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Gut microbiome structure and adrenocortical activity in dogs with aggressive and

phobic behavioral disorders

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Abstract

Accompanying human beings since the Paleolithic period, dogs has been recently regarded as a reliable model for the study of the gut microbiome connections with health and disease. In order to provide some glimpses on the connections between the gut microbiome layout and host behavior, we profiled the phylogenetic composition and structure of the canine gut microbiome of dogs with aggressive (n = 17), phobic (n = 15) and normal behavior (n = 17). According to our findings, aggressive behavioral disorder was found to be characterized by a peculiar gut microbiome structure, with high biodiversity and enrichment in generally subdominant bacterial genera. On the other hand, phobic dogs were enriched in *Lactobacillus*, a bacterial genus with known probiotic and psychobiotic properties. Although further studies are needed to validate our findings, our work supports the intriguing opportunity that different behavioral phenotypes in dogs may be associated with peculiar gut microbiome layouts, suggesting possible connections between the gut microbiome and the central nervous system and indicating the possible adoption of probiotic interventions aimed at restoring a balanced host-symbiont interplay for mitigating behavioral disorders.

Keywords: microbiome, behavioral disorders, aggressive dogs, phobic dogs, hormones, HPA-axis

Introduction

Descending from the gray wolf (*Canis lupus*), dogs were domesticated during the Paleolithic period, accompanying humans across the transition from hunting-gathering to rural agriculture of the Neolithic, to post-industrialized Western lifestyle [1-3]. The frequent sharing of food resources with human beings has been a selective force able to drive changes in the digestive and metabolic system of dogs, enabling them to efficiently adapt to a more starch-enriched diet compared to their wild ancestor, and ultimately influence canine behavior [4,5]. The canine gastrointestinal tract harbors a complex and highly biodiverse microbial ecosystem, whose predominant taxa resemble those typically found in the gut of other omnivorous mammals. However, in comparison to both mice and pigs, the canine gut microbiome (GM) result the most similar to humans [6,7]. Thus, in dogs, the GM-host mutualistic exchange well approximate what has been observed in humans [8-11]. Indeed, the peculiar patterns of dysbiosis observed in dogs with IBD are generally comparable to variations typically found in humans, suggesting that bacterial responses to inflammatory conditions are conserved among the two [12,13]. As observed in humans, the eubiotic and stable configuration of the canine GM is therefore of fundamental importance for the maintenance of a homeostatic gut environment and of the overall host health.

Several recent studies have shown the ability of the mammalian GM to communicate with the host central nervous system (CNS) through several parallel channels, involving the vagus nerve, neuroimmune and neuroendocrine signaling mechanisms, and the production of neuroactive chemicals – i.e. gamma-aminobutyric acid (GABA), serotonin (5-HT), norepinephrine and dopamine [14-17]. Conversely, the CNS can influence GM structure and metabolome, influencing the gut environment, acting on motility, secretion and permeability via the autonomic nervous system (ANS) [18]. It is thus a matter of fact that the GM can influence the host behavior and *vice versa*, exerting a key role in the modulation of the gut-brain axis. The development of researches

76 during the last decades indeed suggests the presence of a bidirectional communication between gut

77 and brain.

78 Despite the high variability and severity of behavioral disorders observed in dogs, the aggressive

79 behavior has been found to be the most common, followed by separation anxiety and phobia [19].

80 Aggressive, anxiety and phobia behavioral disorders can be considered as stress responses with an

81 increase in glucocorticoid (GC) secretion mediated by the hypothalamic-pituitary-adrenal (HPA)

82 axis [20,21]. Although recent works on canine microbiome have investigated potential interactions

83 with aggression, these studies have focused on the variations of their GM profile after targeted

84 dietary interventions to reduce aggressive behaviors [22,23]. To the best of our knowledge, no study

85 has focused on the comparison of the GM structure between dogs exhibiting aggressive, phobic and

86 normal behavior, with specific associations with adrenocortical activity. In order to provide some

87 glimpses in this direction, in our work, 49 dogs – 31 males and 18 females – of different breed, age,

88 and weight, housed in individual boxes were enrolled. Following the behavioral, dogs were

89 classified into three groups: aggressive, phobic and normal behavior. We then profiled the

90 phylogenetic composition and structure of their canine GM, measuring the levels of fecal hormones

91 (i.e. cortisol and testosterone) to evaluate the adrenocortical activity in each study group. Fecal GC

92 and their metabolites may constitute a reliable non-invasive proxy of adrenocortical activity,

93 reflecting long-term stress responses with less interference from acute stressors [24-26].

94 Our data suggest that peculiar microbiome configurations could be connected with dogs' behavioral

95 phenotype, which in turn might be exacerbated by altered proportions of gut symbionts, ultimately

96 triggering a feedback loop. In this scenario, targeted interventions on GM could constitute a

97 valuable tool to restore a proper host-symbiont interplay, finally mitigating behavioral disorders and

98 improving the overall host health.

99

100 **Results**

101

Relative abundance of major gut microbiome components in the enrolled cohort

104 In order to evaluate possible differences in GM communities among the behavioral study groups,

105 we collected fecal samples from 49 dogs. In particular, 31 males and 18 females of different breed,

106 aged between 1 and 13 years, were recruited from three animal shelters located in the metropolitan

107 area of Bologna (Italy). Following the behavioral evaluation performed in accordance with the C-

108 BARQ [27], dogs were grouped based on their behavioral phenotype: 17 were scored as aggressive

109 and 15 as phobic, while 17 exhibited a normal behavior. The 16S rRNA sequencing, performed

110 using the Illumina MiSeq platform, yielded a total of 1,611,153 high-quality sequences, with an

111 average of $32,880 \pm 6,009$ sequences per sample (range 20,664 – 45,486), subsequently clustered in

112 8,460 OTUs with a 97% identity threshold.

113 The most abundant phyla detected within the normal behavior samples were Firmicutes ($68.0 \pm$

114 4.6%), Bacteroidetes ($13.7 \pm 3.6\%$), and Actinobacteria ($9.9 \pm 1.6\%$), with Fusobacteria ($4.8 \pm$

115 1.3%) and Proteobacteria ($2.1 \pm 0.8\%$) as minor components. The aggressive group showed similar

116 proportions among the dominant phyla when compared to the normal behavior group, except for a

117 reduced relative abundance of Bacteroidetes (P-value = 0.02; Wilcoxon test). No significant

118 differences at phylum level were detected between the phobic and the normal behavior groups, as

119 well as between the phobic and the aggressive groups.

120 At family level, Lachnospiraceae, Erysipelotrichaceae and Clostridiaceae constitute the major

121 components of the normal behavior group (relative abundance > 10%). A depletion in the relative

122 abundance of Bacteriodaceae, Alcaligenaceae and [Paraprevotellaceae], as well as an increase in

123 Erysipelotrichaceae (P-value < 0.05) was observed in the aggressive group compared to the normal

124 behavior group. The phobic group was instead characterized by an increase in the relative

125 abundance of the family Rikenellaceae (P-value = 0.04) when compared to the normal behavior

126   group. Aggressive and phobic groups were found to be distinguishable due to different proportions

127   in the relative abundance of the bacterial families [Mogibacteriaceae] and Veillonellaceae,

128   respectively depleted and enriched in the aggressive group ($P < 0.04$) (Fig 1A, 1B).

129   At the genus taxonomic level, *Clostridium*, *Lactobacillus*, *Blautia* and *Collinsella* represent the

130   major portion of the normal behavior group GM (relative abundance > 5%). Several microbial

131   genera were found to be significantly depleted in the aggressive group. The relative abundance of

132   the genera *Oscillospira*, *Peptostreptococcus*, *Bacteroides*, *Sutterella*, and *Coprobacillus* were

133   significantly lower in aggressive compared to normal behavior group, while *Catenibacterium*,

134   *Megamonas* and [*Eubacterium*] showed an opposite trend (P-value < 0.04). At the genus level, no

135   significant differences were detected between the phobic and the normal behavior group. The

136   differences found between phobic and aggressive groups were due to an increased relative

137   abundance of *Catenibacterium* and *Megamonas* in the latter group ($P < 0.007$), in addition to a

138   slightly depletion of the genus *Epulopiscium* ($P = 0.04$) (S3 Fig).

139   **Fig 1. Canine gut microbiome profile of the behavior groups.** (A) Relative abundances of

140   family-level taxa in each subject of the enrolled cohort (barplots) and respective average values of

141   each study group (piecharts). (B) Boxplots showing the distribution of the relative abundances of

142   bacterial families enriched or depleted within the gut microbiota of aggressive or phobic groups.

