

A peer-reviewed version of this preprint was published in PeerJ on 9 January 2019.

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Kirchoff NS, Udell MAR, Sharpton TJ. 2019. The gut microbiome correlates with conspecific aggression in a small population of rescued dogs (*Canis familiaris*) PeerJ 7:e6103 <https://doi.org/10.7717/peerj.6103>

The gut microbiome correlates with conspecific aggression in a small population of rescued dogs (*Canis familiaris*)

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Aggression is a serious behavioral disorder in domestic dogs that endangers both dogs and humans. The underlying causes of canine aggression are poorly resolved and require illumination to ensure effective therapy. Recent research links the compositional diversity of the gut microbiome to behavioral and psychological regulation in other mammals, such as mice and humans. Given these observations, we hypothesized that the composition of the canine gut microbiome could associate with aggression. We analyzed fecal microbiome samples collected from a small population of pit bull type dogs seized from a dogfighting organization. This population included twenty-one dogs that displayed conspecific aggressive behaviors and ten that did not. Beta-diversity analyses support an association between gut microbiome structure and dog aggression. Additionally, we used a phylogenetic approach to resolve specific clades of gut bacteria that stratify aggressive and non-aggressive dogs, including clades within *Lactobacillus*, *Dorea*, *Blautia*, *Turicibacter*, and *Bacteroides*. Several of these taxa have been implicated in modulating mammalian behavior as well as gastrointestinal disease states. Although sample size limits this study, our findings indicate that gut microorganisms are linked to dog aggression and point to an aggression-associated physiological state that interacts with the gut microbiome. These results also indicate that the gut microbiome may be useful for diagnosing aggressive behaviors prior to their manifestation and potentially discerning cryptic etiologies of aggression.

1 **Author Cover Page**

2

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5

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15 **Abstract**

16 Aggression is a serious behavioral disorder in domestic dogs that endangers both dogs
17 and humans. The underlying causes of canine aggression are poorly resolved and require
18 illumination to ensure effective therapy. Recent research links the compositional diversity
19 of the gut microbiome to behavioral and psychological regulation in other mammals,
20 such as mice and humans. Given these observations, we hypothesized that the
21 composition of the canine gut microbiome could associate with aggression. We analyzed
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23 from a dogfighting organization. This population included twenty-one dogs that
24 displayed conspecific aggressive behaviors and ten that did not. Beta-diversity analyses
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27 that stratify aggressive and non-aggressive dogs, including clades within *Lactobacillus*,
28 *Dorea*, *Blautia*, *Turicibacter*, and *Bacteroides*. Several of these taxa have been
29 implicated in modulating mammalian behavior as well as gastrointestinal disease states.
30 Although sample size limits this study, our findings indicate that gut microorganisms are
31 linked to dog aggression and point to an aggression-associated physiological state that
32 interacts with the gut microbiome. These results also indicate that the gut microbiome
33 may be useful for diagnosing aggressive behaviors prior to their manifestation and
34 potentially discerning cryptic etiologies of aggression.

35

37 **Introduction**

38 Domestic dogs (*Canis familiaris*) have coexisted with humans for over 14 thousand years
39 (Nobis, 1979), and remain among the most popular companion animals, especially in the western
40 world where millions can be found living in human homes (National Pet Owners Survey:
41 Industry Statistics & Trends, 2014). Even larger populations of free-roaming and village dogs
42 can be found living among humans around the world (Coppinger & Coppinger, 2001). In recent
43 years, dogs have been studied for their capacity to form strong bonds with humans and other
44 species, resulting in a range of prosocial, cooperative, and communicative behaviors (Udell &
45 Wynne, 2008). However, dog aggression towards humans, other dogs, or other animals remains a
46 common behavioral problem (Bamberger & Houpt, 2006) that can pose serious risks to animals,
47 owners, and other humans including neighbors, friends, or veterinary staff. Aggressive
48 interactions, especially those involving bites, may lead to physical harm, psychological trauma,
49 disease transmission, or even fatality in bitten humans and other dogs (Overall & Love, 2001;
50 Hampson et al., 2009; Brooks, Moxon & England, 2010; Ji et al., 2010). Aggressive behavior
51 also poses risks to the aggressor dog, as aggression is a common reason for relinquishment to
52 animal shelters, where poor progress on mitigation of the behavior, assuming the shelter is even
53 equipped to intervene, often leads to euthanasia (Salman et al., 2000). Consequently,
54 understanding the factors and mechanisms responsible for dog behaviors that are incompatible
55 with success in anthropogenic environments has much potential to benefit both species.

