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Waltham catalogue for the canine gut microbiome: a complete taxonomic and functional catalogue of the canine gut microbiome through novel metagenomic based genome discovery

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Abstract

Background The canine microbiome is a vastly understudied area relative to the importance of dogs in society, particularly given the potential importance of the microbiome in veterinary medicine. This has led to a large knowledge gap in the basic taxonomy and functions of the canine gut microbiome and an overreliance on human databases for canine-specific research. Using a broad sample set, long read sequencing, short read sequencing, and metagenomic assembly approaches, we have produced the most comprehensive microbiome resource in all companion animal research.

Results Here, we describe the recovery of 240 core species that account for > 80% of the canine gut microbiome when tested on an independent validation dataset. We uncovered > 900 new canine-specific strains, 89 novel species, and 10 novel genera, providing a dramatic increase in previous knowledge of the canine microbiome and allowing for mapping rates of up to 95%, a 70% increase on historic mapping rates of ~ 25% using publicly available resources. Through detailed annotation of function, we demonstrate the potential importance of the novel species and genera to health and nutrition and provide evidence of new canine-adapted strains of existing genera and species previously unknown to inhabit canines that provide important metabolic function to the canine host. We discovered the canine microbiome has an expansive ability to metabolize carbohydrates, providing insight into how canines process diverse carbohydrates given their known limited host genomic potential. We uncovered a range of species with abilities to produce butyrate, propionate, and vitamins, highlighting the importance of the canine microbiome to host nutrition. We describe two novel *Peptacetobacter* species that could regulate host bile acid metabolism, an important finding in the context of chronic GI disease in pets. We demonstrated all new species and genera had no known virulence, suggesting they are commensal and, finally, provided a baseline for antimicrobial resistance in the microbiota species of healthy pets.

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Conclusions This work gives entirely new perspectives on the functional capabilities of the canine gut microbiome, suggesting the canine microbiome is distinct, presumably having evolved to its host, diet, and environment over several millennia.

Keywords Dog, Gut microbiome, Metagenomics, Metagenome-assembled genomes

Background

Composed primarily of bacteria and archaea, the gastrointestinal microbiome contributes to essential host metabolic function, immune system education, and pathogen protection [49]. Either directly or indirectly, a healthy and resilient gut microbiome is central to countless physiological processes [3]. Consequently, the role of the microbiome in both human and animal health, as well as in the etiology and progression of various diseases, has become increasingly apparent [38]. Recently, evident correlations have emerged between gut microbial dysbiosis and a number of acute and chronic canine GI disorders, such as chronic enteropathy (CE) [26, 49]. With probiotic candidates, fecal microbiota transplants, and dietary interventions all showing promising results in modulating aspects of canine GI function [4, 32, 57], fully elucidating the microbial species inhabiting this niche and their functional significance is of pressing importance to pet owners, veterinarians, and the pet food industry alike.

Leveraging recent advances in shotgun metagenomic sequencing, the gut microbiome can now be studied at high resolution, annotating both taxonomic presence and functional potential. Commonly, metagenomic data is annotated via the mapping of sequencing reads to databases of known genes or genomes. These databases are comprised mostly of cultivable microbes that have been isolated and sequenced [64]. Consequently, a vast proportion of reads in a metagenomic sequencing run remain unmapped due to the presence of uncultured and unknown microbes [71]. Lloyd et al. estimated that in nonhuman-associated environments, 22% to 87% of genera have not been cultured, compared to 3% to 55% of genera in human-associated environments [37]. These data suggest that current microbiome databases are not only lacking knowledge but are also biased towards human-associated taxa, potentially significantly limiting the insights drawn from research in animal species and environmental samples.

To increase our knowledge of the microbial world, an approach that has proven to be successful is the assembly of genomes from metagenomes. In this strategy, overlapping fragments of DNA sequences from shotgun metagenomics are first assembled into longer contiguous sequences (contigs). Contigs are further grouped or 'binned' based on nucleotide frequency, marker genes phylogenies, and/or DNA sequence coverage to get near

complete metagenome-assembled genomes (MAGs) [9]. After the incorporation of MAGs into reference databases, mapping rates of human gut metagenomic data increased from 67.76% to 87.51% [47]. Beyond their generation within human research, MAG recovery has since been widely applied within the field of animal science. Most notably utilized to study the microbiome of food production species such as pigs [12, 25], cattle [61, 66, 70] and poultry [22, 23], the expansive catalogue of novel MAGs recovered has rapidly increased our taxonomic and functional understanding of these previously unexplored environments. Only recently, and to a far lesser extent, have MAGs been generated from companion animals including horses [21, 34], dogs [1, 10, 14, 15, 76] and cats [10, 39, 52]. In canines, two notable studies have used MAGs to investigate the microbiome. However, these studies have often focused on the linkages with humans rather than the functional role of the microbiome and how it pertains to canine metabolism and health [10, 13]. The latter is incredibly important from a veterinary standpoint, and in this regard, it is key to understand how the microbiome in canines is adapted for the host and its role in health. A large proportion of the canine gut microbiome remains unknown; only 25% of canine metagenomic sequencing reads map to known RefSeq prokaryotic genomes and 40–60% to a curated canine-specific database (MetaGeneCanine™, Diversigen, US). Without the increased database granularity provided by novel species discovery and appropriate description of the roles of these organisms with a focus on the host in question, our understanding of the canine gut microbiome is inherently limited.

Over a number of decades, research has been performed on the nutritional requirements of dogs leading to substantial findings in key areas such as management of disease, growth, and aging [8, 31, 53]. Given the emergence of the microbiome as a key driver of health and disease, we aimed to produce the most comprehensive catalogue of the gut microbiome of dogs to date, including detailed descriptions of the key functions of the canine strains. Using 501 fecal samples collected from 107 dogs from across the USA and Europe and a combination of long-read and short-read sequencing, we describe the reconstruction of 5753 MAGs, dereplicated to 1031 strains, including 982 new canine-specific strains, 89 novel species, and 10 novel genera that provide new

views on the structure, function, role, and evolution of the canine microbiome and provide a resource for use by all researchers in the microbiome space.

