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

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

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## 25 **Summary**

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

## 40 **Introduction**

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

54 The domesticated dog (*Canis lupus familiaris*) is a key companion animal of humans. The possible  
55 impact that the GI microbiota has on canine health and well-being is of broad interest (Kim et al.,  
56 2017; Moon et al., 2018). Along the canine GI tract, the various compartments differ in microbial  
57 composition and total bacterial numbers (Hooda et al., 2012). Notably, the large intestine and feces  
58 possess the highest density and diversity of bacteria, with Firmicutes, Bacteroidetes, Proteobacteria  
59 and Fusobacteria representing the prevalent bacterial phyla (Suchodolski, 2011). In this context, high  
60 throughput sequencing has been used to investigate the taxonomical composition of the intestinal  
61 microbiota of healthy dogs (Suchodolski et al., 2008; Garcia-Mazcorro et al., 2012; Hand et al., 2013;  
62 Omatsu et al., 2018). However, these studies involved a small number of samples belonging to a  
63 single or just a few different breeds. Like other mammals, the canine gut microbiota appears to be  
64 influenced by several factors, such as diet (Wu et al., 2016; Herstad et al., 2017; Kim et al., 2017),

65 age (Masuoka et al., 2017), metabolic disorders including obesity and diabetes (Xu et al., 2016), as  
66 well as intestinal inflammatory diseases (Honneffer et al., 2014).

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

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



## 83 **Results and discussion**

84 **Taxonomic classification of the intestinal microbial community of *Canis lupus*.** In order to  
85 explore the taxonomical composition of the gut microbiota of the mammalian species *Canis lupus*, a  
86 total of 175 fecal samples were collected. In detail, six of these fecal samples belonged to specimens  
87 of the grey wolf, while the other 169 fecal samples belonged to members of 51 canine breeds,  
88 uniformly distributed along the phylogenetic cluster of breeds as reconstructed by Parker *et al.* based  
89 on SNP genotype analysis (Parker *et al.*, 2004). Metadata of these collected samples are reported in  
90 Table S1. Bacterial DNA extracted from the fecal samples was subjected to 16S rRNA gene  
91 sequencing analysis as previously described (Milani *et al.*, 2013). Illumina-mediated sequencing of  
92 the abovementioned samples generated a total of 12,702,820 sequencing reads with an average of  
93 72,588 reads per sample (Table S2). Quality and chimera filtering produced a total of 8,329,451  
94 filtered reads with an average of 47,597 filtered reads per sample (Table S2). Taxonomic  
95 reconstruction of the bacterial population encompassed by each of the analysed samples is reported  
96 in Additional Data File 1. Alpha-diversity analysis, performed through Chao1 index calculation for  
97 10 sub-samplings of sequenced read pools, showed that all curves tend to plateau, thereby indicating  
98 that sample biodiversity was in all cases adequately covered by the applied sequencing depth (Fig.  
99 S1). Moreover, PCoA representation of the unweighted Unifrac distance matrix obtained by analysis  
100 of the datasets generated by this study did not reveal any significant clustering as based on  
101 evolutionary distance between profiled domesticated dog breeds and their wild (i.e. wolf) relative  
102 (Fig. S1). In addition, bioinformatic analyses were performed to evaluate if differences in the canine  
103 gut microbiota may be dependent on canine breed. However, these analyses did not reveal any  
104 statistically significant differences, suggesting that, in this case, host phylogeny divergence plays a  
105 minor role in the modulation of dogs' gut population.

