Metagenomic dissection of the canine gut microbiota: insights into taxonomic, metabolic and nutritional features

Original
Metagenomic dissection of the canine gut microbiota: insights into taxonomic, metabolic and nutritional features / Alessandri, G.; Milani, C.; Mancabelli, L.; Mangifesta, M.; Lugli, G. A.; Viappiani, A.; Duranti, S.; Turroni, F.; Ossiprandi, M. C.; van Sinderen, D.; Ventura, M.. - In: ENVIRONMENTAL MICROBIOLOGY. - ISSN 1462-2912. - 21:4(2019), pp. 1331-1343. [10.1111/1462-2920.14540]

Publisher:
Blackwell Publishing Ltd

Published
DOI:10.1111/1462-2920.14540

environmental microbiology
environmental microbiology reports

Metagenomic dissection of the canine gut microbiota: insights into taxonomic, metabolic and nutritional features

Journal:	*Environmental Microbiology and Environmental Microbiology Reports*
Manuscript ID	EMI-2018-1689.R1
Journal:	Environmental Microbiology
Manuscript Type:	EMI - Research article
Date Submitted by the Author:	n/a
Complete List of Authors:	Alessandri, Giulia; University of Parma, Department of Veterinary Medical Science Milani, Christian Mancabelli, Leonardo; University of Parma, Life Sciences Mangifesta, Marta; University of Parma, Life Sciences Lugli, Gabriele Andrea; University of Parma, Department of Chemistry, Life Sciences and Environmental Sustainability Viappiani, Alice; University of Parma, GenProbio srl Duranti, Sabrina Turroni, Francesca Ossiprandi, Maria; University of Parma, Department of Medical-Veterinary Science van Sinderen, Douwe; National University of Ireland , Department of Microbiology Ventura, Marco; University of Parma, Life Sciences
Keywords:	microbiome, bifidobacteria, metagenomics, dog

Metagenomic dissection of the canine gut microbiota: insights into taxonomic, metabolic and nutritional features

Running title: The canine gut microbiota

Key words: microbiome, bifidobacteria, metagenomics, dog

Giulia Alessandri[1*], Christian Milani[2*], Leonardo Mancabelli[2], Marta Mangifesta[2], Gabriele Andrea Lugli[2], Alice Viappiani[3], Sabrina Duranti[2], Francesca Turroni[2,4], Maria Cristina Ossiprandi[1,4], Douwe van Sinderen[5] and Marco Ventura[2,4]

*These authors contributed equally

Department of Veterinary Medical Science, University of Parma, Parma, Italy[1]; Laboratory of Probiogenomics, Department of Chemistry, Life Sciences, and Environmental Sustainability, University of Parma, Parma, Italy[2]; GenProbio srl, Parma, Italy[3]; Microbiome Research Hub, University of Parma, Parma, Italy[4]; APC Microbiome Institute and School of Microbiology, Bioscience Institute, National University of Ireland, Cork, Ireland[5]

Correspondence. Mailing address for Marco Ventura Laboratory of Probiogenomics, Department of Chemistry, Life Sciences, and Environmental Sustainability, University of Parma, Parma Area delle Scienze 11a, 43124 Parma, Italy. Phone: ++39-521-905666. Fax: ++39-521-905604. E-mail: marco.ventura@unipr.it

Summary

Domestication of dogs from wolves is the oldest known example of ongoing animal selection, responsible for generating more than 300 dog breeds worldwide. In order to investigate the taxonomic and functional evolution of the canine gut microbiota, a multi-omics approach was applied to six wild wolves and 169 dog fecal samples, the latter encompassing 51 breeds, which fully covers currently known canine genetic biodiversity. Specifically, 16S rRNA gene and bifidobacterial Internally Transcribed Spacer (ITS) profiling were employed to reconstruct and then compare the canine core gut microbiota to those of wolves and humans, revealing that artificial selection and subsequent cohabitation of dogs with their owners influenced the microbial population of canine gut through loss and acquisition of specific bacterial taxa. Moreover, comparative analysis of the intestinal bacterial population of dogs fed on Bones and Raw Food (BARF) or commercial food (CF) diet, coupled with shotgun metagenomics, highlighted that both bacterial composition and metabolic repertoire of the canine gut microbiota have evolved to adapt to high-protein or high-carbohydrates intake. Altogether, these data indicate that artificial selection and domestication not only affected the canine genome, but also shaped extensively the bacterial population harbored by the canine gut.

Introduction

The gastrointestinal (GI) microbiota is a large and highly complex community of microorganisms that plays a crucial role in maintaining and promoting host health (Suchodolski et al., 2010; Suchodolski et al., 2012). Historically, characterization of the GI microbiota was performed by means of culture-dependent methods, allowing biochemical and physiological investigations of isolated strains. However, although isolation of novel species is routinely documented, many intestinal microorganisms remain uncultivated and therefore have not been characterized (Furrie, 2006; Deng and Swanson, 2015). In recent years, the availability of constantly advancing next-generation sequencing (NGS) technologies, together with tailor-made bioinformatic tools, have provided novel culture-independent approaches to better assess the composition, functionality and dynamics of this microbial intestinal ecosystem. The high through-put and low cost of NGS technologies has facilitated the study of the intestinal microbiota of not only humans but also of other mammals, including livestock animals (Kim et al., 2011; Ferrario et al., 2017) and companion animals (Suchodolski et al., 2015; Guard et al., 2017).

The domesticated dog (*Canis lupus familiaris*) is a key companion animal of humans. The possible impact that the GI microbiota has on canine health and well-being is of broad interest (Kim et al., 2017; Moon et al., 2018). Along the canine GI tract, the various compartments differ in microbial composition and total bacterial numbers (Hooda et al., 2012). Notably, the large intestine and feces possess the highest density and diversity of bacteria, with Firmicutes, Bacteroidetes, Proteobacteria and Fusobacteria representing the prevalent bacterial phyla (Suchodolski, 2011). In this context, high throughput sequencing has been used to investigate the taxonomical composition of the intestinal microbiota of healthy dogs (Suchodolski et al., 2008; Garcia-Mazcorro et al., 2012; Hand et al., 2013; Omatsu et al., 2018). However, these studies involved a small number of samples belonging to a single or just a few different breeds. Like other mammals, the canine gut microbiota appears to be influenced by several factors, such as diet (Wu et al., 2016; Herstad et al., 2017; Kim et al., 2017), age (Masuoka et al., 2017), metabolic disorders including obesity and diabetes (Xu et al., 2016), as well as intestinal inflammatory diseases (Honneffer et al., 2014).

Despite its original classification as an obligate carnivore, the domestic dog is currently considered omnivorous and able to metabolize a wide variety of dietary carbohydrates that are typically present in commercial pet foods (Swanson et al., 2011). However, in recent years, a novel nutrition for dogs referred to as the Bones and Raw Food (BARF) diet has become rather popular. The BARF diet includes uncooked meat, bones and, though at relatively low levels, vegetables, eggs, and dairy products (van Bree et al., 2018). Although health benefits such as improvement of coat and skin, reduction in dental diseases and alleviation of arthritis have been linked to the consumption of a BARF diet, it has also been demonstrated that this diet is associated with nutritional imbalance and bacterial contamination (Fredriksson-Ahomaa et al., 2017; Kim et al., 2017).

Even though the gut microbiota is a major research topic in microbial ecology, the canine GI microbiota composition is still far from being fully dissected (Hand et al., 2013). In the current study we investigated the taxonomical composition of the canine gut microbiota based on 16S rRNA gene and bifidobacterial ITS profiling, involving a total of six wolves and 169 canine fecal samples belonging to 51 different breeds. Moreover, shotgun metagenomics was employed to assess the metabolic repertoire of the dog gut microbiome fed with two distinct diets in order to shed light on microbial and associated functional changes due to the different protein and carbohydrate intakes.

Results and discussion

Taxonomic classification of the intestinal microbial community of *Canis lupus*. In order to explore the taxonomical composition of the gut microbiota of the mammalian species *Canis lupus*, a total of 175 fecal samples were collected. In detail, six of these fecal samples belonged to specimens of the grey wolf, while the other 169 fecal samples belonged to members of 51 canine breeds, uniformly distributed along the phylogenetic cluster of breeds as reconstructed by Parker *et al.* based on SNP genotype analysis (Parker et al., 2004). Metadata of these collected samples are reported in Table S1. Bacterial DNA extracted from the fecal samples was subjected to 16S rRNA gene sequencing analysis as previously described (Milani et al., 2013). Illumina-mediated sequencing of the abovementioned samples generated a total of 12,702,820 sequencing reads with an average of 72,588 reads per sample (Table S2). Quality and chimera filtering produced a total of 8,329,451 filtered reads with an average of 47,597 filtered reads per sample (Table S2). Taxonomic reconstruction of the bacterial population encompassed by each of the analysed samples is reported in Additional Data File 1. Alpha-diversity analysis, performed through Chao1 index calculation for 10 sub-samplings of sequenced read pools, showed that all curves tend to plateau, thereby indicating that sample biodiversity was in all cases adequately covered by the applied sequencing depth (Fig. S1). Moreover, PCoA representation of the unweighted Unifrac distance matrix obtained by analysis of the datasets generated by this study did not reveal any significant clustering as based on evolutionary distance between profiled domesticated dog breeds and their wild (i.e. wolf) relative (Fig. S1). In addition, bioinformatic analyses were performed to evaluate if differences in the canine gut microbiota may be dependent on canine breed. However, these analyses did not reveal any statistically significant differences, suggesting that, in this case, host phylogeny divergence plays a minor role in the modulation of dogs' gut population.

