

Long-read metagenomics retrieve complete single-contig bacterial genomes from canine feces

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Abstract

Background

Long-read sequencing in metagenomics facilitates the assembly of complete genomes out of complex microbial communities. These genomes include essential biologic information such as the ribosomal genes or the mobile genetic elements, which are usually missed with short-reads. We applied long-read metagenomics with Nanopore sequencing to retrieve high-quality metagenome-assembled genomes (HQ MAGs) from a dog fecal sample.

Results

We used nanopore long-read metagenomics and frameshift aware correction on a canine fecal sample and retrieved eight single-contig HQ MAGs, which were > 90% complete with < 5% contamination, and contained most ribosomal genes and tRNAs. At the technical level, we demonstrated that a high-molecular-weight DNA extraction improved the metagenomics assembly contiguity, the recovery of the rRNA operons, and the retrieval of longer and circular contigs that are potential HQ MAGs. These HQ MAGs corresponded to *Succinivibrio*, *Sutterella*, *Prevotellamassilia*, *Phascolarctobacterium*, *Catenibacterium*, *Blautia*, and *Enterococcus* genera. Linking our results to previous gastrointestinal microbiome reports (metagenome or 16S rRNA-based), we found that some bacterial species on the gastrointestinal tract seem to be more canid-specific – *Succinivibrio*, *Prevotellamassilia*, *Phascolarctobacterium*, *Blautia_A sp900541345-*, whereas others are more broadly distributed among animal and human microbiomes – *Sutterella*, *Catenibacterium*, *Enterococcus*, and *Blautia sp003287895*. *Sutterella* HQ MAG is potentially the first reported genome assembly for *Sutterella stercoricanis*, as assigned by 16S rRNA gene similarity. Moreover, we show that long reads are essential to gain biological insights that are otherwise missed in short-read MAGs catalogs, as shown by the mobilome functions detected in the long-read HQ MAGs.

Conclusions

We recovered eight single-contig HQ MAGs from canine feces of a healthy dog with nanopore long-reads. We also retrieved relevant biological insights from these specific bacterial species previously missed in public databases, such as complete ribosomal operons and mobilome functions. The high-molecular-weight DNA extraction improved the assembly's contiguity, whereas the high-accuracy basecalling, the raw read error correction, the assembly polishing, and the frameshift correction reduced the insertions and deletion errors. Both experimental and analytical steps ensured the retrieval of complete bacterial genomes.

Background

Metagenomics is a powerful and rapidly developing approach that can be used to unravel uncultured microbial diversity and expand the tree of life and give new biological insights into the microbes

inhabiting underexplored environments [1]. When applied to both the canine gastrointestinal (GI) and the fecal microbiomes provides information on health and disease as well as essential clues on how to prevent or treat specific pathologies.

Previous studies have reported similarities between canine and human GI microbiome. In general, different GI diseases relate to an altered GI microbiome that, on the other hand, can be modulated by diet and dietary complements (such as pre- and probiotics) (See [2–5] for extensive reviews). Besides the veterinarian interest itself, dogs are considered closer models to humans than other animal models for GI microbiome studies [6, 7].

Microbiome studies are either marker-specific (e.g., 16S rRNA gene for Bacteria) or whole metagenome sequencing [8]. To date, the canine GI microbiome studies available use next-generation sequencing – short-read sequencing– or earlier technologies and are mostly amplicon-based strategies (16S rRNA gene). Only three studies used shotgun metagenomics with short-read sequencing to characterize the whole microbial community and the gene content in dog feces [7, 9, 10].

The application of long-read sequencing to metagenomics enables retrieving metagenome-assembled genomes (MAGs) with high completeness. The most recent strategy in long-read metagenomics uses the long reads to obtain the raw metagenome assembly –ensuring the greatest contiguity of MAGs– and short reads to polish and improve the overall accuracy. This strategy was applied to assess the human GI microbiome [11], among others –such as mock communities [12], cow rumen [13], natural whey starter cultures [14], or wastewater [15]. Worthy of considering, some authors suggest that we may overcome the need for short reads to polish long-read data by either using correction software, such as frameshift-aware correction [16], or with ultra-deep coverage of the genomes [12].

In our previous work, we used long-read metagenomics to assess the taxonomy and reach species identification on the canine fecal microbiome. Even though we used a low-depth sequencing approach, we assembled a circular contig corresponding to an *uncultured CrAssphage* [17].

In the present study, we use nanopore long-read metagenomics and frameshift aware correction to overcome the need for polishing with short reads. As a result, we retrieve and characterize eight high-quality MAGs and gain new biological insights into the dog fecal microbiome.

Material And Methods

DNA extraction and long-read sequencing

Our study focuses on the analysis of a single fecal sample of a healthy dog. A fresh sample was collected and stored at -80°C until further processing.

We used two different kits from Zymobiomics (Zymo Research) for DNA extraction: the Quick-DNA HMW MagBead for High-Molecular Weight DNA (without bead-beating) and the DNA Miniprep Kit, which is the

standard microbiome DNA extraction with bead-beating. Throughout the manuscript, we use HMW-DNA extraction and non-HMW DNA extraction terms, respectively.

Each DNA extraction was sequenced in a single Flowcell R9.4.1 using MinION™ (Oxford Nanopore Technologies). The Ligation Sequencing Kit 1D (SQK-LSK109; Oxford Nanopore Technologies) was used to prepare both libraries. For non-HMW DNA, we followed the manufacturer's protocol. For the HMW-DNA, we tuned few parameters: i) at DNA repair and end-prep step, we incubated at 20°C for 20 minutes and 65°C for 20 minutes; ii) we extended rotator mixer (Hula mixer) times to 10 minutes; iii) we extended elution time after AMPure XP beads to 10 minutes; iv) final incubation with elution buffer was performed at 37°C for 15 minutes (as recommended for HMW DNA).

Raw reads: pre-processing, quality control, and taxonomic analyses

Raw fast5 files were basecalled using Guppy 3.4.5 (Oxford Nanopore Technologies) with high accuracy basecalling mode (dna_r9.4.1_450bps_hac.cfg). During the basecalling, the reads with an accuracy lower than 7 were discarded.

To obtain the first taxonomic assignment directly from the raw reads, we processed the data using Kraken2 2.0.8 [18] with the maxikraken2 database (Loman Lab, from March 2019) that includes all the genomes from RefSeq. We visualized Kraken2 reports using Sankey diagrams with pavian 1.0.0 R package [19].

We used Nanoplot 1.28 [20] to obtain the run summary statistics, Porechop 0.2.4 [21] for adapters trimming, Nanofilt 2.6.0 [22] to discard reads shorter than 1,000 bp, and different modules of seqkit 0.11.0 [23] to manipulate fastq and fasta files during the whole analysis.

Metagenomics assembly and polishing

Before proceeding with the metagenomics assembly, we performed an error-correction step of the raw nanopore reads using canu 2.0 [24].

We used the corrected reads to perform metagenome assembly with Flye 2.7 [25] (options: `-nano-corr -meta, -genome-size 500 m, -plasmids`). We performed several assemblies with different random amounts of data (100%, 75%, 50%, and HMW) to recover the maximum number of high-quality MAGs (HQ MAGs). We used Bandage 0.8.1 [26] to visualize the metagenome assemblies. We polished the Flye assembly with one round of medaka 1.0.1 [27], including all the raw fastq files as input.