56 Dog aggression is often divided into categories, including dominance aggression, fear
57 aggression, food or possessive aggression, and territorial aggression (Lockwood, 2017; Houpt,
58 2006; Blackshaw, 1991) based on the form of the behavior and the identified or presumed
59 context or consequences associated with specific aggressive acts. However, the factors that

60 predict aggression in one dog, but not in another, under similar conditions (for example, in a
61 standard behavior evaluation) are less well understood. Current research suggests that
62 environmental, experiential, and owner specific variables are important predictors of aggression
63 in dogs (Roll & Unshelm, 1997; Hsu & Sun, 2010). However, underlying biological correlates
64 including genetics, sex, hormone levels, neuter status, nutrition and neurological health have also
65 been identified (Sherman et al., 1996; DeNapoli et al., 2000; Duffy, Hsu & Serpell, 2008;
66 Rosado et al., 2010a). While behavior modification or environmental change can significantly
67 reduce aggressive behavior in at least some contexts (Sherman et al., 1996; Mohan-Gibbons,
68 Weiss & Slater, 2012), underlying physiological mechanisms including pain, elevated stress
69 levels, reduced thresholds for aggression, or impulsivity could impede behavioral treatment or
70 lead to resumption of the behavior if left unidentified. Therefore, further elucidating the
71 physiological underpinnings of aggression in dogs may be critical to mitigating aggressive
72 behavior, especially for situations where other treatment and training options are ineffective on
73 their own. The limited research in this area shows that aggression associates with high levels of
74 cortisol and low levels of serotonin (5HT) (Rosado et al., 2010b; Roth et al., 2016; León et al.,
75 2012). Stress in dogs is often detected by measuring cortisol levels and is thought to be a
76 component associated with behavioral problems such as anxiety as well as aggression (Rooney,
77 Clark & Casey, 2016). Accordingly, many dogs diagnosed with aggression are also diagnosed
78 with anxiety (Bamberger & Houpt, 2006). Unfortunately, there is still much to learn about the
79 underlying causes of aggressive behavior, which limits the development of new preventative
80 strategies, diagnostics, and therapeutic interventions.

81 Emerging evidence suggests that the gut microbiome may interact with mammalian
82 physiology to influence behavior (Cryan & O'Mahony, 2011; Mayer et al., 2014; Foster et al.,

83 2015). These interactions include aspects of physiology that are relevant to mammalian
84 aggression. For example, germ-free and specific-pathogen free mice exhibit different anxiety
85 levels (Heijtz et al., 2011; Neufeld et al., 2011). Other studies have found that specific strains of
86 bacteria can modulate anxiety phenotypes and stress hormones such as glucocorticoids (Bravo et
87 al., 2011; Hsiao et al., 2013). Moreover, gut bacteria can produce neuroactive substances, such as
88 precursors of monoamine neurotransmitters that act on the gut-brain axis to potentially impact
89 behavior, including anxiety (Evrensel & Ceylan, 2015; Diaz Heijtz et al., 2011; Carabotti et al.,
90 2015; O'Mahony et al., 2015). For example, the gut microbiome produces tryptophan, which
91 impacts host serotonin levels and behaviors linked to serotonergic neurotransmission (O'Mahony
92 et al., 2015; Yano et al., 2015). These observations indicate that the gut microbiome and
93 aggressive behavior may be linked in mammals.

94 To date, no studies have investigated the association between the gut microbiome and
95 aggression in dogs, which is a first necessary step towards ultimately ascertaining whether the
96 gut microbiome mediates aggression. Prior work points to a potential interaction between the
97 microbiome and canine aggression. For example, diet is a strong modulator of gut microbial
98 composition in many animals (David et al., 2013) and specific dietary components are associated
99 with aggression including diets that reduce aggressive behaviors in dogs (DeNapoli et al., 2000;
100 Re, Zanoletti & Emanuele, 2008). Additionally, the canine gut microbiome is associated with
101 other health conditions such as inflammatory bowel disease and acute diarrhea (Suchodolski et
102 al., 2012) leading to discomfort or pain that could also contribute to irritability or aggression.
103 Here, we conducted an exploratory analysis of fecal samples originating from a small shelter-
104 housed population of pit bull type dogs seized from organized dogfighting to determine if canine
105 aggression could be predicted based on the composition of the gut microbiome.

106

107 **Materials and Methods**

108 *Sample Collection*

109 A single fecal sample was collected from the kennel of each of thirty-one pit bull type
110 dogs residing at a temporary shelter while in protective custody. This population served as the
111 focus of this pilot study because it enabled control over as many factors as possible, including
112 breed type, environment, diet, and medical care, while providing access to a population with a
113 relatively more frequent aggressive phenotype compared to typical populations. Upon intake into
114 the shelter and prior to the initiation of this study, an animal welfare agency catalogued various
115 parameters of each individual, which were used in this study's analysis as covariate data (Table
116 S1). Animal welfare employees collected feces using aseptic technique within an hour of
117 defecation and immediately froze them at -18C to -20°C to fix bacterial growth and preserve the
118 DNA content. Fecal samples were shipped to Oregon State University and stored at -20°C.
119 Thirty of the dogs were on a diet of Iams Proactive Health minichunks adult kibble (chicken-
120 based formula) and one dog was on a diet of Iams Puppy. Fourteen males and seventeen females
121 were sampled from. Each dog received a behavior evaluation conducted by the animal welfare
122 agency shortly after intake that categorized these dogs as intraspecifically aggressive (n = 21) or
123 non-aggressive (n = 10) based on exhibited aggression towards unfamiliar dogs. Data from these
124 evaluations were sent to Oregon State University along with the stool samples for analysis. With
125 the exception of the collection and processing of fecal material, this study did not involve any
126 manipulation of, measurement of, or contact with dogs that had not already occurred.

