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Optimizing Faecal Sampling and Storage Methods for Microbiome Analysis in Cats and Dogs

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ABSTRACT

Consistency in studies of feline and canine gut microbiomes relies heavily on how faecal samples are collected and preserved. This study evaluated practical strategies for sampling and storing cat and dog faeces, suitable for both laboratory and home settings. We specifically examined whether storing samples at room temperature for up to 12 hours and collecting material from different parts of the stool affected microbial diversity, taxa composition, or DNA quality. Faecal samples were collected from 10 healthy cats and 10 healthy dogs, maintained at 20 °C, and subsampled from the initial, middle, and terminal portions of each stool, at either surface or core, at multiple time points (0–12 h), before stabilization at −80 °C. DNA extraction was performed using Illumina NovaSeq sequencing. Alpha diversity was comparable between canine and feline samples, with Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria being the dominant phyla. Prevotella predominated in both species, while Fusobacterium was absent in cats. Room-temperature storage for up to 12 hours generally did not significantly affect alpha diversity, taxonomic composition, or DNA yield. Sampling location within the stool had minimal effect, although surface samples from cat faeces after 12 hours exhibited increased alpha diversity, with a similar but non-significant trend in dogs. Beta diversity analyses revealed that individual differences were the primary determinant of microbial composition ($R^2 = 0.64$ in dogs, 0.88 in cats), while sampling time and site had smaller but detectable influences. Cat and dog faeces can be stored at room temperature for up to 12 hours without major alterations in microbial composition or DNA recovery. For samples stored longer than 6 hours, core sampling is recommended over surface sampling to maintain representative microbial profiles.

Keywords: Cat, Dog, Microbiome, Gastrointestinal, Faeces, Storage, Temperature

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Background

The gastrointestinal tracts of cats and dogs harbor vast numbers of microorganisms, particularly bacteria, which play a crucial role in maintaining host health [1]. Advances in molecular techniques have enabled increasingly detailed characterization of the feline and canine gut microbiome, shedding light on how factors such as age, diet, medications, and disease influence microbial communities [2–8]. Microbial composition is known to vary along different regions of the gastrointestinal tract, with faecal samples serving as a widely accepted and practical proxy for studying the gut microbiome [9, 10].

Most investigations of cat and dog faecal microbiomes have been limited to small numbers of research colony animals, highlighting the need for studies involving larger, more representative populations. Proper sample

collection, handling, and storage are essential for ensuring accurate and reproducible bacterial DNA analyses, underscoring the importance of standardizing these procedures prior to large-scale studies.

Obtaining fresh faecal samples from privately-owned cats and dogs presents a practical challenge, particularly for cats that may defaecate in litter boxes overnight. Human studies have shown that faecal microbiota composition can change when samples are stored at room temperature for 48 hours [11, 12]. In contrast, studies examining companion animals are limited and typically focus on longer storage periods. For example, one study reported that storing cat faeces at ambient temperature for up to four days did not noticeably affect bacterial composition [13]. Similarly, dog faeces stored in stabilizing solutions at room temperature for 14 days maintained both alpha and beta diversity, whereas unstabilized samples exhibited increased alpha diversity and shifts in dominant bacterial phyla, highlighting the importance of proper stabilization [14].

Evidence from human studies also indicates that rectal swabs can differ in bacterial composition compared with stool samples, likely due to oxygen gradients between mucosal and luminal environments [15, 16]. However, no studies have systematically examined microbial differences across different horizontal locations within faecal stools. These observations suggest that both the sampling location and depth (surface versus core) may influence microbiome analyses. Taken together, these findings emphasize the need for a standardized and validated faecal sampling and processing protocol that can be applied consistently in research settings and is feasible for privately-owned pets.

Study objectives

This study was designed to validate a faecal sampling and processing protocol for cats and dogs using research colony animals, with the goal of identifying a method applicable both in laboratory settings and for privately-owned pets. The investigation focused on evaluating the impact of storing faecal samples at room temperature for up to 12 hours on bacterial DNA concentration, microbial diversity, and relative abundance of taxa. The 12-hour limit reflects the maximum anticipated delay between defecation at home and sample collection and stabilization by pet owners.

Additionally, the study examined how sampling location within the stool—both horizontally (first emitted portion, middle portion, last emitted portion) and in depth (surface vs. core)—affects these outcomes. Two different stabilization solutions were also compared to assess their effect on DNA yield and microbiome composition.

Results

A total of 263 faecal samples, derived from 20 stools from 10 cats and 10 dogs, were processed and sequenced. Two samples were excluded due to insufficient material: the 2-hour timepoint (T2) samples for cats 7 and 8. Stool consistency was acceptable across all samples, with feline samples scoring 2–3 and canine samples 2.5–3 on the grading scale. Sequencing yielded over 90% high-quality reads across all samples.

Species-specific faecal microbiome profiles

Microbial profiles clustered strongly by individual, generating distinct faecal microbial “fingerprints” for nearly all animals. One canine sample (dog 4 at 720 minutes, surface) was markedly different from all other dogs, containing only 14 bacterial species, and was therefore excluded from statistical analyses.

Comparing species, clear differences were observed between feline and canine faecal microbiomes. At the phylum level, cat faeces were predominantly composed of Bacteroidetes, followed by Firmicutes/Actinobacteria and Proteobacteria **(Figure 1; Table 1)**, whereas Spirochaetes and Fusobacteria were absent in cats.