143

Comparison of the overall gut microbiome compositional structure between aggressive, phobic and normal behavior dogs

146   The intra-individual diversity of the canine GM was assessed by means of the phylogenetic metric

147   Observed OTUs and the Shannon biodiversity index at the genus level. While according to the

148   Shannon index the intra-individual GM diversity was comparable between aggressive, phobic and

149   normal behavior groups (mean ± SD, 5.2 ± 0.8, 5.14 ± 0.7, and 5.2 ± 0.7, respectively), the

150   Observed OTUs metric highlighted a statistically significant difference between aggressive and

151   phobic groups (P-value = 0.02; Kruskal-Wallis test). In particular, the aggressive group was

152   characterized by the higher number of different taxa observed (mean ± SD, 583.9 ± 310.6), the

153   phobic group had the lower value (430.07 ± 112.28), and the normal behavior group exhibited

154   intermediate values (454.8 ± 118.3) (Fig 2).

155   According to the Jaccard similarity index, the Principal Coordinates Analysis (PCoA) of the inter-

156   sample variation highlighted a significant separation between the structural composition of the GM

157   among study groups (P-value = 0.02, permutation test with pseudo-*F* ratios) (Fig 2). In order to

158   identify bacterial drivers that contribute to groups clustering (permutation correlation test, P-value <

159   0.001), a superimposition of the genus relative abundance was performed on the PCoA plot. As

160   showed in S1 Fig, major drivers of the normal behavior group segregation were *Faecalibacterium*,

161   *Bacteroides*, *Phascolarctobacterium, Fusobacterium*, *Prevotella*, and [*Prevotella*]. The phobic

162   group was characterized by an enrichment in *Lactobacillus*, while *Dorea*, *Blautia*, *Collinsella*,

163   [*Ruminococcus*], *Slackia*, *Catenibacterium*, and *Megamonas* were more represented within the

164   aggressive group.

165   To dissect discriminant GM component for dogs showing aggressive behavior, we applied the

166   machine learning method Random Forest [28] to the genus level data set. Behavior-discriminatory

167   bacterial genera were identified with distinctive changes in their relative abundances (Fig 3).

168   Specifically, our analysis revealed two 'aggressive-discriminatory' bacterial genera:

169   *Catenibacterium* and *Megamonas*.

170   **Fig 2. Biodiversity of the canine gut microbiome.** Boxplot showing the alpha diversity measures

171   computed with phylogenetic and non-phylogenetic metrics (Shannon diversity index, observed

172   OTUs). Behavior-related groups are identified with colored box and whiskers (orange, Aggressive;

173   blue, Phobic; green, Normal). Significant difference was found between aggressive and phobic

174   groups, according to the observed OTUs metric (P-value = 0.02; Kruskal-Wallis test). Principal

175   coordinate analysis (PCoA) plots showing the beta diversity of the intestinal bacterial communities

176   of the study groups, based on Jaccard similarity index. A significant separation between aggressive

177   and phobic behavior groups was found (P-value = 0.02, permutation test with pseudo-F ratios).

178   **Fig 3. Behavior-related gut microbiome signature.** Top 26 features from the obtained dataset as

179   revealed by Random Forest. Stars denote the bacterial genera discriminant of aggressive group.

180   Boxplots shows the comparison of the relative abundances of these bacterial genera between the

181   study groups.

182

Fecal cortisol and testosterone levels

184   Cortisol and testosterone levels were measured in fecal samples through RIAs. The statistical

185   analysis did not evidence any significant differences between the three groups of dogs for hormonal

186   data. Our results showed that the three study groups had similar median value of fecal cortisol and

187   testosterone levels (Fig 4A). However, it should be noticed that the range of testosterone level in

188   aggressive and phobic populations is largest than in normal, so we can assume that there is a greater

189   variability between subjects (Fig 4B). As consequences of the previously described results, the

190   median of testosterone/cortisol (T/C) ratio were similar in all the three groups, only slightly higher

191   in phobic than in aggressive and normal dogs (Fig 4C).

192   **Fig 4. Fecal hormone levels of the behavior groups.** Boxplots showing levels of cortisol (A) and

193   testosterone (B), and testosterone/cortisol ratio (C) detected in stool samples of the study groups.

194   No significant difference was found among study groups (P-value > 0.05, Kruskal-Wallis test).

195

Discussion

197   Within the present work, we profiled the GM structure and measured the fecal cortisol and

198   testosterone levels of 49 dogs - 31 males and 18 females - of different breed, age, and weight,

199   housed in individual boxes of three animal shelters located in the metropolitan area of Bologna

200   (Italy). Dogs were classified into three study groups based on their behavioral phenotype:

201   aggressive, phobic or normal behavior. The phylogenetic profiles of the canine GM observed in our

202   cohort were found to be in line with those already reported in literature for healthy dogs [9,29,30],

203   but with a slightly higher abundance of Firmicutes and Actinobacteria, as well as a corresponding

204   lower abundance of Bacteroidetes and Proteobacteria. According to our results, the aggressive

205   group GM is characterized by a higher number of observed OTUs compared to both phobic and

206   normal behavior groups. Interestingly, the GM structure of our cohort segregate according to the

207   behavioral disorder of the host, showing a stronger separation of the aggressive group. The latter

208   group seems to be defined by a higher abundance of typically subdominant taxa, such as *Dorea*,

209   *Blautia*, *Collinsella*, [*Ruminococcus*], *Slackia*, *Catenibacterium*, and *Megamonas*. Conversely, the

210   phobic group is characterized by an enrichment of *Lactobacillus*, a bacterial genus comprising well-

211   known GABA producers, the main CNS inhibitory neurotransmitter able to regulate emotional

212   behavior in mice via the vagus nerve [31,32]. The major drivers for the normal behavior group

213   segregation are *Faecalibacterium*, *Bacteroides*, *Phascolarctobacterium*, *Fusobacterium*, *Prevotella*

214   and [*Prevotella*], reflecting the predominance of bacterial genera commonly associated with the

215   GM of healthy dogs [12,33]. Finally, according to the literature, no correlation was observed

216   regarding the phobic behavior in relation to sex or age [19].

217   According to our results, fecal cortisol and testosterone levels of aggressive dogs did not

218   significantly differ from those of phobic and normal dogs. Aggressive dogs are well known to

219   possess higher blood concentrations of cortisol and lower serotonin levels than non-aggressive dogs

220   [20]. However, fecal cortisol levels are not influenced by the activation of HPA axis during the

221   sampling procedure, which is itself stressful for the animal [34]. This can possibly explain the

222   differences in the observed cortisol levels between studies carried out in blood or fecal samples,

223   Testosterone is often correlate with aggressive behaviors in many species [35], but this association

224   is not completely demonstrated in dogs. Indeed, some studies have evidenced that the castration

225   reduce only mildly aggressiveness and that neutered dogs can be more aggressive [36,37]. In

226   contrast with results of Terburg *et al.* [38], who suggested that a high value of T/C ratio may be a

227   predictive factor of aggressive behavior, we found no significant differences between groups.

228   Studies about cortisol in phobic state are confusing, and it seems that cortisol does not increase, or

229   increase only slightly in phobic subjects compared to normal ones [39]. Our results did not show

230   any significant differences between phobic and normal dogs for cortisol concentration. However,

231   the cortisol level of phobic dogs is slightly lower than normal ones. Phobic state can cause a chronic

232   and excessive stress [40], and scientific literature about phobic dogs suggests that they can tend to a

233   depressive state. In human beings and in dogs, depression is characterized by lower cortisol and

234   serotonin levels [41].