Methods

Sample selection

This study is based on metagenomic sequencing data from the Mars Petcare archive (Additional Table S1). Fecal samples were obtained from four cohorts (Table 1): (1) dogs housed in environmentally enriched kennel facilities at the Waltham Petcare Science Institute (Leicestershire, UK) and the Pet Health and Nutrition Center (OH, USA), (2) dogs owned by clients of Mars Veterinary Health (MVH) Hospitals from across the United States of America, and (3) dogs owned by employees of Mars Petcare living in their home environments located in France, United Kingdom, and the United States of America (Fig. 1). In total, 501 fecal samples from 107 dogs were included in the discovery set. All dogs were deemed healthy without any uncontrolled medical condition at the time of sampling and were not selected for breed, age, sex, or neutered status. An additional cohort (4) of 47 dogs (16 beagles, 23 Labrador retrievers, and 8 Norfolk Terriers) housed at the Waltham Petcare Science Institute was used to validate the abundance and prevalence of the MAGs in an independent dataset. All sample collections were approved by the Waltham Petcare Science Institute Animal Welfare and Ethical Review Board. Owners of pets involved were provided with information about the study, including how the samples would be used, and signed a consent form before participation.

Feces collection

Samples from (1) and (4) were kept at 4 °C until aliquoted (200 mg) and frozen at −80°C within 3 h of defecation. Samples from (2) and (3) were placed within 1 h of defecation into Performabiome.GUT collection tubes (DNAGENOTEK) according to the manufacturer’s instructions (~200 mg), shaken for 30 s to mix with

the stabilizing liquid, sent to the laboratory, aliquoted (250 µl), and stored at −80 °C (Fig. 1).

Sequencing

Shotgun metagenomics

All samples were subjected to short-read metagenomics sequencing following one of two different workflows (Fig. 1). For samples from (1) and (2), DNA was extracted with the PowerSoil Pro DNA Isolation Kit (Qiagen) automated for high throughput on the QiaCube HT (Qiagen). Mechanical lysis was completed via bead beating using Powerbead Pro plates (Qiagen), which contain 0.5 mm and 0.1 mm ceramic beads. Genomic DNA was quantified using the Quant-IT PicoGreen dsDNA Assay Kits and Reagents (Thermo Fisher Scientific). Libraries were prepared with a proprietary procedure adapted from the Nextera XT DNA Library Prep Kit (Illumina) and sequenced at Diversigen (USA) on an Illumina NovaSeq 6000 using paired-end sequencing (2×150 bp). Samples from (3) were extracted using the NucleoSpin 96 Soil Kit (MACHEREY-NAGEL) and the standard SL1 buffer according to the manufacturer’s instructions. Genomic DNA was quantified with Qubit dsDNA Quantification Assay Kits (Thermo Fisher Scientific). Libraries were prepared using the NEBNext Ultra DNA Library Kit (New England Biolabs) and sequenced at Eurofins Genomics (Germany) on an Illumina HiSeq 2500 using paired-end sequencing (2×150 bp). DNA sequences were filtered for low quality (Q-Score < 30) and length (< 50 bp), and adapter sequences were trimmed using Cutadapt (v4.6) [40]. Host (CanFam3) sequences were removed using Bowtie2 (v2.5.2) [30]. Samples in which the read depth was less than 30 million were pooled with other samples from the same dog until the 30 million reads were reached. A total of 501 samples were used in the study, and up to 14 samples per dog were pooled into the same FASTQ file for assembly (Additional Table S1).

Table 1 Canine cohorts used in this study

Cohort	Source	Number of dogs (country)
(1)	Dogs housed in environmentally enriched kennel facilities at the Waltham Petcare Science Institute and the Pet Health and Nutrition Center	29 (UK) 23 (USA)
(2)	Dogs owned by clients of MVH in the United States of America	6 (USA)
(3)	Dogs owned by employees of Mars Petcare living in their home environments located in France, United Kingdom or the United States of America	41 (UK) 6 (France) 2 (USA)
(4)	Dogs housed in environmentally enriched kennel facilities at the Waltham Petcare Science Institute	47 (UK)

Long-read metagenomics

Samples from (3) were also subjected to long-read sequencing (Fig. 1). DNA extraction followed the same methodology as samples from (1) and (2). Purified DNA was submitted to the University of Wisconsin-Madison Biotechnology Center. DNA purity and concentration were measured on a NanoDrop One instrument (ThermoFisher Scientific). DNA concentration was verified using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific). DNA size, integrity, and quality were assessed on the Femto Pulse System (Agilent Technologies). Libraries were prepared using the Ligation Sequencing Kit SQK-LSK109 and the Native Barcoding Expansion Kit 96 EXP-NBD196 (Oxford Nanopore Technologies) according to the manufacturer's recommendations. Libraries were quantified using the Qubit dsDNA High Sensitivity Kit (ThermoFisher Scientific). Libraries were sequenced on FLO-PRO002 (R9.4.1) flow cells using the PromethION 24 device. LongQC 1.2.0c [19] was run on the sequencing files, and trimmed reads were used in downstream processes.

Metagenomic assembly

Metagenomic assembly of short sequencing reads was performed individually per pooled sample using MEGAHIT (v1.2.9) (D. [35]) with a minimum length of contigs of 1000 bp. Hybrid assembly of the long-read data and their corresponding short-read data was performed using the hybrid assembler OPERA-MS (v0.8.3) [6] with disabled reference-based clustering and default values.

Binning was performed using MetaBAT 2 (v2.15–25) [29] and followed by an initial round of de-replication, performed by drep (v3.4.2) [44], at 99% average nucleotide identity (ANI) on bins with a minimum completeness of 50% and maximum genome contamination of 25%. Remaining MAGs were decontaminated using Kraken 2 (v2.1.2) [68] and the standard Kraken 2 database as follows. For each MAG, contigs that were not assigned to the Bacteria kingdom using Kraken 2 were removed. The phylum with the maximum total length was determined for each MAG from the Kraken 2 classification, and only contigs belonging to that phylum were retained. Another round of de-replication was performed using drep (v3.4.2) with a completeness threshold of >50% and contamination <10% before de-replication of the remaining MAGs at 99% ANI.

Taxonomic assignment

For each assembly with completeness >50% and contamination <10%, the contigs were concatenated with 10 Ns inserted between them. MAGs were classified using GTDB-Tk (v1.7.0) [11] against the Genome Taxonomy

Database (GTDB) database release 202. Unclassified MAGs were assigned as candidate novel taxa. First, we applied hierarchical clustering with average linkage on the all-versus-all MASH distances of all genomes, including identified and unidentified MAGs, using the fast-cluster python package [43]. The resulting dendrogram was divided with cutoffs at 5%, 15%, and 30% genetic distance to define clusters of species-level genome bins (SGBs), genus-level genome bins (GGBs), and family-level genome bins (FGBs), respectively [7]. Each of the resulting clusters at the different cutoffs was given a cluster number for identification (e.g., SGB1, GGB1, FGB1). ANI at 0.95 was used to refine SGBs; these were merged if any pair of their members showed ANI >0.95. All MAGs in a cluster were given the taxonomy of the most common GTDB assignment. Clusters that did not contain any GTDB assignment at a certain taxonomic level were given a *Candidatus* assignment. Linnaean binomials were generated using the Great Automatic Nomenclator (GAN) [46].