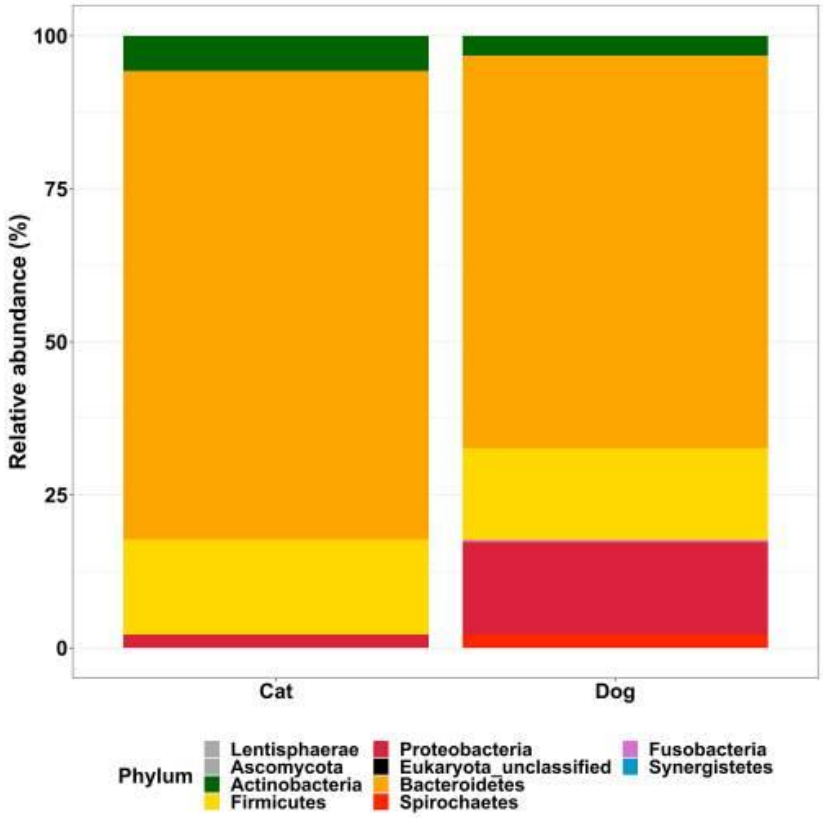

Figure 1. Relative abundance of predominant phyla in faecal samples from 10 healthy cats and 10 healthy dogs

Table 1. Prevalence and relative abundance of bacterial phyla in dog and cat faecal samples selected according to criteria (Ozyme samples, at T0, Core and Middle samples)

Phylum	Animal species	Prevalence (%)	Mean relative abundance (%) (SD)
Spirochaetes	Cat	0.0	0.0
	Dog	38.1	2.2 (5.6)
Proteobacteria	Cat	96.1	2.2 (5.99)
	Dog	97.8	15.1 (15.1)
Fusobacteria	Cat	0.0	0.0
	Dog	10.4	0.3 (1.2)
Firmicutes	Cat	100	15.6 (8.3)
	Dog	100	15.0 (15.2)
Bacteroidetes	Cat	100	76.5 (16.0)
	Dog	100	64.1 (22.4)
Actinobacteria	Cat	100	5.7 (8.6)
	Dog	100	3.2 (3.4)

Values for > 10% prevalence shown, based on faecal samples from ten healthy cats and ten healthy dogs

In the canine samples, the majority of dogs exhibited a gut microbiome dominated by Bacteroidetes, followed in abundance by Firmicutes, Proteobacteria, and Actinobacteria **(Figure 1; Table 1)**, with only two individuals deviating from this pattern. Minor phyla such as Spirochaetes and Fusobacteria were detected in some dogs, with overall occurrence rates of 38.1% and 10.4%, respectively. Across both cats and dogs, Bacteroidetes, Actinobacteria, and Firmicutes were consistently present in all animals.

At the genus level, Prevotella emerged as the most prevalent in cats, comprising roughly two-thirds (66.7%) of all classified reads **(Figure 2; Table 2)**. In dogs, Prevotella also dominated but represented a smaller portion of the microbiome, averaging 49.0% of reads **(Figure 2; Table 3)**. Both species exhibited 100% prevalence of this genus. Bacteroides followed in abundance, also present in all samples, contributing an average of 14.7% in cats and 7.8% in dogs. Other dominant genera differed between species: in cats, Collinsella accounted for about 4.0% and Megasphaera for 3.6% of reads, while Bifidobacteria made up 1.7%. In dogs, unclassified Proteobacteria

represented 7.1% on average, with Escherichia and Streptococcus contributing 5.4% and 3.6%, respectively, whereas Megasphaera was absent and Bifidobacteria were nearly undetectable.

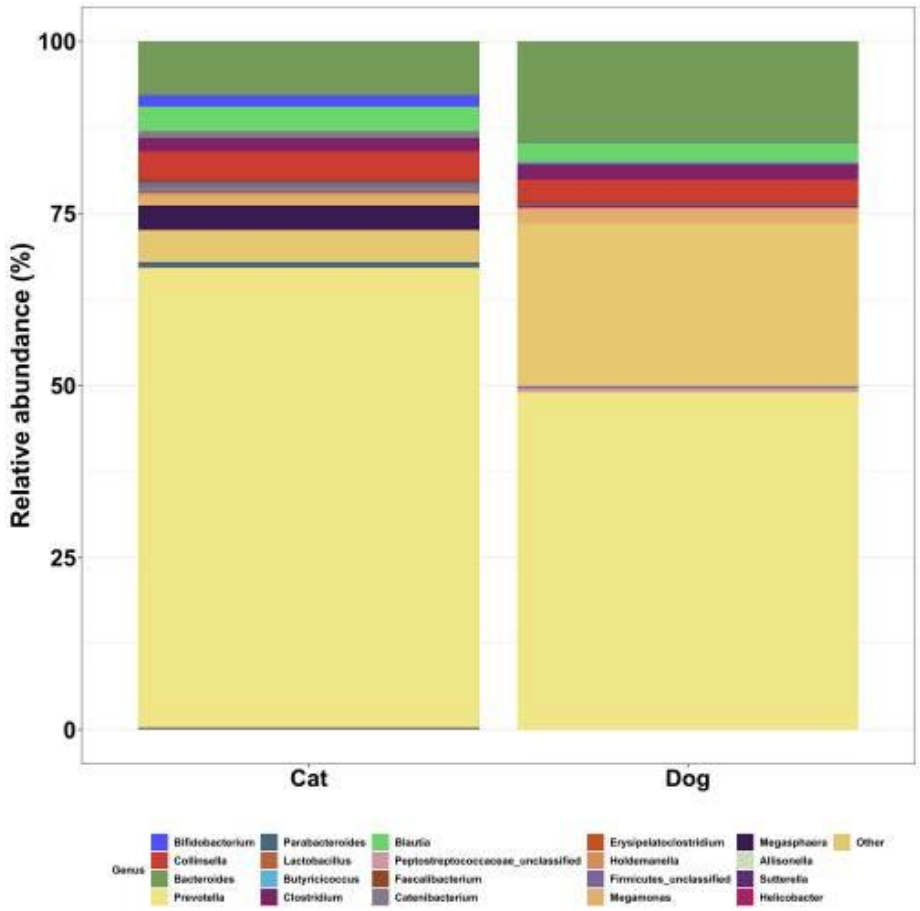

Figure 2. Relative abundance of predominant genera in faecal samples from 10 healthy cats and 10 healthy dogs

Table 2. Prevalence and relative abundance of bacterial genera in dog and cat faecal selected according to criteria (Ozyme samples, at T = 0, Core and Middle samples)