The next step for the HQ MAGs was to correct the frameshift errors, as described in [16], using Diamond 0.9.32 [28] and MEGAN-LR 6.19.1 [29]. We used ideel [30] to visualize the number of truncated open-reading frames (ORF).

To assess the quality of the MAGs, we used CheckM 1.1.1 [31] to retrieve completeness and contamination. Considering MIMAG criteria, MAGs are classified as: high-quality, with > 90%

completeness, < 5% contamination, and presence of rRNAs genes and tRNAs; medium-quality, with > 50% completeness and < 10% contamination and low-quality, the remaining ones [32].

Characterization of the high-quality MAGs

GTDB-tk 1.3.0 [33] with GTDB taxonomy release 95 [34] were used to assess the novelty and the taxonomy of HQ MAGs. We used PROKKA 1.13.4 [35] to annotate the MAGs. We used FastANI 1.3 [36] to confirm a potentially new species by determining the average nucleotide identity (ANI) between the most related genomes.

We extracted the 16S rRNA genes from the HQ MAGs before the frameshift correction step using Anvi'o 6.1 [37]. The 16S rRNA genes were analyzed using MOLE-BLAST tool in NCBI website [38] to obtain a phylogenetic tree. Mafft [39] in the EBI website was used to align 16S rRNA gene sequences from *Sutterella* HQ MAG and obtain an identity matrix.

Abricate 0.9.8 [40] was used to detect antimicrobial resistance genes using CARD database [41]. OriTfinder [42] was used to identify the origin of transfer (oriT) and conjugative machinery of mobile genetic elements and SnapGene Viewer 5.0.7 [43] to visualize the results.

Functional and Pangenomics analysis of the HQ MAGs

We compared the HQ MAGs obtained to previously reported MAGs from two recent gastrointestinal collections: i) the animal gut metagenome [10] and ii) the Unified Human Gastrointestinal Genome (UHGG) [44].

We retrieved MAGs that represented the same species as our HQ MAGs by keeping: i) those with > 95% of ANI [36] for the animal gut metagenome; and ii) those with the same species-level taxonomy as stated by GTDB-tk for UHGG.

We performed a pangenome analysis for those bacterial species with more than 10 representatives using Anvi'o 6.2 [37]. When many MAGs were available, only some random representatives with > 90% completeness were kept. Within Anvi'o pangenomics workflow [45], Prodigal [46] was used as a gene caller to identify open reading frames, whereas genes were functionally annotated using blastp against NCBI COGs database [47] (cog2003-2014). The pangenome database was created using NCBI's blastp to calculate each amino acid sequence's similarity in every genome against every other amino acid sequence across all genomes to resolve gene clusters. MCL inflation parameter that was set to 10 (Additional File 1 for detailed steps).

Results

We characterized the fecal microbiome of a healthy dog using long-read metagenomics with Nanopore sequencing. An overview of the complete experimental design is presented on Fig. 1. We obtained a total

of 16.94 million reads (36.05 Gb), after two runs corresponding to the HMW and non-HMW DNA extractions.

After high accuracy basecalling and error correction, we performed several metagenomics assembly strategies to retrieve eight single-contig high-quality MAGs (HQ MAGs), which were > 90% complete with < 5% contamination and contained most ribosomal genes and tRNAs, and three medium-quality ones (MQ MAGs). We further corrected the HQ MAGs for frameshifts errors and compared them at the functional level with those previously identified in other gastrointestinal catalogs.

HMW DNA extraction for longer reads and larger contigs

HMW sequencing produced 5.81 million reads with N50 of 4,369 bp and a median length of 2,312 bp (total throughput: 18.76 Gb), whereas non-HMW produced 11.13 million reads with N50 of 2,102 bp and a median length of 1,093 bp (total throughput: 17.29 Gb).

We taxonomically classified all the uncorrected raw reads with Kraken2 and found 81.8% of classified reads in HMW vs. 70.8% in non-HMW. More than 99% of total reads corresponded to Bacteria. The most abundant phylum was Bacteroidetes (~ 80% of total reads), followed in abundance by Firmicutes (12.5% in HMW vs. 8.9% in non-HMW), Proteobacteria (~ 5%), and Fusobacteria (1.9% in HMW vs. 3.9% in non-HMW). At the genus level, this dog fecal microbiome was rich in *Prevotella* (> 50%) and *Bacteroides* (> 20%). Moreover, it also contains *Fusobacterium*, *Megamonas*, *Sutterella*, and other fecal-related genera, representing each one of them less than 5% of the total bacterial composition (Additional File 2).

The metagenomics assembly with the HMW-DNA dataset is more contiguous, presenting fewer and longer contigs than the non-HMW DNA one (contigs: 1,898 vs. 2,944; N50: 187,680 vs. 94,109 bp) (Additional File 3). Moreover, HMW-DNA metagenomics assembly retrieves six HQ MAGs, yet only one HQ MAGs are retrieved from the non-HMW DNA assembly (Fig. 2 and Additional File 3).

In summary, HMW DNA extraction improved the taxonomic classification of the raw unassembled reads (less unclassified reads), the metagenomics assembly contiguity, and the retrieval of longer and circular contigs (potential HQ MAGs). Thus, HMW DNA extraction becomes the preferred choice to recover HQ MAGs directly from complex metagenomics samples.

Metagenomics assembly with different subsets followed by frameshift aware correction retrieved eight high-quality MAGs

To ensure the highest coverage and consensus accuracies for the retrieved MAGs, we further merged and assembled the HMW and the non-HMW datasets (100% dataset; 16.94 million reads, 36.05 Gb). As we aimed to retrieve the maximum number of HQ MAGs, we performed extra metagenomics assemblies using 75% and 50% data subsets from that merged dataset (Additional File 3).

After assigning taxonomy and comparing among assemblies, we identified non-redundant MAGs: eight HQ MAGs, and three MQ MAGs (Table 1). When compared to HMW assembly, we retrieved two new MQ MAGs from the 100% data assembly (the HMW and the non-HMW datasets together). Moreover, two MQ MAGs from HMW and 100% datasets were recovered as HQ MAGs from the 75% dataset. None of the performed assemblies alone retrieved the eight HQ MAGs.

Table 1

High quality (HQ) and medium quality (mq) single-contig MAGs retrieved in each metagenome assembly. Taxonomy assigned using the GTDB database release 95. Q is the MAG quality. Cov. is the coverage from Flye. **Blautia_A* sp900541345 and *g__*Sutterella* HQ MAGs after correction of the indels.