Functional annotation

Genes present in the MAGs were annotated using Prokka (v1.14.6) [58] to Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology groups (KOs) downloaded in 2020 [28]. Antimicrobial resistance genes (AMRs) were annotated using AMRFinderPlus (v3.11.4) [18]. Nucleotide sequences of MAGs as well as protein sequences characterized by Prokka were used as the inputs of AMRFinderPlus (<https://github.com/ncbi/amr/wiki>). AMRFinderPlus was run in the plus mode. Carbohydrate-Active enZYme (CAZyme) annotation was performed with dbcan (v3.0.6) [75]. From these annotations, inference of specific functions of interest was made according to the presence/absence of marker genes within the genomes of species representatives [56]. Butyrate production potential was determined according to the presence of acetate-CoA/acetoacetate-CoA-transferase (K01034 and K01035), butyrate kinase (K00929), and/or butyryl-CoA dehydrogenase (K00248). Propionate production was assessed by the presence of enzymes within the succinate pathways, these being succinyl-CoA synthetase (K01902 and K01903), methylmalonyl-CoA (K01847), and methylmalonyl-CoA decarboxylase (K11264). Secondary bile acid conversion was assessed by the presence of Bile Salt Hydrolase (K01442) and genes within the *bai* operon (*baiB* (K15868), *baiE* (K15872), and *baiF* (K15871)). The presence of CAZymes responsible for the breakdown of different carbohydrate substrate classes (cellulose, starch, chitin, and xylan) was determined as described by [59], requiring the presence of both a backbone CAZyme for the specific substrate and an oligo-cleaving CAZyme within each species.

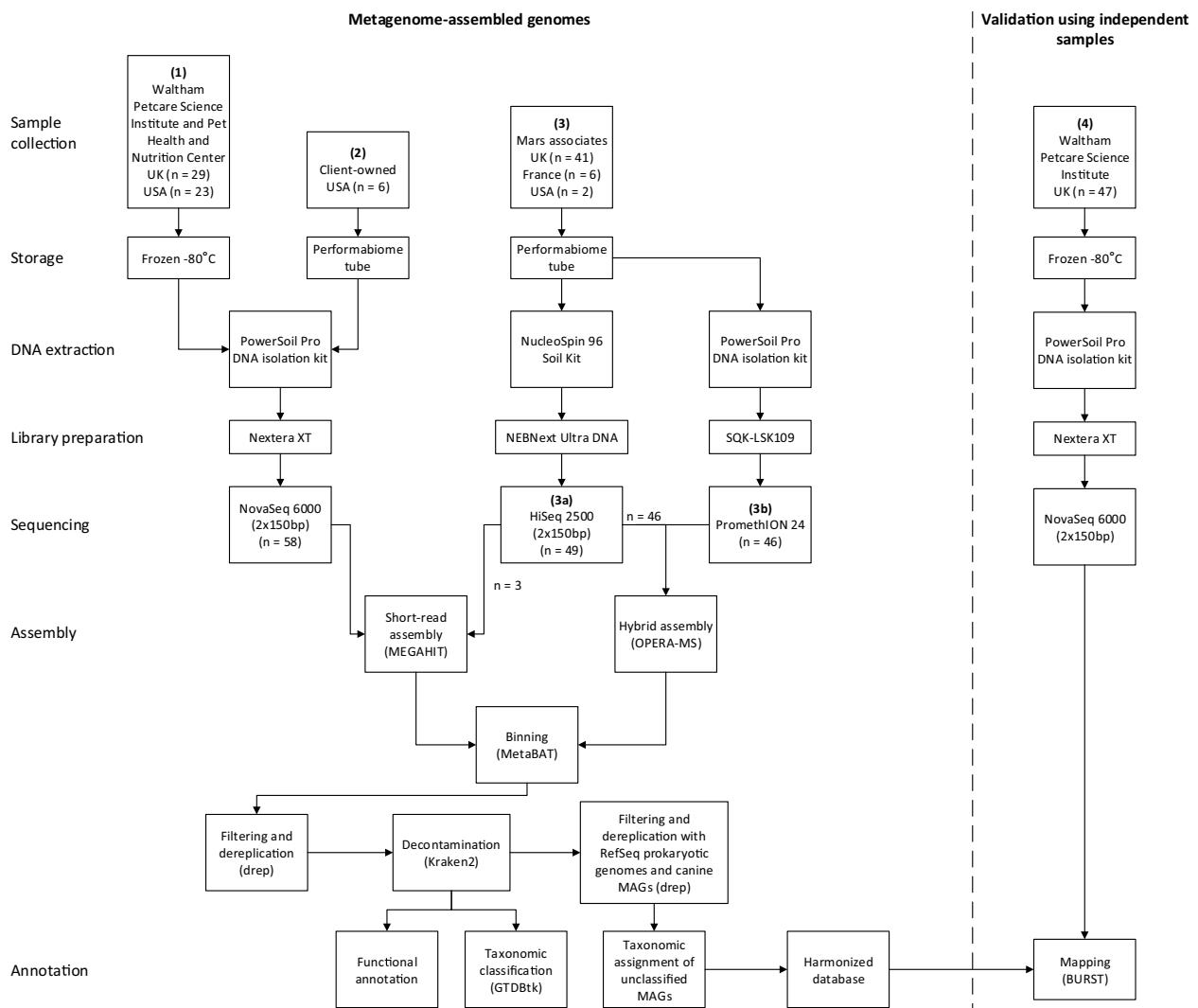


Fig. 1 Flowchart of the study design from sample collection to metagenome-assembled genomes assembly and validation using an independent set of dogs. *N* = number of dogs

Mapping of an independent dataset

The generated MAGs were combined with RefSeq prokaryotic genomes and canine MAGs derived from publicly available data [73] and de-replicated at 99% ANI. Metagenomic data were aligned to this combined database using fully gapped alignment with BURST at an identity threshold of 97%. Each sequencing read was assigned to the lowest common ancestor that was consistent across at least 80% of all reference sequences tied for best hit. KOs were observed via alignment to a gene database derived from the previously mentioned combined database. Again, all sequencing reads were aligned to all reference gene sequences at an identity threshold of 97% using fully-gapped alignment with BURST. Ambiguously mapped reads were excluded.