Genus	Animal species	Prevalence (%)	Mean relative abundance (%) (SD)
Streptococcus	Cat	2.3	< 0.1
	Dog	49.2	3.6 (7.5)
Proteobacteria	Cat	0.0	0.0
	Dog	38.8	7.1 (12.1)
Prevotella	Cat	100.0	66.7 (17.3)
	Dog	100.0	49.0 (28.4)
Megasphaera	Cat	100.0	3.6 (4.2)
	Dog	0.0	0.0
Escherichia	Cat	7.0	< 0.1
	Dog	76.1	5.4 (11.3)
Collinsella	Cat	100.0	4.0 (7.1)
	Dog	100.0	3.2 (3.5)
Blautia	Cat	100.0	3.6 (3.0)
	Dog	100.0	2.6 (4.05)
Bifidobacteria	Cat	88.3	1.7 (3.3)
	Dog	35.8	< 0.1
Bacteroides	Cat	100.0	7.8 (6.12)
	Dog	100.0	14.7 (12.7)

Top five most abundant genera for each species shown, based on faecal samples from ten healthy cats and ten healthy dogs

Table 3. Most prevalent bacterial species in cat and dog faeces samples selected according to the folloeing criteria: Ozyme tube samples, at T = 0, core and middle samples

Bacterial species	Adjusted p-value	Significance level	Mean relative abundance cat (%) (SD)	Mean relative abundance dog (%) (SD)
Prevotella copri	0.02	*	65.1 (16.7)	29.5 (27.3)
Holdemanella biformis	0.01	*	0.39 (0.43)	0.06 (0)
Firmicutes bacterium CAG646	0.03	*	0.21 (0.0)	0.05 (0)
Firmicutes bacterium CAG424	0.005	**	0.34 (0.0)	0.05 (0)
Collinsella stercoris	0.003	**	2.16 (3.42)	0.09 (0.08)
Collinsella intestinalis	0.003	**	0.18 (0.22)	3.06 (3.44)
Clostridium hiranonis	0.008	**	0.10 (0.1)	0.67 (0.8)
Catenibacterium mitsuokai	0.02	*	0.97 (1.04)	0.22 (0.5)
Butyricicoccus pullicaecorum	0.03	*	0.03 (0.05)	0.22 (0.2)

Significant differences in relative abundance between cat and dog core faeces sample taken at timepoint T0. Adjusted *p* values refer to Benjamini-Hochberg corrected Wilcoxon rank sum test comparisons
Comparisons of relative abundance by genus across individuals demonstrated individual variability **(Figure 3)**.

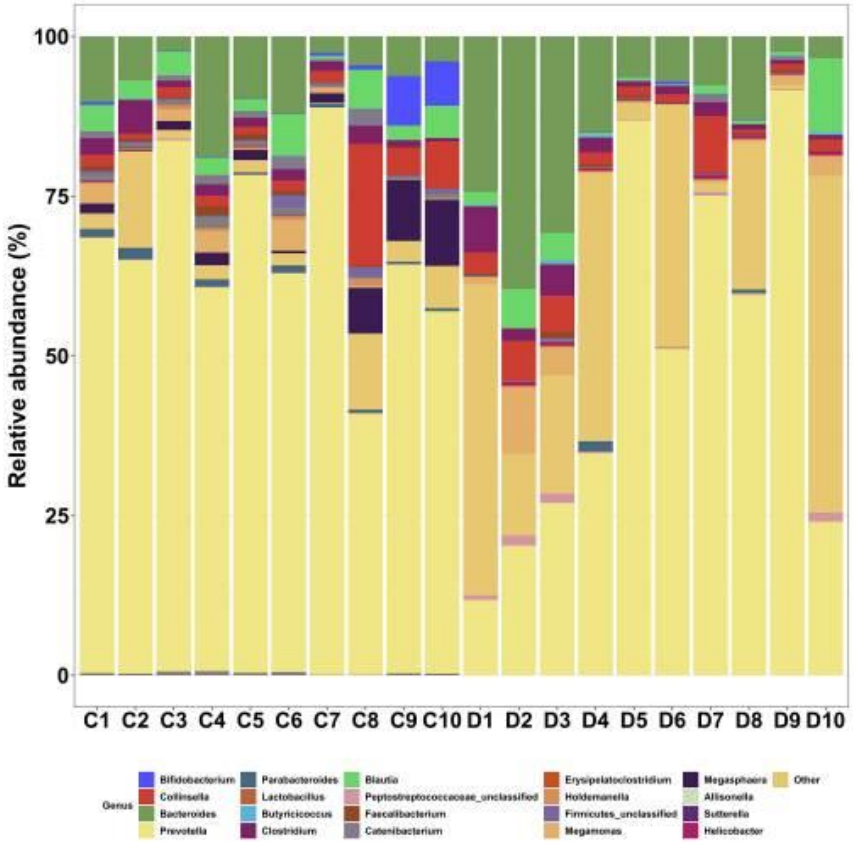

Figure 3 presents the predominant bacterial genera found in individual faecal samples from 10 cats (C) and 10 dogs (D). Several species were detected almost exclusively in cats, such as *Blautia wexlerae*, *Collinsella aerofaciens*, *Flavonifractor plautii*, *Megasphaera elsdenii*, *Ruminococcaceae bacterium D16*, *Fusicatenibacter saccharivorans*, and *Bifidobacterium pullorum*. Conversely, *Gemmiger* sp. An194 and *Helicobacter bilis* appeared only in canine samples

When evaluating species present in at least 60% of samples from both hosts, statistical analysis revealed significant differences for five species (adjusted $p < 0.05$), with four species showing even stronger significance (adjusted $p < 0.01$) as listed in **Table 3**.
Alpha diversity was assessed using the core portion of the first-emitted stool (T0) to standardize sampling. Cats and dogs exhibited similar bacterial richness, with cats averaging 43.4 ± 6.3 species (median 45.5) and dogs

41.4 ± 11.5 species (median 39.0). No significant difference was observed between the two species (adjusted p = 0.20; **Figure 4**), indicating comparable levels of microbial diversity at the individual sample level.