Genus-level core gut microbiota of the *Canis lupus familiaris*. Reconstruction of a core microbiota, which represents bacterial taxa that are shared across samples of a defined cohort (Salonen et al., 2012), allows identification of dominant and prevalent bacterial species that have been preserved during co-evolution of the intestinal community and its host (Tap et al., 2009; Salonen et al., 2012). In order to determine the core gut bacterial community of the collected fecal samples, bacterial genera present in at least 80 % of the samples and with at least an average relative abundance of >0.01 % were considered. Based on these criteria, we identified 43 bacterial genera (Figure 1). In detail, at phylum level the core microbiota was dominated by taxa belonging to Bacteroidetes (total average abundance 33.68 %), followed by Fusobacteria (25.53 %), Firmicutes (23.56 %), Proteobacteria (6.29 %) and Actinobacteria (0.93 %) (Figure 1). As could be expected, the core microbiota includes genera of the five dominant phyla generally found in the canine fecal microbiota (Hand et al., 2013; Moon et al., 2018). Furthermore, at genus level, a particular representative of the Fusobacteria phyla, i.e., *Fusobacterium*, was shown to be present at the highest average relative abundance (25.36 %) among all domesticated dog breeds (Figure 1), suggesting extensive co-evolution between this taxon and the canine GI. Moreover, *Prevotella* 9 and *Bacteroides*, which both belong to the Bacteroidetes phylum, were second and third most abundant genera in the canine microbiota (13.86 % and 13.43 %, respectively). In a human context, *Bacteroides* and *Prevotella* have been linked to a vegan or vegetarian diet (De Filippo et al., 2010). Therefore, the high abundance of these two genera in the canine gut microbiota may be due to the transition from a carnivorous diet typical of wolves to the omnivorous diet of domestic dogs (see below).

Role of diet as modulator of the canine core gut microbiota. As reported in Table S1, the collected samples belonged to dogs that followed different diets: 141 dogs had been fed with commercial food preparations, typically produced to guarantee a balanced nutritional intake, with a high abundance of fibres and carbohydrates generally higher than 3 % and 30 %, respectively. In contrast, the diet of 28 dogs was based on BARF. Therefore, in order to determine whether and to what extent diet may modulate the canine core gut microbiota, the collected samples were divided into two groups, encompassing dogs fed with commercial food (CF group) and dogs following a BARF diet (BARF group).

Evaluation of the bacterial biodiversity of the two diet groups was performed through the Chao1 index calculated for 10 sub-samplings of sequenced read pools obtained for each sampled dog up to a maximum of 30,000 reads. The two curves, corresponding to the average observed for the CF and BARF groups, are significantly different based on Student's t-test statistical analysis calculated at 30,000 reads (p-value < 0.01) (Fig. 2a). Interestingly, the average rarefaction curves showed a higher level of complexity of the CF group gut microbiota compared to the BARF group. Moreover, the β-diversity was analysed based on unweighted UniFrac and represented through Principal Coordinate Analysis (PCoA) (Fig 2b). The predicted PCoA exhibited partial clustering of CF and BARF groups (P-value < 0.01), supporting the notion that the two distinct diets indeed cause differences in the canine gut microbiota. In addition, analysis of the predicted taxonomic profiles at phylum level revealed that the average abundance of three of the five phyla that are present in the canine core gut microbiota appeared to be altered by diet. Specifically, Fusobacteria and Actinobacteria were significantly increased in dogs fed on a BARF diet, while Bacteroidetes showed an opposite trend (Fig. 2; Table S3). An in-depth inspection at genus level revealed that 14 of the 43 core genera are significantly affected by diet (Fig. 2c). Interestingly, the two most representative genera of the core microbiota, i.e., *Fusobacterium* and *Bacteroides*, did not significantly fluctuate in the two assessed canine groups (Fig. 2c). Otherwise, *Prevotella 9*, *Faecalibacterium* and *Sutterella* significantly decreased in the BARF group compared to the CF group (Fig. 2c; Table S3). In this context, it has been shown that a high abundance of *Prevotella* in the human gut microbiota correlates with a fiber-based diet, due to the capability of members of this microbial genus to degrade simple carbohydrates (David et al., 2014; Schnorr et al., 2014), while it is known that *Faecalibacterium* spp. and *Sutterella* spp. can also metabolize a wide range of different carbohydrates (Lopez-Siles et al., 2012; Liu et al., 2016). Therefore, dogs fed with commercial pet foods, which are typically enriched in fibers and carbohydrates, are associated with a higher abundance of these saccharolytic species, as compared with dogs of the BARF group whose diet was based on a high abundance of animal proteins and fats. Notably, in humans, *Faecalibacterium*, and in particular *Faecalibacterium prausnitzii*, is associated with a healthy microbiota (Lopez-Siles et al., 2018). Indeed, as a butyrogenic bacterium, this commensal species has been reported to possess anti-inflammatory features and to positively influence the gut physiology (Sokol et al., 2008). In this context, the reduction of *Faecalibacterium* spp. in the BARF group indicates that a meat-based diet is less protective against inflammatory activity in the canine gut.

Effect of artificial selection and close contact with humans on the canine gut microbiota evolution. The dog was the first animal species to be domesticated from wild grey wolves over 15,000 years ago (Savolainen et al., 2002), thus becoming a very coveted companion animal of humans. In this context, it has been demonstrated that man-made selection of canine breeds generated both phenotypic and genotypic changes in dogs (Savolainen et al., 2002). In order to assess if artificial selection and close contact with humans may have impacted on the canine gut microbiota, the latter was compared to the wolf gut bacterial community. Due to the difficulty of collecting feces of wolves living in wild conditions, we were able to retrieve six fecal samples. Thus, it's worth to underline that additional samples may improve accuracy of the comparative analysis. Considering only the bacterial genera with a prevalence > 80 %, the analysis showed that the wolf gut microbiota consists of 39 bacterial genera, while 43 bacterial taxa were commonly found in all dog samples. Interestingly, *Bacteroides*, U.m. of Lachnospiraceae family, *Faecalibacterium*, *Anaerostipes*, *Fusobacterium* and *Ruminococcus gnavus* group were shared among all investigated dog and wolf samples (Fig. 3). An additional 17 genera were present in all wolf fecal samples and in more than 80 % of the assessed fecal samples from dogs (Fig. 3), suggesting that these 23 bacterial taxa have co-evolved with the species *Canis lupus*, regardless of human intervention. Interestingly, six genera of the core gut microbiota of wolves, i.e., *Alistipes*, *Pseudomonas*, *Slackia*, *Subdoligranulum*, *Eubacterium coprostanoligenes* group and *Barnesiella*, were not represented in the canine core (Fig. 3), suggesting that modifications in the animal lifestyle and the human influence, i.e., domestication, have promoted a modulation of the gut microbiota of dogs when compared to their wild ancestors.

In addition, as predators, the diet of wolves is almost exclusively based on raw meat. Comparison of the BARF and CF groups' gut microbiota to that of wolves further support what is reported above, showing a statistically significant progressive increase in the relative abundance of carbohydrate-degrading taxa such as *Prevotella 9* and *Sutterella,* moving from a raw-meat based diet typical of wolves and BARF dogs to a CF diet. (Figure S2). Conversely, *Parabacteroides* and Ruminococcaceae UCG-005 exhibited an opposite trend, displaying a significant reduction in relative abundance in the CF group as compared to wolves and dogs belonging to the BARF group whose diet is based on raw-meat (Figure S2).