235   Our results suggest that dysbiotic GM configurations in a long-term stress levels scenario might

236   influence the local gut environment through the release of potentially neuroactive microbial by-

237   products, probably affecting the behavior of the host mainly as a side effect. In particular, dogs

238   exhibiting aggressive behavioral disorders were characterized by a peculiar GM structure, a high

239   biodiversity and an enrichment in generally subdominant bacterial genera. We then applied a

240   machine learning method (Random Forest) to our genus level data set, identifying *Catenibacterium*

241   and *Megamonas* as bacterial discriminants of aggressive behavior. Respectively belonging to the

242   families Erysipelotrichaceae and Veillonellaceae, *Catenibacterium* and *Megamonas* have been

243   recently correlated with primary bile acid metabolism and abdominal pain in humans [42-44],

244   suggesting a possible connection between a dysbiotic GM profile and behavioral disorders. We

245   found no alteration within the GM of dogs with phobic behavioral disorder except for an increase in

246   *Lactobacillus*, bacterial genera with well-known probiotic properties. Interestingly, chronic

247   treatment with *L.rhamnosus* can influence anxiety- and depression-related behavior by modulating

248   GABA receptor mRNA expression in specific brain regions [31]. Even if it is impossible to dissect

249   the factors supporting the increase of *Lactobacillus* observed in phobic dogs, it is tempting to

250   speculate that a higher abundance of this psychobiotics [45,46] could contribute to the

251   establishment of peculiarities of the phobic behavioral phenotype. However, further studies are

252   required to validate our findings. Indeed, the limitations due to the small number of enrolled

253   animals imply a limited statistical power. Further, invasive blood sampling will be necessary,

254   allowing to match blood and fecal cortisol and testosterone levels to find robust connections with

255   the GM state. Nonetheless, our study support the intriguing opportunity that different behavioral

256   phenotypes in dogs associate with peculiar GM layouts. Particularly, aggressive dogs possess

257   dysbiotic GM configuration, possibly exacerbating the host aggressiveness and supporting the

258   adoption probiotic interventions aimed at restoring a balanced host-symbiont interplay, improving

259   the overall host health and eventually mitigating behavioral disorders. This preliminary research can

260   thus be considered a starting point for future studies of clinical interest, deepening the

261   understanding of the mechanisms underlying the relationship between behavioral disorders and the

262   GM, ultimately providing new insight into veterinary behavioral medicine and facilitating a

263   predictive diagnosis of canine behavior.

264

Materials and methods

266

Enrolled animals, behavioral evaluation and sample collection

268   The entire study was previously evaluated and approved by the Scientific Ethic Committee for

269   Animal Experimentation (University of Bologna). All the procedures were monitored by the

270   responsible of the Department of Veterinary Medical Science (DIMEVET) for animal welfare.

271   In the study, 49 dogs (31 males and 18 females) of different breed, age, and weight, housed in

272   individual boxes of three different animal shelters located in the metropolitan area of Bologna

273   (Italy) were enrolled (S1 Table). The animals were fed on a mixed diet, including wet and dry

274   commercial feed and additional homemade food.

275   For each animal, a behavioral evaluation was performed according to the Canine Behavioral

276   Assessment and Research Questionnaire (C-BARQ) [27], classifying the dogs enrolled based on

277   their behavioral phenotype. Seventeen animals were scored as aggressive and 15 as phobic, while

278   17 animals exhibited a normal behavior. According to C-BARQ, behaviors placed within the

279   aggressive category consisted of barking, growling and baring teeth, as well as snapping, lunging,

280   biting, or attempting to bite [27]. On the other hand, phobic components consisted of excessive fear

281   when exposed to particular sounds, objects or situations, often associated with exaggerated

282   cowering, and/or vigorous attempts to escape, retreat or hide from the feared stimuli [27]. Phobia

283   and aggressiveness are both expression of anxiety disorders and they are strictly correlate to distress

284   conditions of animals [20,47].

285   Fecal samples were collected from each animal immediately after the evacuation, between 7:00 and

286   12:00 am, avoiding debris and cross-contaminations. Specimens were frozen with liquid nitrogen

287   and transported to the laboratory, then stored at -80°C until DNA extraction and sequencing. The

288   remaining part of fecal samples was picked up through non-sterile bags and frozen at -20°C until

289   cortisol and testosterone assay.

290

Fecal cortisol and testosterone radioimmunoassays

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292   Cortisol and testosterone concentrations were determined by radioimmunoassays (RIAs) based on

293   binding of ^{3}H-steroid by competitive adsorption [48]. All concentrations were expressed in pg/mg

294   of fecal matter. Extraction methodology was modified from Schatz & Palme [21]. Cortisol and

295   testosterone were extracted from fecal specimens (500 mg, wet weight) with methanol-water

296   solution (5 ml, v/v 4:1) and ethyl ether (5 ml). The portion of ether was vaporized under an

297   airstream suction hood at 37° C. Dry residue was finally dissolved again into 0.5 ml PBS (0.05 M,

298   pH 7.5), adding 125, 250, 500, or 1000 pg of ^{3}H-cortisol or ^{3}H-testosterone to 500 mg of feces and

299   incubating for 30 min at room temperature, performing a recovery test on five replicates. The

300   extraction was performed as described before, yielding a mean percentage recovery of 87.5 ± 2.4

301   and 89.3 ± 2.1 for cortisol and testosterone, respectively. Cortisol and testosterone metabolites

302   assay in feces were carried out according to Tamanini [49] and Gaiani [50], respectively. Analysis

303 were performed in duplicates. The cortisol RIA was performed using an antiserum to cortisol-21-

304 hemisuccinate-BSA (anti- rabbit), at a working dilution of 1:20 000 and ^{3}H-cortisol (30 pg/tube

305 vial) as tracer. The testosterone RIA was performed using an antiserum to testosterone-3-

306 carboxymethyloxime-BSA (anti- rabbit), at a working dilution of 1:35 000 and ^{3}H-testosterone (31

307 pg/tube vial) as tracer. Fecal cortisol and testosterone samples containing high concentrations of

308 endogenous steroids (100 μl) were serially diluted through PBS (0.05 M, pH 7.5) in volumes of 50,

309 25, 10 and 5 ml, in order to determine the parallelism between cortisol and testosterone standards.

310 Parallelism was assessed between these serial dilutions of standards (ranging from 7.8 to 1000

311 pg/100 ml tube vial). Validation parameters of analysis were: sensitivity 0.19 pg/mg, intra-assay

312 variability 5.9%, inter-assay variability 8.7%, for cortisol; sensitivity 1.1 pg/mg, intra-assay

313 variability 6.2%, inter-assay variability 9.6%, for testosterone. Radioactivity was determined using

314 a liquid scintillation β counter and a linear standard curve, ad hoc designed by a software program

315 [51].

316

Bacterial DNA extraction from stool samples

318 Total microbial DNA was extracted from each fecal sample using the DNeasy Blood & Tissue kit

319 (QIAGEN), with the modified protocol described by Turroni *et al.* [52]. Briefly, 250 mg of feces

320 were resuspended in 1 ml of lysis buffer (500 mM NaCl, 50 mM Tris-HCl pH 8, 50 mM EDTA, 4%

321 SDS). Fecal samples were added with four 3-mm glass beads and 0.5 g of 0.1-mm zirconia beads

322 (BioSpec Products, Bartlesville, USA) and homogenized with 3 bead-beating steps using the

323 FastPrep instrument (MP Biomedicals, Irvine, CA) at 5.5 movements/s for 1 min, keeping the

324 samples on ice for 5 min after each treatment. Samples were subsequently heated at 95°C for 15

325 min and centrifuged to pellet stool particles. Supernatants were added with 260 μl of 10 M

326 ammonium acetate, centrifuged for 10 min at full speed, and incubated in ice for 30 min with one

327 volume of isopropanol. Nucleic acids were collected by centrifugation, washed with 70% ethanol

328   and resuspended in 100 µl of TE buffer. RNA and protein removal was performed by incubating the

329   samples with DNase-free RNase (10 mg/ml) at 37°C for 15 min and protease K at 70°C for 10 min,

330   respectively. Subsequently, DNA purification with QIAmp Mini Spin columns were performed as

331   per manufacturers instruction. The extracted bacterial DNA was quantified using the NanoDrop

332   ND-1000 spectrophotometer (NanoDrop Technologies).

333

PCR amplification and sequencing

335   The V3-V4 region of the 16S rRNA was amplified with PCR using 200 nmol/l of S-D-Bact-0341-

336   b-S-17/S-D-Bact-0785-a-A-21 primers [53] with Illumina overhang adapter sequences, in a final

337   volume of 25 µl containing 12.5 ng of genomic DNA and 2X KAPA HiFi HotStart ReadyMix

338   (Kapa Biosystems). PCR reactions were performed in a Thermal Cycle T gradient (Biometra

339   GmbH) using the following thermal program: 3 min at 95°C for the initial denaturation, followed

340   by 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for

341   30 sec, and a final extension step at 72°C for 5 min. PCR products of about 460 bp were purified

342   using a magnetic bead-based system (Agencourt AMPure XP; Beckman Coulter) and sequenced on

343   Illumina MiSeq platform using the 2 x 250 bp protocol, according to the manufacturer's instructions

344   (Illumina). The libraries were pooled at equimolar concentrations, denatured and diluted to 6 pmol/l

345   before loading onto the MiSeq flow cell.