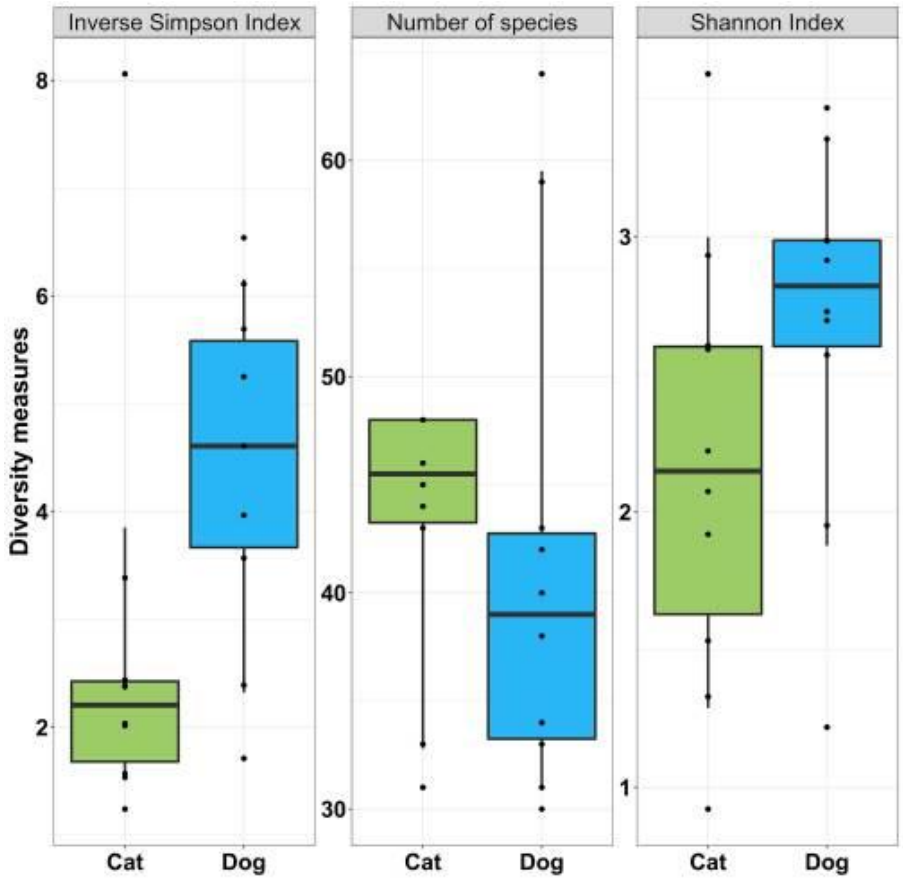

Figure 4. illustrates faecal microbial diversity in cats and dogs, assessed using three commonly applied metrics, with samples collected at T0 from the middle portion (region ii) of the stool. While differences were not statistically significant, dogs tended to show higher alpha diversity. The Inverse Simpson Index averaged 4.4 ± 1.6 in dogs compared with 2.7 ± 2.0 in cats (adjusted p = 0.05). Similarly, Shannon Index values were generally higher in dogs, though this difference did not reach statistical significance (adjusted p = 0.18). Overall, no significant divergence in alpha diversity was observed between species, but the data suggested a modest trend toward greater diversity in dogs

To investigate whether sampling location within the stool influenced microbiome measures, T0 core samples were analyzed from three horizontal positions. Across both cats and dogs, the choice of sampling site did not significantly affect microbial diversity. This held true for the Inverse Simpson Index (cats adjusted p = 0.06; dogs adjusted p = 0.81), total species richness (cats adjusted p = 0.85; dogs adjusted p = 0.81), and Shannon Index (cats adjusted p = 0.06; dogs adjusted p = 0.81) **(Figure 5)**. These findings indicate that, at least for standardized core samples, horizontal variation within the stool has minimal impact on overall microbial diversity or the number of taxa detected.

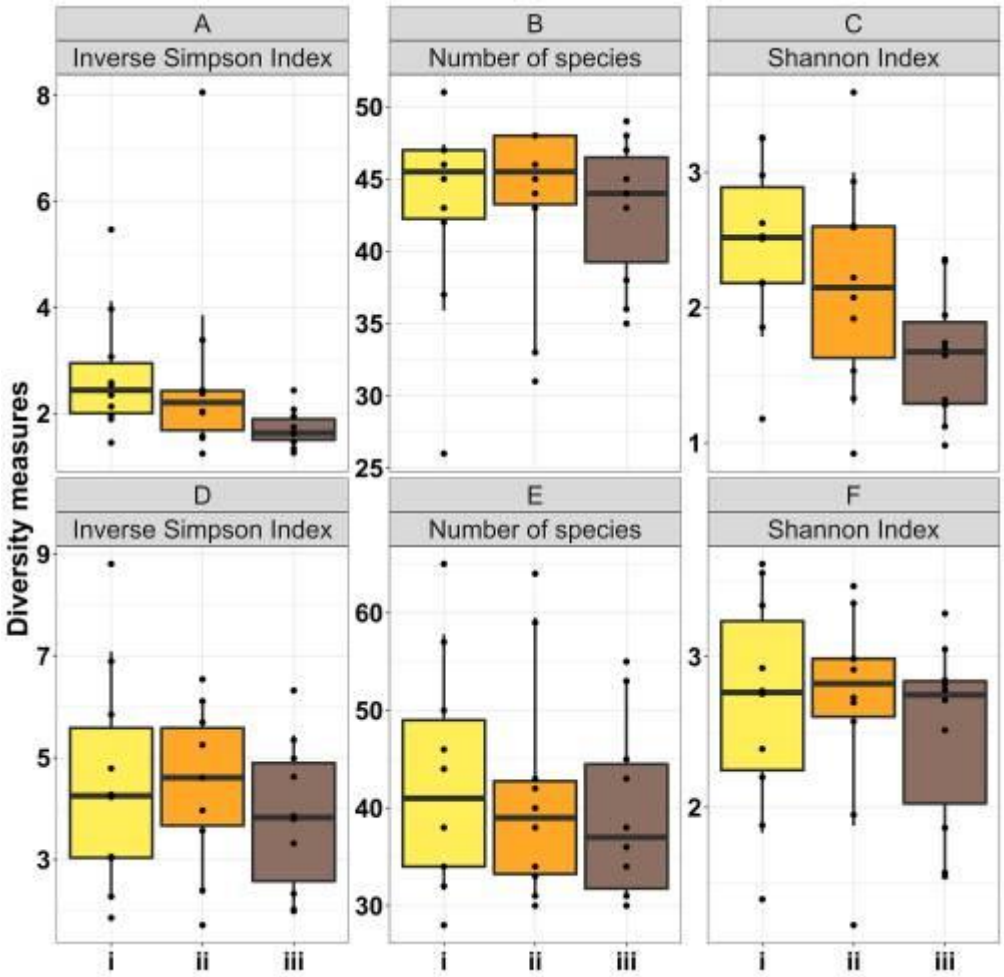

Figure 5. Illustrates how microbial diversity varies across different regions of cat and dog faecal samples at the initial timepoint (T0). When examining samples from the middle portion (region ii), no meaningful differences were observed between surface and core material for either species, suggesting that the depth of sampling has little effect on microbial richness or diversity immediately after defecation