127

128 *Ethical Statement*

129 No animal subjects, animal handling or study specific animal interactions were required
130 for the purpose of this study. Dog fecal samples were collected from shelter kennels after natural
131 deposit. Previously collected behavioral data from the animal welfare agency's records were
132 used in analysis. Therefore, this study was determined to be exempt from institutional animal
133 care and use review by Oregon State University's ethical review board.

134

135 ***Fecal DNA Extraction and 16S Sequencing***

136 DNA was extracted from fecal samples using the QIAGEN PowerSoil DNA isolation kit
137 as per manufacturer instruction with the exception of an additional heat incubation of 10 minutes
138 at 65°C immediately before the bead beating step. The 16S rRNA gene was amplified from the
139 extracted DNA with PCR and primers designed to target the V4 region (Caporaso et al., 2012).
140 Amplicons were subsequently quantified using the Qubit HS kit and then pooled and cleaned
141 using the UltraClean PCR clean-up kit. These cleaned amplicons were then sequenced on an
142 Illumina MiSeq (v3 chemistry) instrument. This sequencing generated 3.31 million 150bp single
143 end reads (median reads per sample = 78,272).

144

145 ***Bioinformatic and Statistical Analyses***

146 The QIIME (v1.8.0) bioinformatics pipeline was used to quality control raw sequences as
147 well as quantify the diversity of microorganisms isolated from the fecal samples. The Illumina-
148 generated sequences were demultiplexed and quality filtered (i.e., sequences with a Phred quality
149 score less than 20 were removed) with the QIIME script `split_libraries_fastq.py`. The
150 `pick_open_reference_otus.py` script then assigned sequences to Operational Taxonomic Units
151 (OTUs) based on the alignment of sequences to the Greengenes (v13_8) reference database using

152 a 97% similarity threshold with the UCLUST algorithm (v1.2.22). With the
153 `core_diversity_analysis.py` script, samples were subject to rarefaction through random sub-
154 sampling of sequences at a depth of 40,000 reads, which corresponded to the lowest sequencing
155 depth obtained across samples. The BIOM table generated from the `core_diversity_analysis.py`
156 script was imported into R and potentially spurious OTUs were filtered by removing those that
157 (1) were found in fewer than three samples and (2) were observed fewer than 20 times across all
158 samples from all subsequent analyses. The resulting OTU matrix was subsequently processed
159 using the `beta_diversity.py` script to calculate the weighted and unweighted UniFrac distances
160 between all pairs of samples (Lozupone & Knight, 2005). Alpha diversity was calculated in R
161 (v3.2.3) using the `diversity` function in the `vegan` package (v2.3-3).

162 Intersample similarity was visualized using principal coordinates analysis (PCoA) based
163 on the Bray-Curtis dissimilarity index using the `vegan` (v2.3-3) package in R (v3.2.3). The
164 association between sample covariates, including dog aggression, and intersample similarity was
165 quantified with the `envfit` function in the `vegan` package. Kruskal-Wallis tests, as implemented
166 by the `coin` package (version 1.1-2), were used to identify OTUs and phylotypes that stratify
167 samples by covariate factors. Phylogenetic clades that associate with aggression were identified
168 by assembling a reference-guided 16S sequence phylogeny via `FastTree` as previously described
169 (O'Dwyer, Kembel & Green, 2012), using `Claatu` to resolve monophyletic clades that are
170 conserved in aggressive or non-aggressive dogs ($fdr < 0.05$) (Gaulke et al., 2017), and Kruskal-
171 Wallis tests to ascertain if these conserved clades are differentially abundant across these
172 populations. The taxonomy of these clades was determined by identifying the most resolved
173 taxonomy label that is shared among all members of the clade. Multiple tests were corrected

174 using the qvalue package (version 2.2.2). Phylotypes or clades with a p-value less than 0.05 and
175 a q-value less than 0.2 were designated as those that stratify samples.

176

177 **Results**

178 To determine possible differences in gut microbial composition between aggressive and
179 non-aggressive dogs, we compared stool microbiomes that were sampled from 21 aggressive
180 dogs and 10 non-aggressive dogs. A Principal Coordinates Analysis (PCoA) using the weighted
181 UniFrac metric shows separation of the aggressive and non-aggressive samples based on 95%
182 confidence interval ellipses (Fig. 1). The separation between aggressive and non-aggressive
183 samples in the PCoA plot was confirmed by environmental fit ($p = 0.0250$, $R^2 = 0.1297$) and
184 PERMANOVA ($p = 0.0346$, $R^2 = 0.0349$) analyses. Alternative measures of beta-diversity
185 marginally support these results. For example, using a Bray-Curtis dissimilarity metric finds a
186 similar trend (PERMANOVA, $p = 0.0957$, $R^2 = 0.0573$). Unlike these differences in beta-
187 diversity, no significant differences were detected in alpha diversity based on the Shannon index
188 when comparing behavioral groups ($p = 0.5258$).

