability to provide direct functional insights from bacterial genomes reconstructed from metagenomic data. For instance, short-chain fatty acids (SCFAs) stood out among the affected biosynthetic pathways, with capacities for synthesising all three major SCFAs—namely butyrate, acetate, and propionate—being enriched in feral cats. SCFAs are produced by bacteria in the lower intestine from indigestible carbohydrates and peptides. Beyond their significance for animal health (Frost et al., 2014; Goswami et al., 2018; Hu et al., 2022; Rowe et al., 2024), SCFAs could also influence observed behaviours by altering brain function via immune, endocrine, and vagal pathways, directly by crossing the blood-brain barrier, or acting as endogenous ligands (Dalile et al., 2019; Kratsman et al., 2016; Mirzaei et al., 2021; Silva et al., 2020). As an example of their relevance in brain function, butyrate has been used to restore cognitive function in neurodegeneration models (Ferrante et al., 2003; Govindarajan et al., 2011; Ryu et al., 2005), and acetate has been shown to offer protection against cognitive impairment in mice (Erny et al., 2021; Zheng et al., 2021). Acetate can also be incorporated into the glutamate–glutamine transcellular cycle and influence hypothalamic neurotransmission, as well as acting as an epigenetic modulator that could have contributed to gene expression changes during domestication (Anastasiadi et al., 2022; Bélteky et al., 2018; Nätt et al., 2012). In fact, acetate synthesis has been recently found to be enriched in the microbiomes of foxes genetically selected for aggressive behaviour (Puetz et al., 2024), which reinforces the possible link between microbiome functions and behaviour in feral cats.

Additionally, the enhanced capacities for synthesising amino acids and derivatives also hold the potential to influence brain function. The capacity to biosynthesise proline, a dietary factor with a significant impact on psychotic disorders (Mayneris-Perxachs et al., 2022; Savio et al., 2012; Yao and Han, 2022), was among the most significantly enriched functions in feral cats. Similarly, the amino acid derivative spermidine has been recently shown to improve cognitive function (Flory et al., 2023; Schroeder et al., 2021). Higher intestinal bacterial degradation of threonine and tryptophan can partially reduce their availability in the brain (Pardridge, 1979; Tovar et al., 1988) thereby influencing the production of key neurotransmitters such as glycine and serotonin, which have important brain functions (Boehm et al., 1998; Gao et al., 2020). Finally, the ability to synthesise cobalamin (vitamin B12) exhibited one of the most significant enrichments in feral over domestic cats. Commonly supplemented in commercial diets, Vitamin B12 can modulate behaviour, its deficiency leading to cognitive impairment and a range of neurological disorders (Kang et al., 2024).

Insights into the role of microbiomes in feralisation and domestication processes

Feralisation is often recognised as the inverse process of domestication (Price, 1984), where many changes induced by domestication are expected to revert (Henriksen et al., 2018). Despite the broad geographical range screened, our results consistently showed that feral cats gained more microbial functions than their domestic counterparts, confirming our prediction that feral cats would exhibit an enriched functional

repertoire compared to domestic cats. Community-level multivariate modelling unveiled significant functional changes in the absence of broad-resolution microbiota markers such as diversity metrics, highlighting the value of statistical tools such as Hmsc for functional microbiome research (Koziol et al., 2023). Although without faecal microbiota transplants and controlled behavioural experiments, we cannot determine causal relationships between microbiota, behaviour and feralisation (Moeller and Sanders, 2020), we conclude that our results point towards a favourable contribution of microbiome changes in the acquisition of physiological and behavioural traits beneficial for a feral lifestyle, as well as a likely effect on the increased aggressiveness and elusiveness observed in the sampled feral cats.