Results and discussion

Reconstruction of bacterial strains and discovery of novel taxa

To create a comprehensive canine microbiome catalogue, we reconstructed 5753 MAGs comprising 1031 bacterial strains (de-replicated at ANI ≥ 99%), with completeness > 50% and contamination < 10% from 501 samples across a cohort of 107 dogs comprising different breeds and life stages across the USA and Europe (Additional Table S2). The median completeness and contamination were 78.65% and 0.81%, respectively. Out of this, 291 MAGs had a completeness ≥ 90% and contamination ≤ 5%, 9 had a completeness of 100%, and 298 had a contamination of 0. Genome sizes ranged from 459 kbp to 4.9 Mbp with a median of 1.8 Mbp.

These genomes were assigned to 9 phyla, 10 classes, 24 orders, 43 families, 121 genera, and 240 species (Fig. 2; Additional Table S2). In total, 20 MAGs remained unclassified by GTDB-tk at the genus level and 229 at the species level. To assign taxonomy to such MAGs, we integrated them with all prokaryotic RefSeq genomes and applied hierarchical clustering with average linkage on the all-versus-all MASH distances of all genomes, including classified and unclassified MAGs. The resulting dendrogram was divided with cutoffs at 5%, 15%, and 30% genetic distance to define species, genera, and families, respectively. We defined 10 candidate novel genera and 89 candidate novel species for which we assigned *Candidatus* names (Fig. 3; Additional Table S3). This is an unprecedented discovery of novel taxa in the companion animal space, with the discovery likely aided by the dual approach of using short-read and long-read sequencing. The novel genera belonged to the families Erysipelotrichaceae, Erysipelotrichaceae, Atopobiaceae, Anaeroplasmataceae, Lachnospiraceae, and Anaerovoracaceae. The most represented genera with novel species were *Sutterella*, *Collinsella*, and *Faecalimonas*. While these families and genera are known to inhabit the gut microbiome in mammals, the recovery of these novel species suggests the existence of canine-specific taxa. Within known species, we discovered 822 new

strains, suggesting a significant amount of canine adaptation even for well-described taxa.

We assigned the majority of the 1031 MAGs to Firmicutes_A ($n=422$; 41.0%), Actinobacteriota ($n=136$; 13.2%), Firmicutes ($n=128$; 12.4%), Bacteroidota ($n=114$; 11.1%), Proteobacteria ($n=98$; 9.5%), Firmicutes_C ($n=49$; 4.8%), and Fusobacteriota ($n=43$; 4.2%). Members of these phyla are all known important inhabitants of the mammalian gut [16]. Notably, there was an absence of the phylum Verrucomicrobiota, whose member *Akkermansia muciniphila* has shown a protective role in the pathogenesis of cardiovascular disease in mice and humans [48]. The reasons for this are unknown but have been reported previously [20]. Understanding the biological reasons is an important direction for future research, particularly considering Akkermansia's proposed role in health and Akkermansia supplements becoming increasingly available.

The remaining MAGs were assigned to the phyla Campylobacterota ($n=39$; 3.8%) and Spirochaetota ($n=2$; 0.2%), whose most known species are considered pathogens [50]. *Campylobacter* spp. and *Helicobacter* spp. have been found in dogs with a history of diarrhea or vomiting [33, 50]. *Spirochaetota* spp. have been reported in the canine oral microbiome [54], which supports the idea of an oral–gut microbiome crosstalk.

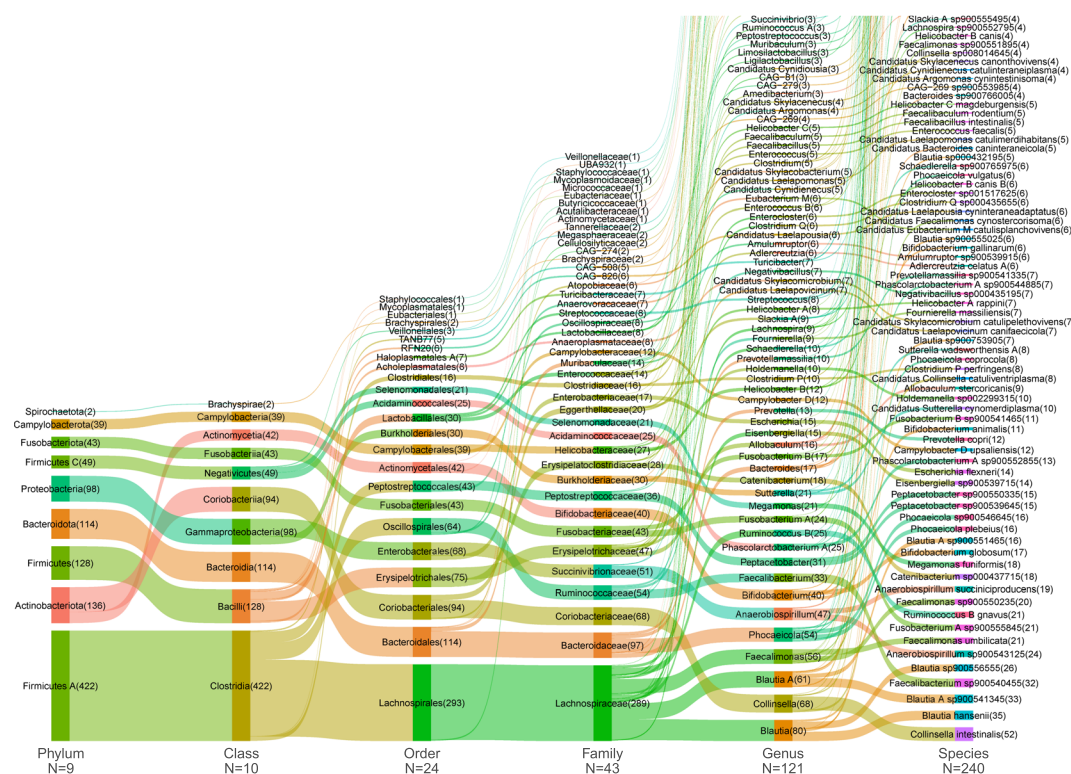


Fig. 2 Sankey diagram of the generated MAGs. Genus and species levels have been truncated for representation purposes