346

Bioinformatics and statistics

348   Raw sequences were processed using a pipeline combining PANDAseq [54] and QIIME [55]. The

349   UCLUST software [56] was used to bin high-quality reads into operational taxonomic units (OTUs)

350   through an open-reference strategy at a 0.97 similarity threshold. Taxonomy was assigned using the

351   RDP classifier and the Greengenes database as a reference (release May 2013). Chimera filtering

352   was performed discarding all singleton OTUs. Alpha rarefaction was evaluated by using the

353   Observed OTUs metric, and the Shannon biodiversity index, which aims to measure diversity based

354   on evenness, while beta diversity was estimated according to the Jaccard similarity. Random

355   Forests and all statistical analysis was computed using R software (version 3.1.3) and the packages

356   randomForest, vegan and made4. The significance of data separation on the PCoA was tested using

357   a permutation test with pseudo-F ratios (function adonis of vegan package). Non-parametric and

358   correlation tests were achieved with Wilcoxon rank-sum or Kruskal-Wallis test and the Kendall tau,

359   respectively. Cortisol and testosterone concentrations, as well as T/C ratio was analyzed using the

360   normality test of Shapiro-Wilk, in order to establish the distribution of each variable in the

361   population. P-values < 0.05 were considered statistically significant.

362

References

364   1.   Frantz LA, Mullin VE, Pionnier-Capitan M, Lebrasseur O, Ollivier M, Perri A, et al.

365   Genomic and archaeological evidence suggest a dual origin of domestic dogs. Science.

366   2016;352(6290):1228-31. doi: 10.1126/science.aaf3161. PubMed PMID: 27257259.

367   2.   Wang GD, Zhai W, Yang HC, Fan RX, Cao X, Zhong L, et al. The genomics of selection in

368   dogs and the parallel evolution between dogs and humans. Nat Commun. 2013;4:1860. doi:

369   10.1038/ncomms2814. PubMed PMID: 23673645.

370   3.   Larson G, Karlsson EK, Perri A, Webster MT, Ho SY, Peters J, et al. Rethinking dog

371   domestication by integrating genetics, archeology, and biogeography. Proc Natl Acad Sci U S A.

372   2012;109(23):8878-83. doi: 10.1073/pnas.1203005109. PubMed PMID: 22615366; PubMed

373   Central PMCID: PMCPMC3384140.

374   4.   Ollivier M, Tresset A, Bastian F, Lagoutte L, Axelsson E, Arendt ML, et al. Amy2B copy

375   number variation reveals starch diet adaptations in ancient European dogs. R Soc Open Sci.

376   2016;3(11):160449. doi: 10.1098/rsos.160449. PubMed PMID: 28018628; PubMed Central

377   PMCID: PMCPMC5180126.

378   5.   Axelsson E, Ratnakumar A, Arendt ML, Maqbool K, Webster MT, Perloski M, et al. The

379   genomic signature of dog domestication reveals adaptation to a starch-rich diet. Nature.

380   2013;495(7441):360-4. doi: 10.1038/nature11837. PubMed PMID: 23354050.

381   6.   Coelho LP, Kultima JR, Costea PI, Fournier C, Pan Y, Czarnecki-Maulden G, et al.

382   Similarity of the dog and human gut microbiomes in gene content and response to diet.

383   Microbiome. 2018;6(1):72. doi: 10.1186/s40168-018-0450-3. PubMed PMID: 29669589; PubMed

384   Central PMCID: PMCPMC5907387.

385   7.   Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, et al. Evolution of

386   mammals and their gut microbes. Science. 2008;320(5883):1647-51. doi: 10.1126/science.1155725.

387   PubMed PMID: 18497261; PubMed Central PMCID: PMCPMC2649005.

388   8.   Honneffer JB, Minamoto Y, Suchodolski JS. Microbiota alterations in acute and chronic

389   gastrointestinal inflammation of cats and dogs. World J Gastroenterol. 2014;20(44):16489-97. doi:

390   10.3748/wjg.v20.i44.16489. PubMed PMID: 25469017; PubMed Central PMCID:

391   PMCPMC4248192.

392   9.   Swanson KS, Dowd SE, Suchodolski JS, Middelbos IS, Vester BM, Barry KA, et al.

393   Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals

394   similarities with humans and mice. ISME J. 2011;5(4):639-49. doi: 10.1038/ismej.2010.162.

395   PubMed PMID: 20962874; PubMed Central PMCID: PMCPMC3105739.

396   10.   Song SJ, Lauber C, Costello EK, Lozupone CA, Humphrey G, Berg-Lyons D, et al.

397   Cohabiting family members share microbiota with one another and with their dogs. Elife.

398   2013;2:e00458. doi: 10.7554/eLife.00458. PubMed PMID: 23599893; PubMed Central PMCID:

399   PMCPMC3628085.

400   11.   Hayward JJ, Castelhano MG, Oliveira KC, Corey E, Balkman C, Baxter TL, et al. Complex

401   disease and phenotype mapping in the domestic dog. Nat Commun. 2016;7:10460. doi:

402   10.1038/ncomms10460. PubMed PMID: 26795439; PubMed Central PMCID: PMCPMC4735900.

403   12.   Vazquez-Baeza Y, Hyde ER, Suchodolski JS, Knight R. Dog and human inflammatory

404   bowel disease rely on overlapping yet distinct dysbiosis networks. Nat Microbiol. 2016;1:16177.

405   doi: 10.1038/nmicrobiol.2016.177. PubMed PMID: 27694806.

406   13.   Minamoto Y, Otoni CC, Steelman SM, Buyukleblebici O, Steiner JM, Jergens AE, et al.

407   Alteration of the fecal microbiota and serum metabolite profiles in dogs with idiopathic

408   inflammatory bowel disease. Gut Microbes. 2015;6(1):33-47. doi: 10.1080/19490976.2014.997612.

409   PubMed PMID: 25531678; PubMed Central PMCID: PMCPMC4615558.

410   14.   Forsythe P, Bienenstock J, Kunze WA. Vagal pathways for microbiome-brain-gut axis

411   communication. Adv Exp Med Biol. 2014;817:115-33. doi: 10.1007/978-1-4939-0897-4_5.

412   PubMed PMID: 24997031.

413   15.   Fung TC, Olson CA, Hsiao EY. Interactions between the microbiota, immune and nervous

414   systems in health and disease. Nat Neurosci. 2017;20(2):145-55. doi: 10.1038/nn.4476. PubMed

415   PMID: 28092661.

416   16.   Neuman H, Debelius JW, Knight R, Koren O. Microbial endocrinology: the interplay

417   between the microbiota and the endocrine system. FEMS Microbiol Rev. 2015;39(4):509-21. doi:

418   10.1093/femsre/fuu010. PubMed PMID: 25701044.

419   17.   Lyte M. Microbial endocrinology in the microbiome-gut-brain axis: how bacterial

420   production and utilization of neurochemicals influence behavior. PLoS Pathog.

421   2013;9(11):e1003726. doi: 10.1371/journal.ppat.1003726. PubMed PMID: 24244158; PubMed

422   Central PMCID: PMCPMC3828163.

423   18.   Mayer EA. Gut feelings: the emerging biology of gut-brain communication. Nat Rev

424   Neurosci. 2011;12(8):453-66. doi: 10.1038/nrn3071. PubMed PMID: 21750565; PubMed Central

425   PMCID: PMCPMC3845678.

426   19.   Yalcin E, Batmaz H. Signalment Factors, Comorbidity in Behavior Diagnoses in Dogs in

427   Bursa Region, Turkey (2000 - 2004) Acta Vet. Brno. 2007; 76: 445-450. doi:

428   10.2754/avb200776030445.

429   20.   Rosado B, Garcia-Belenguer S, Leon M, Chacon G, Villegas A, Palacio J. Blood

430   concentrations of serotonin, cortisol and dehydroepiandrosterone in aggressive dogs. Appl Anim

431   Behav Sci. 2010;123(3-4):124-30. doi: 10.1016/j.applanim.2010.01.009. PubMed PMID:

432   WOS:000275677500006.

433   21.   Schatz S, Palme R. Measurement of faecal cortisol metabolites in cats and dogs: a non-

434   invasive method for evaluating adrenocortical function. Vet Res Commun. 2001;25(4):271-87.

435   PubMed PMID: 11432429.

436   22.   Kirchoff NS, Udell MAR, Sharpton TJ. The gut microbiome correlates with conspecific

437   aggression in a small population of rescued dogs (Canis familiaris). PeerJ Preprints. 2018;

438   6:e26990v1. doi: 10.7287/peerj.preprints.26990v1.

439   23.   Re S, Zanoletti M, Emanuele E. Aggressive dogs are characterized by low omega-3

440   polyunsaturated fatty acid status. Vet Res Commun. 2008;32(3):225-30. doi: 10.1007/s11259-007-

441   9021-y. PubMed PMID: 17891468.