	HMW data		100% data		75% data		50% data	
Taxonomy (GTDB)	Q	Cov.	Q	Cov.	Q	Cov.	Q	Cov.
HQ MAG								
<i>g__Succinivibrio</i>	HQ	47X	HQ	101X	mq	82X	HQ	50X
<i>g__Sutterella*</i>	mq	95X	mq	159X	HQ	123X	mq	87/80X
<i>Prevotellamassilia</i> sp900541335	HQ	394X	HQ	577X	HQ	430X	HQ	282X
<i>Phascolarctobacterium</i> sp900544885	HQ	87X	HQ	205X	HQ	155X	mq	98X
<i>Catenibacterium</i> sp000437715	HQ	13X	mq	24X	HQ	17X	mq	11X
<i>Blautia_A</i> sp003287895	-	-	mq	38X	HQ	31X	mq	18X
<i>Enterococcus_B</i> hirae	HQ	17X	HQ	42X	HQ	31X	HQ	22X
<i>Blautia_A</i> sp900541345*	HQ	44X	-	-	mq	45X	-	-
MQ MAG								
<i>Phocaeicola</i> plebeius	mq	126X	mq	234X	mq	168X	-	-
<i>g__Bacteroides</i>	mq	206X	mq	368X	mq	282X	mq	196X
<i>g__Phocaeicola</i>	-	-	mq	271X	-	-	-	-

For each HQ MAG, we selected the representative with the highest coverage – and subsequent highest consensus accuracy – for further analyses. We performed an extra step of frameshift aware correction that reduced the insertions and deletions (indels), which are the most abundant nanopore sequencing error type. The frameshift correction resulted in fewer predicted coding sequences (CDS) (Fig. 3, and Additional File 4). This correction step transformed two MQ MAGs into HQ MAGs: *Blautia* sp900541345 on the HMW-only assembly (from MQ MAG with 84.99% completeness to HQ MAG with 93.86% completeness) and the *Sutterella* MAG on the 75% assembly (from MQ MAG with 84.88% completeness to HQ MAG with 95.49% completeness) (Fig. 3). On the other HQ MAGs, completeness remained constant or increased after applying the frameshift correction, except for one of the contigs (*Enterococcus hirae*,

47X coverage; completeness of 99.69–99.13% after the indel correction). The differences in applying frameshift correction were more evident in contigs with low coverage than in those with high coverage.

High-quality MAGs of the canine fecal microbiome improved previous genome assemblies

From a single canine fecal sample, we obtained eight HQ MAGs that were single-contig, > 90% complete with < 5% contamination, and contained most ribosomal genes and tRNAs (Table 2). Thus, they represent HQ MAGs, without gaps or unplaced scaffolds regarding MIMAG criteria [32]. We used GTDB-tk to assign the taxonomy and assess the potential novelty. The ANI values serve to identify potential novel taxa (> 95% ANI are considered as the same species [36, 48]).

Table 2

Summary of genome statistics for High-quality MAGs comparison when compared to representatives on the public datasets. Completeness (% Compl.) values come from CheckM; tRNAs and rRNA values from PROKKA. MAGs in public databases with > 95 ANI represent the same species. Ref, reference, complete genome. *Despite *Succinivibrio* and *Sutterella* were potential novel species regarding GTDB, we found a single MAG > 95% ANI on the animal gut metagenome and UHGG catalog, respectively.

HQ MAG	Length (Mbp)	% Compl.	tRNAs	rRNAs	Contiguity level
<i>Succinivibrio sp.*</i>	2.04	98.68	77	22	Complete
> 95% ANI to <i>Succinivibrio</i> MAG in dog GI	1.74	97.5	32	0	185 contigs
<i>Sutterella sp.*</i>	2.7	95.49	67	18	Complete
> 95% ANI to <i>Sutterella</i> MAG in human GI	1.14	78.72	37	0	24 contigs
<i>Prevotellamassilia sp900541335</i>	2.72	97.65	72	21	Complete
> 95% ANI to GCA_900541335.1	2.42	96.13	16	0	95 contigs
<i>Phascolarctobacterium sp900544885</i>	2.09	99.85	58	15	Complete
> 95% ANI to GCA_900544885.1	1.75	98.65	18	1	87 contigs
<i>Catenibacterium sp000437715</i>	2.53	98.5	76	21	Complete
> 95% ANI to GCF_004168205.1	2.54	100	20	2	212 contigs
<i>Blautia sp900541345</i>	2.44	93.86	53	18	Complete
> 95% ANI to GCA_900541345.1	2.69	95.85	16	0	160 contigs
<i>Enterococcus_B hirae</i>	2.78	99.13	69	18	Complete
Ref: GCF_000271405.2	2.83	99.63	71	18	Complete
<i>Blautia sp003287895 (Blautia argi)</i>	2.96	92.78	58	10	Complete
Ref: GCF_003287895.1	3.3	97.64	57	14	Complete

Despite *Sutterella* and *Succinivibrio* were considered novel by GTDB-tk, we found one MAG for each in human and dog GI datasets, respectively, that presented > 95% ANI to our HQ MAGs. Similarly, *Prevotellamassilia* sp900541335, *Phascolarctobacterium* sp900544885, *Catenibacterium* sp000437715, and *Blautia* sp900541345 HQ MAGs were representing bacterial species previously retrieved from metagenomes. In contrast, *Enterococcus_B hirae* and *Blautia* sp003287895 HQ MAGs were representing bacterial species that have complete reference genomes. In fact, *Blautia* sp003287895 –proposed name *Blautia argii*– was first isolated and characterized from dog feces [49]. *Enterococcus_B hirae* and *Blautia* sp003287895 HQ MAGs were aligned against their respective reference genomes to prove and validate the results (Additional File 5).

To conclude, six out of eight HQ MAGs represented bacterial species that lack a complete genome reference. Their current representatives are MAGs retrieved with short-read data, so highly fragmented and containing only a few ribosomal genes (if any) (Table 2).

Screening of previous microbiome studies revealed the first potential genome assembly for *Sutterella stercoricanis*

We assessed the prevalence of the HQ MAGs retrieved in the present study among several GI microbiome surveys, either using whole-genome data (metagenome surveys) or the 16S rRNA genes data (amplicon surveys).

On the one hand, we assessed the prevalence of our HQ MAGs in humans' [44] and animals' [10] gastrointestinal metagenome catalogs (Table 3). We identified that some of the bacterial species represented by the HQ MAGs from this study seem to be more canid-specific – *Blautia_A* sp900541345, *Phascolarctobacterium* sp900544885, *Prevotellamassilia* sp900541335, *Succinivibrio* –, whereas others are more broadly distributed among animal microbiomes – *Catenibacterium* sp000437715, *Enterococcus_B hirae*, *Blautia* sp003287895, and *Sutterella*–.

On the other hand, we took advantage of the fact that long-read sequencing allows retrieving complete ribosomal genes, which are universal taxonomic markers for Bacteria. So, we further extracted the 16S rRNA genes of the HQ MAGs to link them to 16S rRNA gene-based microbiome studies (Fig. 4, and Additional File 6) –most of the microbiome studies use this genetic marker. We found out that the *Sutterella* HQ MAG is potentially the first high-quality genome assembly for *Sutterella stercoricanis* since its 16S rRNA genes presented identities > 98% with the previously reported 16S rRNA gene reference (NR_025600.1) (Fig. 4). *S. stercoricanis* was first isolated in feces from a healthy dog and was characterized using microbiological methods and 16S rRNA gene sequencing [50].

For the other five HQ MAGs without a reference genome, we identified that their 16S rRNA genes were closely related to others previously identified in wolves' distal gut microbiome [51] (*Succinivibrio* HQ MAG and *Prevotellamassilia* HQ MAG), canine intestinal microbiome [52] (*Phascolarctobacterium* HQ MAG), and human GI microbiome [53] (*Catenibacterium* and *Blautia* sp900541345 HQ MAG) (Additional File 6).

Table 3

Prevalence of the bacterial species identified in public microbiome surveys. For human-derived MAGs, the Unified Human Gut Genome database was used [44]. For animal-derived MAGs, the animal gut metagenome catalog [10] was used. If no MAG belonged to that bacterial species, we further screened GTDB [34]. For further detail on 16S rRNA gene phylogenies, see Additional File 6.