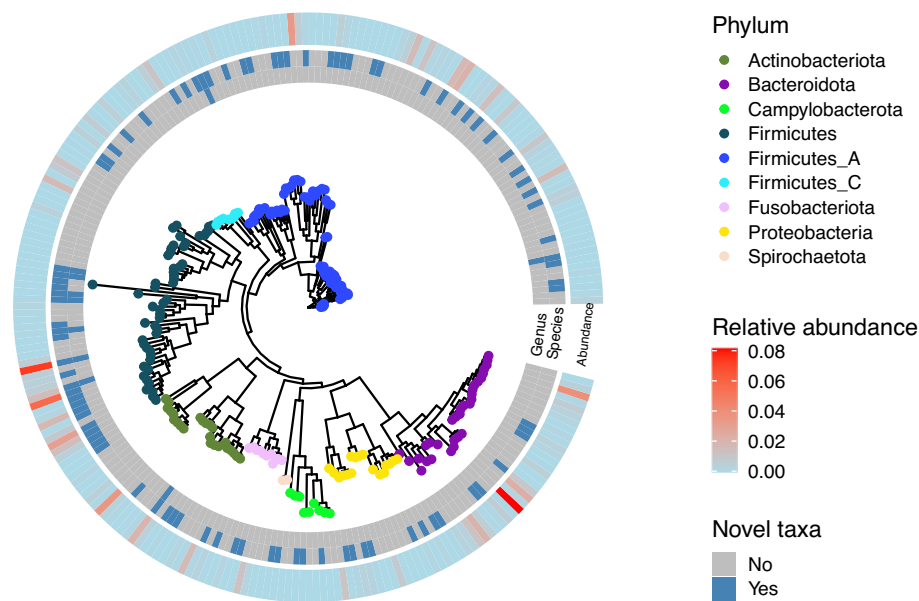


Fig. 3 Phylogenetic tree of all recovered MAGs at species taxonomic level with assigned GTDBtk bacterial phylum denoted by colored range. Inner heatmap shows candidate novel taxa at the genus and species levels (blue). Outer heatmap shows relative abundance levels of the 240 species in an independent dataset of 47 dogs (cohort 4)

At genus level, the genera with the highest number of representative MAGs were *Blautia* ($n=80$; 7.8%), *Collinsella* ($n=68$; 6.6%), *Blautia A* ($n=61$; 5.9%), *Fecalimonas* ($n=56$; 5.4%), and *Phocaeicola* ($n=54$; 5.2%), all of which have been found in mammalian gut microbiomes. *Blautia hansenii* has been inversely associated with obesity in mice [60]. *Collinsella intestinalis* has not been described previously in the canine gut microbiome but is known to be capable of degrading potentially harmful processed food components [67]. *Fecalimonas umbilicata* is an acetate-producing bacterium proposed as a novel species of a novel genus in 2017 [55] and *Phocaeicola spp.* are considered mutualistic bacteria in the human gut microbiome [24].

Independent dataset reveals high abundance and prevalence of candidate novel taxa

We next wanted to understand the relative proportions of the newly discovered bacteria across the canine microbiome and demonstrate applicability in an independent cohort. We therefore performed short-read metagenomic sequencing of 47 dogs from Waltham Petcare Science Institute, composed of 16 beagles, 23 Labrador retrievers, and 8 Norfolk Terriers. Metagenomes from these individuals were mapped to a reference database combining our generated MAGs with RefSeq prokaryotic genomes and MAGs derived from publicly available data [73]. The 240 species covered by our generated MAGs accounted on average for 95% of

the reads mapped at the species level of the combined database, with novel species accounting for 32.6%. Mapping rates at any taxonomic level ranged from 60 to 94% with a median of 87%. At the species level, the median mapping rate was 75%. This suggests that the 240 species found in this study represent the core canine gut microbiome, covering on average ~83% of the taxa in the canine microbiome in terms of relative abundance. This is a significant step-change increase in our resolution of the canine microbiome compared to mapping rates close to 25% when mapping to RefSeq genomes. This highlights previous knowledge gaps in the canine gut microbiome and the importance of this resource for canine microbiome research.

The most abundant species was *Prevotella copri* (8.1%) followed by two novel species, *Candidatus Skylacomonas catulintestiniplasma* (7.3%) and *Candidatus Ileibacterium canenteradaptatus* (5.7%). *Prevotella copri* is also the most abundant bacterium in the gut microbiome of humans [72]. It has been described as both beneficial and detrimental to human health as it has been associated with high fiber, low fat diets and improved glucose metabolism, but also with hypertension and persistent gut inflammation [72]. Both novel species, *Candidatus Skylacomonas catulintestiniplasma* and *Candidatus Ileibacterium canenteradaptatus*, belong to the family Erysipelotrichaceae, a highly abundant family in the mammalian gut microbiome [69]. This family, in mammalian hosts, has been associated with metabolic

disorders [27]. In dogs specifically, Erysipelotrichaceae has shown positive correlations with levels of acetate, propionate, and butyrate, and negative correlations with crude protein and fat digestibility [5].

Relative abundances >1% were observed in 26 species, eight of which were candidate novel species (*Candidatus Skylacomonas catulintestiniplasma*, *Candidatus Ileibacterium canenteradaptatus*, *Candidatus Allobaculum canaphodoplasma*, *Candidatus Laelapomicrobium cynegerieisoma*, *Candidatus Allobaculum canexcrementisoma*, *Candidatus Blautia_A catulifaecisoma*, *Candidatus Laelapobacterium catulisplanchochola*, and *Candidatus Allobaculum catulistercoricola*), the majority of these belonging to the phylum Firmicutes. The average abundance of the 89 candidate novel species ranged from 1×10^{-6} to 7.3% with a mean of 0.35%. In terms of prevalence, 131 out of the 240 species covered by our generated MAGs (54.6%) and 41 out of the 89 novel species (46.1%) were present in all 47 independent samples. *Candidatus Skylacomonas catulintestiniplasma* was present in 46 samples (98%), while *Candidatus Ileibacterium canenteradaptatus* was present in 41 samples (87.2%).