By 12 hours (T12), however, subtle changes emerged in the terminal portion (region iii) of cat faeces. Surface samples showed slightly higher alpha diversity than core samples, with Shannon Index values rising from 1.8 ± 0.7 in the core to 2.3 ± 0.7 at the surface (adjusted $p = 0.018$), and the Inverse Simpson Index increasing from 2.3 ± 1.8 to 2.7 ± 1.6 (adjusted $p = 0.048$). Interestingly, the total number of detected species remained statistically similar (adjusted $p = 0.06$).

Dogs displayed a comparable, though less pronounced, pattern at T12. Surface samples from region iii exhibited a small increase in Shannon diversity (2.6 ± 0.7) compared to the corresponding core samples (2.4 ± 0.7; adjusted $p = 0.027$). In contrast, differences in Inverse Simpson Index and species count were minimal and not statistically significant ($p = 0.09$ and 0.37, respectively). These observations indicate that after extended room-temperature storage, microbial diversity can slightly increase at the stool surface, particularly in the last-emitted portion, but the effect is modest and varies depending on the diversity metric used.

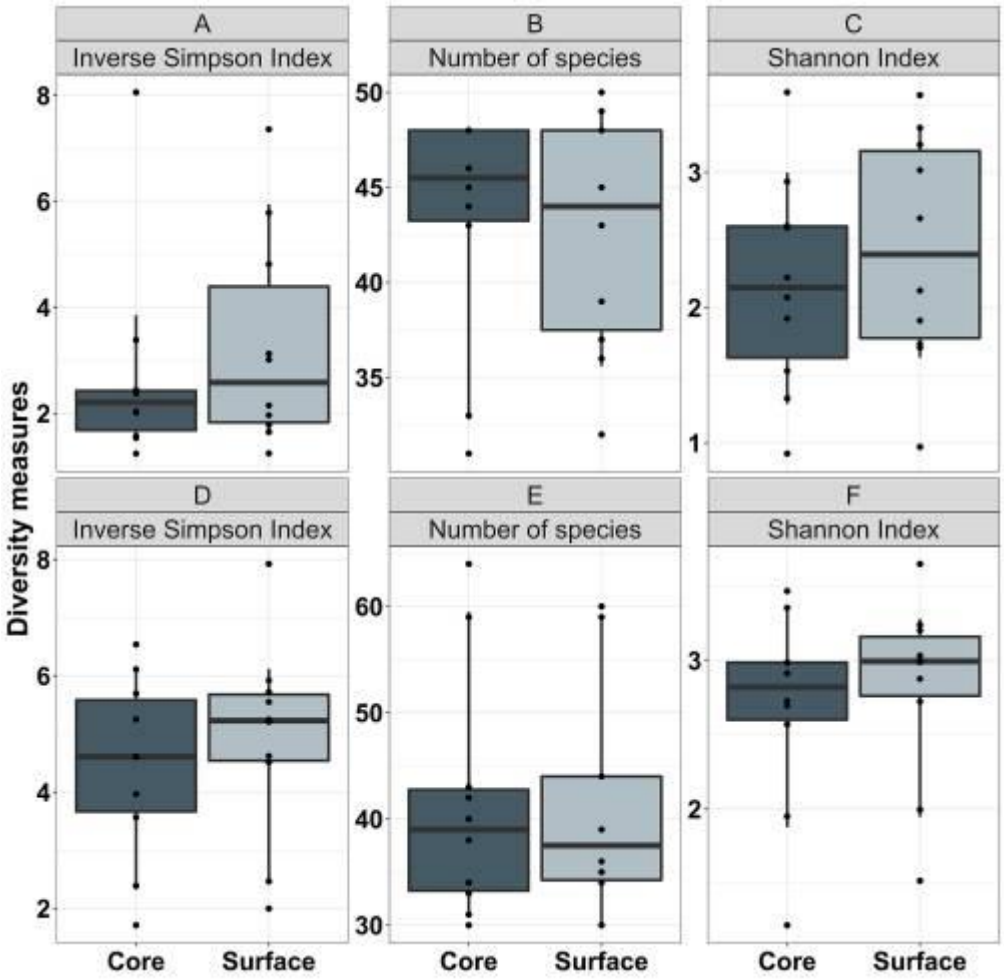

Figure 6. compares microbial diversity between core and surface samples from healthy cat (A–C) and dog (D–F) faeces after 12 hours of storage at room temperature. Analyses revealed that keeping samples at ambient temperature for this duration did not meaningfully affect alpha diversity in either species. Kruskal-Wallis testing confirmed no significant changes, with all adjusted p-values equal to 0.74 for cats and 1.00 for dogs across all diversity metrics

Similarly, the type of stabilization buffer used had no detectable effect on microbial diversity within dogs; comparisons across both buffers showed no significant differences for any alpha diversity measure (adjusted $p = 0.5$).

Beta diversity analysis, summarized in **Figure 7**, highlighted that individual animal identity was the dominant factor shaping faecal microbial composition. PERMANOVA results indicated strong effects of individuality, with R^2 values of 0.64 for cats and 0.88 for dogs ($Prf < 0.001$). In cats, sampling depth and horizontal stool location also reached statistical significance ($Prf < 0.001$ for both), but the magnitude of their contributions was relatively small ($R^2 = 0.027$ and 0.037, respectively). Time of sampling had no significant effect on beta diversity in cats ($Prf = 0.07$), indicating that short-term storage at room temperature preserves the overall microbial structure.