189 The bacterial phylotypes that were observed across the dog fecal samples were compared
190 between behavioral groups to resolve those phylotypes that vary in association with aggression
191 (Fig. 2). Firmicutes, Fusobacteria, Bacteroidetes, and Proteobacteria were the dominant phyla in
192 all fecal samples, which is consistent with dominant bacterial phyla discovered in previous
193 canine gut microbiome studies (Deng & Swanson, 2015). While Proteobacteria, Fusobacteria,
194 and Firmicutes predominated all samples, the relative abundances of these phyla significantly
195 differed across aggressive and non-aggressive dogs ($p < 0.05$, $q < 0.1$). Specifically,
196 Proteobacteria and Fusobacteria manifested higher relative abundance in non-aggressive dogs,

197 while Firmicutes was relatively more abundant in aggressive dogs. These trends were driven by
198 variation in a small number of more granular phylotypes (Fig. 3). The family Lactobacillaceae
199 was more abundant in aggressive dogs, while the family Fusobacteriaceae was more abundant in
200 non-aggressive dogs ($p < 0.05$, $q < 0.2$). Consistently, the genus *Lactobacillus* was more
201 abundant in aggressive dogs, while the genus *Fusobacteria* was more abundant in non-
202 aggressive dogs ($p < 0.05$, $q < 0.2$). Additional separation between aggressive and non-
203 aggressive dogs is observed at the OTU level. Specifically, seven OTUs significantly differed
204 between aggressive and non-aggressive dogs ($p < 0.05$, $q < 0.1$), including four OTUs from the
205 genus *Dorea*, two OTUs from the genus *Lactobacillus*, and one OTU from *Turicibacter*. All of
206 the phylotypes and OTUs that significantly associated with aggression are included in Table S2
207 (phylotypes) and Table S3 (OTUs).

208 To better resolve taxa that stratify aggressive and nonaggressive dogs, we used a
209 phylogenetic approach that defines taxa as monophyletic clades of bacteria that are prevalently
210 observed across members of the aggressive or nonaggressive populations. These clades represent
211 evolutionary groupings of bacteria that often correspond to intermediate levels of taxonomy
212 (e.g., between species and genus) that are defined by the shared ancestry and ecology of clade
213 members. Moreover, by focusing on prevalent clades, which are those that are observed in more
214 individuals within a population than expected by chance (Gaulke et al., 2017), we are able to
215 resolve bacterial taxa that are especially common to at least one population. This property of a
216 high prevalence of behavior-stratifying gut microbes may be a desirable characteristic when
217 searching for potentially diagnostic indicators of aggression status.

218 Of the 578 clades that are prevalent in either aggressive or non-aggressive dogs, 96
219 significantly differ in abundance between the two populations ($q < 0.2$). Of these clades, 39 have

220 a mean relative abundance that is significantly higher in the gut microbiomes of aggressive dogs,
221 while 57 have a higher relative abundance in non-aggressive dog microbiomes. A complete list
222 of clades that associate with behavior can be found in Table S4. Of particular note is our finding
223 that nine clades with the genus *Bacteroides* are elevated in the gut microbiomes of non-
224 aggressive dogs compared to aggressive dogs. This finding indicates that the relative abundance
225 of these lineages within *Bacteroides* may predict aggression status and that their depletion may
226 contribute to aggression. We also find that the genus *Lactobacillus* contains 25 clades that are
227 relatively abundant in aggressive canines. Similar patterns are observed for clades within the
228 family Paraprevotellaceae. These observations indicate that aggression may be associated with
229 an increase in specific lineages within *Lactobacillus* and Paraprevotellaceae and they may
230 express traits that interact with aggression-associated aspects of canine physiology. Moreover,
231 we find that the genus *Turicibacter* contains both aggression-elevated and aggression-depleted
232 clades (Fig. S1), indicating that descendants of this genus may have recently evolved traits that
233 contribute to their differential association with canine behavior. This observation underscores
234 prior work that indicates that the functional associations between gut bacteria and mammals may
235 rapidly evolve among bacteria (Conley et al., 2016a).

236

237 **Discussion**

238 Accumulating evidence indicates that the gut microbiome acts as an agent of the nervous
239 system and influences affective disorders such as anxiety and depression (Clapp et al., 2017).
240 However, it is unknown if the gut microbiome similarly relates to animal aggression. Our
241 exploratory analysis of a population of rescued, sheltered-housed dogs links the composition of
242 the gut microbiome to conspecific aggression in canines. While this associative study cannot
243 disentangle cause and effect, it holds important implications for clinical practices surrounding

244 canines, as its results indicate that (a) the gut microbiome may contribute to aggression or its
245 severity, and that manipulation of the microbiome may alleviate the behavior; (b) the physiology
246 of aggressive dogs results in different gut microbiome compositions, indicating that the
247 microbiome may facilitate predictive diagnosis of aggressive behavior and preventative
248 intervention; or (c) aggression and the gut microbiome are similarly associated with a cryptic
249 physiological or environmental covariate, such as inflammation or cortisol levels, which may
250 help discern the physiological underpinnings of canine aggression. Future studies should build
251 upon this exploratory investigation to discern the mechanisms underlying the relationship
252 between canine aggression and the gut microbiome.