Materials and Methods

Sample collection

Veterinary clinics and shelters with trap-neuter-return programmes from six geographically separated countries (Aruba, Brazil, Cabo Verde, Denmark, Malaysia, and Spain) participated in this study. In each location, faecal samples of cats defined as feral (i.e., individuals that had been originally captured in the streets/nature by the Trap-Neuter-Return type programmes) and domesticated (i.e., individuals living with humans prior to entering the clinic or shelter) were collected. To ensure standardised faecal sample collections, sampling kits were sent to each collaborator, including sampling instructions, cat metadata sheets, FTA cards (Whatman FTA Classic), sterile swabs, plastic bags and desiccant packs. The collaborator collected a fresh scat using a sterile swab to transfer some faecal material onto the FTA card, which was then left to dry. Two circles on the filter paper FTA card were collected for each cat faecal sample. The dry FTA card was then stored in a plastic bag until all samples were collected. To characterise the behaviour of the cats categorised as domestic and feral, information about the cats' behavioural traits towards humans in each facility such as bites, hissing, retreating from, or avoiding human contact was recorded. Cats' age, sex, date of entry to the facility, date of sampling, and use of antibiotics in the animal were also noted. Once all samples were collected, they were shipped to Denmark for storage at room temperature along with a new desiccant pack.

DNA extraction

DNA was extracted from 8 feral and 8 domesticated cats from each sampling site following (Bolt Botnen et al., 2023). Briefly, approximately 1/4th of a circle was cut out by a scalpel and placed in a Monarch spin column. 175 µl of elution buffer was added to each spin column before incubating at 37°C for 1 hour. After incubation, each tube was centrifuged at 13,000 x g for 10 minutes. The supernatant was placed in a new Eppendorf tube, ready for storage at -20°C or bead purification with SPRI beads. Bead purification followed

the AMPure protocol for a DNA:Bead ratio of 1:1.4 and eluted in 50 µl of elution buffer. DNA was fragmented to an average length of 400 nucleotides using a Covaris M220 ultrasonicator with 50 µl DNA in the microTUBE-50 (Peal power 30, duty factor 20, cycles per burst 50, treatment time 60 seconds).

Library building

DNA extracts were quantified using a Qubit and then diluted to approximately 100 ng in 16 µl for the library building. Libraries were built using the BEST ligation-based library preparation protocol (Carøe et al., 2018). Per reaction, the end repair master mix contained 2 µl T4 DNA ligase buffer, 1 µl reaction enhancer, 0.5 µl T4 PNK, 0.2 µl T4 polymerase, and 0.2 µl dNTP 25 mM. 3.9 µl of the master mix was added to each sample before incubating at 20□ for 30 minutes and 65□ for 30 minutes. The ligation step master mix contained 3 µl PEG 4000 50%, 0.5 µl T4 DNA ligase buffer, and 0.5 µl T4 DNA ligase, per reaction. To each sample 1 µl adapter (50 µM) was added followed by 4 µl of the ligation master mix. The samples were then incubated at 20°C for 30 minutes and 65°C for 10 minutes. Fill-in master mix contained, per reaction, 3 µl ddH_2O, 1 µl Isothermal buffer, 0.2 µl dNTP 25 mM, and 0.8 µl Bst 2.0 polymerase. 5 µl of fill-in master mix was added to each sample followed by incubation at 65□ for 15 min and 80□ for 15 minutes. Each library was then diluted 1:2 with molecular-grade water. Bead purification followed the AMPure purification protocol, substituted with SPRI beads, at a ratio of 1:1.4 and eluted in 30 µl of elution buffer.