Evaluation of shared and unique bacterial genera of the canine core intestinal community as compared to the human core gut microbiota. To assess if domestication of dogs and their cohabitation with humans has allowed microbiota exchanges, we compared the canine core gut microbiota with that of humans. In order to include comparable 16S rRNA gene microbiota profiling data, the reconstruction of the human core gut microbiota was assessed through the re-analysis of 79 fecal samples of healthy adult individuals used as control group in a previous study where the experimental procedures were the same of this study (Mancabelli et al., 2017). Interestingly, of the six bacterial genera common to all canine samples, *Fusobacterium* and *Ruminococcus gnavus* group were not represented in the human microbiota (Fig. 3), indicating that these microbial taxa are typical inhabitants of the canine gut. Moreover, domestication seemed to have caused the loss of six bacterial genera in dogs with respect to its wild relative (Fig. 3), while just five microbial taxa were specifically shared between human and canine core gut microbiota (Fig. 3). Indeed, *Dorea*, *Parabacteroides*, *Streptococcus*, U. m. of Bacteroidales order and U. m. of Clostridiales order were present in both the human and dog core gut microbiota yet were absent in the core gut microbiota of wolves (Fig. 3). These data therefore suggest that the shift from a natural, undomesticated life style to that which involved cohabitation with humans has caused major changes in the bacterial composition of domesticated dogs.

The effect of aging on canine core gut microbiota. Canine life stage classification is known to be affected by both breed and size of dogs (Greer et al., 2007; Fleming et al., 2011; Bartges et al., 2012). In order to evaluate age-related changes in the canine core microbiota, samples were divided into 4 age groups, while disregarding their breed, including puppies (20 dogs, 0 – 8 months old), junior (27 dogs, 9 – 24 months old), adult (104 dogs, 25 – 96 months old) and senior (18 dogs, > 97 months old). Grey wolves' fecal samples were excluded from this analysis as their age was unknown. Considering the canine core genus microbiota, U. m. of Bacteroidales order, *Phascolarctobacterium*, *Roseburia* and *Fusobacterium* significantly differ among the four age groups (ANOVA P-value < 0.01) (Fig. S3). Interestingly, a higher level of *Fusobacterium* was reached in canine adulthood (relative abundance 28.80 %), as compared to the junior (20.75 %) and senior (17.58 %) groups. Furthermore, *Roseburia* significantly increased in the senior group, while U. m. of Bacteroidales order was more abundant in puppies when compared to the junior and adult groups. In addition, *Phascolarctobacterium* was shown to be present at a higher abundance in the junior group when compared to the other assessed age groups (Fig. S3)

Moreover, a significant reduction in the abundance of the *Bifidobacterium* genus was apparent in adult and senior groups (average relative abundance of 0.21 % in both cases) when compared to puppies (0.57 %) (P-value < 0.05) (Fig. S3). Therefore, these data suggest that the bifidobacterial population in the canine gut microbiota exhibits a similar trend to that observed in the human intestinal microbiota (Arboleya et al., 2016; Milani et al., 2017; Turroni et al., 2018).

Profiling of the bifidobacterial community harbored by the canine gut microbiota. In order to further investigate the bifidobacterial communities harbored by the canine gut microbiota, a recently developed pipeline based on genus-specific primers targeting the hypervariable ITS region was applied to all 175 collected samples (Milani et al., 2014). Bifidobacterial ITS microbial profiling produced a total of 12,702,820 reads that were quality-filtered obtaining a total of 8,393,755 reads with an average of 47,964 filtered reads per sample (Table S4). Taxonomic reconstruction of the bifidobacterial population harbored by the analyzed samples is reported in Additional File 2. The obtained ITS data revealed that *Bifidobacterium breve* (25.55 %), *Bifidobacterium pseudolongum* subsp. *globosum* (16.05 %), *Bifidobacterium longum* subsp. *longum* (15.38 %), *Bifidobacterium adolescentis* (9.75 %) and *Bifidobacterium pseudolongum* subsp. *pseudolongum* (6.68 %) were both the most abundant as well as the most prevalent (98.80 %, 99.40 %, 98.80 %, 100 %, 99.40 %, respectively) bifidobacterial taxa in the canine gut microbiota (Fig. S4). Moreover, seven additional bifidobacterial species, i.e., *Bifidobacterium bifidum*, *Bifidobacterium longum* subsp. *suis*, *Bifidobacterium dentium*, *Bifidobacterium animalis* subsp. *lactis*, *Bifidobacterium magnum*, *Bifidobacterium catenulatum* and *Bifidobacterium pseudocatenulatum*, were found to be among the most prevalent (>90 %) canine gut commensals despite showing lower relative abundance (<4.00 %), suggesting their adaptation to colonization of the canine gut. Interestingly, these species were reported to be among the most dominant bifidobacterial taxa in the gut of all mammals (Milani et al., 2017), with the exception of *B. breve*, *B. catenulatum* and *B. pseudocatenulatum.* In fact, the latter species are considered to be more typically associated with the human gut (Turroni et al., 2018). Therefore, the high prevalence of *B. breve*, *B. catenulatum* and *B. pseudocatenulatum* in the canine gut microbiota suggests that human influence, such as artificial selection, dietary contents and subsequent co-habitation of dogs with their owners, may have promoted adaptation and colonization of these bacterial species in the dog GI tract. In this context, comparison of the bifidobacterial population between domesticated dogs and wolves highlighted that the domesticated canine gut is colonized by both a higher average relative abundance (0.25 % and 0.01 % in dogs and wolves, respectively) and a higher level of diversity in terms of number of different bifidobacterial species. Indeed, when considering only known species with an average relative abundance of > 0.01 %, 44 bifidobacterial taxa were detected in the domesticated canine microbiota, while only 14 were found in that of their wild relatives. Moreover, only three of these 14 bifidobacterial taxa, i.e., *B. adolescentis*, *B. longum* subsp. *longum* and *Bifidobacterium dentium* showed a prevalence of > 80 %, thus supporting the notion that a close interaction with humans, a domesticated lifestyle and corresponding diet may have favored horizontal transmission, sub-sequent colonization and persistence of bifidobacterial members from Hominidae to Canidae.

As described above, diet is a contributory factor in modulating the microbial community of the canine gut microbiota. Thus, in order to evaluate the impact that different diets may have on the bifidobacterial population, the obtained ITS sequences of the BARF and CF groups were compared. Of the five most abundant bifidobacterial species of canine gut microbiota, only *B. pseudolongum* subsp. *pseudolongum* was significantly different between BARF and CF dogs (11.48 % and 5.71 % in BARF and CF groups, respectively, p-value = 0.014). Conversely, *B. breve*, *B. pseudolongum* subsp. *globosum*, *B. longum* subsp. *longum* and *B. adolescentis* showed no significant differences, indicating that these species are resilient to dietary changes, probably due to extensive co-evolution with the host. Nevertheless, the abundance of other, less represented bifidobacterial species appears to be modulated by diet. Indeed, *Bifidobacterium animalis* subsp. *animalis* and *Bifidobacterium choerinum*, which represent two bifidobacterial species typically found in the mammalian gut, showed an increased relative abundance in the BARF group relative to the CF group. In contrast, *B. catenulatum*, *B. magnum* and *B. pseudocatenulatum* decreased in the BARF group respect to CF group. Interestingly, both *B. animalis* subsp. *animalis* and *B. coherinum* both displayed a prevalence of 100 % in the BARF group, while in the CF group they were prevalent at just 18.84 % and 47.10 %, respectively. The reduced relative abundance and prevalence of these latter taxa points to the possibility that they are selected by a meat-based diet.

Notably, the presence of putative bifidobacterial novel species in the canine gut microbiota was evaluated following the protocol previously described by Milani *et al*. (Milani et al., 2014; Milani et al., 2017). Interestingly, among the detected putative bifidobacterial novel species, one putative new bacterial taxon, previously named new_taxa_43 (Milani et al., 2014), was present at a prevalence of >80 % in both wolves and dogs, suggesting that this new taxon has co-evolved with the *Canis lupus* species.

Functional characterization of the fecal microbiomes of BARF and CF dogs. Shotgun metagenomic data allows the assessment of the metabolic repertoire of an entire complex microbial population through analysis of all coding genes, i.e. the microbiome (Quince et al., 2017). Therefore, in order to evaluate possible differences in the microbiomes of BARF and CF groups, a BARF sample (C99) and a CF sample (C41) were subjected to shotgun metagenomic sequencing. Selection of these particular two samples was based on the 16S rRNA microbial profiling in order to select the closest canine fecal samples to the average of their corresponding group. Shotgun metagenomic sequencing generated 9,706,454 reads for the CF sample and 13,531,961 reads for the BARF sample, that were then analyzed using the METAnnotatorX software pipe line (Milani et al., 2018).