HQ MAG	Dog	Human	Other animals	Closest 16S	Main host
<i>Blautia_A sp900541345</i>	35	1	0	Human gut	Dog
<i>Phascolarctobacterium sp900544885</i>	12	1	0	Dog gut	Dog
<i>Prevotellamassilia sp900541335</i>	7	1	0	Wolves' gut	Canids
<i>g_Succinivibrio</i>	1	0	0	Wolves' gut	Canids
<i>Catenibacterium sp000437715</i>	27	691	2	Human gut	Human, animal
<i>Enterococcus_B hirae</i>	1	35	3	Multiple	Human, animal
<i>Blautia sp003287895</i>	1	6	1	Dog gut	Human, animal
<i>g_Sutterella</i>	0	1	0	Multiple carnivora	Human, animal

Finally, we performed a pangenome analysis among the HQ MAGs from our study and other genomes from the same bacterial species inhabiting different hosts to assess functional and genomic similarities (Additional File 7). We included only those in which more than 10 representative genomes were available: *Blautia_A sp900541345* (Additional File 7A), *Catenibacterium sp000437715* (Additional File 7B), *Enterococcus_B hirae* (Additional File 7C), *Phascolarctobacterium sp900544885* (Additional File 7D). Based on the ANI values, the HQ MAGs clustered with dog MAGs for *Blautia*, with a human MAG for *Phascolarctobacterium*, and with MAGs from mixed host origins for *Catenibacterium* and *Enterococcus hirae* (Additional File 7). The number of gene clusters belonging to the accessory genome was the highest for *Catenibacterium* (84%) when compared to *Enterococcus hirae* (66%), *Phascolarctobacterium sp900544885* (60%), and *Blautia_A sp900541345* (50%). Altogether, these results coincide with the fact that *Catenibacterium* and *Enterococcus hirae* seem to be more broadly distributed among different hosts (Table 3).

Long reads provide genomic context and enable capturing mobilome functions and antimicrobial-resistant genes

Long-reads enable to retrieve complete genes and their genomic context within a single read. Therefore, both the mobile genetic elements and the antimicrobial resistance genes assemble easily within the correct MAG.

We compared each HQ MAG's functional potential to previously published MAGs from the same bacterial species found in GI microbiome of dogs, humans, or other animals (Fig. 5). The main difference between the long-read HQ MAGs and other genomes from the same species in the public database is the overrepresentation of the COG category corresponding to Mobilome, except for *Blautia argii* and *Enterococcus hirae*, both with a reference genome in the database (Fig. 5B). Conversely to the MAGs from both UHGG and the animal gut metagenome catalogs obtained using exclusively short reads, the long-read metagenomic approach can retrieve mobile genetic elements and assemble them to the proper contig.

Finally, we further characterized the HQ MAGs to assess their potential antimicrobial resistance. Tetracycline resistance genes were detected in *Enterococcus hirae* (*tetM* gene), *Catenibacterium* sp000437715 (*tetM* gene), *Blautia* sp900541345 (*tet(O)* gene), and *Blautia* sp003287895 (*tet(32)* and *tet(40)* genes). Moreover, *Enterococcus hirae* also harbored *aac(6')-lid* gene conferring resistance to aminoglycosides. *Prevotellamassilia* HQ MAG harbored *Mef(En2)* gene, which encodes for an efflux pump that exports macrolides. *Phascolarctobacterium* HQ MAG harbored two copies of *Inu(C)* gene conferring resistance to lincosamide. Each *Inu(C)* gene was located in an *ISSag10* mobile element, allowing it to transpose. *Succinivibrio* and *Sutterella* HQ MAGs did not contain any antimicrobial resistance genes.

As an example of the potential of long-reads for providing genomic context, we were able to identify that *tetM* gene in *Enterococcus hirae* was in a region identified as a conjugative element (Tn916) integrated into the chromosome. This region encoded for a transposase, type 4 secretion system (T4SS), type 4 coupling protein, oriT, and relaxase (Additional File 8).

Discussion

Metagenomics approaches can provide new biological insights into the microbes inhabiting underexplored environments, such as the canine fecal microbiome. Here, we applied nanopore long-read metagenomics and frameshift aware correction to a fecal sample of a healthy dog and retrieved eight HQ MAGs and three MQ MAGs.

At the technical level, we compared an HMW and non-HMW DNA extraction to perform long-read metagenomics and confirmed that an HMW DNA extraction was the best choice. For analyses using unassembled raw reads, it improved the taxonomic classification and gave less unclassified reads. For metagenomics assembly, it improved the contiguity and increased the retrieval of longer and circular contigs (potential HQ MAGs). We tested several metagenomics assembly strategies (using HMW data only, 100%, 75%, and 50% of the total data) to retrieve the highest number of different HQ MAGs. The HMW data and the 75% data retrieved the highest number of HQ MAGs, but none of the performed assemblies alone retrieved the eight HQ MAGs.

The HQ MAGs belonged to *Succinivibrio*, *Sutterella*, *Prevotellamassilia*, *Phascolarctobacterium*, *Enterococcus*, *Blautia*, and *Catenibacterium* genera. The HQ MAGs presented > 90% completeness and <

5% contamination, improved the contiguity of previous MAGs in databases (single-contigs vs. multiple contigs), and contained the full-length ribosomal genes. Thus, our MAGs met MIMAG criteria for high-quality [32]. This fact is challenging when using shotgun metagenomics (with short-read technologies).

For *Sutterella* HQ MAG, we suggest that it is potentially the first reported high-quality genome assembly for *Sutterella stercoricanis*, which can be used as a representative genome for this bacterial species. It was first isolated in feces from a healthy dog, and it was defined as a novel species phenotypically and with full-length 16S rRNA sequencing [50]. Since the reference isolate lacks additional genome information, we compared the full-length 16S rRNA gene sequences to identify the bacterial species. Both the classical threshold of 97% identity and the updated one of 99% identity were met in this case [54]: the nine 16S rRNA genes presented identities from 99.04–98.69% against *S. stercoricanis* 16S ribosomal RNA (NR_025600.1). Whole-genome sequencing of the reference isolate and comparison to the HQ MAG could confirm if they represent the same species.

Despite humans and dogs share similar microbial signatures on the GI microbiome [6, 7], we found that *Succinivibrio*, *Prevotellamassilia* sp900541335, *Phascolarctobacterium* sp900544885, *Blautia_A* sp90054134 seem more canid-specific, whereas *Sutterella*, *Catenibacterium* sp000437715, *Enterococcus_B hirae*, and *Blautia* sp003287895 are more broadly distributed among human and animal gastrointestinal microbiomes. These findings highlight the need for building and using niche-specific databases to accurately map and classify new reads from a particular environment and understand the overall biological significance [13, 55].

The genera *Succinivibrio*, *Prevotella*, *Phascolarctobacterium*, *Catenibacterium*, and *Blautia*, are recognized short-chain fatty acid (SCFA) producers [56–58]. These molecules provide multiple gut health benefits, from reducing inflammation and tumorigenesis to increasing gut motility and secretory activity [2, 57, 59]. In the dog GI microbiome, different diets and dietary interventions can modulate their abundances to promote gut health [7, 60–65]. Moreover, several studies on dog GI microbiome identified *Blautia* genus – among others – as a microbial marker for health and had targeted it to assess differences with disease status [66–69]. Thus, in-depth characterization of these genera is of most relevance to defining a healthy GI microbiome in dogs.

Sutterella stercoricanis was isolated from the feces of a healthy dog [50]. However, the increase of the genus *Sutterella* was associated with detrimental effects rather than health. Dogs with acute hemorrhagic diarrhea presented higher *Sutterella* [66], and some diets aiming to promote health benefits observed its decrease [70, 71]. Further metagenomics studies are needed to identify the different *Sutterella* species on dog feces and correlate their abundances to health or disease status.