106 **Genus-level core gut microbiota of the *Canis lupus familiaris*.** Reconstruction of a core microbiota,  
107 which represents bacterial taxa that are shared across samples of a defined cohort (Salonen *et al.*,  
108 2012), allows identification of dominant and prevalent bacterial species that have been preserved

109 during co-evolution of the intestinal community and its host (Tap et al., 2009; Salonen et al., 2012).  
110 In order to determine the core gut bacterial community of the collected fecal samples, bacterial genera  
111 present in at least 80 % of the samples and with at least an average relative abundance of >0.01 %  
112 were considered. Based on these criteria, we identified 43 bacterial genera (Figure 1). In detail, at  
113 phylum level the core microbiota was dominated by taxa belonging to Bacteroidetes (total average  
114 abundance 33.68 %), followed by Fusobacteria (25.53 %), Firmicutes (23.56 %), Proteobacteria (6.29  
115 %) and Actinobacteria (0.93 %) (Figure 1). As could be expected, the core microbiota includes genera  
116 of the five dominant phyla generally found in the canine fecal microbiota (Hand et al., 2013; Moon  
117 et al., 2018). Furthermore, at genus level, a particular representative of the Fusobacteria phyla, i.e.,  
118 *Fusobacterium*, was shown to be present at the highest average relative abundance (25.36 %) among  
119 all domesticated dog breeds (Figure 1), suggesting extensive co-evolution between this taxon and the  
120 canine GI. Moreover, *Prevotella 9* and *Bacteroides*, which both belong to the Bacteroidetes phylum,  
121 were second and third most abundant genera in the canine microbiota (13.86 % and 13.43 %, respectively).  
122 In a human context, *Bacteroides* and *Prevotella* have been linked to a vegan or  
123 vegetarian diet (De Filippo et al., 2010). Therefore, the high abundance of these two genera in the  
124 canine gut microbiota may be due to the transition from a carnivorous diet typical of wolves to the  
125 omnivorous diet of domestic dogs (see below).

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



134 Evaluation of the bacterial biodiversity of the two diet groups was performed through the Chao1 index  
135 calculated for 10 sub-samplings of sequenced read pools obtained for each sampled dog up to a  
136 maximum of 30,000 reads. The two curves, corresponding to the average observed for the CF and  
137 BARF groups, are significantly different based on Student's t-test statistical analysis calculated at  
138 30,000 reads (p-value < 0.01) (Fig. 2a). Interestingly, the average rarefaction curves showed a higher  
139 level of complexity of the CF group gut microbiota compared to the BARF group. Moreover, the  $\beta$ -  
140 diversity was analysed based on unweighted UniFrac and represented through Principal Coordinate  
141 Analysis (PCoA) (Fig 2b). The predicted PCoA exhibited partial clustering of CF and BARF groups  
142 (P-value < 0.01), supporting the notion that the two distinct diets indeed cause differences in the  
143 canine gut microbiota. In addition, analysis of the predicted taxonomic profiles at phylum level  
144 revealed that the average abundance of three of the five phyla that are present in the canine core gut  
145 microbiota appeared to be altered by diet. Specifically, Fusobacteria and Actinobacteria were  
146 significantly increased in dogs fed on a BARF diet, while Bacteroidetes showed an opposite trend  
147 (Fig. 2; Table S3). An in-depth inspection at genus level revealed that 14 of the 43 core genera are  
148 significantly affected by diet (Fig. 2c). Interestingly, the two most representative genera of the core  
149 microbiota, i.e., *Fusobacterium* and *Bacteroides*, did not significantly fluctuate in the two assessed  
150 canine groups (Fig. 2c). Otherwise, *Prevotella* 9, *Faecalibacterium* and *Sutterella* significantly  
151 decreased in the BARF group compared to the CF group (Fig. 2c; Table S3). In this context, it has  
152 been shown that a high abundance of *Prevotella* in the human gut microbiota correlates with a fiber-  
153 based diet, due to the capability of members of this microbial genus to degrade simple carbohydrates  
154 (David et al., 2014; Schnorr et al., 2014), while it is known that *Faecalibacterium* spp. and *Sutterella*  
155 spp. can also metabolize a wide range of different carbohydrates (Lopez-Siles et al., 2012; Liu et al.,  
156 2016). Therefore, dogs fed with commercial pet foods, which are typically enriched in fibers and  
157 carbohydrates, are associated with a higher abundance of these saccharolytic species, as compared  
158 with dogs of the BARF group whose diet was based on a high abundance of animal proteins and fats.  
159 Notably, in humans, *Faecalibacterium*, and in particular *Faecalibacterium prausnitzii*, is associated

160 with a healthy microbiota (Lopez-Siles et al., 2018). Indeed, as a butyrogenic bacterium, this  
161 commensal species has been reported to possess anti-inflammatory features and to positively  
162 influence the gut physiology (Sokol et al., 2008). In this context, the reduction of *Faecalibacterium*  
163 spp. in the BARF group indicates that a meat-based diet is less protective against inflammatory  
164 activity in the canine gut.