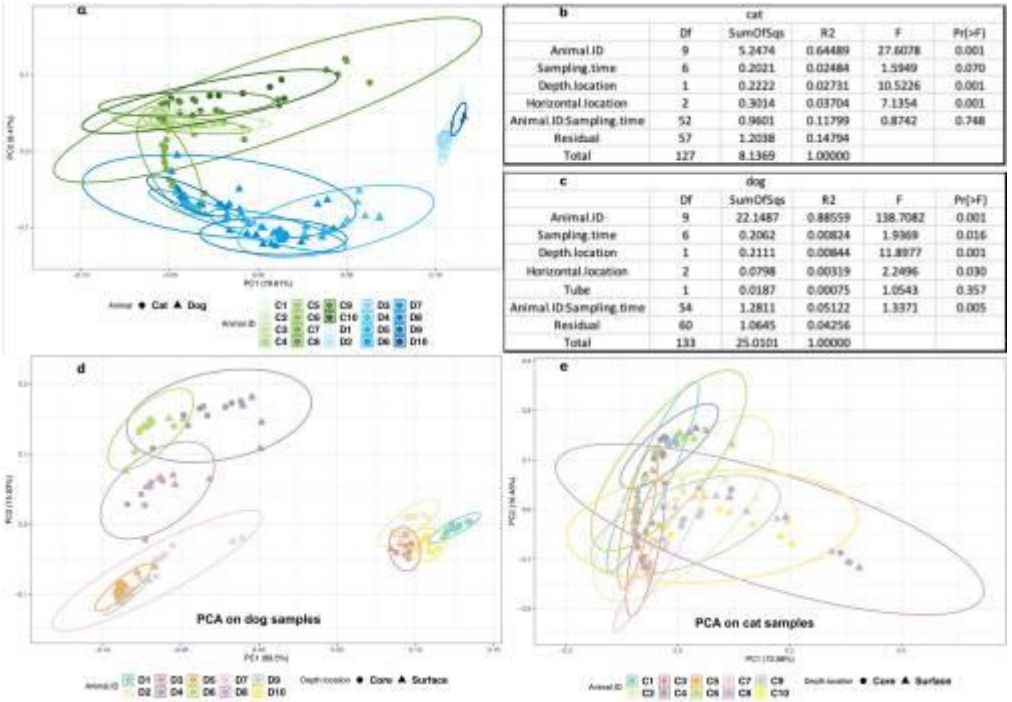

Figure 7 presents beta diversity analyses using Principal Component Analysis (PCA) and PERMANOVA. Panel A shows PCA for all samples, while Panels B and C display PERMANOVA results for cats and dogs, respectively. Separate PCA plots for dog and cat samples are shown in Panels D and E

For dogs, the depth of sampling within the stool was the second most influential factor on microbial composition, although its impact was substantially smaller than the effect of individual identity (Prf = 0.001; R^2 = 0.008). Sampling time also reached significance at a conventional threshold (Prf = 0.016), with an effect size similar to sampling depth (R^2 = 0.008). In contrast, the type of stabilization buffer did not noticeably affect beta diversity (PERMANOVA $p > 0.3$), and any minor differences observed between buffers followed the same trends across both conditions.

Impact of experimental variables on DNA yield

DNA concentration varied among individual animals, as illustrated in pooled T0 data **(Figure 8)**. Overall, dog faecal samples contained significantly more DNA than cat samples, with mean concentrations of 6.3 ± 3.9 ng/µl versus 3.1 ± 1.9 ng/µl (adjusted p = 0.016; **Figure 9**). Notably, DNA yield in dogs showed a wider range compared with cats, highlighting greater inter-individual variability in canine samples.

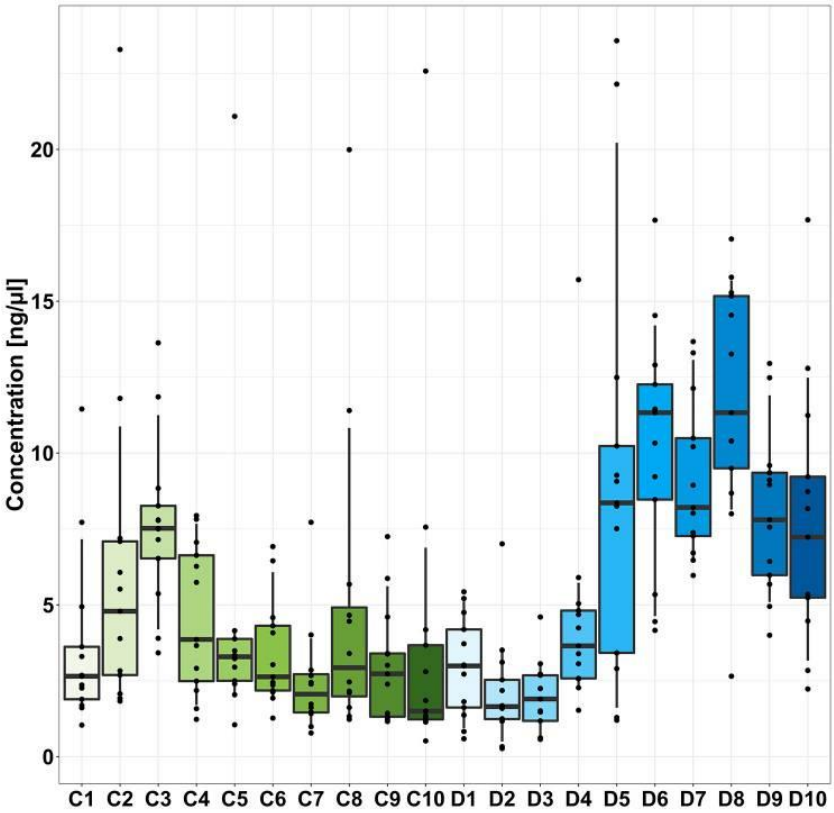

Figure 8. DNA concentration in T0 samples for 10 individual healthy cats (**C**) and 10 individual healthy dogs [10]

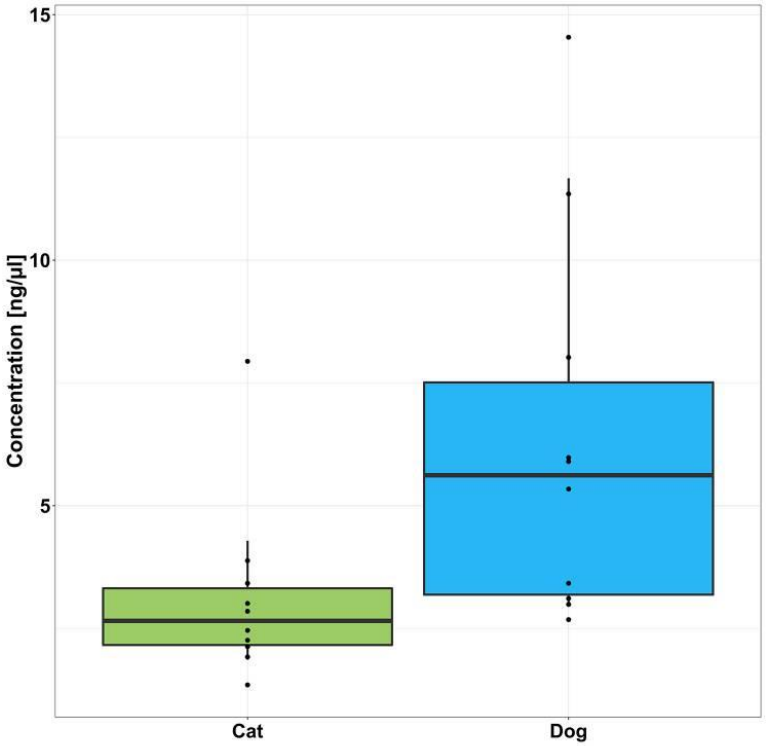

Figure 9.