Dilutions (1:100) of each library were prepared for qPCR. A qPCR master mix was prepared with 11.8 µl ddH_2O, 2.5 µl 10x buffer, 2.5 µl $MgCl_2$ 25mM, 0.5 µl BSA, 0.5 µl SYBR, 0.2 µl dNTP 25 mM, 0.5 forward primer (BGI-F 10 µm) and 0.5 µl TaqGold, per reaction. Each reaction consisted of 10 µl diluted DNA, 1 µl reverse primer (BGI-R 10 µM) and 19 µl master mix. qPCR settings were as follows: 95°C for 10 minutes, 30 cycles of 95°C for 20 seconds, 60°C for 30 seconds, 72°C for 40 seconds, followed by a final 72°C for 7 minutes and a 4°C hold. The cycle threshold determined the number of cycles required for indexing. For indexing, a master mix was prepared with 12.3 µl ddH_2O, 2.5 µl 10x buffer, 2.5 µl $MgCl_2$ 25mM, 0.5 µl BSA, 0.2 µl dNTP 25 mM, and 0.5 µl TaqGold, per reaction. Each reaction consisted of 10 µl undiluted DNA, 1 µl of each indexed primer (BGI-R and BGI-F 10 µM) and 19 µl master mix. PCR settings were as follows: 95°C for 10 minutes, 7-11 cycles of 95°C for 20 seconds, 60°C for 30 seconds, 72°C for 40 seconds, followed by a final 72°C for 7 minutes and a 4°C hold. Libraries were then bead purified with a ratio of 1:1.4 and eluted in 30 µl elution buffer and then quantified on the Qubit and 2-3 µl aliquoted for fragment analysis. Libraries were pooled together based on country. Libraries from five locations were sequenced on a DNBseq platform at BGI Denmark, while one location (Denmark) was sequenced using Illumina technology at a NovaSeq 6000 platform at Novogene (UK), after ensuring that both platforms are compatible for combined microbiome analyses (Mak et al., 2017; Smith et al., 2019).

Bioinformatics

Using a custom snakemake-based (Mölder et al., 2021) pipeline, paired-end reads were trimmed and quality controlled using fastp v0.20.1 (Chen et al., 2018), with the following options: —trim_poly_g, —trim_poly_x, —n_base_limit 5, —qualified_quality_phred 20, —length_required 35. Processed reads were then mapped to the concatenated host reference genome assemblies (Human; GRCh38.p13), Cat; F.catus_Fca126_mat1.0) using Bowtie2 (Langmead and Salzberg, 2012) and samtools (Li et al., 2009) (default settings). Unaligned reads from each geographic location were then pooled and coassembled using metaSPAdes (Nurk et al., 2017) with the following kmer sizes: 21,29,39,59,79,99,119. Coassembled contigs shorter than 1,500 bp were removed. Each sample's reads were then mapped its appropriate coassembly using Bowtie2, and the resulting BAMs were used as input to MetaWRAP's (Uritskiy et al., 2018) binning module, and the coassembled contigs binned using CONCOCT (Alneberg et al., 2014), MaxBin2 (Wu et al., 2016), and MetaBAT2 (Kang et al., 2019). The output bins were automatically refined using MetaWRAP's bin_refinement module, with a minimum CheckM (Parks et al., 2015) completeness score of 70%, and a minimum checkM contamination score of 10%. dRep (Olm et al., 2017) was then used to dereplicate the refined bins, first into primary clusters >90% average nucleotide identity (ANI) using MASH (Ondov et al., 2016), then into secondary clusters >98% ANI with ANImf (Kurtz et al., 2004; Richter and Rosselló-Móra, 2009). The non-host quality-controlled reads were then mapped against this dereplicated MAG catalogue as above. The resulting BAMs were then profiled using CoverM (https://github.com/wwood/CoverM) to create the final sample count table. The dereplicated MAGs were taxonomically annotated using the GTDB-tk (Chaumeil et al., 2019; Parks et al., 2020), which uses pplacer (Matsen et al., 2010), prodigal (Hyatt et al., 2010), HMMER3 (Eddy, 2011), FastANI (Jain et al., 2018), and FastTree (Price et al., 2010). The MAGs were also functionally annotated using the DRAM pipeline (Shaffer et al., 2020), which searches predicted proteins against multiple databases (El-Gebali et al., 2019; Kanehisa et al., 2017; Rawlings et al., 2010; Suzek et al., 2015) using MMseqs2 (Steinegger and Söding, 2017). Finally, gene annotations were distilled into Genome-Inferred Functional Traits (GIFTs) using distillR (https://github.com/anttonalberdi/distillR), producing biologically meaningful annotations that highlight each bacterial genome's potential to degrade or synthesise compounds relevant to host metabolism. DistillR utilises a curated database of over 300 metabolic pathways, employing KEGG and Enzyme Commission (EC) identifiers to calculate standardised GIFT values. These values range from 0 to 1, where 0 signifies the absence of all genes associated with a particular metabolic pathway, and 1 indicates the presence of all necessary genes. For example, if a pathway step requires two specific identifiers, it is deemed complete when both are present, half-complete when only one is present, and empty if neither is present.