#### 165 **Effect of artificial selection and close contact with humans on the canine gut microbiota**

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

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

193 **Evaluation of shared and unique bacterial genera of the canine core intestinal community as**

194 **compared to the human core gut microbiota.** To assess if domestication of dogs and their

195 cohabitation with humans has allowed microbiota exchanges, we compared the canine core gut

196 microbiota with that of humans. In order to include comparable 16S rRNA gene microbiota profiling

197 data, the reconstruction of the human core gut microbiota was assessed through the re-analysis of 79

198 fecal samples of healthy adult individuals used as control group in a previous study where the

199 experimental procedures were the same of this study (Mancabelli et al., 2017). Interestingly, of the

200 six bacterial genera common to all canine samples, *Fusobacterium* and *Ruminococcus gnavus* group

201 were not represented in the human microbiota (Fig. 3), indicating that these microbial taxa are typical

202 inhabitants of the canine gut. Moreover, domestication seemed to have caused the loss of six bacterial

203 genera in dogs with respect to its wild relative (Fig. 3), while just five microbial taxa were specifically

204 shared between human and canine core gut microbiota (Fig. 3). Indeed, *Dorea*, *Parabacteroides*,

205 *Streptococcus*, U. m. of Bacteroidales order and U. m. of Clostridiales order were present in both the

206 human and dog core gut microbiota yet were absent in the core gut microbiota of wolves (Fig. 3).

207 These data therefore suggest that the shift from a natural, undomesticated life style to that which

208 involved cohabitation with humans has caused major changes in the bacterial composition of

209 domesticated dogs.

210 **The effect of aging on canine core gut microbiota.** Canine life stage classification is known to be  
211 affected by both breed and size of dogs (Greer et al., 2007; Fleming et al., 2011; Bartges et al., 2012).  
212 In order to evaluate age-related changes in the canine core microbiota, samples were divided into 4  
213 age groups, while disregarding their breed, including puppies (20 dogs, 0 – 8 months old), junior (27  
214 dogs, 9 – 24 months old), adult (104 dogs, 25 – 96 months old) and senior (18 dogs, > 97 months  
215 old). Grey wolves' fecal samples were excluded from this analysis as their age was unknown.  
216 Considering the canine core genus microbiota, U. m. of Bacteroidales order, *Phascolarctobacterium*,  
217 *Roseburia* and *Fusobacterium* significantly differ among the four age groups (ANOVA P-value <  
218 0.01) (Fig. S3). Interestingly, a higher level of *Fusobacterium* was reached in canine adulthood  
219 (relative abundance 28.80 %), as compared to the junior (20.75 %) and senior (17.58 %) groups.  
220 Furthermore, *Roseburia* significantly increased in the senior group, while U. m. of Bacteroidales  
221 order was more abundant in puppies when compared to the junior and adult groups. In addition,  
222 *Phascolarctobacterium* was shown to be present at a higher abundance in the junior group when  
223 compared to the other assessed age groups (Fig. S3)

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

229 **Profiling of the bifidobacterial community harbored by the canine gut microbiota.** In order to  
230 further investigate the bifidobacterial communities harbored by the canine gut microbiota, a recently  
231 developed pipeline based on genus-specific primers targeting the hypervariable ITS region was  
232 applied to all 175 collected samples (Milani et al., 2014). Bifidobacterial ITS microbial profiling  
233 produced a total of 12,702,820 reads that were quality-filtered obtaining a total of 8,393,755 reads  
234 with an average of 47,964 filtered reads per sample (Table S4). Taxonomic reconstruction of the  
235 bifidobacterial population harbored by the analyzed samples is reported in Additional File 2. The

--



261 favored horizontal transmission, sub-sequent colonization and persistence of bifidobacterial members  
262 from Hominidae to Canidae.