442   24.   Van der Weyde LK, Martin GB, Paris MC. Monitoring stress in captive and free-ranging

443   African wild dogs (Lycaon pictus) using faecal glucocorticoid metabolites. Gen Comp Endocrinol.

444   2016;226:50-5. doi: 10.1016/j.ygcen.2015.12.022. PubMed PMID: 26723188.

445   25.   Kersey DC, Dehnhard M. The use of noninvasive and minimally invasive methods in

446   endocrinology for threatened mammalian species conservation. Gen Comp Endocrinol.

447   2014;203:296-306. doi: 10.1016/j.ygcen.2014.04.022. PubMed PMID: 24798579.

448   26.   Sheriff MJ, Krebs CJ, Boonstra R. Assessing stress in animal populations: Do fecal and

449   plasma glucocorticoids tell the same story? Gen Comp Endocrinol. 2010;166(3):614-9. doi:

450   10.1016/j.ygcen.2009.12.017. PubMed PMID: 20051245.

451   27.   Hsu Y, Serpell JA. Development and validation of a questionnaire for measuring behavior

452   and temperament traits in pet dogs. J Am Vet Med Assoc. 2003.

453   28.   Breiman, L. Random Forests. Mach Learn. 2001; 45: 5-32.

454   29.   Omatsu T, Omura M, Katayama Y, Kimura T, Okumura M, Okumura A, et al. Molecular

455   diversity of the faecal microbiota of Toy Poodles in Japan. J Vet Med Sci. 2018;80(5):749-54. doi:

456   10.1292/jvms.17-0582. PubMed PMID: 29643280; PubMed Central PMCID: PMCPMC5989017.

457   30.   Sandri M, Dal Monego S, Conte G, Sgorlon S, Stefanon B. Raw meat based diet influences

458   faecal microbiome and end products of fermentation in healthy dogs. BMC Vet Res. 2017;13(1):65.

459   doi: 10.1186/s12917-017-0981-z. PubMed PMID: 28245817; PubMed Central PMCID:

460   PMCPMC5331737.

461   31.   Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion

462   of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a

463   mouse via the vagus nerve. Proc Natl Acad Sci U S A. 2011;108(38):16050-5. doi:

464   10.1073/pnas.1102999108. PubMed PMID: 21876150; PubMed Central PMCID:

465   PMCPMC3179073.

466   32.   Lyte M. Probiotics function mechanistically as delivery vehicles for neuroactive

467   compounds: Microbial endocrinology in the design and use of probiotics. Bioessays.

468   2011;33(8):574-81. doi: 10.1002/bies.201100024. PubMed PMID: 21732396.

469   33.   Garcia-Mazcorro JF, Dowd SE, Poulsen J, Steiner JM, Suchodolski JS. Abundance and

470   short-term temporal variability of fecal microbiota in healthy dogs. Microbiologyopen.

471   2012;1(3):340-7. doi: 10.1002/mbo3.36. PubMed PMID: 23170232; PubMed Central PMCID:

472   PMCPMC3496977.

473   34.   Farca AM, Cavana P, Badino P, Barbero R, Odore R, Pollicino P. Measurement of faecal

474   corticoid metabolites in domestic dogs. Schweiz Arch Tierheilkd. 2006;148(12):649-55. doi:

475   10.1024/0036-7281.148.12.649. PubMed PMID: 17263082.

476   35.   Archer J. The Behavioural Biology of Aggression, Vol. 1. Cambridge (UK): CUP Archive.

477   Cambridge University Press. 1988.

478   36.   Neilson JC, Eckstein RA, Hart BL. Effects of castration on problem behaviors in male dogs

479   with reference to age and duration of behavior. J Am Vet Med Assoc. 1997;211(2):180-2. PubMed

480   PMID: 9227747.

481   37.   Guy NC, Luescher UA, Dohoo SE, Spangler E, Miller JB, Dohoo IR, et al. Demographic

482   and aggressive characteristics of dogs in a general veterinary caseload. Appl Anim Behav Sci.

483   2001;74(1):15-28. doi: Doi 10.1016/S0168-1591(01)00153-8. PubMed PMID:

484   WOS:000170357100002.

485   38.   Terburg D, Morgan B, van Honk J. The testosterone-cortisol ratio: A hormonal marker for

486   proneness to social aggression. Int J Law Psychiatry. 2009;32(4):216-23. doi:

487   10.1016/j.ijlp.2009.04.008. PubMed PMID: 19446881.

488   39.   Fyer AJ. Current approaches to etiology and pathophysiology of specific phobia. Biol

489   Psychiatry. 1998;44(12):1295-304. PubMed PMID: 9861472.

490   40.   Schull-Selcer EA, Stagg W. Advances ad phobias. An update on separation anxiety and

491   noise aversion. Vet. Clin. Small Animal 1991; 38:1081-1106. doi: 10.1016/j.cvsm.2008.04.012.

492   41.   Cocchi M, Gabrielli F, Tonello L, Delogu M, Beghelli V, Mattioli M, Accorsi PA.

493   Molecular contiguity between human and animal consciousness through evolution: some

494   considerations. J. Phylogen. & Evolution Biol. 2013; 1:1-8. doi: 10.4172/2329-9002.1000119.

495   42.   Aleman JO, Bokulich NA, Swann JR, Walker JM, De Rosa JC, Battaglia T, et al. Fecal

496   microbiota and bile acid interactions with systemic and adipose tissue metabolism in diet-induced

497   weight loss of obese postmenopausal women. J Transl Med. 2018;16(1):244. doi: 10.1186/s12967-

498   018-1619-z. PubMed PMID: 30176893; PubMed Central PMCID: PMCPMC6122649.

499   43.   Sakon H, Nagai F, Morotomi M, Tanaka R. Sutterella parvirubra sp. nov. and Megamonas

500   funiformis sp. nov., isolated from human faeces. Int J Syst Evol Microbiol. 2008;58(Pt 4):970-5.

501   doi: 10.1099/ijs.0.65456-0. PubMed PMID: 18398204.

502   44.   Yusof N, Hamid N, Ma ZF, Lawenko RM, Wan Mohammad WMZ, Collins DA, et al.

503   Exposure to environmental microbiota explains persistent abdominal pain and irritable bowel

504   syndrome after a major flood. Gut Pathog. 2017;9:75. doi: 10.1186/s13099-017-0224-7. PubMed

505   PMID: 29255490; PubMed Central PMCID: PMCPMC5729606.

506   45.   Dinan TG, Stanton C, Cryan JF. Psychobiotics: a novel class of psychotropic. Biol

507   Psychiatry. 2013;74(10):720-6. doi: 10.1016/j.biopsych.2013.05.001. PubMed PMID: 23759244.

508   46.   Sarkar A, Lehto SM, Harty S, Dinan TG, Cryan JF, Burnet PWJ. Psychobiotics and the

509   manipulation of bacteria-gut-brain signals. Trends Neurosci. 2016;39(11):763-81. doi:

510   10.1016/j.tins.2016.09.002. PubMed PMID: 27793434; PubMed Central PMCID:

511   PMCPMC5102282.

512   47.   Appleby D, Pluijmakers J. Separation anxiety in dogs: The function of homeostasis in its

513   development and treatment. Clin Tech Small Anim Pract. 2004;19(4):205-15. doi:

514   10.1053/j.ctsap.2004.10.002. PubMed PMID: 18371317.

515   48.   Fenske M, Schonheiter H. Thin-layer chromatography on silica-coated aluminium sheet as

516   an adjunct to radioimmunoassay of steroids. J Chromatogr. 1991;563(1):178-83. PubMed PMID:

517   1648105.

518   49.   Tamanini C, Giordano N, Chiesa F, Seren E. Plasma cortisol variations induced in the

519   stallion by mating. Acta Endocrinol (Copenh). 1983;102(3):447-50. PubMed PMID: 6681927.

520   50.   Gaiani R, Chiesa F, Mattioli M, Nannetti G, Galeati G. Androstenedione and testosterone

521   concentrations in plasma and milk of the cow throughout pregnancy. J Reprod Fertil.

522   1984;70(1):55-9. PubMed PMID: 6694152.

523   51.   Motta M, Esposti AD. A computer program for mathematical treatment of data in

524   radioimmunoassay. Comput Programs Biomed. 1981.

525   52.   Turroni S, Rampelli S, Biagi E, Consolandi C, Severgnini M, Peano C, et al. Temporal

526   dynamics of the gut microbiota in people sharing a confined environment, a 520-day ground-based

527   space simulation, MARS500. Microbiome. 2017.