DNA Yield across sampling locations and timepoints

Analysis of DNA concentration in core samples from the middle region (region ii) at T0 revealed notable differences between surface and core material in cats. Surface samples contained higher DNA amounts (5.8 ± 1.5 ng/µl) compared with core samples (2.8 ± 2.2 ng/µl; adjusted $p = 0.044$). Beyond this observation, neither horizontal sampling position nor depth significantly affected DNA concentration in either cats or dogs.

Over the course of the study, DNA levels in region ii core samples remained stable from T0 to T6 for both species (adjusted $p = 0.99$). By T12, cat surface samples exhibited a substantial increase in DNA concentration (from 3.5 ± 2.1 ng/µl at T0 to 10.7 ± 7.6 ng/µl; adjusted $p = 0.045$), whereas cat core samples and all canine samples showed no significant temporal change (all adjusted $p > 0.05$).

Discussion

Overall, the findings indicate that short-term room-temperature storage (up to 12 hours), horizontal sampling location, sampling depth, and the choice between two stabilization solutions have minimal influence on alpha diversity, taxa abundance, or DNA yield in cat and dog faeces. Beta diversity analysis using the Bray-Curtis metric reinforced that inter-individual differences are far more influential than any methodological variable. Sampling time had a minor effect on canine samples but not feline samples, and horizontal location within the stool contributed little to variability in either species. The type of collection device for dog samples did not alter beta diversity, and PCA and PERMANOVA analyses confirmed that samples consistently clustered by animal identity, underscoring the limited impact of sampling factors.

These results support prioritizing core sampling of cat stools to avoid potential contamination from litter substrates. Interestingly, horizontal location had little effect on microbial profiles, contrary to expectations based on intestinal oxygen gradients. It appears that faecal samples predominantly reflect transient luminal bacteria rather than mucosa-associated populations, which are more sensitive to oxygen exposure along the colorectum.

From a practical standpoint, these findings are relevant for collecting samples from privately-owned pets. Dogs can generally provide fresh samples during walks, but cat stools may remain in litter boxes for several hours. The study indicates that room-temperature storage for up to 12 hours is generally acceptable for downstream microbiome analyses. However, the observed rise in alpha diversity and DNA concentration in cat surface samples at 12 hours—and a similar trend in dogs—suggests that stools exposed to ambient conditions for more than six hours should ideally be sampled from the core to avoid potential bias.

Molecular analyses of faecal samples provided detailed insight into differences in bacterial communities between healthy cats and dogs, particularly regarding the prevalence and relative abundance of specific taxa. In both species, the dominant phyla were Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria, consistent with previous studies [4, 7, 17–21]. Our findings also align with earlier work reporting higher Proteobacteria abundance in canine faeces, while Actinobacteria were more prominent in cats [22, 23]. Interestingly, Fusobacteria were absent in the feline samples, which contrasts with some reports describing this phylum as common in cat faeces; however, abundance in healthy cats is typically very low (<0.5%) [8, 18–20]. Dietary factors may account for this observation: short-term feeding studies have shown that cats consuming dry diets have markedly lower Fusobacteria levels (0.3%) compared with wet diets (23.1%) [24]. All cats in this study were primarily fed dry food, with only one receiving a daily portion of wet food. Additionally, dietary protein content has been linked to Fusobacteria abundance, with moderate-protein diets associated with reduced levels [25].

At the genus level, Prevotella was universally present in both cats and dogs, with mean relative abundances of 66.7% and 49.0%, respectively. This prevalence likely reflects the capacity of Prevotella to degrade a wide array of polysaccharides, consistent with the high fiber and carbohydrate content common in commercial pet foods [26]. Bacteroides was the second most abundant genus in both species (14.7% in cats, 7.8% in dogs). In dogs, Bacteroides and Prevotella abundances appear inversely related to Fusobacteria levels, suggesting potential niche competition [9]. In the present study, cats exhibited a combined Prevotella and Bacteroides abundance of 74.5%, which may partly explain the absence of Fusobacteria.

At the species level, the most notable difference between cats and dogs was the abundance of *Prevotella copri*. Although prevalence was high in both species (100% in cats, 82.1% in dogs), mean relative abundance was markedly higher in cats (65.1% versus 29.5%). This observation exceeds values reported in previous studies, such as a whole-genome metagenomic analysis that found *P. copri* represented 12.9% of the healthy feline gut microbiome [27]. While *P. copri* abundance has been associated with obesity, none of the animals in the current

study were obese [27, 28]. The dominance of *P. copri* in cats may have influenced overall microbial diversity, contributing to the trend toward slightly lower alpha diversity in cats compared with dogs, although differences were not statistically significant. Prior research has sometimes reported higher gut microbiome diversity in cats, highlighting the need for further investigation [7, 22, 29].

Considerable inter-individual variation in microbial composition was observed, consistent with known influences of age, breed, diet, and other host-specific factors on faecal microbiota [7, 9, 30–33]. The study deliberately included a heterogeneous cohort to capture a broader picture of bacterial diversity; however, the lack of standardization for factors such as breed and age may have contributed to the observed variation.

Preliminary associations between dietary fiber type or quantity and faecal microbial composition were noted (data not shown), in agreement with prior studies [34–36]. Given the small sample size and diversity of diets in this study, additional research is needed to clarify these relationships.

Limitations

Several limitations should be acknowledged when interpreting these findings. The study was exploratory in nature, with a relatively small number of animals, and variables such as diet, age, and breed were not controlled. Consequently, the results may primarily reflect cats and dogs fed dry, commercial diets, and caution is warranted when extrapolating to animals consuming alternative diet formats or to broader populations, including privately-owned pets. Additionally, the methods used in this study did not assess the functional characteristics, viability, or full genomic features of the bacterial strains identified. Finally, estimation of diversity using rarefaction curves was not possible, as MetaPhlAn3 relies on relative abundance rather than raw sequencing counts.