253 Our investigation finds that the composition of the gut microbiome differs between
254 aggressive and non-aggressive dogs in the population that we studied. The rescued, shelter-
255 housed dogs included in this investigation proved useful for this study because they included
256 aggressive and non-aggressive individuals and were taken into the shelter at the same time,
257 maintained in the same facility, exposed to the same diet, and generally of consistent breed type.
258 Despite our attempt to homogenize the sources of variation amongst these dogs, we observed
259 extensive variation in the composition of the gut microbiome within each behavioral cohort. This
260 intra-cohort variation indicates that the stool samples we studied are subject to cryptic factors
261 that associate with microbiome composition (e.g., age of host (Conley et al., 2016a)). This is
262 unsurprising given that individuals living outside of a laboratory setting (including pet and
263 shelter dogs, as well as humans) are subject to genetic and environmental diversity that cannot
264 fully be controlled for. That said, the identification of significant differences between these
265 populations under naturalistic conditions heightens the applied value of these findings. Future

266 efforts should consider larger populations and measure more diverse covariates per individual to
267 clarify the relationship between microbiome composition and the gut microbiome.

268 Several taxa significantly differ in their relative abundance between aggressive and
269 nonaggressive dogs. For example, we find that that lineages within the genus *Bacteroides* are
270 elevated in non-aggressive dogs, which might be expected given that species within this genus,
271 such as *Bacteroides fragilis*, have been shown to modulate mammalian behavior in prior
272 investigations (Hsiao et al., 2013). Moreover, the genus *Dorea* elevates in non-aggressive dogs
273 compared to aggressive dogs, which is notable because *Dorea* manifests a reduced abundance in
274 dogs afflicted with inflammatory bowel disease (Jergens et al., 2010) and other enteropathies
275 (Suchodolski, 2011), and because psychological disorders are frequently comorbid with
276 gastrointestinal inflammation (Bannaga & Selinger, 2015; Clapp et al., 2017). However, our
277 observations of which taxa stratify these cohorts are not always consistent with prior
278 investigations of microbial taxa that associate with mammalian behavior. As an example, we find
279 that members of *Lactobacillus* are more abundant in the gut microbiomes of aggressive dogs,
280 which might defy expectations given that prior research of specific strains of *Lactobacillus*
281 *rhamnosus* have been found to reduce stress-associated corticosterone levels and anxiety related
282 behavior in mice and is known to produce GABA neurotransmitters (Bravo et al., 2011).
283 Similarly, the genus *Fusobacterium* is typically thought to elicit pro-inflammatory effects inside
284 the gut (Bashir et al., 2016); here, we find that *Fusobacterium* is more abundant in the stool of
285 non-aggressive dogs. That said, it is challenging to determine the physiological role of specific
286 microbiota from 16S sequences given that an organism's interaction with its host may be context
287 dependent (Schubert, Sinani & Schloss, 2015) and may rapidly diversify (Conley et al., 2016b).
288 Indeed, our analysis of monophyletic clades of gut bacteria that associate with aggression finds

289 that closely related clades can manifest opposite patterns of association with behavior, such as
290 that of *Turicibacter*. Additionally, the limited population size may challenge the discovery of
291 taxa that statistically stratify cohorts. Despite this, these taxa represent compositional distinctions
292 between aggressive and non-aggressive dogs in our population. Further study of their physiology
293 role may help clarify whether or how they influence canine aggression.

294 **Conclusions**

295 Our results indicate that there are statistical associations between aggression status and
296 the gut microbiome. For example, microbial composition differs based on aggressive and non-
297 aggressive evaluations. Additionally, the relative abundances of specific bacterial taxa and
298 lineages are different across aggressive and non-aggressive groups. These observation are
299 important because they indicate that either (a) aggressive dogs manifest physiological conditions
300 in the gut that influence the composition of the gut microbiome, (b) the composition of the gut
301 microbiome may influence aggressive behavior, or (c) that aggressive dogs are subject to some
302 biased covariate relative to non-aggressive dogs that also influences the gut microbiome. Future
303 studies should seek to confirm that these findings are consistent in additional populations of
304 dogs, and seek to discriminate between these possibilities. Additionally, future studies should
305 focus on expanding the size of the populations being studied, labor to measure a diverse array of
306 physiological covariates to tease out aggression-specific effects and discern mechanisms of
307 interactions, and consider using metagenomic analyses to deduce the potential functional role of
308 the microbiome in these interactions.

309 Ultimately, our results indicate that the composition of the gut microbiome associates
310 with conspecific canine aggression in this group of dogs. These results pave the way for future
311 investigations to ascertain whether similar results are seen in other dog populations and if the

312 microbiome can be used to develop diagnostics, preventative strategies, and therapeutics of
313 aggression.

314

315 **Acknowledgements**

316 We thank Dr. Christopher A. Gaulke and Dr. Yuan Jiang for their helpful comments.

317

318 **References**

319 Bamberger M., Houpt KA. 2006. Signalment factors, comorbidity, and trends in behavior diagnoses in
320 dogs: 1,644 cases (1991–2001). *Journal of the American Veterinary Medical Association*
321 229:1591–1601. DOI: 10.2460/javma.229.10.1591.

322 Bannaga AS., Selinger CP. 2015. Inflammatory bowel disease and anxiety: links, risks, and challenges
323 faced. *Clinical and experimental gastroenterology* 8:111–7. DOI: 10.2147/CEG.S57982.