528   53.   Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of

529   general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based

530   diversity studies. Nucleic Acids Res. 2013;41(1):e1. doi: 10.1093/nar/gks808. PubMed PMID:

531   22933715; PubMed Central PMCID: PMCPMC3592464.

532   54.   Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD. PANDAseq: paired-

533   end assembler for illumina sequences. BMC Bioinformatics. 2012;13:31. doi: 10.1186/1471-2105-

534   13-31. PubMed PMID: 22333067; PubMed Central PMCID: PMCPMC3471323.

535   55.   Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al.

536   QIIME allows analysis of high-throughput community sequencing data. Nat Methods.

537   2010;7(5):335-6. doi: 10.1038/nmeth.f.303. PubMed PMID: 20383131; PubMed Central PMCID:
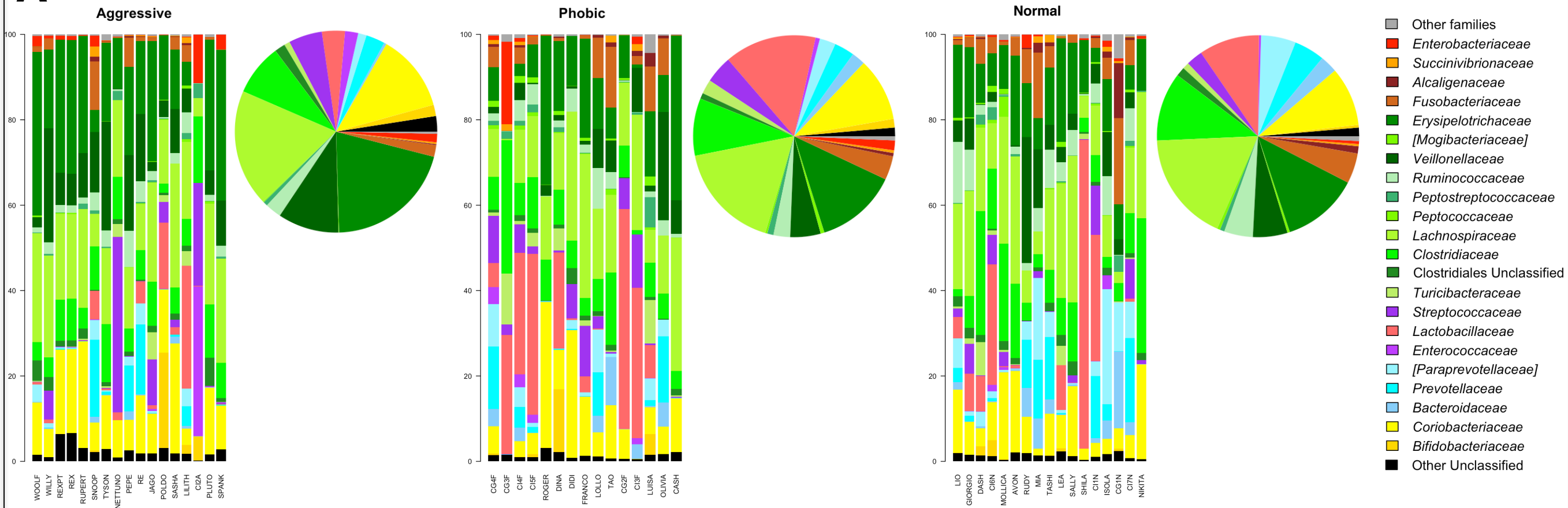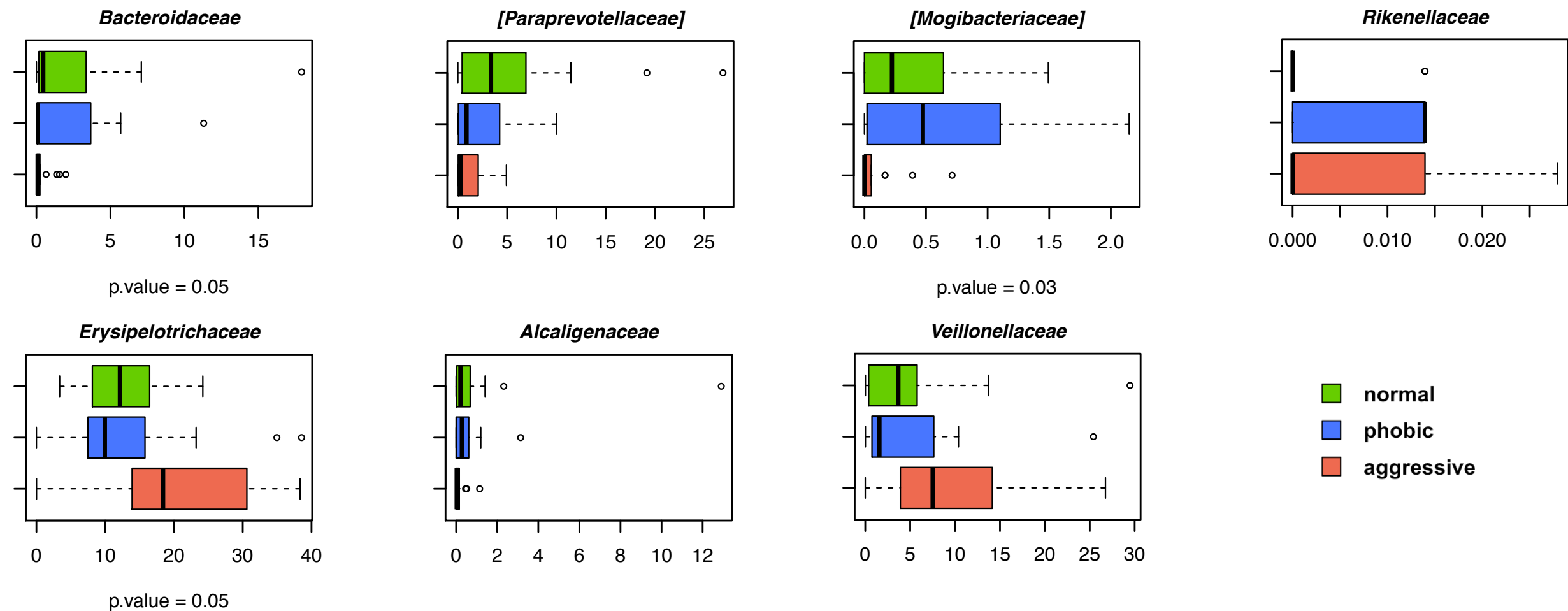
538   PMCPMC3156573.

539   56.   Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics.