Statistics

Behavioural phenotypes in domestic and feral cats

We follow the earlier definitions of feral cats (Crowley et al., 2020; Gosling et al., 2013), defining a feral cat as an unowned specimen capable of surviving with or without direct human intervention, and additionally showing fearful or defensive behaviour upon human contact. In each location, cats captured in the streets or nature were classified as feral, while those living in a residence with their owners were classified as domestic. To ensure the origin of the cats correlated with the behavioural phenotype, we analysed the differences in cats' fearfulness or defensive behaviour towards humans using chi-tests.

Alpha and beta diversities

Alpha and beta diversity analyses were performed within the Hill numbers framework (Alberdi and Gilbert, 2019), using different combinations of orders of diversity (q) and regularity in Hilldiv2 R package (https://github.com/anttonalberdi/Hilldiv2). 'Richness' refers to the neutral Hill number of q=0, which is limited to the counts of MAGs. 'Neutral' refers to the neutral Hill number of q=1, in which the MAGs are weighted according to their relative representation. In the case of alpha diversity, it equals the exponential of the Shannon index, also known as Shannon diversity. 'Phylogenetic' refers to the phylogenetic Hill number of q=1, which accounts for the phylogenetic relationships between MAGs derived from the phylogenetic tree. 'Functional' refers to the functional Hill number of q=1, which accounts for the functional distances between MAGs derived from the GIFT table. Compositional differences were quantified using pairwise dissimilarity estimation via the Jaccard-type turnover metric (S) derived from the Hill numbers beta diversity. All four metrics were employed for alpha and beta diversity comparisons. Differences in alpha diversity between locations were assessed using linear models, while differences between origins were tested using linear mixed models with location as a random effect. PERMANOVA analysis was performed to compare the beta diversity differences between locations and origins using vegan::adonis2 function.

Hierarchical modelling of species communities

To understand taxonomic and functional responses to the analysed variables, we built models of species communities using Hmsc, a multivariate hierarchical generalised linear model that uses Bayesian inference (Tikhonov et al., 2020). In the species matrix Y, which typically includes species abundance or occurrence values, we included the genome-size normalised and log-transformed counts of MAGs. In the environmental variable matrix X, we included the categorical variables origin and sex, as well as the log-transformed sequencing depth varying sequencing efforts across samples. Geographic location was included as a random effect to account for the non-independence of samples collected from the same location. Additionally, we

included the MAG phylogeny in the matrix C to quantify the degree of phylogenetic signal in the MAG's responses to the variables.

We fitted the models assuming default priors and sampled the posterior distribution running four Markov Chain Monte Carlo (MCMC) chains, each running for 3,750 iterations with 1,250 discarded as burn-in. We thinned by 10 to obtain a total of 250 posterior samples per chain and 1,000 total posterior samples. To test for MCMC convergence we measured the potential scale reduction factor for the beta (response to variables) and rho parameters (phylogenetic signal). We then performed five-fold cross-validation to evaluate the predictive performance of the models for the analysed MAGs in terms of R^2. Once the models were fitted, we quantified the posterior estimates for the variable to origin to identify MAGs were considered significantly associated with either feral or domestic origins when 90% of the posterior credible interval of the beta parameter measuring the effect of origin was either positive or negative (i.e. using a significance threshold of 0.9 posterior probability). To estimate functional differences between microbial communities associated with feral or domestic origins, we compared the community-level functional attributes of the subset of MAGs with significant association with origin. To minimise the risk of false positives, we filtered out MAGs with low predictive capacity related to origin. This filtering was based on a metric calculated as the product of the variance attributed to origin (derived from variance partitioning) and the R^2 value obtained from five-fold cross-validation.

All statistical analyses performed in this study are fully reproduced in a Rmarkdown webbook (https://alberdilab.github.io/domestic_feral_cat_metagenomics).

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