Finally, *Enterococcus hirae* is a prevalent Enterococci species of the GI microbiome of healthy dogs. However, Enterococci species usually carry antimicrobial-resistant genes and virulence factors and are potential antimicrobial-resistant gene reservoirs that could be transferred to people [72–76]. *Enterococcus* HQ MAG harbors *aac(6′)-lid* gene, which conferred resistance to aminoglycosides. Besides, it harbors a *tetM* gene within the Tn916 conjugative element, which was first reported in *Enterococcus faecalis* [77,

78]. The use of long-reads enables the retrieval of complete genes and their genomic context within a single read, which facilitates the location of antimicrobial resistance genes within the proper MAG and the evaluation of its mobilization mechanisms [79, 80].

Tetracycline resistance genes were found not only in the genome of *Enterococcus hirae*, but also in *Catenibacterium* and both *Blautia* HQ MAGs and could be linked to a previous antimicrobial exposure that selected the resistant bacteria [81]. Three years before sampling, this dog was treated with doxycycline – tetracycline-class antibiotic– for 15 days due to excess secretion of mucus and saliva. Whole resistome analyses are needed to determine the AMR genes within the fecal microbiome in healthy dogs and evaluate all the bacterial species and their mobile genetic elements that could act as a reservoir for AMR genes.

At the functional level, we detected an overrepresentation of the Mobilome COG category within most of the HQ MAGs retrieved here when compared to other MAGs –not when compared to reference genomes. Long-reads allow retrieving complete mobile genetic elements together with their genomic context facilitating its assembly to the proper MAG. This advantage was also reported in metagenomics studies that include long-reads in their assemblies (hybrid assemblies) [11, 13, 82].

Apart from eight HQ MAGs, we recovered three different MQ MAGs from potentially new species of the *Bacteroides* and *Phocaeicola* genera and *Phocaeicola plebeius*. Our next step is to apply proximity ligation to link all contigs among them and recover new HQ MAGs and MQ MAGs and link antimicrobial resistance genes, mobile genetic elements, and bacteriophages to their bacterial host [83].

A limitation of this study is the use of nanopore-only data since it can compromise the accuracy of the HQ MAGs. However, the combination of high-accuracy basecallers and raw reads correction, followed by further polishing of the metagenome assemblies increased the consensus accuracy to levels suitable to retrieve metagenome-assembled genomes from a single fecal sample. In our case, we applied Guppy for basecalling, Canu for raw reads correction, and Medaka for polishing the assembled metagenomes. To ensure reducing the insertion and deletion error type, we further applied a frameshift-aware correction step [16] that improved the completeness and reduced the CDS number. Long-read metagenomics sequencing can be harnessed to build comprehensive and curated databases for in-depth characterization of novel bacterial diversity in the canine fecal microbiome or any other underexplored environment.

Abbreviations

GI

Gastrointestinal

HMW

high-molecular weight DNA

Non-HMW

non high-molecular weight DNA

MAG
metagenome-assembled genome
HQ MAG
high-quality metagenome-assembled genome
MQ MAG
medium-quality metagenome-assembled genome
Indels
insertions and deletions
AMR
antimicrobial resistance

Declarations

Ethics approval and consent to participate: The owner gave written consent to the collection of samples.

Consent for publication: not applicable.

Availability of data and materials: The raw assemblies, the metagenome-assembled genomes, and an overview of the scripts used are available on Zenodo: <https://zenodo.org/record/3982645>. An overview of the scripts used to analyze the data is at Additional File 1.

Competing interests: AC, NF, and JV work for Vetgenomics, SL. The other authors declare that they have no competing interests.

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Authors' contributions: OF and AC conceptualized the study. OF and AC designed the experiment. DP extracted the DNA, performed the sequencing libraries and the nanopore sequencing. AC performed the metagenome assembly and correction. AC analyzed and interpreted the data. JV analyzed the antimicrobial-resistance genes. NF and DP performed the pangenome analysis. AC wrote the main manuscript text. OF, NF, DP and JV substantially revised the work. All the authors have approved the submitted version.

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Figures

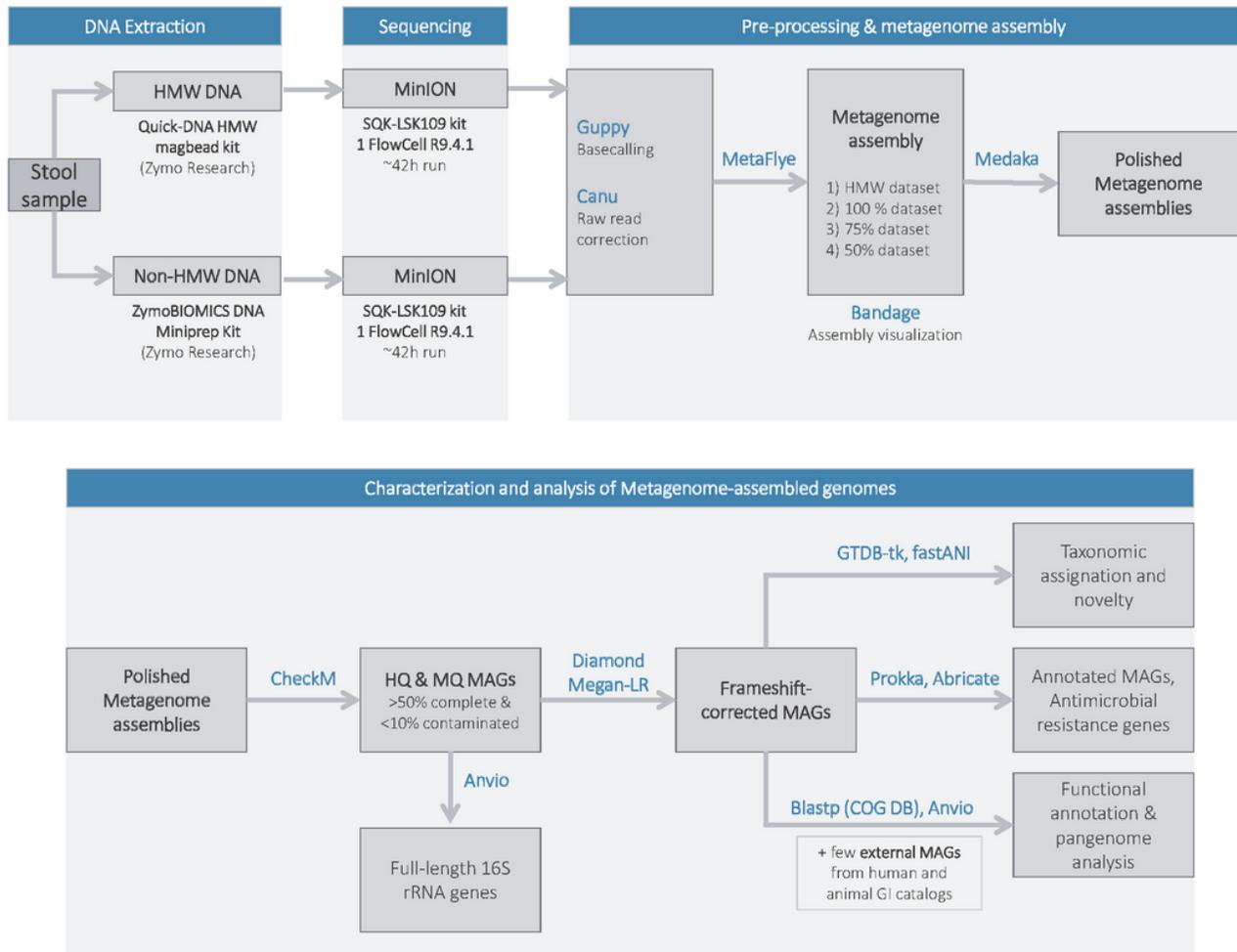
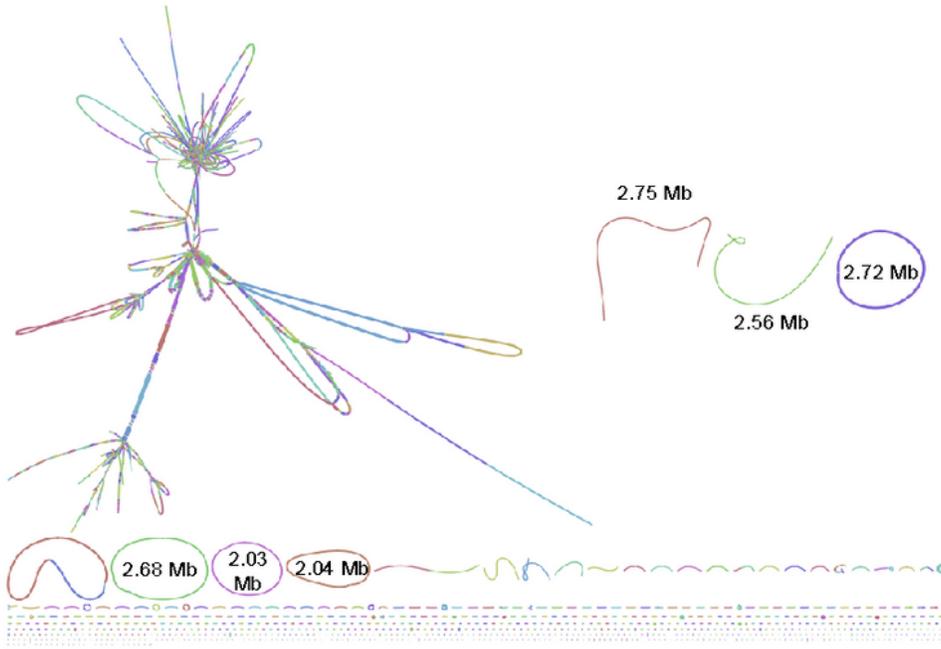