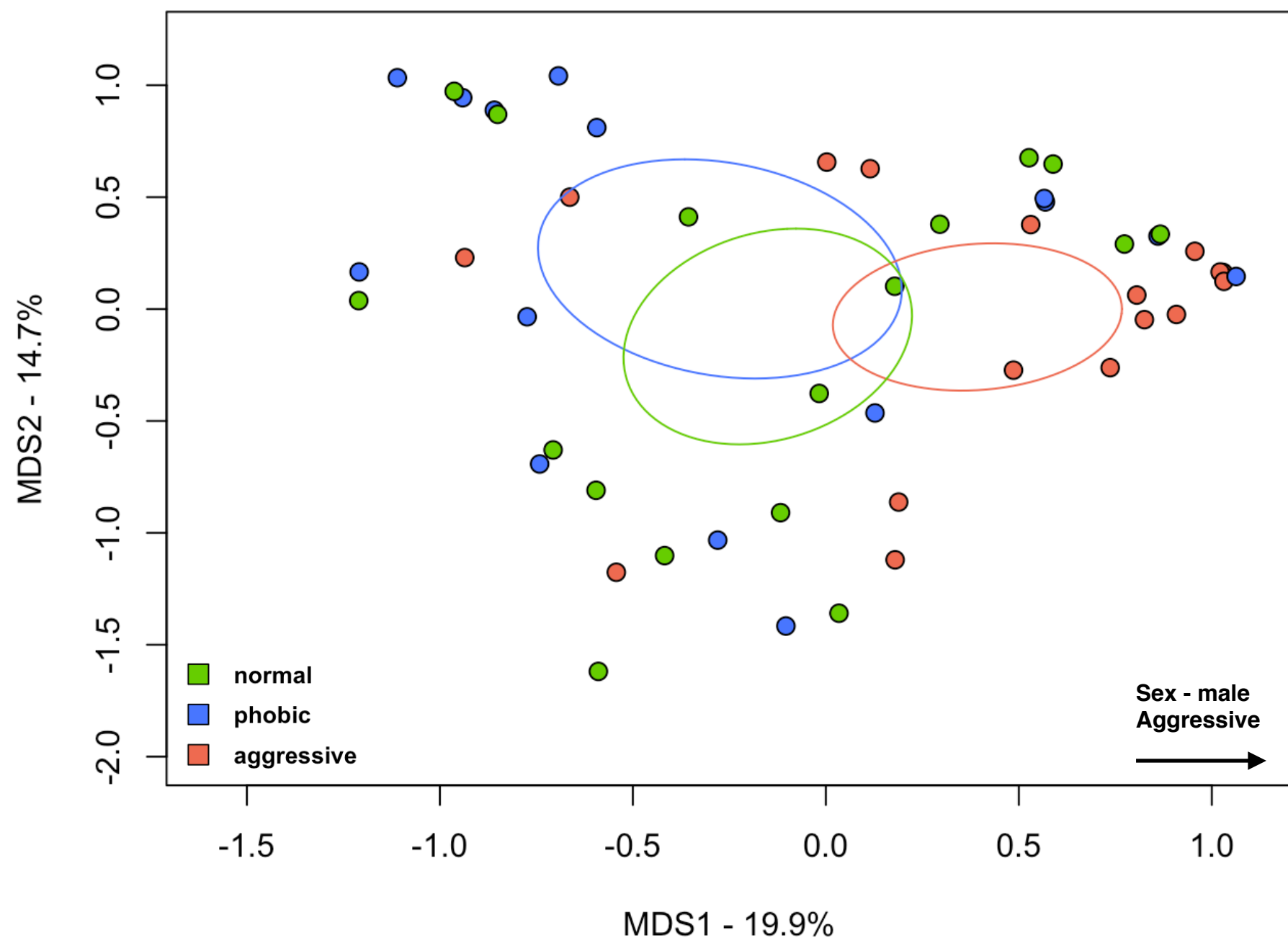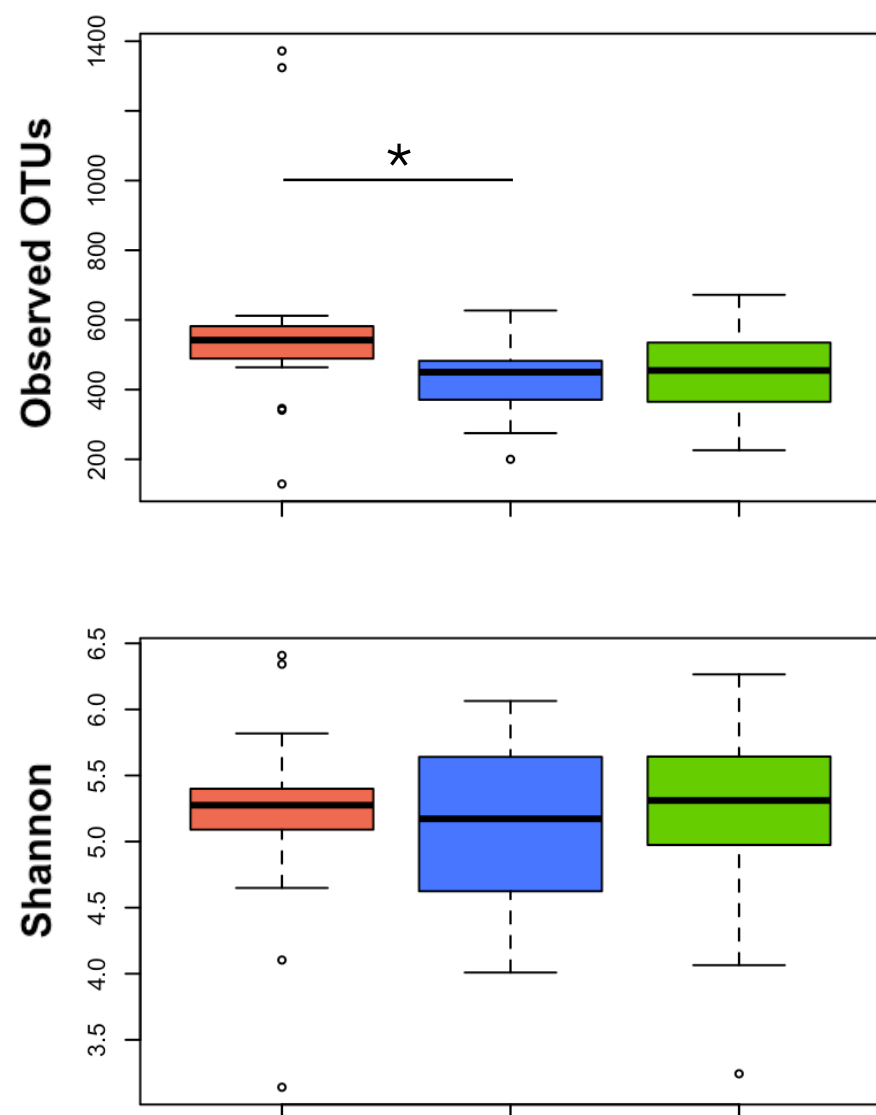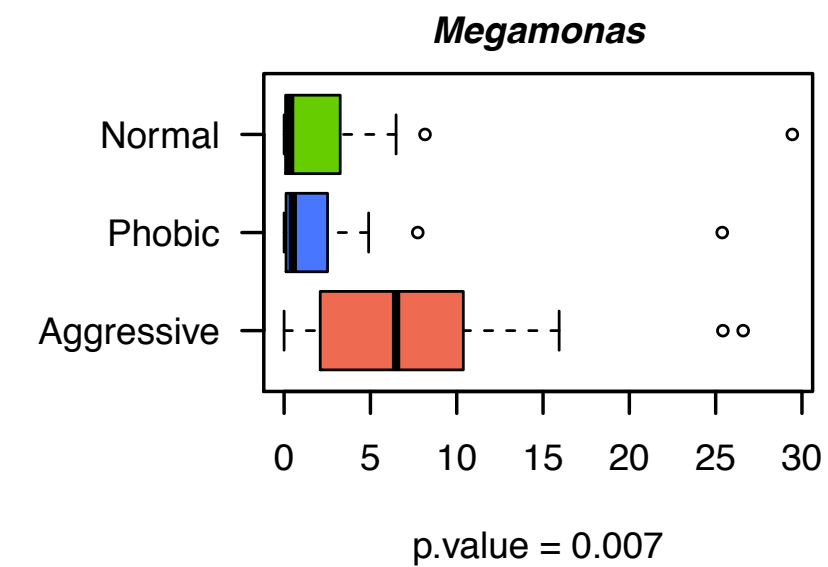540   2010;26(19):2460-1. doi: 10.1093/bioinformatics/btq461. PubMed PMID: 20709691.