263 As described above, diet is a contributory factor in modulating the microbial community of the canine  
264 gut microbiota. Thus, in order to evaluate the impact that different diets may have on the  
265 bifidobacterial population, the obtained ITS sequences of the BARF and CF groups were compared.  
266 Of the five most abundant bifidobacterial species of canine gut microbiota, only *B. pseudolongum*  
267 subsp. *pseudolongum* was significantly different between BARF and CF dogs (11.48 % and 5.71 %  
268 in BARF and CF groups, respectively, p-value = 0.014). Conversely, *B. breve*, *B. pseudolongum*  
269 subsp. *globosum*, *B. longum* subsp. *longum* and *B. adolescentis* showed no significant differences,  
270 indicating that these species are resilient to dietary changes, probably due to extensive co-evolution  
271 with the host. Nevertheless, the abundance of other, less represented bifidobacterial species appears  
272 to be modulated by diet. Indeed, *Bifidobacterium animalis* subsp. *animalis* and *Bifidobacterium*  
273 *choerinum*, which represent two bifidobacterial species typically found in the mammalian gut,  
274 showed an increased relative abundance in the BARF group relative to the CF group. In contrast, *B.*  
275 *catenulatum*, *B. magnum* and *B. pseudocatenulatum* decreased in the BARF group respect to CF  
276 group. Interestingly, both *B. animalis* subsp. *animalis* and *B. coherinum* both displayed a prevalence  
277 of 100 % in the BARF group, while in the CF group they were prevalent at just 18.84 % and 47.10  
278 %, respectively. The reduced relative abundance and prevalence of these latter taxa points to the  
279 possibility that they are selected by a meat-based diet.

280 Notably, the presence of putative bifidobacterial novel species in the canine gut microbiota was  
281 evaluated following the protocol previously described by Milani *et al.* (Milani *et al.*, 2014; Milani *et*  
282 *al.*, 2017). Interestingly, among the detected putative bifidobacterial novel species, one putative new  
283 bacterial taxon, previously named new\_taxa\_43 (Milani *et al.*, 2014), was present at a prevalence of  
284 >80 % in both wolves and dogs, suggesting that this new taxon has co-evolved with the *Canis lupus*  
285 species.

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

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

312

313 **Conclusions**

314 The canine gut microbiota has previously been explored through analysis of a limited number of  
315 samples, while did not take the genetic variability into account as introduced by artificial selection of  
316 the various breeds. In the current study, metagenomic approaches based on 16S rRNA gene and ITS  
317 bifidobacterial profiling, combined with shotgun metagenomics were employed to investigate the gut  
318 microbiota of a large number of healthy dogs, representing 51 different breeds, covering the canine  
319 genetic biodiversity as highlighted by a previous SNP genotype analysis (Parker et al., 2004). Our  
320 detailed reconstruction of the core gut microbiota based on metagenomic data revealed that  
321 Bacteroidetes, Fusobacteria, Firmicutes, Proteobacteria and Actinobacteria were the dominant phyla  
322 of the canine core intestinal population, which encompasses 43 shared bacterial genera, with  
323 *Fusobacterium* as the most abundant genus. Our results provide evidence of extensive co-evolution  
324 between a dog and its gut microbiota, and of resilience to artificial selection. Moreover, 16S rRNA  
325 microbial profiling data highlighted that diet plays an important role in modulating the canine core  
326 gut microbiota, leading to higher bacterial diversity in the CF group when compared to that of the  
327 BARF group. In addition, when comparing the intestinal core microbial community of dogs fed on a  
328 BARF diet with that of CF-fed dogs, we observed an alteration in the average relative abundance of  
329 14 of the 43 core microbial genera. Interestingly, bacterial genera, such as *Faecalibacterium*,  
330 *Sutterella* and *Prevotella*, which are known to be able to degrade a diverse range of carbohydrates,  
331 were more abundant in the CF group, whose diet is typically enriched in carbohydrates and fibers.  
332 Furthermore, comparison of the core gut microbiota of dogs vs. that of wolves and human beings  
333 highlighted that the domesticated canine core gut microbial community appears to have lost six  
334 bacterial genera typical of the wolf core microbiota, yet, at the same time, has acquired five taxa that  
335 are also present in the human core gut microbiota. Thus, these data suggest that the canine gut  
336 microbiota has co-evolved with its host so as to adapt to and gain resilience against dietary changes  
337 induced by co-habitation with humans. This notion was further supported by analysis of the canine