In silico characterization of putative GHs (Glycosyl Hydrolases), i.e., enzymes that hydrolyze complex carbohydrates into mono- or oligomeric glycan constituents, showed that CF microbiome datasets possessed proportionately more reads classified as GHs (5.31 % and 2.72 % in CF and BARF group, respectively) (Table S5). More specifically, genes encoding members of GH families GH2, GH31, GH92 and GH97, which include β-galactosidase, α-glucosidase, α- mannosidase and α-galactosidase activities, respectively, constituted 0.74 % of the CF samples and 0.08 % of the BARF samples (Fig. S4). Similarly, GH families involved in the breakdown of complex polysaccharides derived from plants such as GH3 (L-arabinofuranosidase), GH43 (xylanase), GH51 (endoglucanase)and GH77 (amylomaltase) (Matsuzawa et al., 2015), were more represented in the CF datasets, corresponding with 0.95 % and 0.26 % of the CF and BARF samples, respectively (Fig. S5). These differences may be explained by the increased intake of carbohydrates and fibers of vegetable origin by the CF group (when compared to the BARF group), indicating that the gut microbial glycobiome of dogs is influenced by diet. In parallel, analysis of predicted bacterial metabolic pathways based on MetaCyc classification revealed that genes involved both in amino acid degradation pathways and fatty acid and lipid degradation are more abundant in the BARF sample (Fig. S5), suggesting that an increased animal fat and protein intake favors colonization of the canine gut by microorganisms with an enriched repertoire of amino acid and lipid degradation pathways.

Conclusions

The canine gut microbiota has previously been explored through analysis of a limited number of samples, while did not take the genetic variability into account as introduced by artificial selection of the various breeds. In the current study, metagenomic approaches based on 16S rRNA gene and ITS bifidobacterial profiling, combined with shotgun metagenomics were employed to investigate the gut microbiota of a large number of healthy dogs, representing 51 different breeds, covering the canine genetic biodiversity as highlighted by a previous SNP genotype analysis (Parker et al., 2004). Our detailed reconstruction of the core gut microbiota based on metagenomic data revealed that Bacteroidetes, Fusobacteria, Firmicutes, Proteobacteria and Actinobacteria were the dominant phyla of the canine core intestinal population, which encompasses 43 shared bacterial genera, with *Fusobacterium* as the most abundant genus. Our results provide evidence of extensive co-evolution between a dog and its gut microbiota, and of resilience to artificial selection. Moreover, 16S rRNA microbial profiling data highlighted that diet plays an important role in modulating the canine core gut microbiota, leading to higher bacterial diversity in the CF group when compared to that of the BARF group. In addition, when comparing the intestinal core microbial community of dogs fed on a BARF diet with that of CF-fed dogs, we observed an alteration in the average relative abundance of 14 of the 43 core microbial genera. Interestingly, bacterial genera, such as *Faecalibacterium*, *Sutterella* and *Prevotella*, which are known to be able to degrade a diverse range of carbohydrates, were more abundant in the CF group, whose diet is typically enriched in carbohydrates and fibers. Furthermore, comparison of the core gut microbiota of dogs vs. that of wolves and human beings highlighted that the domesticated canine core gut microbial community appears to have lost six bacterial genera typical of the wolf core microbiota, yet, at the same time, has acquired five taxa that are also present in the human core gut microbiota. Thus, these data suggest that the canine gut microbiota has co-evolved with its host so as to adapt to and gain resilience against dietary changes induced by co-habitation with humans. This notion was further supported by analysis of the canine bifidobacterial community. Indeed, ITS bifidobacterial profiling highlighted that the canine gut microbiota was colonized by some of the most dominant bifidobacterial taxa of the mammalian gut, but also by certain *Bifidobacterium* species that are typical of the human microbiota. Moreover, we observed a lower relative abundance of the *Bifidobacterium* genus in the wolf gut microbiota when compared to that of domesticated dogs. Notably, the relative abundance of bifidobacteria is known to decrease with aging in humans (Arboleya et al., 2016). Nevertheless, we could not exclude age-related biases due to the fact that the sampled wolves were all adults of unknown age and gender. This reinforces the idea that co-habitation of dogs with humans has directed the evolution of the canine gut microbiota through horizontal transmission and sub-sequent colonization of human commensals in the domesticated dog GI tract. However, no statistically significant differences in canine gut microbiota composition were observed when analyzing metagenomics data based on dog breeds. Probably this is due to the above-mentioned high impact of age and diet that prevents from assessing potential differences in the microbial intestinal population of different canine breeds.

Moreover, *in silico* functional characterization of the canine gut microbiome of BARF and CF groups showed that CF diet, typically enriched in plant carbohydrates selects for an intestinal community characterized by a more extensive and diverse repertoire of genes encoding glycan-degrading enzymes. At the same time, prediction of bacterial metabolic pathways revealed that genes involved in amino acid, fatty acid and lipid degradation are more abundant in gut microbiomes of dogs fed on a BARF diet as compared to that of dogs from the CF group. This therefore suggests that these distinct diets influence and modulate the metabolic pathway arsenal of the canine gut microbiome. However, because of the limited number of samples employed for our shotgun metagenomics analysis further investigation with a larger sample set is required to characterize differences in the metabolic repertoire of BARF and CF groups in a statistically robust manner.

Altogether, the metagenomic investigations presented in this study revealed that, while maintaining common characteristics with its wild relative in terms of taxonomic composition and metabolic potential, the domesticated canine gut microbiota has been extensively shaped by artificial selection, altered diet and close contact with humans.

Experimental procedures

Ethical statement. This study was performed in compliance with the rules, regulations and recommendations of the ethical Committee of the University of Parma. The corresponding protocols were approved by the 'Comitato di Etica Università degli Studi di Parma', Italy. All animal procedures were carried out in accordance with national guidelines (Decreto legislativo 26/2014).

Sample collection and DNA extraction. For the purpose of the current study, a total of 169 canine stool samples were collected through a collaboration with several Italian dog breeders in the north and centre of Italy (Table S1). To be included in the study, dogs had to be healthy, not having undergone treatment with any probiotics or drugs, such as antibiotics, during the six previous months. For each sample, breed, gender, weight, age and diet were noted (Table S1). In addition, fecal samples from six wolves were recovered from the National Park of Abruzzo, Italy, where wolves live under wild conditions. Therefore, information about wolf gender, weight, age and diet was unknown. In all cases, stool samples were collected immediately after defecation, kept on ice and shipped to the laboratory under frozen conditions where they were preserved at -20 °C, until they were processed. Samples were subjected to DNA extraction using the QIAmp DNA Stool Mini kit following the manufacturer's instructions (Qiagen, Germany).

16S rRNA/ITS Microbial Profiling. Partial 16S rRNA gene sequences were amplified from extracted DNA using primer pair Probio_Uni/Probio_Rev, targeting the V3 region of the 16S rRNA gene sequence (Milani et al., 2013). Partial ITS sequences were amplified from extracted DNA using the primer pair Probio-bif_Uni/Probi-bif_Rev, which targets the spacer region between the 16S rRNA and the 23S rRNA genes within the ribosomal RNA (rRNA) locus (Milani et al., 2014). Illumina adapter overhang nucleotide sequences were added to the partial 16S rRNA gene-specific amplicons and to the generated ITS amplicons of approximately 200 bp, which were further processed using the

16S Metagenomic Sequencing Library Preparation Protocol (Part No. 15044223 Rev. B—Illumina). Amplifications were carried out using a Verity Thermocycler (Applied Biosystems). The integrity of the PCR amplicons was analyzed by electrophoresis on a 2200 Tape Station Instrument (Agilent Technologies, USA). DNA products obtained following PCR-mediated amplification of the 16S rRNA gene sequences were purified by a magnetic purification step involving the Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) in order to remove primer dimers. DNA concentration of the amplified sequence library was determined by a fluorimetric Qubit quantification system (Life Technologies, USA). Amplicons were diluted to a concentration of 4 nM, and 5 µL quantities of each diluted DNA amplicon sample were mixed to prepare the pooled final library. 16S rRNA gene and ITS bifidobacterial sequencing were performed using an Illumina MiSeq sequencer with MiSeq Reagent Kit v3 chemicals.

16S rRNA/ITS microbial profiling analysis. The fastq files were processed using QIIME2 software (Bokulich et al., 2018). Paired-end reads were merged and quality control retained sequences with a length between 140 and 400 bp, mean sequence quality score >25 and with truncation of a sequence at the first base if a low quality rolling 10 bp window was found. Sequences with mismatched forward and/or reverse primers were omitted. In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were defined at ≥99 % sequence homology using DADA2 (Callahan et al., 2016) and OTUs with less than 2 sequences in at least one sample were removed. All reads were classified to the lowest possible taxonomic rank using QIIME2 (Caporaso et al., 2010) and a reference dataset from the SILVA database (Quast et al., 2013). Biodiversity of the samples (alpha-diversity) was calculated with Chao1 index, while similarity between samples (beta-diversity) was calculated by unweighted uniFrac (Lozupone and Knight, 2005). The similarity range is calculated between the values 0 and 1. PCoA representations of beta-diversity were performed using QIIME2 (Caporaso et al., 2010).

Shotgun metagenomics. The extracted DNA was fragmented to 550-650 bp using a BioRuptor machine (Diagenode, Belgium). Samples were prepared following the TruSeq Nano DNA Samples

Preparation Guide (Part#15041110Rev.D). Sequencing was performed using an Illumina NextSeq 500 sequencer with NextSeq Mid Output v2 Kit Chemicals.