541

542

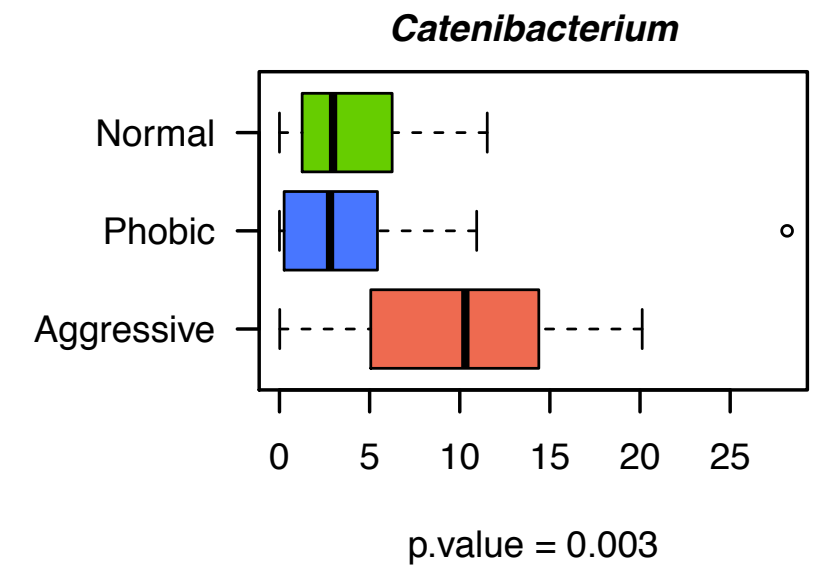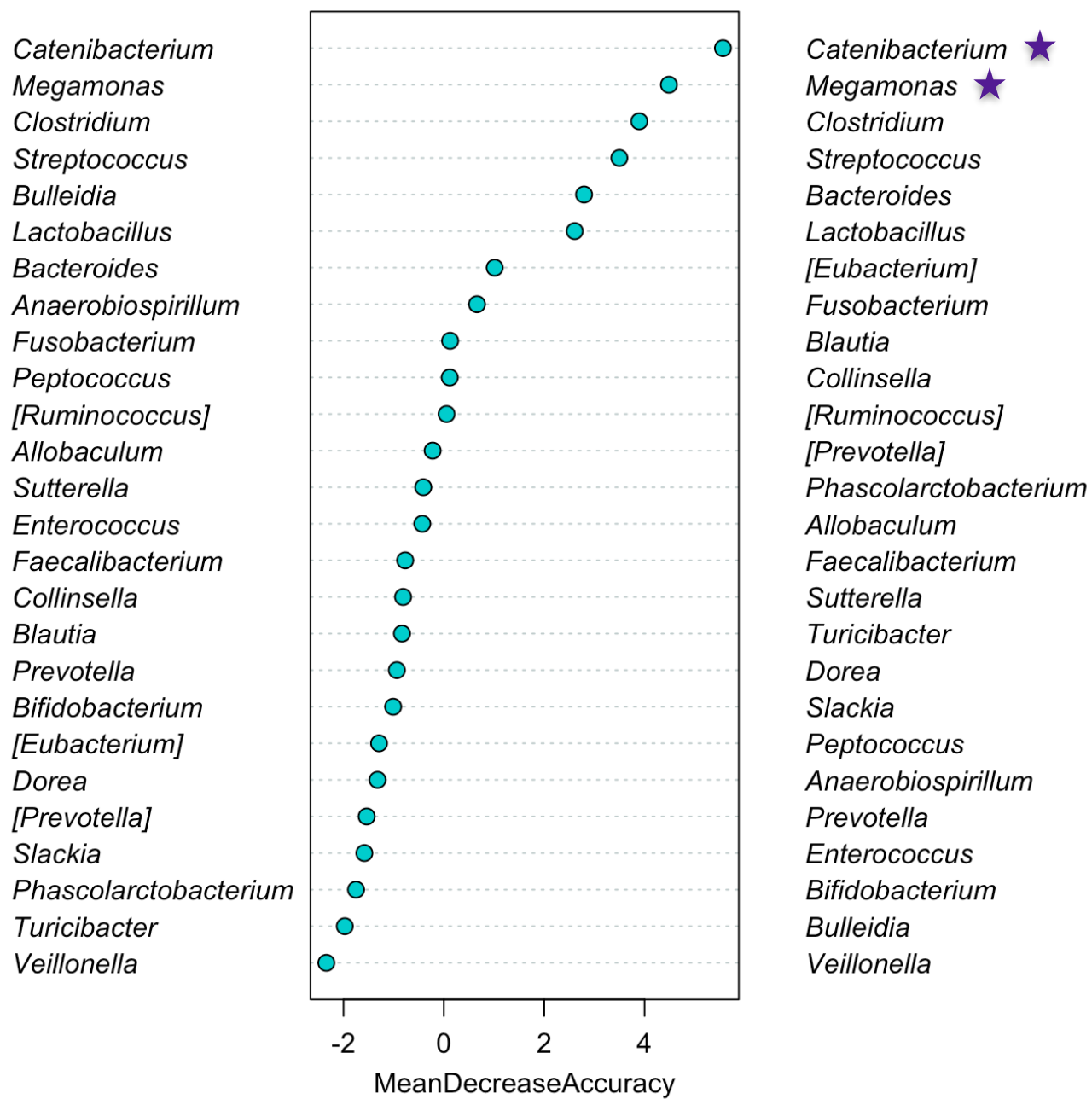## Supporting information

544   **S1 Table. Metadata of the enrolled cohort.**
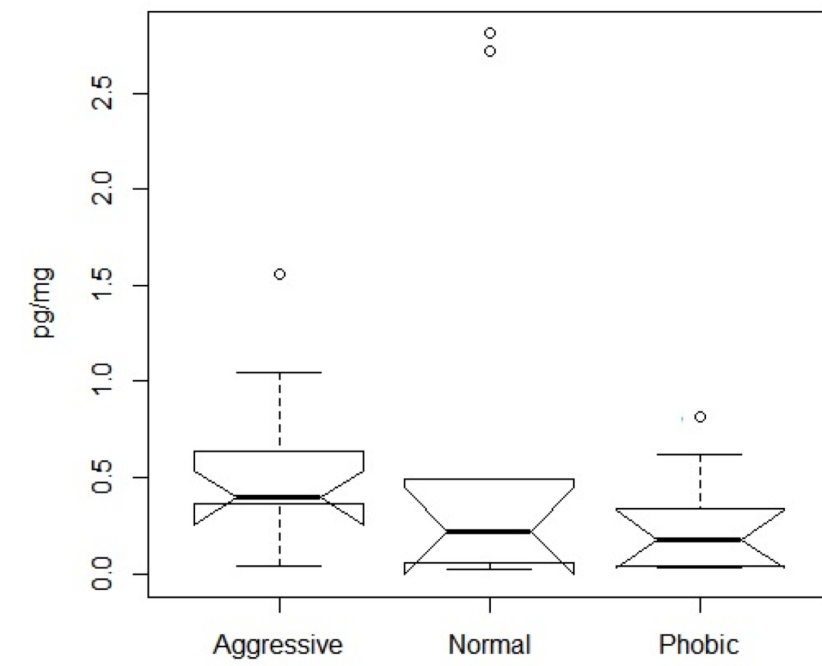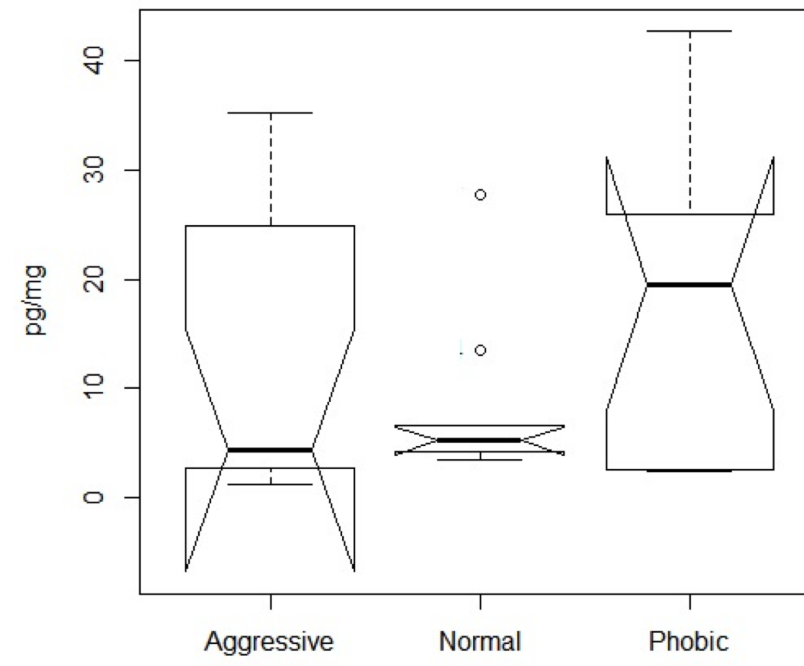
545   **S1 Fig. Superimposition of the genus relative abundance on the PCoA plot.** Arrows represent

546   the direction of significant correlations (permutation correlation test, P-value < 0.001).

547   **S2 Fig. Main bacterial genera represented within the canine gut microbiome.**

A Aggressive | Phobic | Normal

Legend:
- Other families
- *Enterobacteriaceae*
- *Succinivibrionaceae*
- *Alcaligenaceae*
- *Fusobacteriaceae*
- *Erysipelotrichaceae*
- *[Mogibacteriaceae]*
- *Veillonellaceae*
- *Ruminococcaceae*
- *Peptostreptococcaceae*
- *Peptococcaceae*
- *Lachnospiraceae*
- *Clostridiaceae*
- Clostridiales Unclassified
- *Turicibacteraceae*
- *Streptococcaceae*
- *Lactobacillaceae*
- *Enterococcaceae*
- *[Paraprevotellaceae]*
- *Prevotellaceae*
- *Bacteroidaceae*
- *Coriobacteriaceae*
- *Bifidobacteriaceae*
- Other Unclassified

B

Bacteroidaceae | *[Paraprevotellaceae]* | *[Mogibacteriaceae]* | *Rikenellaceae*

p.value = 0.05   p.value = 0.03

Erysipelotrichaceae | *Alcaligenaceae* | *Veillonellaceae*

- normal
- phobic
- aggressive

p.value = 0.05

A Cortisol

B Testosterone

C Testosterone/Cortisol