338 bifidobacterial community. Indeed, ITS bifidobacterial profiling highlighted that the canine gut  
339 microbiota was colonized by some of the most dominant bifidobacterial taxa of the mammalian gut,  
340 but also by certain *Bifidobacterium* species that are typical of the human microbiota. Moreover, we  
341 observed a lower relative abundance of the *Bifidobacterium* genus in the wolf gut microbiota when  
342 compared to that of domesticated dogs. Notably, the relative abundance of bifidobacteria is known to  
343 decrease with aging in humans (Arboleya et al., 2016). Nevertheless, we could not exclude age-  
344 related biases due to the fact that the sampled wolves were all adults of unknown age and gender.  
345 This reinforces the idea that co-habitation of dogs with humans has directed the evolution of the  
346 canine gut microbiota through horizontal transmission and sub-sequent colonization of human  
347 commensals in the domesticated dog GI tract. However, no statistically significant differences in  
348 canine gut microbiota composition were observed when analyzing metagenomics data based on dog  
349 breeds. Probably this is due to the above-mentioned high impact of age and diet that prevents from  
350 assessing potential differences in the microbial intestinal population of different canine breeds.  
351 Moreover, *in silico* functional characterization of the canine gut microbiome of BARF and CF groups  
352 showed that CF diet, typically enriched in plant carbohydrates selects for an intestinal community  
353 characterized by a more extensive and diverse repertoire of genes encoding glycan-degrading  
354 enzymes. At the same time, prediction of bacterial metabolic pathways revealed that genes involved  
355 in amino acid, fatty acid and lipid degradation are more abundant in gut microbiomes of dogs fed on  
356 a BARF diet as compared to that of dogs from the CF group. This therefore suggests that these distinct  
357 diets influence and modulate the metabolic pathway arsenal of the canine gut microbiome. However,  
358 because of the limited number of samples employed for our shotgun metagenomics analysis further  
359 investigation with a larger sample set is required to characterize differences in the metabolic  
360 repertoire of BARF and CF groups in a statistically robust manner.  
361 Altogether, the metagenomic investigations presented in this study revealed that, while maintaining  
362 common characteristics with its wild relative in terms of taxonomic composition and metabolic

363 potential, the domesticated canine gut microbiota has been extensively shaped by artificial selection,  
364 altered diet and close contact with humans.

365

## 366 **Experimental procedures**

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

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

382 **16S rRNA/ITS Microbial Profiling.** Partial 16S rRNA gene sequences were amplified from  
383 extracted DNA using primer pair Probio\_Uni/Probio\_Rev, targeting the V3 region of the 16S rRNA  
384 gene sequence (Milani et al., 2013). Partial ITS sequences were amplified from extracted DNA using  
385 the primer pair Probio-bif\_Uni/Probi-bif\_Rev, which targets the spacer region between the 16S rRNA  
386 and the 23S rRNA genes within the ribosomal RNA (rRNA) locus (Milani et al., 2014). Illumina  
387 adapter overhang nucleotide sequences were added to the partial 16S rRNA gene-specific amplicons  
388 and to the generated ITS amplicons of approximately 200 bp, which were further processed using the