Figure 1

Experimental design overview. A single fecal sample from a healthy dog was extracted using a HMW and a non-HMW DNA extraction. Samples were sequenced using a nanopore sequencing. Raw reads were basecalled and corrected prior to assembly. Four different data subsets were assembled to retrieve the maximum number of high-quality MAGs. Those MAGs were frameshift-corrected and further analyzed.

A) HMW DNA metagenome assembly



B) Non-HMW DNA metagenome assembly

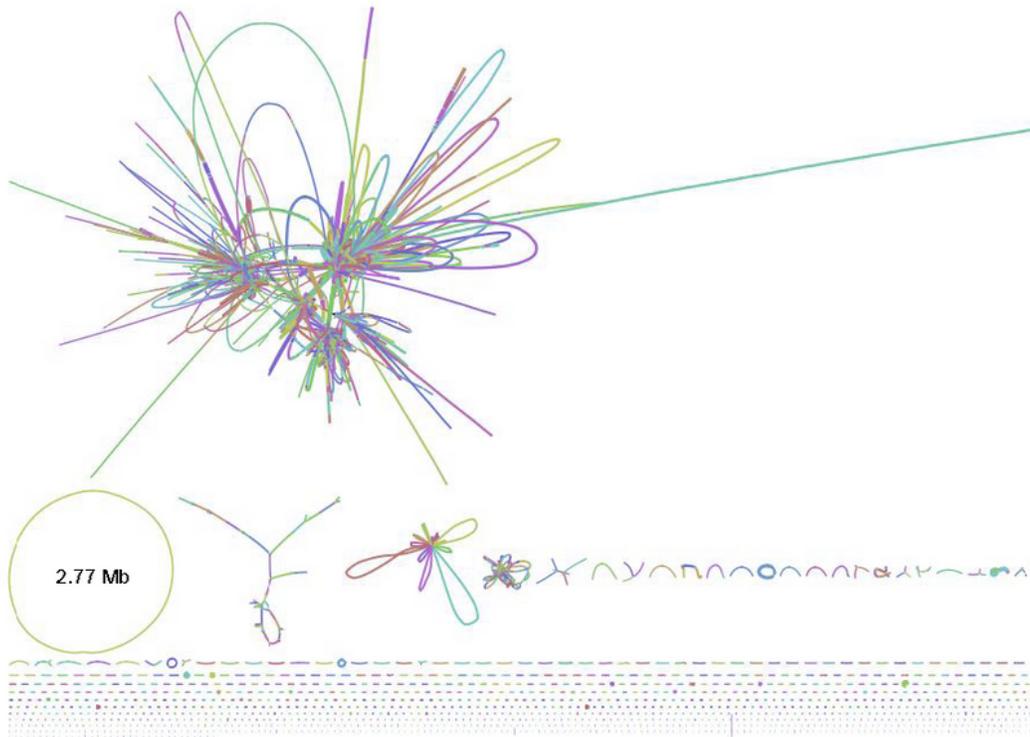
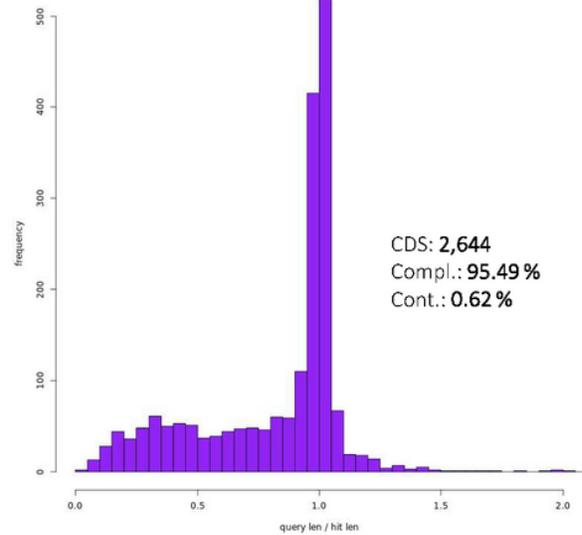
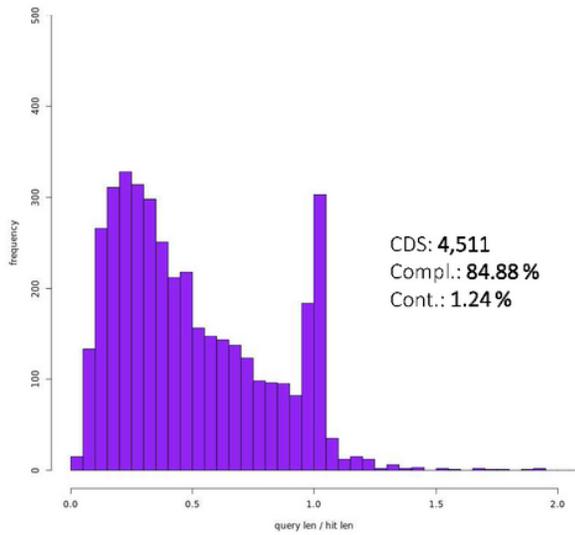


Figure 2

HMW-DNA vs. non-HMW DNA metagenomics assembly from the fecal sample of a healthy dog. Bandage plots of A) HMW-DNA assembly and B) non-HMW DNA assembly. HMW-DNA allows the recovery of long, circular contigs, which can potentially represent complete closed MAGs. We report the longest contig in both datasets (Mb).