Results here demonstrate that we have uncovered a significant number of novel taxa in the canine microbiome that have yet to be described elsewhere and are highly abundant in dogs, supporting our hypothesis that there are significant gaps in the canine microbiome knowledge. The majority of the newly discovered genera and species belong to families that are known to be associated with other mammalian microbiomes. We propose these may have evolved in concert with the canine host and diet, given their absence in any other database (i.e., novel), though they may be present in other less well-studied animal species. Finally, we demonstrate that certain paradigms for health, such as *Akkermansia*, are absent from the canine microbiome. Collectively, this suggests that despite some similarities across mammals, the canine microbiome is distinct, presumably having evolved to its host, diet, and environment over several millennia. We therefore next endeavored to uncover the functions of the canine-specific genera, species, and strains that we have described, as well as the wider core microbiome of 240 species.

Functional description of the canine gut microbiome uncovered through metagenomics

Using Kyoto Encyclopedia of Genes and Genomes (KEGG) and Carbohydrate-Active enZymes (CAZymes), we performed functional annotation of predicted genes within our canine MAG catalogue, discovering 4182 KEGG orthologous groups (KOs) and 244 CAZymes. These could be grouped into a further 187 KEGG

pathways and all six CAZyme families, encompassing wide functional capacities. KEGG functional pathways were primarily associated with metabolic systems, with carbohydrate (7.7–29.2%), lipid (1.6–6.3%), and amino acid metabolism (4.3–20.7%) predominant, accounting for an average of 32.3% of total KOs per genome. These observations likely reflect the role of the microbiome in canine diet metabolism, with a canine omnivorous diet mainly consisting of cereals, animal proteins, and vegetables [2]. A summary of key function presence for each identified species can be found in Fig. 4. Notably, the most abundant new species, *Candidatus Skylacomonas catulintestiniplasma*, was enriched for butyrate production, chitin degradation, and cellulose degradation, and the second most abundant new species, *Candidatus Ileibacterium canenteradaptatus*, was enriched for butyrate production and lysine biosynthesis, with both enriched in CAZymes. This suggests these species are important for carbohydrate metabolism and, as a result, may influence host health.

A new view of carbohydrate metabolism capacity of the canine gut microbiome

In spite of being a crucial energy source, the canine genome encodes few genes with carbohydrate metabolism functionality [51]. We therefore aimed to understand the contribution the microbiome makes in the capacity to metabolize carbohydrates in dogs. The carbohydrate-degrading enzymes produced by bacterial species (CAZymes) are crucial to the breakdown of simple and complex carbohydrates that are often present in commercial canine diets into components that can be absorbed by the intestinal epithelium. Here, across all MAGs, we describe an average of 71.3 ± 51.7 CAZymes per species, with glycoside hydrolases (GHs, $n=113$) consistently accounting for the largest proportion of identified CAZymes per genome (46.1%), as is evident in other omnivorous mammals [25, 45]. This was followed by glycosyltransferases ($n=47$; 36.8%), carbohydrate esterases ($n=17$; 9.1%), carbohydrate-binding modules ($n=37$; 5.8%), polysaccharide lyases ($n=24$; 1.3%), and auxiliary activities ($n=6$; 0.9%). Species encoding the greatest number of CAZymes, and likely showing high carbohydrate breakdown function, belong to the fibrolytic Bacteroidaceae family; of which four are candidate novel species with a combined relative abundance of 1.94% within our mapped cohort (*Candidatus Bacteroides caniventradaptatus*, 0.24%; *Candidatus Cynidiobacterium catulicopradaptatus*, 0.06%; *Candidatus Paraprevotella catulicaccoccola*, 0.67%; and *Candidatus Prevotellamassilia catulegerieihabitans*, 0.97%). Primarily attributed to GH families GH2, GH3, GH20, GH29, GH43, and GH92, it is likely that these species are key

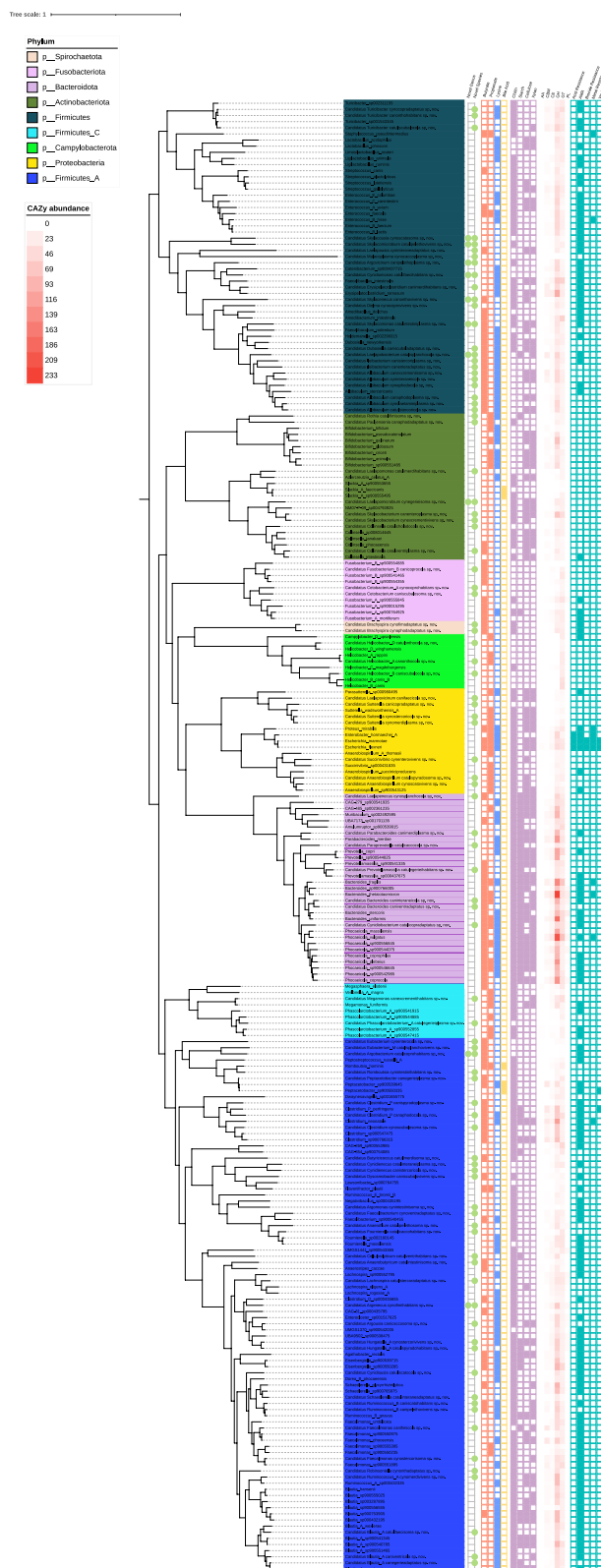


Fig. 4 Phylogenetic tree displaying 240 recovered bacterial species from the canine gut microbiome. Phylogenetic tree has been visualized using the online iTOLv5.7 tool, with assigned GTDBtk bacterial phylum denoted by colored range. The presence (filled) or absence (hollow) of genes associated with metabolite biosynthesis (KEGG) or enzymes associated with substrate degradation (CAZyme) are reported in the binary plot. Abundance of specific CAZyme classes is depicted as a heatmap. AA, Auxiliary Activity; CBM, Carbohydrate-Binding Module; CE, Carbohydrate Esterase; GH, Glycoside Hydrolase; GT, GlycosylTransferase; PL, Polysaccharide Lyase. Additional Table S2 can be used to convert bin names to species names