--

389 16S Metagenomic Sequencing Library Preparation Protocol (Part No. 15044223 Rev. B—Illumina).  
390 Amplifications were carried out using a Verity Thermocycler (Applied Biosystems). The integrity of  
391 the PCR amplicons was analyzed by electrophoresis on a 2200 Tape Station Instrument (Agilent  
392 Technologies, USA). DNA products obtained following PCR-mediated amplification of the 16S  
393 rRNA gene sequences were purified by a magnetic purification step involving the Agencourt AMPure  
394 XP DNA purification beads (Beckman Coulter Genomics GmbH, Bernried, Germany) in order to  
395 remove primer dimers. DNA concentration of the amplified sequence library was determined by a  
396 fluorimetric Qubit quantification system (Life Technologies, USA). Amplicons were diluted to a  
397 concentration of 4 nM, and 5  $\mu$ L quantities of each diluted DNA amplicon sample were mixed to  
398 prepare the pooled final library. 16S rRNA gene and ITS bifidobacterial sequencing were performed  
399 using an Illumina MiSeq sequencer with MiSeq Reagent Kit v3 chemicals.

400 **16S rRNA/ITS microbial profiling analysis.** The fastq files were processed using QIIME2 software  
401 (Bokulich et al., 2018). Paired-end reads were merged and quality control retained sequences with a  
402 length between 140 and 400 bp, mean sequence quality score  $>25$  and with truncation of a sequence  
403 at the first base if a low quality rolling 10 bp window was found. Sequences with mismatched forward  
404 and/or reverse primers were omitted. In order to calculate downstream diversity measures (alpha and  
405 beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were  
406 defined at  $\geq 99$  % sequence homology using DADA2 (Callahan et al., 2016) and OTUs with less than  
407 2 sequences in at least one sample were removed. All reads were classified to the lowest possible  
408 taxonomic rank using QIIME2 (Caporaso et al., 2010) and a reference dataset from the SILVA  
409 database (Quast et al., 2013). Biodiversity of the samples (alpha-diversity) was calculated with Chao1  
410 index, while similarity between samples (beta-diversity) was calculated by unweighted uniFrac  
411 (Lozupone and Knight, 2005). The similarity range is calculated between the values 0 and 1. PCoA  
412 representations of beta-diversity were performed using QIIME2 (Caporaso et al., 2010).

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

415 Preparation Guide (Part#15041110Rev.D). Sequencing was performed using an Illumina NextSeq  
416 500 sequencer with NextSeq Mid Output v2 Kit Chemicals.

417 **Analysis of metagenomic datasets.** The obtained fastq files were filtered for reads with a quality of  
418 < 25, for reads > 80 and for sequences of canine DNA. Moreover, bases were removed from the end  
419 of the reads unless the average quality score was > 25, in a window of 5 bp. Only paired data were  
420 used to further analysis. Investigation of Glycosyl Hydrolase (GH) profiles together with the  
421 reconstruction of bacterial metabolic pathways and evaluation of their abundance in the shotgun  
422 metagenomics datasets were assessed using custom scripts based on RapSearch2 software (Zhao et  
423 al., 2012), htseq-count (Anders et al., 2015) and the CAZy database or the MetaCyc database (Caspi  
424 et al., 2012), respectively.

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

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

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572

573 **Figure legends**

574

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

581

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

590

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



597 **Supplementary figure legends**

598 **Figure S1.** Evaluation of  $\alpha$ - and  $\beta$ - diversity of the assessed microbiota from collected fecal samples.