Sutterella, 50X coverage



Blautia sp900541345, 44X coverage

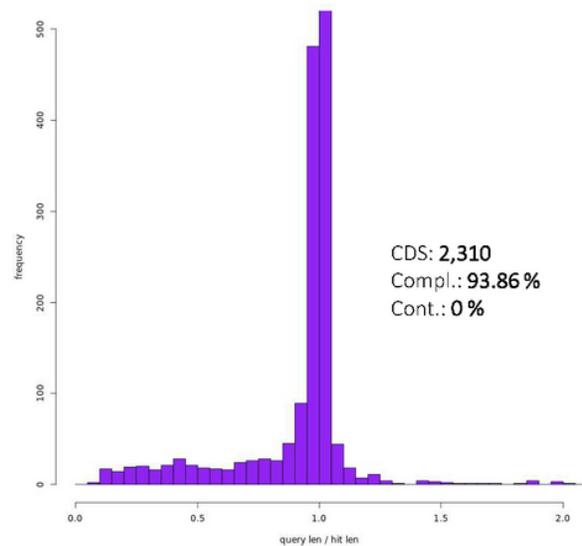
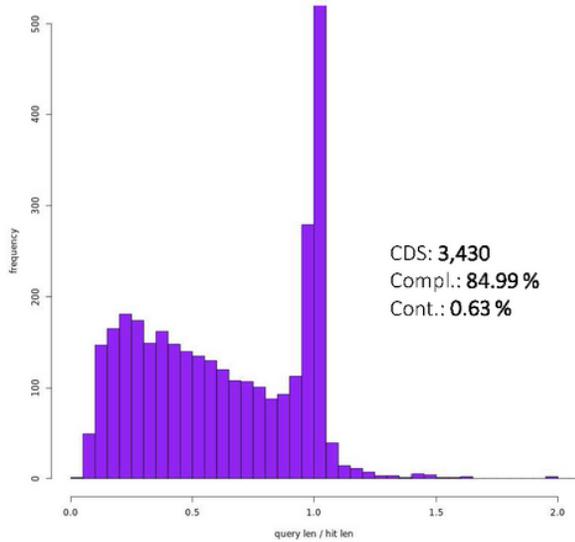
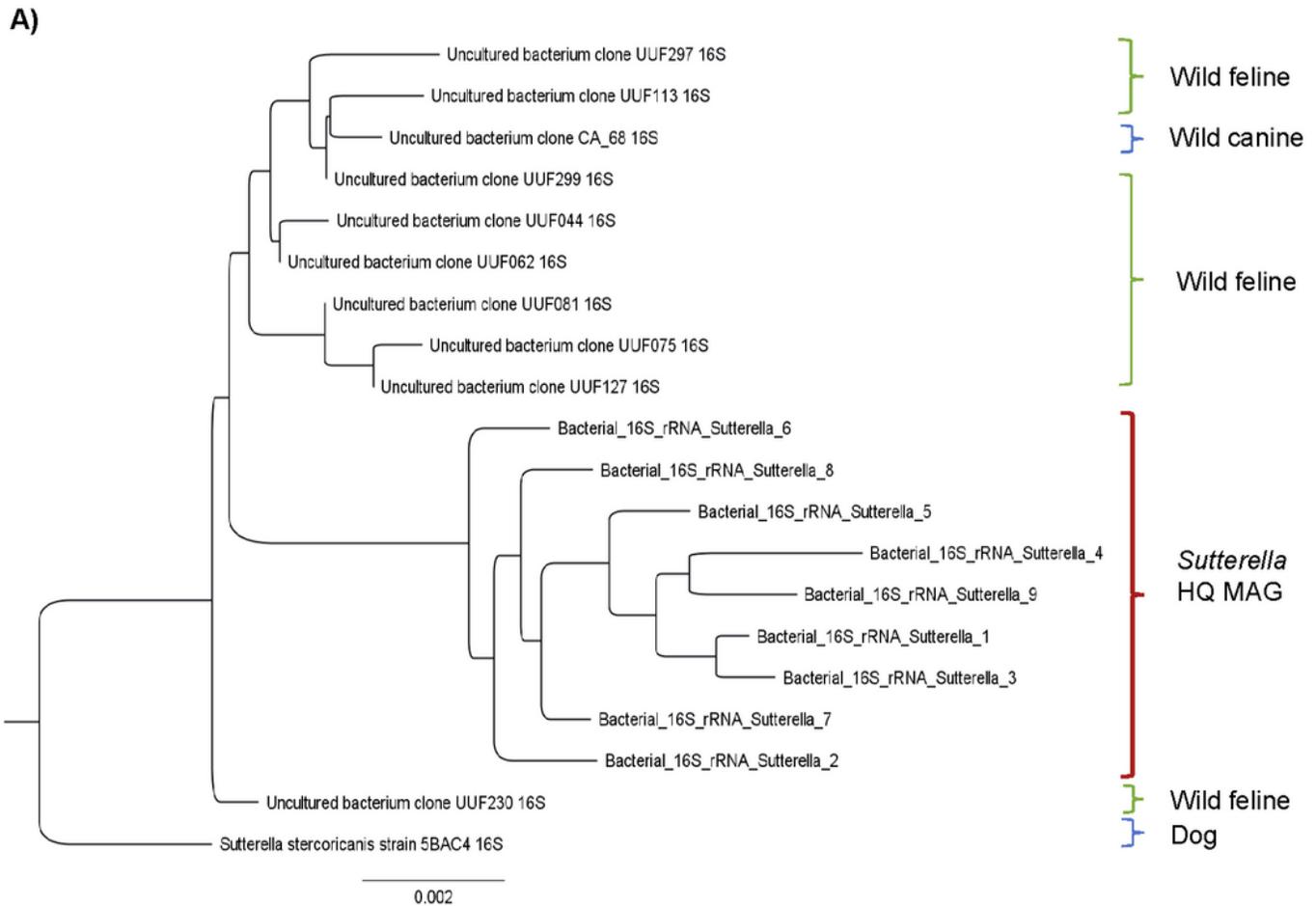


Figure 3

Histograms of the insertions and deletions in medium-quality MAGs (left) transformed into high-quality MAGs, after frameshift correction (right). The number of CDS, completeness, and contamination are also included to evaluate the quality. Y-axis scale is 500 for better visualization of the insertions and deletions.