Analysis of metagenomic datasets. The obtained fastq files were filtered for reads with a quality of < 25, for reads > 80 and for sequences of canine DNA. Moreover, bases were removed from the end of the reads unless the average quality score was > 25, in a window of 5 bp. Only paired data were used to further analysis. Investigation of Glycosyl Hydrolase (GH) profiles together with the reconstruction of bacterial metabolic pathways and evaluation of their abundance in the shotgun metagenomics datasets were assessed using custom scripts based on RapSearch2 software (Zhao et al., 2012), htseq-count (Anders et al., 2015) and the CAZy database or the MetaCyc database (Caspi et al., 2012), respectively.

Statistical analyses. All statistical analysis, i.e., ANOVA, PERMANOVA and Student's t-test, were performed with SPSS software (www.ibm.com/software/it/analytics/spss/).

Data deposition. Raw sequences of 16S rRNA gene profiling and bifidobacterial ITS profiling together with shotgun metagenomics data are accessible through SRA study accession number PRJNA504009.

Acknowledgements

This work was primarily funded by the EU Joint Programming Initiative – A Healthy Diet for a Healthy Life (JPI HDHL, http://www.healthydietforhealthylife.eu/) to DvS (in conjunction with Science Foundation Ireland [SFI], Grant number 15/JP-HDHL/3280) and to MV (in conjunction with MIUR, Italy). The study is supported by Fondazione Cariparma, under TeachInParma Project (DV). GA is supported by Fondazione Cariparma, Parma, Italy. We furthermore thank GenProbio srl for financial support of the Laboratory of Probiogenomics. This research benefited from the HPC (High Performance Computing) facility of the University of Parma, Italy. The authors declare that they have no competing interests.

References

Anders, S., Pyl, P.T., and Huber, W. (2015) HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**: 166-169.

Arboleya, S., Watkins, C., Stanton, C., and Ross, R.P. (2016) Gut Bifidobacteria Populations in Human Health and Aging. *Front Microbiol* **7**: 1204.

Bartges, J., Boynton, B., Vogt, A.H., Krauter, E., Lambrecht, K., Svec, R., and Thompson, S. (2012) AAHA canine life stage guidelines. *J Am Anim Hosp Assoc* **48**: 1-11.

Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R. et al. (2018) Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* **6**: 90.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., and Holmes, S.P. (2016) DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* **13**: 581-583.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K. et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**: 335-336.

Caspi, R., Altman, T., Dreher, K., Fulcher, C.A., Subhraveti, P., Keseler, I.M. et al. (2012) The MetaCyc database of metabolic pathways and enzymes and the BioCyc collection of pathway/genome databases. *Nucleic Acids Res* **40**: D742-753.

David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E. et al. (2014) Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**: 559-563.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J.B., Massart, S. et al. (2010) Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci U S A* **107**: 14691-14696.

Deng, P., and Swanson, K.S. (2015) Gut microbiota of humans, dogs and cats: current knowledge and future opportunities and challenges. *Br J Nutr* **113 Suppl**: S6-17.

Ferrario, C., Alessandri, G., Mancabelli, L., Gering, E., Mangifesta, M., Milani, C. et al. (2017) Untangling the cecal microbiota of feral chickens by culturomic and metagenomic analyses. *Environ Microbiol* **19**: 4771-4783.

Fleming, J.M., Creevy, K.E., and Promislow, D.E. (2011) Mortality in north american dogs from 1984 to 2004: an investigation into age-, size-, and breed-related causes of death. *J Vet Intern Med* **25**: 187-198.

Fredriksson-Ahomaa, M., Heikkila, T., Pernu, N., Kovanen, S., Hielm-Bjorkman, A., and Kivisto, R. (2017) Raw Meat-Based Diets in Dogs and Cats. *Vet Sci* **4**.

Furrie, E. (2006) A molecular revolution in the study of intestinal microflora. *Gut* **55**: 141-143.

Garcia-Mazcorro, J.F., Dowd, S.E., Poulsen, J., Steiner, J.M., and Suchodolski, J.S. (2012) Abundance and short-term temporal variability of fecal microbiota in healthy dogs. *Microbiologyopen* **1**: 340-347.

Greer, K.A., Canterberry, S.C., and Murphy, K.E. (2007) Statistical analysis regarding the effects of height and weight on life span of the domestic dog. *Res Vet Sci* **82**: 208-214.

Guard, B.C., Mila, H., Steiner, J.M., Mariani, C., Suchodolski, J.S., and Chastant-Maillard, S. (2017) Characterization of the fecal microbiome during neonatal and early pediatric development in puppies. *PLoS One* **12**: e0175718.

Hand, D., Wallis, C., Colyer, A., and Penn, C.W. (2013) Pyrosequencing the canine faecal microbiota: breadth and depth of biodiversity. *PLoS One* **8**: e53115.

Herstad, K.M.V., Gajardo, K., Bakke, A.M., Moe, L., Ludvigsen, J., Rudi, K. et al. (2017) A diet change from dry food to beef induces reversible changes on the faecal microbiota in healthy, adult client-owned dogs. *BMC Vet Res* **13**: 147.

Honneffer, J.B., Minamoto, Y., and Suchodolski, J.S. (2014) Microbiota alterations in acute and chronic gastrointestinal inflammation of cats and dogs. *World J Gastroenterol* **20**: 16489-16497.

Hooda, S., Minamoto, Y., Suchodolski, J.S., and Swanson, K.S. (2012) Current state of knowledge: the canine gastrointestinal microbiome. *Anim Health Res Rev* **13**: 78-88.

Kim, H.B., Borewicz, K., White, B.A., Singer, R.S., Sreevatsan, S., Tu, Z.J., and Isaacson, R.E. (2011) Longitudinal investigation of the age-related bacterial diversity in the feces of commercial pigs. *Vet Microbiol* **153**: 124-133.

Kim, J., An, J.U., Kim, W., Lee, S., and Cho, S. (2017) Differences in the gut microbiota of dogs (Canis lupus familiaris) fed a natural diet or a commercial feed revealed by the Illumina MiSeq platform. *Gut Pathog* **9**: 68.

Liu, J.P., Zou, W.L., Chen, S.J., Wei, H.Y., Yin, Y.N., Zou, Y.Y., and Lu, F.G. (2016) Effects of different diets on intestinal microbiota and nonalcoholic fatty liver disease development. *World J Gastroenterol* **22**: 7353-7364.

Lopez-Siles, M., Khan, T.M., Duncan, S.H., Harmsen, H.J., Garcia-Gil, L.J., and Flint, H.J. (2012) Cultured representatives of two major phylogroups of human colonic Faecalibacterium prausnitzii can utilize pectin, uronic acids, and host-derived substrates for growth. *Appl Environ Microbiol* **78**: 420-428.

Lopez-Siles, M., Enrich-Capo, N., Aldeguer, X., Sabat-Mir, M., Duncan, S.H., Garcia-Gil, L.J., and Martinez-Medina, M. (2018) Alterations in the Abundance and Co-occurrence of Akkermansia muciniphila and Faecalibacterium prausnitzii in the Colonic Mucosa of Inflammatory Bowel Disease Subjects. *Front Cell Infect Microbiol* **8**: 281.

Lozupone, C., and Knight, R. (2005) UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology* **71**: 8228-8235.

Mancabelli, L., Milani, C., Lugli, G.A., Turroni, F., Mangifesta, M., Viappiani, A. et al. (2017) Unveiling the gut microbiota composition and functionality associated with constipation through metagenomic analyses. *Sci Rep* **7**: 9879.

Masuoka, H., Shimada, K., Kiyosue-Yasuda, T., Kiyosue, M., Oishi, Y., Kimura, S. et al. (2017) Transition of the intestinal microbiota of dogs with age. *Biosci Microbiota Food Health* **36**: 27-31.

Matsuzawa, T., Kaneko, S., and Yaoi, K. (2015) Screening, identification, and characterization of a GH43 family beta-xylosidase/alpha-arabinofuranosidase from a compost microbial metagenome. *Appl Microbiol Biotechnol* **99**: 8943-8954.

Milani, C., Lugli, G.A., Turroni, F., Mancabelli, L., Duranti, S., Viappiani, A. et al. (2014) Evaluation of bifidobacterial community composition in the human gut by means of a targeted amplicon sequencing (ITS) protocol. *FEMS Microbiol Ecol* **90**: 493-503.

Milani, C., Mangifesta, M., Mancabelli, L., Lugli, G.A., James, K., Duranti, S. et al. (2017) Unveiling bifidobacterial biogeography across the mammalian branch of the tree of life. *ISME J* **11**: 2834-2847.