324 Bashir A., Miskeen AY., Hazari YM., Asrafuzzaman S., Fazili KM. 2016. Fusobacterium nucleatum,
325 inflammation, and immunity: the fire within human gut. *Tumor Biology* 37:2805–2810. DOI:
326 10.1007/s13277-015-4724-0.

327 Blackshaw JK. 1991. An overview of types of aggressive behaviour in dogs and methods of treatment.
328 *Applied Animal Behaviour Science* 30:351–361. DOI: 10.1016/0168-1591(91)90140-S.

329 Bravo JA., Forsythe P., Chew M V., Escaravage E., Savignac HM., Dinan TG., Bienenstock J., Cryan JF.
330 2011. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor
331 expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences of*
332 *the United States of America* 108:16050–5. DOI: 10.1073/pnas.1102999108.

333 Brooks A., Moxon R., England GCW. 2010. Incidence and impact of dog attacks on guide dogs in the
334 UK. *Veterinary Record* 166:778–781. DOI: 10.1136/vr.b4855.

335 Caporaso JG., Lauber CL., Walters WA., Berg-Lyons D., Huntley J., Fierer N., Owens SM., Betley J.,
336 Fraser L., Bauer M., Gormley N., Gilbert JA., Smith G., Knight R. 2012. Ultra-high-throughput

337 microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal*
338 6:1621–4. DOI: 10.1038/ismej.2012.8.

339 Carabotti M., Scirocco A., Maselli MA., Severi C. 2015. The gut-brain axis: interactions between enteric
340 microbiota, central and enteric nervous systems. *Annals of Gastroenterology : Quarterly*
341 *Publication of the Hellenic Society of Gastroenterology* 28:203–209.

342 Clapp M., Aurora N., Herrera L., Bhatia M., Wilen E., Wakefield S. 2017. Gut microbiota’s effect on
343 mental health: The gut-brain axis. *Clinics and practice* 7:987. DOI: 10.4081/cp.2017.987.

344 Conley MN., Wong CP., Duyck KM., Hord N., Ho E., Sharpton TJ. 2016a. Aging and serum MCP-1 are
345 associated with gut microbiome composition in a murine model. *PeerJ* 2016. DOI:
346 10.7717/peerj.1854.

347 Conley MN., Wong CP., Duyck KM., Hord N., Ho E., Sharpton TJ. 2016b. Aging and serum MCP-1 are
348 associated with gut microbiome composition in a murine model. *PeerJ* 4:e1854. DOI:
349 10.7717/peerj.1854.

350 Coppinger R., Coppinger L. 2001. *Dogs: A Startling New Understanding of Canine Origin, Behavior &*
351 *Evolution*. New York: Scribner.

352 Cryan JF., O’Mahony SM. 2011. The microbiome-gut-brain axis: from bowel to behavior.
353 *Neurogastroenterology and motility : the official journal of the European Gastrointestinal*
354 *Motility Society* 23:187–92. DOI: 10.1111/j.1365-2982.2010.01664.x.

355 David LA., Maurice CF., Carmody RN., Gootenberg DB., Button JE., Wolfe BE., Ling AV., Devlin AS.,
356 Varma Y., Fischbach MA., Biddinger SB., Dutton RJ., Turnbaugh PJ. 2013. Diet rapidly and
357 reproducibly alters the human gut microbiome. *Nature* 505:559. DOI: 10.1038/nature12820.

358 DeNapoli JS., Dodman NH., Shuster L., Rand WM., Gross KL. 2000. Effect of dietary protein content
359 and tryptophan supplementation on dominance aggression, territorial aggression, and
360 hyperactivity in dogs. *Journal of the American Veterinary Medical Association* 217:504–508.

361 Deng P., Swanson KS. 2015. Gut microbiota of humans, dogs and cats: current knowledge and future
362 opportunities and challenges. *The British Journal of Nutrition* 113 Suppl:S6-17. DOI:
363 10.1017/S0007114514002943.

364 Diaz Heijtz R., Wang S., Anuar F., Qian Y., Björkholm B., Samuelsson A., Hibberd ML., Forsberg H.,
365 Pettersson S. 2011. Normal gut microbiota modulates brain development and behavior.
366 *Proceedings of the National Academy of Sciences of the United States of America* 108:3047–
367 3052. DOI: 10.1073/pnas.1010529108.

368 Duffy DL., Hsu Y., Serpell JA. 2008. Breed differences in canine aggression. *Applied Animal Behaviour*
369 *Science* 114:441–460. DOI: 10.1016/j.applanim.2008.04.006.

370 Evrensel A., Ceylan ME. 2015. The Gut-Brain Axis: The Missing Link in Depression. *Clinical*
371 *Psychopharmacology and Neuroscience* 13:239–244. DOI: 10.9758/cpn.2015.13.3.239.

372 Foster JA., Lyte M., Meyer E., Cryan JF. 2015. Gut Microbiota and Brain Function: An Evolving Field in
373 Neuroscience. *The international journal of neuropsychopharmacology / official scientific journal*
374 *of the Collegium Internationale Neuropsychopharmacologicum (CINP)*. DOI:
375 10.1093/ijnp/pyv114.