to dietary and host-derived glycan breakdown in the dog, as has been described previously for *Bacteroidaceae* spp. in humans [45]. More broadly, CAZymes involved in the breakdown of commonly used dietary fibers were identified in a number of species; specifically, cellulose (present in 35.83% [$n=86$] of species), starch (22.08%, $n=52$), chitin (75.42%; $n=181$), or xylan (hemicellulose) (36.67%; $n=88$). For many identified species, this is the first description of their ability to degrade such complex dietary fibers within the canine gut microbiome (Fig. 4). The high percentage of metabolic pathways attributed to carbohydrate metabolism within our recovered species and absence in the canine genome indicates a strong host reliance on the commensal bacteria of the gut to perform this critical metabolic function.

Uncovering the SCFA producing potential of the canine gut microbiome

Using KEGG annotation of detected species, we can identify the presence of KOs crucial in the production of well-described health-promoting metabolites generated as a product of the carbohydrate degradation pathways described above. Short-chain fatty acids (SCFAs) from dietary fiber degradation have anti-inflammatory properties and are an energy source for colonocytes [65]. In dogs, dysregulation of these pathways in the gut microbiome is associated with a number of enteropathologies, including canine chronic enteropathy [41]. SCFAs comprise three major forms: acetate (60%), propionate (20%), and butyrate (20%), with only specific, phylogenetically diverse bacterial groups possessing the ability to form butyrate and propionate [42]. The majority of microbial butyrate is synthesized via the acetyl-CoA pathway utilizing butyryl-CoA dehydrogenase (*bcd*), butyryl-CoA: acetate CoA-transferase (*but*), and butyrate kinase (*buk*) as enzymes involved in the terminal steps of metabolite synthesis. Here, genes for *bcd* (K00248), *but* (K01034 and K01035), or *buk* (K00929) were identified in 37.5% ($n=90$) of recovered

species (collective relative abundance 45.59%). While the majority of these species are previously recognized species, their presence, and as such, likely contribution to butyrate production within the canine gut microbiome has been otherwise poorly described. Such species include *Bacteroides uniformis* and *Romboutsia hominis*, as well as almost all recovered *Allobaculum* spp., *Bacteroides* spp., and *Phocaeicola* spp. Thirty-four of the butyrate-producing species identified are candidate novel species from butyrate-producing genera (e.g., *Candidatus Anaerobutyricum catulintestinisoma*, *Candidatus Clostridium cynoscubalosoma*) and together contribute an average relative abundance of 24.6% within the canine gut. Of note, we identified one novel genus and 12 candidate novel species showing butyrate-producing capacity within the Erysipelotrichaceae family, with *Candidatus Skylacomonas catulintestiniplasma*, *Candidatus Ileibacterium canenteradaptatus*, and *Candidatus Allobaculum canaphodoplasma* being particularly prominent members of the healthy canine gut microbiome (relative abundance of 7.28%, 5.74%, and 3.08%, respectively). With increasing links between members of this bacterial family and mammalian metabolic disorders being described [27], the value of uncovering such novelty and abundance within the canine gut is of pressing importance. The widespread nature of these pathways across a number of species supports the importance of microbial butyrate production for canine gut health.

While three propionate production pathways exist within the intestinal microbiome, the succinate (suc) pathway predominates, with three key enzymes used in the production process: methylmalonyl-CoA mutase (*mut*), methylmalonyl-CoA decarboxylase (*mmcD*), and succinyl-CoA synthetase (*sucC* and *sucD*). Propionate production potential through detection of these enzymes was determined for 18.8% ($n=45$) of recovered species in the canine gut (collective relative abundance 11.40%). Known *Bifidobacterium* spp., *Phascolarctobacterium* spp., *Megamonas* spp., *Bacteroides* spp., and *Anaerobiospirillum* spp. account for the vast majority of propionate production potential within our species catalogue ($n=19$; collective relative abundance 2.02%). However, similar to findings for butyrate production, a number of known species with no previous description within the canine gut (e.g., *Phocaeicola* sp900546645; relative abundance 3.93%) alongside novel species (e.g., *Candidatus Bacteroides caninteraneicola*; relative abundance 0.42%) encode key propionate-producing enzymes and likely account for a fundamental portion of propionate production within the healthy dog. Therefore, this resource dramatically expands our understanding of bacterial species capable of supporting SCFA production within the

healthy canine gut, particularly pertaining to butyrate production. This highlights the importance of having a canine-specific catalogue for interpreting the key bacteria involved in critical metabolic function.

Microbial synthesis of amino acids to support dietary intake

The gut microbiome also plays an important role in host amino acid homeostasis. Recent evidence demonstrates that the gut microbiota in the small and large intestine plays a role not only in the breakdown of proteins to amino acids but also in the synthesis of amino acids themselves, with these subsequently utilized by the host. The amino acids produced by specific bacterial species may contribute to the availability of essential amino acids, particularly for lysine. Lysine biosynthesis occurs through the metabolism of aspartate through the diaminopimelate pathway (DAP). At least one variant of the complete lysine biosynthesis DAP pathway (succinyl, acetyl, dehydrogenase, or aminotransferase) was present in 42.1% (101/240) of species, indicating their contribution to this function within the canine gut microbiome. The vast majority of bacterial species containing complete DAP modules show no previous description within the canine gut.