Milani, C., Hevia, A., Foroni, E., Duranti, S., Turroni, F., Lugli, G.A. et al. (2013) Assessing the fecal microbiota: an optimized ion torrent 16S rRNA gene-based analysis protocol. *PLoS One* **8**: e68739.

Milani, C., Casey, E., Lugli, G.A., Moore, R., Kaczorowska, J., Feehily, C. et al. (2018) Tracing mother-infant transmission of bacteriophages by means of a novel analytical tool for shotgun metagenomic datasets: METAnnotatorX. *Microbiome* **6**: 145.

Moon, C.D., Young, W., Maclean, P.H., Cookson, A.L., and Bermingham, E.N. (2018) Metagenomic insights into the roles of Proteobacteria in the gastrointestinal microbiomes of healthy dogs and cats. *Microbiologyopen* **7**: e00677.

Omatsu, T., Omura, M., Katayama, Y., Kimura, T., Okumura, M., Okumura, A. et al. (2018) Molecular diversity of the faecal microbiota of Toy Poodles in Japan. *J Vet Med Sci* **80**: 749-754.

Parker, H.G., Kim, L.V., Sutter, N.B., Carlson, S., Lorentzen, T.D., Malek, T.B. et al. (2004) Genetic structure of the purebred domestic dog. *Science* **304**: 1160-1164.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P. et al. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* **41**: D590-596.

Quince, C., Walker, A.W., Simpson, J.T., Loman, N.J., and Segata, N. (2017) Shotgun metagenomics, from sampling to analysis. *Nat Biotechnol* **35**: 833-844.

Salonen, A., Salojarvi, J., Lahti, L., and de Vos, W.M. (2012) The adult intestinal core microbiota is determined by analysis depth and health status. *Clin Microbiol Infect* **18 Suppl 4**: 16-20.

Savolainen, P., Zhang, Y.P., Luo, J., Lundeberg, J., and Leitner, T. (2002) Genetic evidence for an East Asian origin of domestic dogs. *Science* **298**: 1610-1613.

Schnorr, S.L., Candela, M., Rampelli, S., Centanni, M., Consolandi, C., Basaglia, G. et al. (2014) Gut microbiome of the Hadza hunter-gatherers. *Nat Commun* **5**: 3654.

Sokol, H., Pigneur, B., Watterlot, L., Lakhdari, O., Bermudez-Humaran, L.G., Gratadoux, J.J. et al. (2008) Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A* **105**: 16731-16736.

Suchodolski, J.S. (2011) Companion animals symposium: microbes and gastrointestinal health of dogs and cats. *J Anim Sci* **89**: 1520-1530.

Suchodolski, J.S., Camacho, J., and Steiner, J.M. (2008) Analysis of bacterial diversity in the canine duodenum, jejunum, ileum, and colon by comparative 16S rRNA gene analysis. *FEMS Microbiol Ecol* **66**: 567-578.

Suchodolski, J.S., Xenoulis, P.G., Paddock, C.G., Steiner, J.M., and Jergens, A.E. (2010) Molecular analysis of the bacterial microbiota in duodenal biopsies from dogs with idiopathic inflammatory bowel disease. *Vet Microbiol* **142**: 394-400.

Suchodolski, J.S., Foster, M.L., Sohail, M.U., Leutenegger, C., Queen, E.V., Steiner, J.M., and Marks, S.L. (2015) The fecal microbiome in cats with diarrhea. *PLoS One* **10**: e0127378.

Suchodolski, J.S., Markel, M.E., Garcia-Mazcorro, J.F., Unterer, S., Heilmann, R.M., Dowd, S.E. et al. (2012) The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. *PLoS One* **7**: e51907.

Swanson, K.S., Dowd, S.E., Suchodolski, J.S., Middelbos, I.S., Vester, B.M., Barry, K.A. et al. (2011) Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. *ISME J* **5**: 639-649.

Tap, J., Mondot, S., Levenez, F., Pelletier, E., Caron, C., Furet, J.P. et al. (2009) Towards the human intestinal microbiota phylogenetic core. *Environ Microbiol* **11**: 2574-2584.

Turroni, F., Milani, C., Duranti, S., Ferrario, C., Lugli, G.A., Mancabelli, L. et al. (2018) Bifidobacteria and the infant gut: an example of co-evolution and natural selection. *Cell Mol Life Sci* **75**: 103-118.

van Bree, F.P.J., Bokken, G., Mineur, R., Franssen, F., Opsteegh, M., van der Giessen, J.W.B. et al. (2018) Zoonotic bacteria and parasites found in raw meat-based diets for cats and dogs. *Vet Rec* **182**: 50.

Wu, G.D., Compher, C., Chen, E.Z., Smith, S.A., Shah, R.D., Bittinger, K. et al. (2016) Comparative metabolomics in vegans and omnivores reveal constraints on diet-dependent gut microbiota metabolite production. *Gut* **65**: 63-72.

Xu, J., Verbrugghe, A., Lourenco, M., Janssens, G.P., Liu, D.J., Van de Wiele, T. et al. (2016) Does canine inflammatory bowel disease influence gut microbial profile and host metabolism? *BMC Vet Res* **12**: 114.

Zhao, Y., Tang, H., and Ye, Y. (2012) RAPSearch2: a fast and memory-efficient protein similarity search tool for next-generation sequencing data. *Bioinformatics* **28**: 125-126.

Figure legends

Figure 1. Taxonomic distribution of the 43 core bacterial genera of the canine gut microbiota. The heat map shows the relative abundance of the 43 bacterial genera that constitute the canine core gut microbiota of the 175 analyzed samples. On the left-hand side, sample breed is reported and samples were ordered as indicated in Supplementary Table S1. In the upper part of the heat map, numbers correspond to the 43 core bacterial genera listed on the right-hand side together with the corresponding prevalence.

Figure 2. Evaluation of α- and β- diversity in BARF- and CF-fed dog fecal samples. Panel a shows the representation of α-diversity trough average rarefaction curves on the left-side, and Box and Whisker graphic on the right-side. Average rarefaction curves represent variation of the Chao1 index at increasing sequencing depth of BARF and CF samples. Panel b displays the predicted PCoA encompassing the 169 domesticated canine fecal samples through a three-dimensional image and three two-dimensional sections. Panel c displays the relative abundance variation of significantly different genera between the BARF and CF groups together with their corresponding phylum, absolute percentage and p-value.

Figure 3. Comparison of core gut microbiota of humans, dogs and wolves. Panel a represents the heat map reporting the bacterial genera that constitute the core gut microbiota of humans, dogs and wolves. The symbol – indicates that the relative bacterial genus is not represented or at least present with a prevalence of < 80 %. Panel b displays the Venn-diagram related to the heat map showing the number of genera that are shared and unique in the core gut microbiota of the three compared mammalian species.

Supplementary figure legends

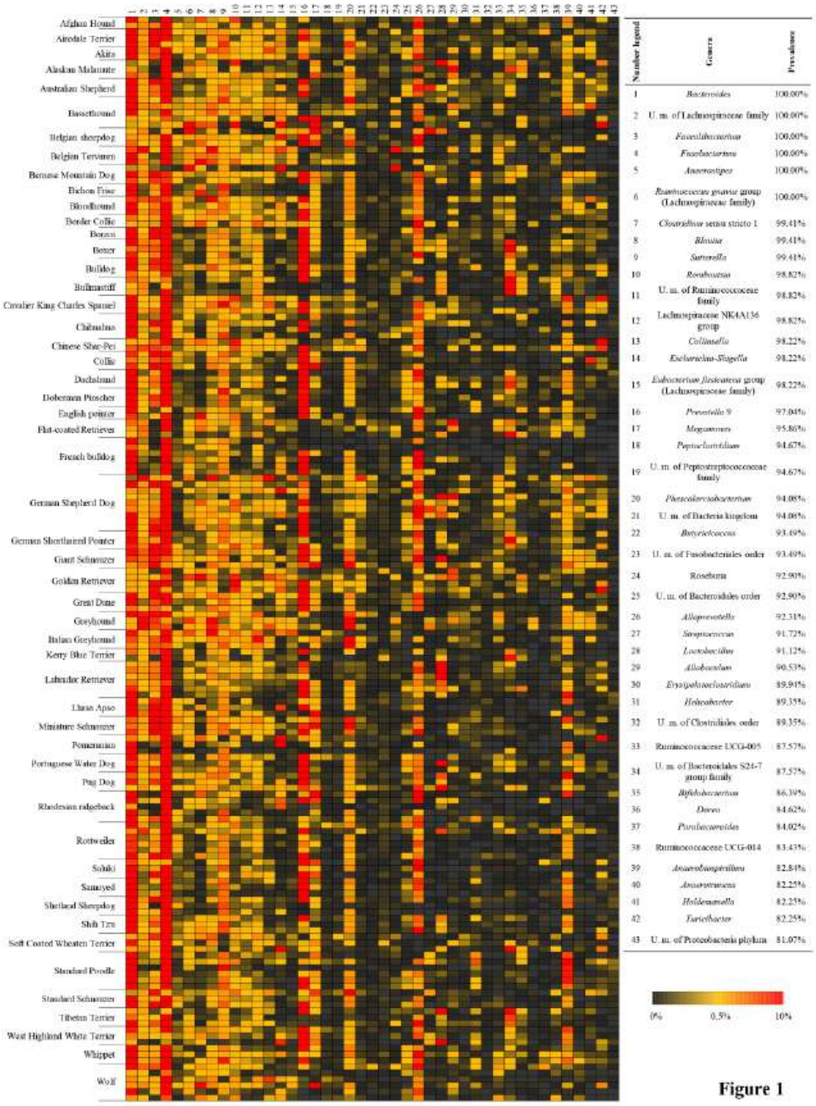
Figure S1. Evaluation of α- and β- diversity of the assessed microbiota from collected fecal samples. Panel a shows rarefaction curves representing variation of Chao1 index at increasing depth of each collected sample. Panel b displays the predicted PCoA encompassing all samples through a three-dimensional image as based on evolutionary distances that were determined by SNP analysis (Parker et al., 2004). Samples were divided in 5 groups: wolves (group 1, red), asian/ancient dogs (group 2, blue), herding dogs (group 3, orange), hunting dogs (group 4, green) and mastiff dogs (group 5, purple).