376 Foster ZSL., Sharpton TJ., Grünwald NJ., Lefort M., Malumbres-Olarte J., Vink C. 2017. Metacoder: An
377 R package for visualization and manipulation of community taxonomic diversity data. *PLOS*
378 *Computational Biology* 13:e1005404. DOI: 10.1371/journal.pcbi.1005404.

379 Gaulke CA., Arnold HK., Kembel SW., O’Dwyer JP., Sharpton TJ. 2017. Ecophylogenetics Reveals the
380 Evolutionary Associations between Mammals and their Gut Microbiota. *doi.org*:182212. DOI:
381 10.1101/182212.

382 Hampson K., Dushoff J., Cleaveland S., Haydon DT., Kaare M., Packer C., Dobson A. 2009.
383 Transmission Dynamics and Prospects for the Elimination of Canine Rabies. *PLOS Biol*
384 7:e1000053. DOI: 10.1371/journal.pbio.1000053.

385 Heijtz RD., Wang S., Anuar F., Qian Y., Björkholm B., Samuelsson A., Hibberd ML., Forsberg H.,
386 Pettersson S. 2011. Normal gut microbiota modulates brain development and behavior.

387 *Proceedings of the National Academy of Sciences* 108:3047–3052. DOI:
388 10.1073/pnas.1010529108.

389 Houpt KA. 2006. Terminology Think Tank: Terminology of aggressive behavior. *Journal of Veterinary*
390 *Behavior: Clinical Applications and Research* 1:39–41. DOI: 10.1016/j.jveb.2006.04.006.

391 Hsiao EY., McBride SW., Hsien S., Sharon G., Hyde ER., McCue T., Codelli JA., Chow J., Reisman SE.,
392 Petrosino JF., Patterson PH., Mazmanian SK. 2013. Microbiota modulate behavioral and
393 physiological abnormalities associated with neurodevelopmental disorders. *Cell* 155:1451–63.
394 DOI: 10.1016/j.cell.2013.11.024.

395 Hsu Y., Sun L. 2010. Factors associated with aggressive responses in pet dogs. *Applied Animal Behaviour*
396 *Science* 123:108–123. DOI: 10.1016/j.applanim.2010.01.013.

397 Jergens AE., Nettleton D., Suchodolski JS., Wymore M., Wilke V., Dowd S., Steiner JM., Wang C.,
398 Wannemuehler MJ. 2010. Relationship of Mucosal Gene Expression to Microbiota Composition
399 in Dogs with Inflammatory Bowel Disease. *Journal of Veterinary Internal Medicine* 24:725–725.

400 Ji L., Xiaowei Z., Chuanlin W., Wei L. 2010. Investigation of posttraumatic stress disorder in children
401 after animal-induced injury in China. *Pediatrics* 126:e320-324. DOI: 10.1542/peds.2009-3530.

402 León M., Rosado B., García-Belenguer S., Chacón G., Villegas A., Palacio J. 2012. Assessment of
403 serotonin in serum, plasma, and platelets of aggressive dogs. *Journal of Veterinary Behavior:*
404 *Clinical Applications and Research* 7:348–352. DOI: 10.1016/j.jveb.2012.01.005.

405 Lockwood R. 2017. *Ethology, ecology and epidemiology of canine aggression (Chapter 9) - The*
406 *Domestic Dog*. Cambridge University Press.

407 Lozupone C., Knight R. 2005. UniFrac: a new phylogenetic method for comparing microbial
408 communities. *Applied and environmental microbiology* 71:8228–8235. DOI:
409 10.1128/AEM.71.12.8228.

410 Mayer EA., Knight R., Mazmanian SK., Cryan JF., Tillisch K. 2014. Gut Microbes and the Brain:
411 Paradigm Shift in Neuroscience. *The Journal of Neuroscience* 34:15490–15496. DOI:
412 10.1523/JNEUROSCI.3299-14.2014.

413 Mohan-Gibbons H., Weiss E., Slater M. 2012. Preliminary Investigation of Food Guarding Behavior in
414 Shelter Dogs in the United States. *Animals (2076-2615)* 2:331–346. DOI: 10.3390/ani2030331.

415 National Pet Owners Survey: Industry Statistics & Trends 2014.

416 Neufeld KM., Kang N., Bienenstock J., Foster JA. 2011. Reduced anxiety-like behavior and central
417 neurochemical change in germ-free mice. *Neurogastroenterology & Motility* 23:255-e119. DOI:
418 10.1111/j.1365-2982.2010.01620.x.

419 Nobis G. 1979. Der älteste Haushunde lebte vor 14000 Jahren. [The oldest domestic dog lived 14,000
420 years ago]. *Umschau* 79:610.

421 O’Dwyer JP., Kembel SW., Green JL. 2012. Phylogenetic diversity theory sheds light on the structure of
422 microbial communities. *PLoS computational biology* 8:e1002832. DOI:
423 10.1371/journal.pcbi.1002832.

424 O’Mahony SM., Clarke G., Borre YE., Dinan TG., Cryan JF. 2015. Serotonin, tryptophan metabolism
425 and the brain-gut-microbiome axis. *Behavioural Brain Research* 277:32–48. DOI:
426 10.1016/j.bbr.2014.07.027.