Conclusions

This study demonstrates that, in both cats and dogs, faecal samples remain largely stable for up to 12 hours at room temperature in terms of DNA concentration and alpha diversity. Sampling from different horizontal regions of the stool or from the surface versus core generally had minimal impact on microbial profiles, with individual animal identity emerging as the primary determinant of observed diversity. Surface samples from faeces stored at room temperature for more than six hours may show modest alterations, suggesting core sampling is preferable in these circumstances. The lack of influence from the choice of stabilization buffer provides flexibility for future studies.

These findings provide a foundation for designing both in-house research and studies involving privately-owned pets, supporting reliable microbiome analyses under practical sampling conditions while informing best practices for sample collection and handling.

Methods

Study animals

The experiment involved ten healthy adult cats (average age 3.8 ± 2.7 years; range 2.2–11.1 years) and ten healthy adult dogs (average age 3.2 ± 1.5 years; range 1.5–5.1 years). The study was conducted in July 2021 **(Table 1)**. Health status was confirmed through clinical examinations and standard hematology and biochemistry panels. Cats were obtained from Isoquimen Company, and dogs were sourced from breeders certified by the French Research Ministry. Animals were selected based on regular defecation of stools ≥15 cm in length. Animals were excluded if they had received any medication in the preceding 14 days or antibiotics within the past six weeks. All animals were treated with routine antiparasitic and deworming protocols.

Housing was provided at the Royal Canin Research Centre, Aimargues, France, in accordance with European animal welfare regulations, and all procedures were approved by the Royal Canin Ethics Committee. Dogs were individually housed indoors with unrestricted access to outdoor runs. Indoor temperatures ranged from 22.3 to 22.9 °C, and lighting included natural and artificial sources from 06:30 to 18:30. Dogs were provided daily exercise outdoors (40–60 minutes) and 20 minutes of human interaction. Cats were housed in groups in social rooms with ambient temperatures between 22.2 and 23.6 °C, received daily social interaction, and had access to enclosed outdoor spaces. Cats used litter boxes filled with Cat's Best® Comfort non-clumping wood-based litter.

Animals were fed nutritionally complete dry diets tailored to individual energy requirements, with one cat receiving a daily wet food supplement.

Faecal sample collection

Freshly voided faeces were collected from each animal and kept at room temperature (20 °C) in sterile containers. Cat stools were collected prior to contact with litter substrate. Faeces were assessed using a modified nine-point scale based on Moxham (2001), where 1 indicates hard and crumbly stool and 5 indicates liquid. Stools scoring 2–3.5 were considered acceptable for inclusion.

Approximately 1 g of faeces was sampled at multiple timepoints: 0, 0.5, 1, 2, 3, 6, and 12 hours after collection. At the initial timepoint (T0), three horizontal regions of the stool were sampled: first emitted (i), middle (ii), and last emitted (iii). Samples were taken from both the surface and the core of each region, resulting in six samples per animal at T0. Surface and core were distinguished visually, with the first portion being drier and the last portion softer and moister. Surface samples from cat faeces were taken only from areas not in contact with litter. Between T0.5 and T6, only core samples from the middle region (ii) were collected. At T12, both surface and core samples were collected from the last portion (iii). All samples were preserved at −80 °C using DNA/RNA™ Shield (Ozyme).

To evaluate the effect of stabilisation buffer, selected aliquots from five dog samples (first emitted part i, core, T0–T1) were stored in PERFORMABiome™ 200 (DNAgenotek®, Ottawa, Canada). All samples were coded individually and analyzed blinded to ensure unbiased results.

DNA extraction

DNA was isolated using a magnetic bead-based protocol at Eurofins Genomics Europe (Germany), incorporating an additional shredding step before lysis. Extracted DNA was stored in 1.5 mL microcentrifuge tubes at −80 °C until downstream sequencing analysis.

DNA preparation and sequencing

Shotgun metagenomic libraries were generated using the NEBNext Ultra II FS DNA Library Prep Kit (New England Biolabs) with enzymatic fragmentation optimized for Illumina sequencing. Sequencing was carried out on an Illumina NovaSeq 6000 platform, producing paired-end reads of approximately 150 base pairs in length. Each sample yielded a minimum of 20 million paired-end reads.

Bioinformatics processing and statistical analysis

Raw paired-end reads were first quality filtered to remove low-quality sequences, defined as reads with a Phred score below 15. Taxonomic profiling was performed using MetaPhlAn 3.0 [38] with the ChocoPhlAn database (version mpa_v30_ChocoPhlAn_201901). MetaPhlAn utilizes clade-specific marker genes to estimate the relative abundance of microbial taxa, which were then visualized as bar plots at the phylum, genus, and species levels.

Alpha diversity was quantified using the Shannon Index [39] and the Inverse Simpson Index [40], both of which incorporate species richness and evenness, with higher values indicating greater diversity. Beta diversity was assessed using the Bray-Curtis dissimilarity metric based on species-level relative abundances. Principal Component Analysis (PCA) was employed to visualize similarities among samples, with one PCA including all cat and dog samples and separate PCA plots for each species.

The contribution of experimental variables to observed beta diversity was further evaluated using PERMANOVA (adonis2 function, R package vegan v2.6-4), assessing factors such as individual animal identity, sampling time, depth (surface versus core), horizontal stool region, and stabilization tube.

For statistical comparisons, non-parametric tests were applied. Wilcoxon rank-sum tests were used for unpaired two-group comparisons, and Wilcoxon signed-rank tests for paired comparisons. For analyses involving more than two groups, Kruskal-Wallis rank-sum tests were applied. Multiple testing correction was performed using the Benjamini-Hochberg method. Statistical significance was set at $p < 0.05$. Data management and all analyses were conducted in R version 4.0.3.

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Conflict of Interest: Xavier Langon is an employee of Royal Canin, with an interest in petfood manufacture.

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Ethics Statement: All the pets (dogs and cats) are our animals from our facilities. All experiments are performed in accordance with relevant guidelines and regulations. This study was conducted in accordance with the Mars Animal Research Policy (www.mars.com), adhering to the 3Rs approach to animal research as described by Robinson (2005) [41] and complies with the ARRIVE guidelines [42]. All studies were approved by the Royal Canin Ethic Committee.

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