Figure S2. Variation of genera in the canine gut microbiota in fecal samples obtained from BARF, CF and wolf groups. Panel a depicts the heat map with the average relative abundances of bacterial genera that change among CF, BARF and wolf groups, reporting p-value, absolute and relative variance. Statistically significant differences are indicated in bold. Panel b shows bar plots indicating bacterial taxa modulated by diet.

Figure S3. Bar plots representing average relative abundance of bacterial genera that significantly vary in the four age groups.

Figure S4. Bifidobacterial ITS profiling of the 175 analyzed fecal samples. The bar plots represent the percentage of the total bifidobacterial community found in each collected sample.

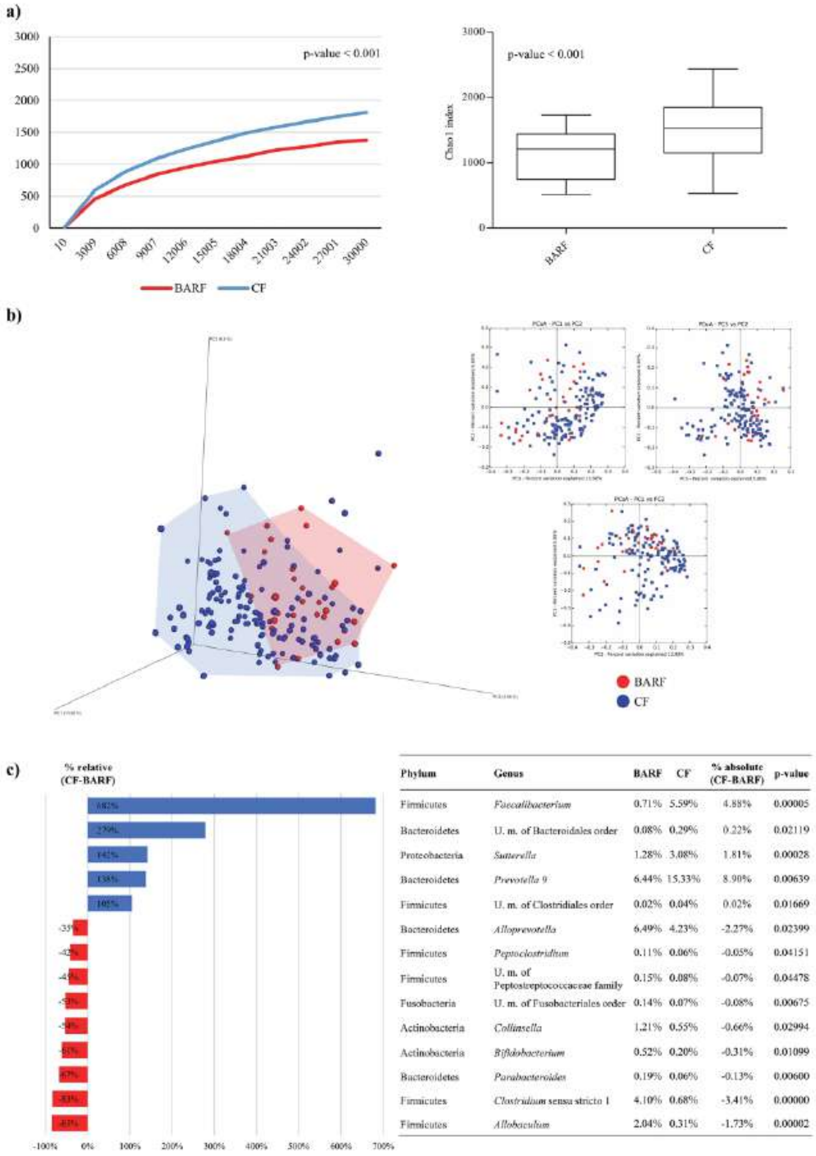
Figure S5. Changes in GH families involved in fiber and plant-derived carbohydrate degradation.



Number legend	Genera	Prevalence
1	*Bacteroides*	100.00%
2	U. m. of Lachnospiraceae family	100.00%
3	*Faecalibacterium*	100.00%
4	*Fusobacterium*	100.00%
5	*Anaerostipes*	100.00%
6	*Ruminococcus gnavus* group (Lachnospiraceae family)	100.00%
7	*Clostridium* sensu stricto 1	99.41%
8	*Blautia*	99.41%
9	*Sutterella*	99.41%
10	*Romboutsia*	98.82%
11	U. m. of Ruminococcaceae family	98.82%
12	Lachnospiraceae NK4A136 group	98.82%
13	*Collinsella*	98.22%
14	*Escherichia-Shigella*	98.22%
15	*Eubacterium fissicatena* group (Lachnospiraceae family)	98.22%
16	*Prevotella* 9	97.04%
17	*Megamonas*	95.86%
18	*Peptoclostridium*	94.67%
19	U. m. of Peptostreptococcaceae family	94.67%
20	*Phascolarctobacterium*	94.08%
21	U. m. of Bacteria kingdom	94.08%
22	*Butyricicoccus*	93.49%
23	U. m. of Fusobacteriales order	93.49%
24	Roseburia	92.90%
25	U. m. of Bacteroidales order	92.90%
26	*Alloprevotella*	92.31%
27	*Streptococcus*	91.72%
28	*Lactobacillus*	91.12%
29	*Allobaculum*	90.53%
30	*Erysipelatoclostridium*	89.94%
31	*Helicobacter*	89.35%
32	U. m. of Clostridiales order	89.35%
33	Ruminococcaceae UCG-005	87.57%
34	U. m. of Bacteroidales S24-7 group family	87.57%
35	*Bifidobacterium*	86.39%
36	*Dorea*	84.62%
37	*Parabacteroides*	84.02%
38	Ruminococcaceae UCG-014	83.43%
39	*Anaerobiospirillum*	82.84%
40	*Anaerotruncus*	82.25%
41	*Holdemanella*	82.25%
42	*Turicibacter*	82.25%
43	U. m. of Proteobacteria phylum	81.07%