B)

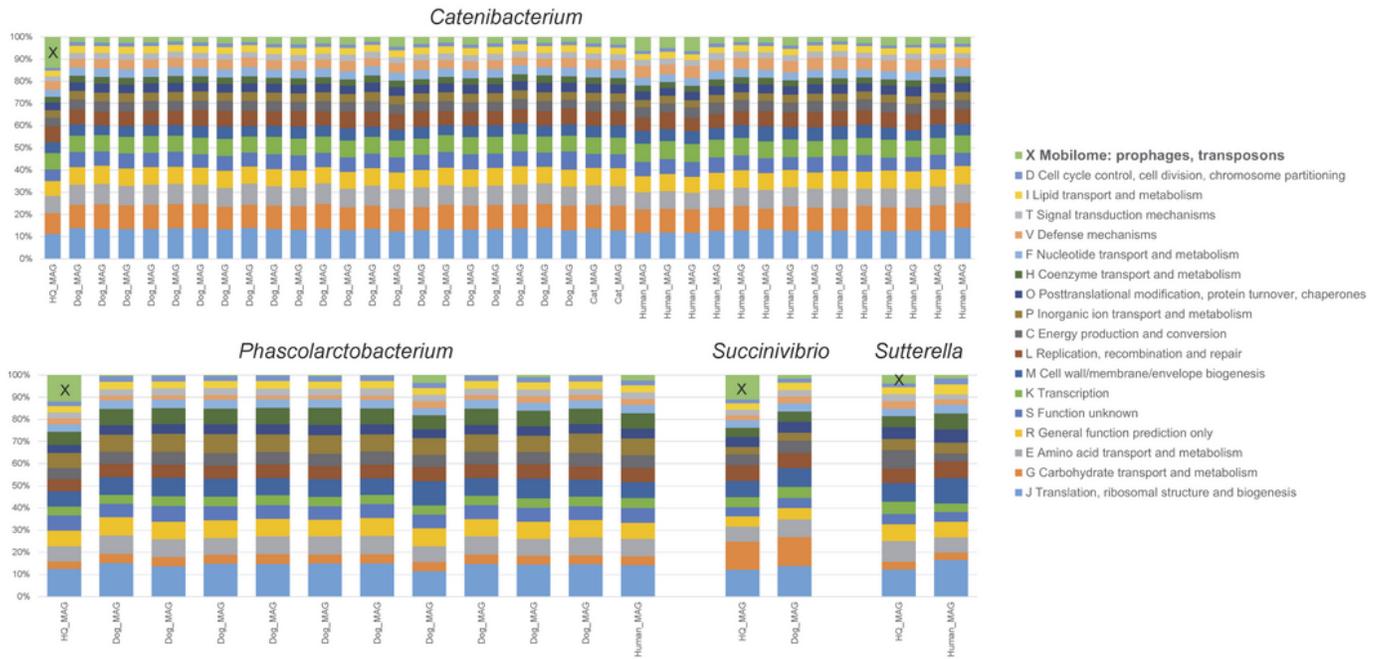
<i>Sutterella stercoricanis</i> (NR_025600.1)	100													
16S_1_ <i>Sutterella</i> _HQ-MAG	98.83	100												
16S_3_ <i>Sutterella</i> _HQ-MAG	98.76	99.87	100											
16S_2_ <i>Sutterella</i> _HQ-MAG	98.97	99.54	99.41	100										
16S_4_ <i>Sutterella</i> _HQ-MAG	98.69	99.54	99.54	99.35	100									
16S_6_ <i>Sutterella</i> _HQ-MAG	99.04	99.48	99.35	99.67	99.41	100								
16S_9_ <i>Sutterella</i> _HQ-MAG	98.69	99.67	99.67	99.61	99.61	99.54	100							
16S_5_ <i>Sutterella</i> _HQ-MAG	98.76	99.8	99.74	99.61	99.61	99.54	99.61	100						
16S_8_ <i>Sutterella</i> _HQ-MAG	98.9	99.61	99.54	99.8	99.41	99.74	99.67	99.67	100					
16S_7_ <i>Sutterella</i> _HQ-MAG	98.97	99.67	99.61	99.74	99.61	99.8	99.61	99.74	99.8	100				

Figure 4

Similarity of 16S rRNA gene from *Sutterella* HQ MAGs to public datasets. The 16S rRNA gene comparison from *Sutterella* HQ MAGs suggested it is the genome assembly for *Sutterella stercoricanis*. A) Phylogenetic 16S rRNA gene tree of *Sutterella* HQ MAGs. It presents high similarity to uncultured bacterium clone with codes UUF from *Panthera uncia* (wild feline); uncultured bacterium clone CA_68 from *Cuon alpinus* (wild canid) (JN559525.1), and *S. stercoricanis* from dog feces [50]. B) Identity matrix

of 16S rRNA genes of Sutterella HQ MAG against *S. stercoricanis* (NR_025600.1). Sutterella HQ MAG contained nine 16S rRNA genes that were more than 98% identical to NR_025600.1 (reference). Specifically, 16S_6 presented more than 99% of identity.

A) Functional profile considering the more abundant COG categories



B) Mobilome COG category distribution

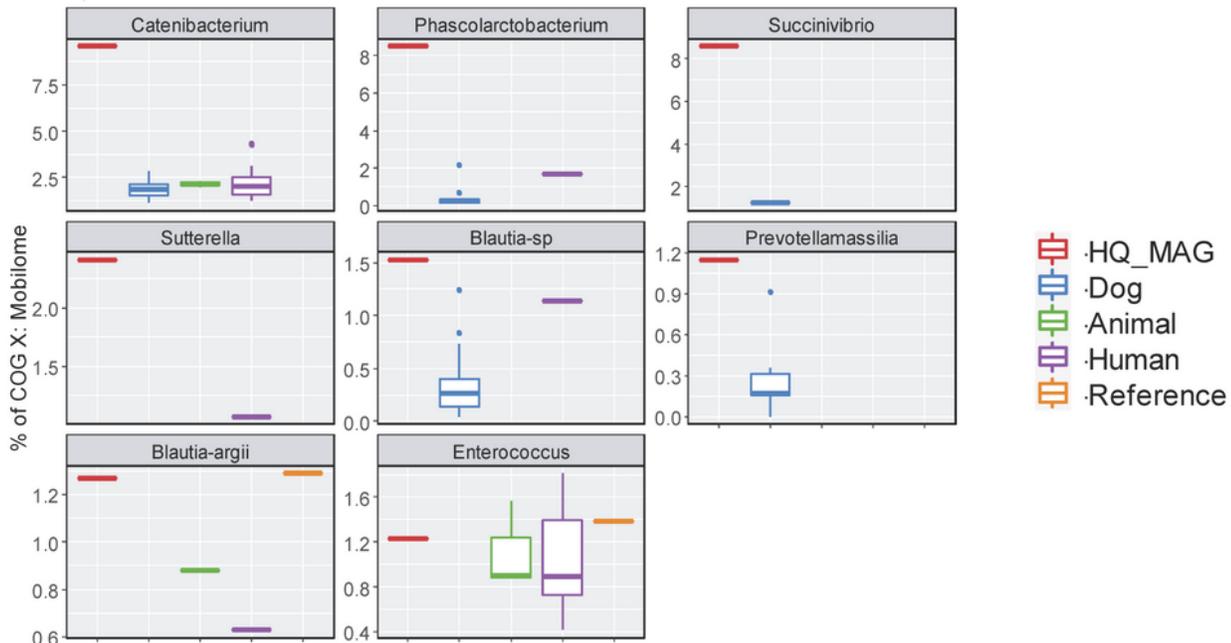


Figure 5

Functional analysis and comparison of HQ MAGs and published bacterial species using the COG database. A) Stacked bar plots representing the 18 more abundant COG categories for Catenibacterium,

Phascolarctobacterium, Succinivibrio, and Sutterella representatives (HQ MAGs with more Mobilome functions). Y-axis is escalated to 100% for visualization purposes. B) Boxplots representing the actual percentage of mobilome COG category in each of the HQ MAGs and the bacterial species present in the databases grouped by origin. Y-axis presents different values.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Suppinformation.pdf](#)