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

605

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

611

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

614

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

617

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

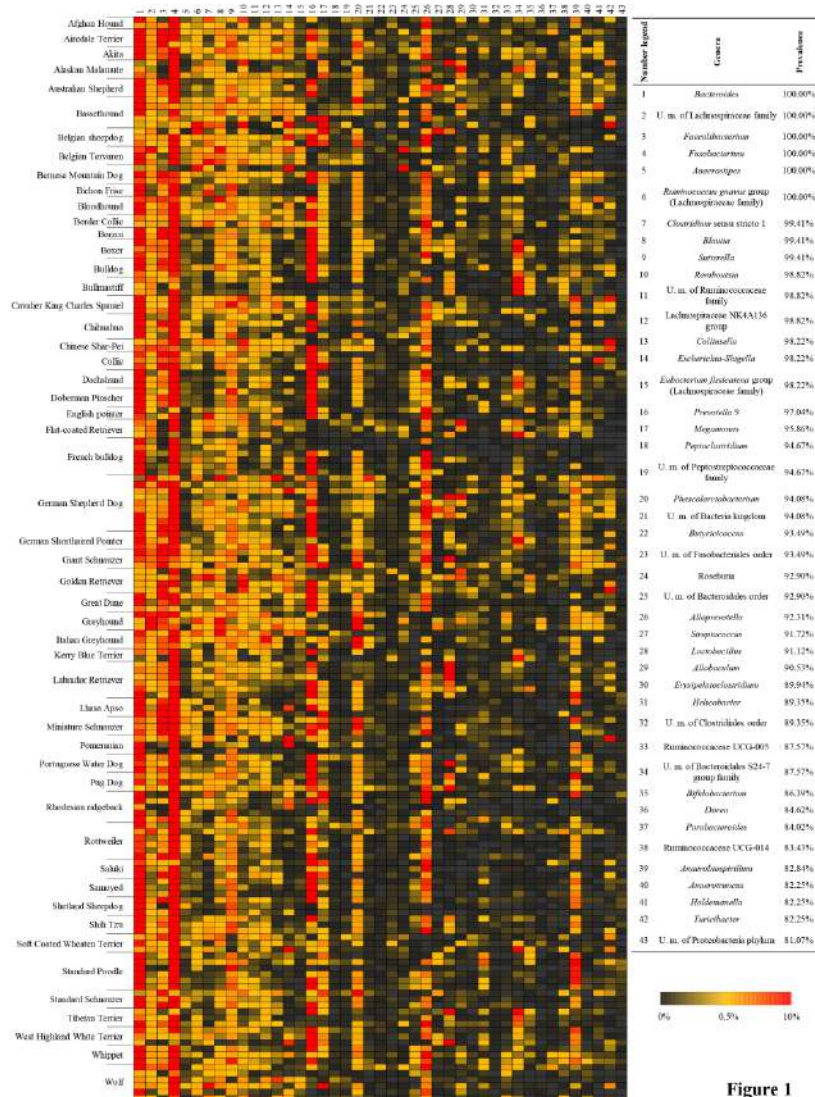


Figure 1

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.