Figure 1

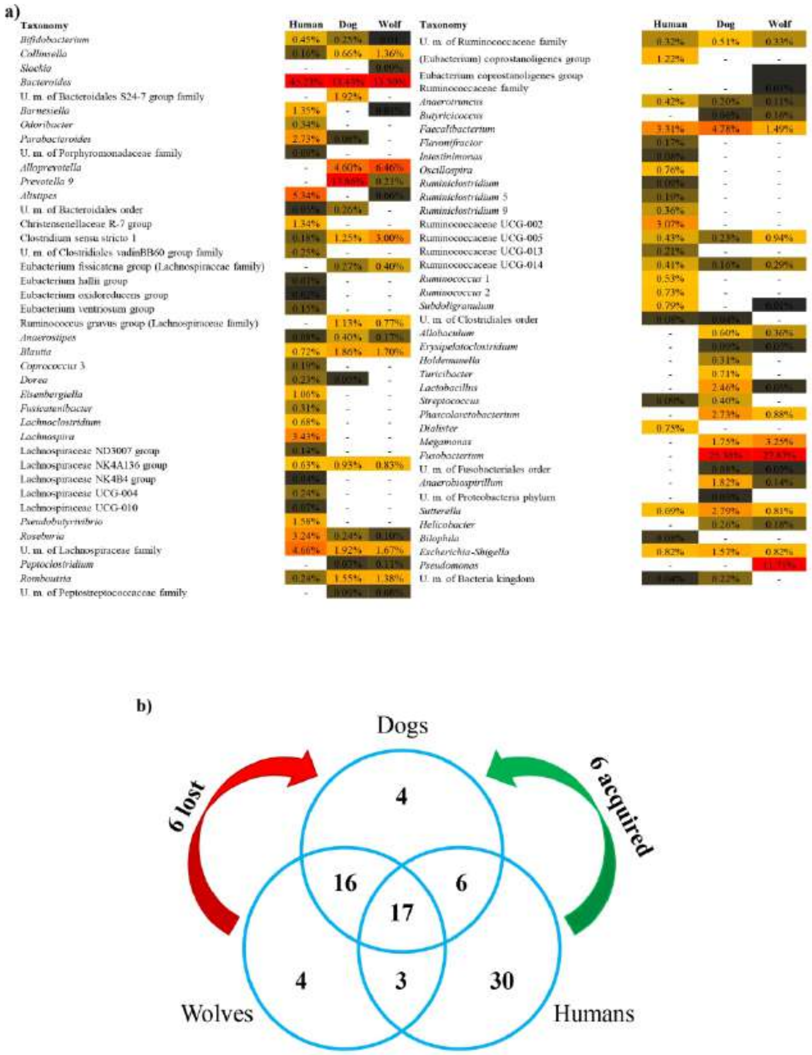
Figure 1. Taxonomic distribution of the 43 core bacterial genera of the canine gut microbiota. The heat map shows the relative abundance of the 43 bacterial genera that constitute the canine core gut microbiota of the 175 analyzed samples. On the left-hand side, sample breed is reported and samples were ordered as indicated in Supplementary Table S1. In the upper part of the heat map, numbers correspond to the 43 core bacterial genera listed on the right-hand side together with the corresponding prevalence.



Phylum	Genus	BARF	CF	% absolute (CF-BARF)	p-value
Firmicutes	*Faecalibacterium*	0.71%	5.59%	4.88%	0.00005
Bacteroidetes	U. m. of Bacteroidales order	0.08%	0.29%	0.22%	0.02119
Proteobacteria	*Sutterella*	1.28%	3.08%	1.81%	0.00028
Bacteroidetes	*Prevotella 9*	6.44%	15.33%	8.90%	0.00639
Firmicutes	U. m. of Clostridiales order	0.02%	0.04%	0.02%	0.01669
Bacteroidetes	*Alloprevotella*	6.49%	4.23%	-2.27%	0.02399
Firmicutes	*Peptoclostridium*	0.11%	0.06%	-0.05%	0.04151
Firmicutes	U. m. of Peptostreptococcaceae family	0.15%	0.08%	-0.07%	0.04478
Fusobacteria	U. m. of Fusobacteriales order	0.14%	0.07%	-0.08%	0.00675
Actinobacteria	*Collinsella*	1.21%	0.55%	-0.66%	0.02994
Actinobacteria	*Bifidobacterium*	0.52%	0.20%	-0.31%	0.01099
Bacteroidetes	*Parabacteroides*	0.19%	0.06%	-0.13%	0.00600
Firmicutes	*Clostridium* sensu stricto 1	4.10%	0.68%	-3.41%	0.00000
Firmicutes	*Allobaculum*	2.04%	0.31%	-1.73%	0.00002

Figure 2

Figure 2. Evaluation of α- and β- diversity in BARF- and CF-fed dog fecal samples. Panel a shows the representation of α-diversity trough average rarefaction curves on the left-side, and Box and Whisker graphic on the right-side. Average rarefaction curves represent variation of the Chao1 index at increasing sequencing depth of BARF and CF samples. Panel b displays the predicted PCoA encompassing the 169 domesticated canine fecal samples through a three-dimensional image and three two-dimensional sections. Panel c displays the relative abundance variation of significantly different genera between the BARF and CF groups together with their corresponding phylum, absolute percentage and p-value.



Taxonomy	Human	Dog	Wolf
Bifidobacterium	0.45%	0.25%	[illegible]
Collinsella	0.16%	0.66%	1.36%
Slackia	-	-	0.09%
Bacteroides	[illegible]	[illegible]	[illegible]
U. m. of Bacteroidales S24-7 group family	-	1.92%	-
Barnesiella	1.35%	-	[illegible]
Odoribacter	0.34%	-	-
Parabacteroides	2.73%	[illegible]	-
U. m. of Porphyromonadaceae family	[illegible]	-	-
Alloprevotella	-	4.60%	6.46%
Prevotella 9	-	[illegible]	0.21%
Alistipes	5.34%	-	[illegible]
U. m. of Bacteroidales order	[illegible]	0.26%	-
Christensenellaceae R-7 group	1.34%	-	-
Clostridium sensu stricto 1	0.18%	1.25%	3.00%
U. m. of Clostridiales vadinBB60 group family	0.25%	-	-
Eubacterium fissicatena group (Lachnospiraceae family)	-	0.27%	0.40%
Eubacterium hallii group	[illegible]	-	-
Eubacterium oxidoreducens group	[illegible]	-	-
Eubacterium ventriosum group	0.15%	-	-
Ruminococcus gravus group (Lachnospiraceae family)	-	1.13%	0.77%
Anaerostipes	[illegible]	0.40%	0.17%
Blautia	0.72%	1.86%	1.70%
Coprococcus 3	0.19%	-	-
Dorea	0.23%	[illegible]	-
Eisenbergiella	1.06%	-	-
Fusicatenibacter	0.31%	-	-
Lachnoclostridium	0.68%	-	-
Lachnospira	3.43%	-	-
Lachnospiraceae ND3007 group	0.14%	-	-
Lachnospiraceae NK4A136 group	0.63%	0.93%	0.83%
Lachnospiraceae NK4B4 group	[illegible]	-	-
Lachnospiraceae UCG-004	0.24%	-	-
Lachnospiraceae UCG-010	[illegible]	-	-
Pseudobutyrivibrio	1.58%	-	-
Roseburia	3.24%	0.24%	0.10%
U. m. of Lachnospiraceae family	4.66%	1.92%	1.67%
Peptoclostridium	-	0.07%	0.11%
Romboutsia	0.24%	1.55%	1.38%
U. m. of Peptostreptococcaceae family	-	[illegible]	[illegible]
U. m. of Ruminococcaceae family	0.32%	0.51%	0.33%
(Eubacterium) coprostanoligenes group	1.22%	-	-
Eubacterium coprostanoligenes group Ruminococcaceae family	-	-	[illegible]
Anaerotruncus	0.42%	0.20%	0.11%
Butyricicoccus	-	[illegible]	0.16%
Faecalibacterium	3.31%	4.78%	1.49%
Flavonifractor	0.17%	-	-
Intestinimonas	[illegible]	-	-
Oscillospira	0.76%	-	-
Ruminiclostridium	[illegible]	-	-
Ruminiclostridium 5	0.19%	-	-
Ruminiclostridium 9	0.36%	-	-
Ruminococcaceae UCG-002	3.07%	-	-
Ruminococcaceae UCG-005	0.43%	0.23%	0.94%
Ruminococcaceae UCG-013	0.21%	-	-
Ruminococcaceae UCG-014	0.41%	0.16%	0.29%
Ruminococcus 1	0.53%	-	-
Ruminococcus 2	0.73%	-	-
Subdoligranulum	0.79%	-	[illegible]
U. m. of Clostridiales order	[illegible]	[illegible]	-
Allobaculum	-	0.60%	0.36%
Erysipelatoclostridium	-	[illegible]	[illegible]
Holdemanella	-	0.31%	-
Turicibacter	-	0.71%	-
Lactobacillus	-	2.46%	[illegible]
Streptococcus	[illegible]	0.40%	-
Phascolarctobacterium	-	2.73%	0.88%
Dialister	0.75%	-	-
Megamonas	-	1.75%	3.25%
Fusobacterium	-	[illegible]	[illegible]
U. m. of Fusobacteriales order	-	[illegible]	[illegible]
Anaerobiospirillum	-	1.82%	0.14%
U. m. of Proteobacteria phylum	-	[illegible]	
Sutterella	0.69%	2.79%	0.81%
Helicobacter	-	0.26%	0.18%
Bilophila	[illegible]	-	-
Escherichia-Shigella	0.82%	1.57%	0.82%
Pseudomonas	-	-	[illegible]
U. m. of Bacteria kingdom	[illegible]	0.22%	-

Figure 3

Figure 3. Comparison of core gut microbiota of humans, dogs and wolves. Panel a represents the heat map reporting the bacterial genera that constitute the core gut microbiota of humans, dogs and wolves. The symbol – indicates that the relative bacterial genus is not represented or at least present with a prevalence of < 80 %. Panel b displays the Venn-diagram related to the heat map showing the number of genera that are shared and unique in the core gut microbiota of the three compared mammalian species.