Expanded potential for secondary bile acid conversion by *Peptacetobacter* species within the canine gut

Bile acids aid in digestion and absorption of lipids in the GI tract, with the microbiome being critical for primary to secondary bile acid conversion, itself a key step in bile acid host homeostasis. It is proposed that secondary bile acids provide further capability in inhibiting the growth of certain pathogenic bacterial species in dogs, namely *E. coli* and *C. perfringens*. Primary bile acids are converted to secondary bile acids by bacteria with 7 α -dehydroxylation capabilities, a capability possessed by a relatively limited set of bacteria that encode the bile acid inducible (*bai*) operon. Species were examined for the presence of genes involved in the conversion of primary bile acid to secondary bile acid, being bile salt hydrolase (BSH; K01442), alongside the seven *bai* operon enzyme genes (*baiA*–*F*). Of the *bai* operon genes, only *baiB* (K15868), *baiE* (K15872), and *baiF* (K15871) were detected within our species catalogue, with these likely retained during assembly and binning due to higher gene abundance. Genes for both BSH and the *bai* operon were present in two *Peptacetobacter* species (*P. sp900550335* (relative abundance 1.13%) and *P. sp900539645* (0.56%)), with these uncultured species only previously identified in the human gut. Functional characterization of these species and description of their presence within the canine gut microbiome expands our understanding of the repertoire of bacteria

that could influence secondary bile acid concentration during healthy and dysbiotic states beyond the well-described *P. hiranonis* (*Clostridium hiranonis*) within the same genera [63].

At least 54 candidate novel species show relative abundance of key butyrate, propionate, lysine, or secondary bile acid biosynthesis genes and, alongside known species otherwise undescribed in the canine gut microbiome, have the potential to be key contributors to these functions of known health impact within the dog. Collectively, this demonstrates the compiled MAG catalogue comprises species with key functionality in supporting metabolic function of the canine gut, many of which were novel or previously unknown to the canine gut or were unknown to carry such functional potential. We believe this will be a critical resource for all future microbiome work in the companion animal space, but also shows the importance of having a host or environment-specific taxa and gene catalogue to interpret studies from.

Antimicrobial resistance, stress resistance, and virulence in the canine gut microbiome

Although the microbiome has important beneficial roles, it's also important to understand the relative potential for pathogenicity. AMRFinderPlus was used to identify the presence of antimicrobial resistance (AMR), stress resistance, and virulence genes in the reconstructed genomes. Virulence factors were found in four species, three of which are known pathogens, *Clostridium_P perfringens*, *Escherichia flexneri*, *Escherichia marmotae*, and *Peptacetobacter sp900550335*, but none in the candidate novel species. This is important as it suggests all novel discovered species are commensal rather than pathobionts or pathogens, which was expected given the healthy cohort. Among the AMR genes, *abc-f*, known to provide resistance to a broad range of clinically used antibiotic classes [17], was found in 49 (20.4%) of the recovered species. This was followed by *vanR* ($n=41$; 17.1%), which plays a role in the resistance to the last resort antibiotic vancomycin [62] and *blaRI* ($n=23$; 9.6%) involved in resistance to β -lactam antibiotics [36]. AMR genes were found present mainly in members of the genera *Fecalimonas* ($n=8$), *Blautia_A* ($n=6$), and *Blautia* ($n=6$), which are members of the fibrolytic Lachnospiraceae family and known to harbor conjugative transposons containing antibiotic resistance genes [74]. Stress resistance genes were found in 10 of the recovered species; eight of them contained genes conferring resistance to arsenicals, one to copper, and one to tellurite.

Conclusion

The canine microbiome is a vastly understudied area relative to the importance of dogs in society, particularly given the potential importance of the microbiome in veterinary

medicine. Generally, microbiome research has been heavily biased toward human studies, consequently limiting the understanding of the canine gut microbiome mostly to species previously found in humans, which may go some way to explaining prior reports of marked similarities between the human and canine microbiomes [13]. Using a broad sample set, long-read sequencing, short-read sequencing, and metagenomic assembly approaches, we have produced the most comprehensive resource in all companion animal research. We have managed to describe 240 species that account for ~83% of the canine gut microbiome and uncovered >900 new canine-specific strains, 89 novel species, and 10 novel genera that we believe are unique to dogs. This is a dramatic increase in previous knowledge of the canine microbiome, with traditional mapping rates of ~25% using publicly available RefSeq genomes. Through detailed annotation of function, we demonstrate the potential of these novel species to influence host health and nutrition, with genomes enriched in genes for SCFA production, carbohydrate metabolism, bile acid metabolism, and amino acid synthesis. We further give new insights into known species not previously known to be present in the canine gut by providing genomes for canine-specific strains and giving novel insights into function.

As our recovered catalogue spans multiple cohorts of pet dogs across different countries and living in a range of environments, the taxa and functions presented here provide the highest resolution image of the healthy pet dog microbiome to date. By providing the MAGs themselves as well as details on their taxonomic lineage and functional annotation, this resource can be used by all researchers working on canines across breed, age, sex, or geography, expanding opportunities to understand the role of the microbiome in canine health and disease, as well as more completely map the taxonomic and functional impact of dietary interventions.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40168-025-02265-w>.

Supplementary Table S1: Signalment information for all dogs from which MAGs were generated (Cohort 1-3) and for all dogs used as part of validation checks (cohort 4).

Supplementary Table S2: Genome information for all bacterial strains recovered as part of this study, including taxonomic assignment according to GTDB-Tk.

Supplementary Table S3: Protologues for new Candidatus taxa identified from metagenomic analysis of canine gut samples.

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Authors' contributions

R.W.H., P.W. and G.C.A.A. conceived the project. J.C-F., R.B.J., R.W.H., and G.C.A.A. designed the study. J.C-F. and R.G. performed the data analyses. G.C.A.A., M.J.W., and P.W. supervised the study. G.C.A.A., J.C-F. and R.G. wrote the manuscript and all authors reviewed and approved the final manuscript.

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Data availability

Sequencing data and constructed MAGs generated in this study are available under BioProject PRJNA1222153.

Declarations

Ethics approval and consent to participate

All samples included in this study were approved by the Waltham Petcare Science Institute Animal Welfare and Ethical Review Board. For animal cohorts 2 and 3, all study participants provided informed, written consent at the beginning of the study and were advised of their rights to withdraw at any time.

Consent for publication

N/A.

Competing interests

J.C-F., R.G., M.J.W., G.C.A.A. and P.W. are employees of Mars Petcare, a manufacturer of pet food and provider of veterinary services. R.B.J. and R.W.H. were employees of NomNomNow, a company owned by Mars Petcare, at the time of the study. Funding was provided by Mars Petcare. Mars Petcare has filed patents relating to this research. G.C.A.A. is a senior editor of the journal 'microbiome'.

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