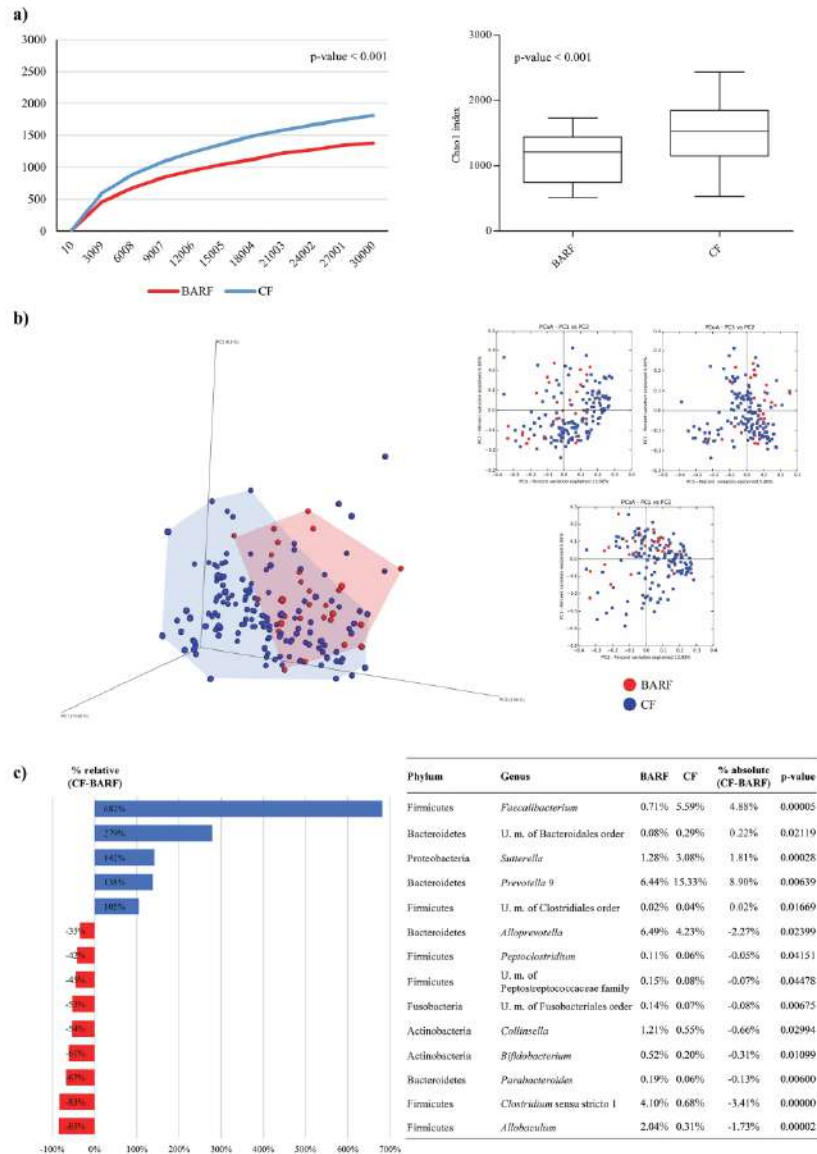


Figure 2

Figure 2. Evaluation of  $\alpha$ - and  $\beta$ - diversity in BARF- and CF-fed dog fecal samples. Panel a shows the representation of  $\alpha$ -diversity through 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.

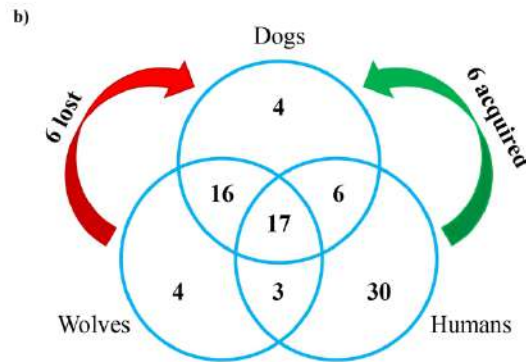
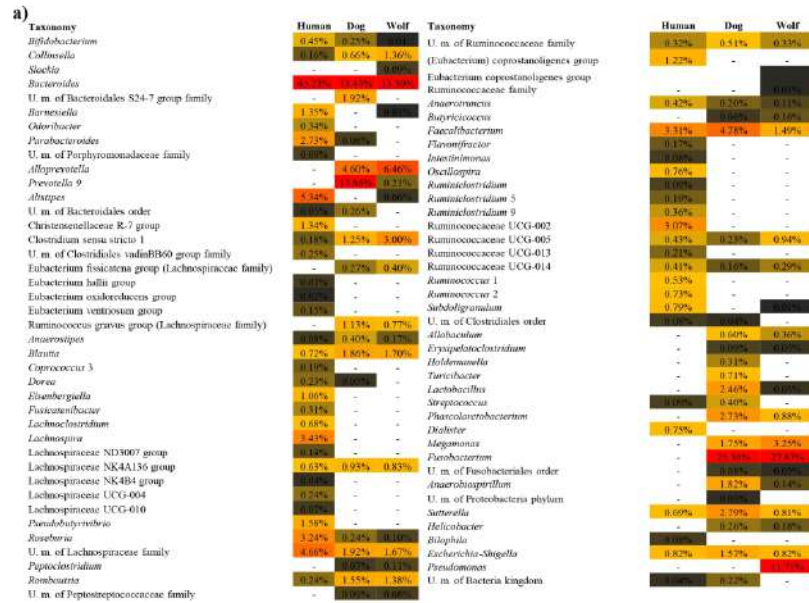


Figure 3

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.