427 Overall KL., Love M. 2001. Dog bites to humans—demography, epidemiology, injury, and risk. *Journal*
428 *of the American Veterinary Medical Association* 218:1923–1934. DOI:
429 10.2460/javma.2001.218.1923.

430 Re S., Zanoletti M., Emanuele E. 2008. Aggressive dogs are characterized by low omega-3
431 polyunsaturated fatty acid status. *Veterinary Research Communications* 32:225–230. DOI:
432 10.1007/s11259-007-9021-y.

433 Roll A., Unshelm J. 1997. Aggressive conflicts amongst dogs and factors affecting them. *Applied Animal*
434 *Behaviour Science* 52:229–242. DOI: 10.1016/S0168-1591(96)01125-2.

435 Rooney NJ., Clark CCA., Casey RA. 2016. Minimizing fear and anxiety in working dogs: A review.
436 *Journal of Veterinary Behavior: Clinical Applications and Research* 16:53–64. DOI:
437 10.1016/j.jveb.2016.11.001.

438 Rosado B., García-Belenguer S., León M., Chacón G., Villegas A., Palacio J. 2010a. Blood
439 concentrations of serotonin, cortisol and dehydroepiandrosterone in aggressive dogs. *Applied*
440 *Animal Behaviour Science* 123:124–130. DOI: 10.1016/j.applanim.2010.01.009.

441 Rosado B., García-Belenguer S., León M., Chacón G., Villegas A., Palacio J. 2010b. Blood
442 concentrations of serotonin, cortisol and dehydroepiandrosterone in aggressive dogs. *Applied*
443 *Animal Behaviour Science* 123:124–130. DOI: 10.1016/j.applanim.2010.01.009.

444 Roth LSV., Faresjö Å., Theodorsson E., Jensen P. 2016. Hair cortisol varies with season and lifestyle and
445 relates to human interactions in German shepherd dogs. *Scientific Reports* 6:19631. DOI:
446 10.1038/srep19631.

447 Salman MD., Hutchison J., Ruch-Gallie R., Kogan L., Jr JCN., Kass PH., Scarlett JM. 2000. Behavioral
448 Reasons for Relinquishment of Dogs and Cats to 12 Shelters. *Journal of Applied Animal Welfare*
449 *Science* 3:93–106. DOI: 10.1207/S15327604JAWS0302_2.

450 Schubert AM., Sinani H., Schloss PD. 2015. Antibiotic-Induced Alterations of the Murine Gut Microbiota
451 and Subsequent Effects on Colonization Resistance against *Clostridium difficile*. *mBio* 6:e00974-
452 15-. DOI: 10.1128/mBio.00974-15.

453 Sherman CK., Reisner IR., Taliaferro LA., Houpt KA. 1996. Characteristics, treatment, and outcome of
454 99 cases of aggression between dogs. *Applied Animal Behaviour Science* 47:91–108. DOI:
455 10.1016/0168-1591(95)01013-0.

456 Suchodolski JS. 2011. Intestinal microbiota of dogs and cats: a bigger world than we thought. *The*
457 *Veterinary Clinics of North America. Small Animal Practice* 41:261–272. DOI:
458 10.1016/j.cvsm.2010.12.006.

459 Suchodolski JS., Markel ME., Garcia-Mazcorro JF., Unterer S., Heilmann RM., Dowd SE., Kachroo P.,
460 Ivanov I., Minamoto Y., Dillman EM., Steiner JM., Cook AK., Toresson L. 2012. The fecal
461 microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. *PloS one*
462 7:e51907. DOI: 10.1371/journal.pone.0051907.

463 Udell MAR., Wynne CDL. 2008. A Review of Domestic Dogs' (*Canis Familiaris*) Human-Like
464 Behaviors: Or Why Behavior Analysts Should Stop Worrying and Love Their Dogs. *Journal of*
465 *the Experimental Analysis of Behavior* 89:247–261. DOI: 10.1901/jeab.2008.89-247.

466 Yano JM., Yu K., Donaldson GP., Shastri GG., Ann P., Ma L., Nagler CR., Ismagilov RF., Mazmanian
467 SK., Hsiao EY. 2015. Indigenous bacteria from the gut microbiota regulate host serotonin
468 biosynthesis. *Cell* 161:264–276. DOI: 10.1016/j.cell.2015.02.047.

469

Figure 1(on next page)

Aggressive and non-aggressive dogs differ in beta-diversity using the weighted UniFrac metric.

Visualization of the phylogenetic differences in fecal microbiota of aggressive (green) and non-aggressive (purple) dogs using principal coordinates analysis (PCoA) of OTU abundances and weighted UniFrac distance. The separation between aggressive and non-aggressive samples in the PCoA plot was confirmed with an environmental fit analysis ($p = 0.0250$, $R^2 = 0.1297$), which supports aggression status as being the variable that is separating the microbial composition of the samples. The gut microbiome structure of aggressive and non-aggressive dogs is also significantly different with the weighted UniFrac metric using PERMANOVA ($p = 0.0346$, $R^2 = 0.0349$).

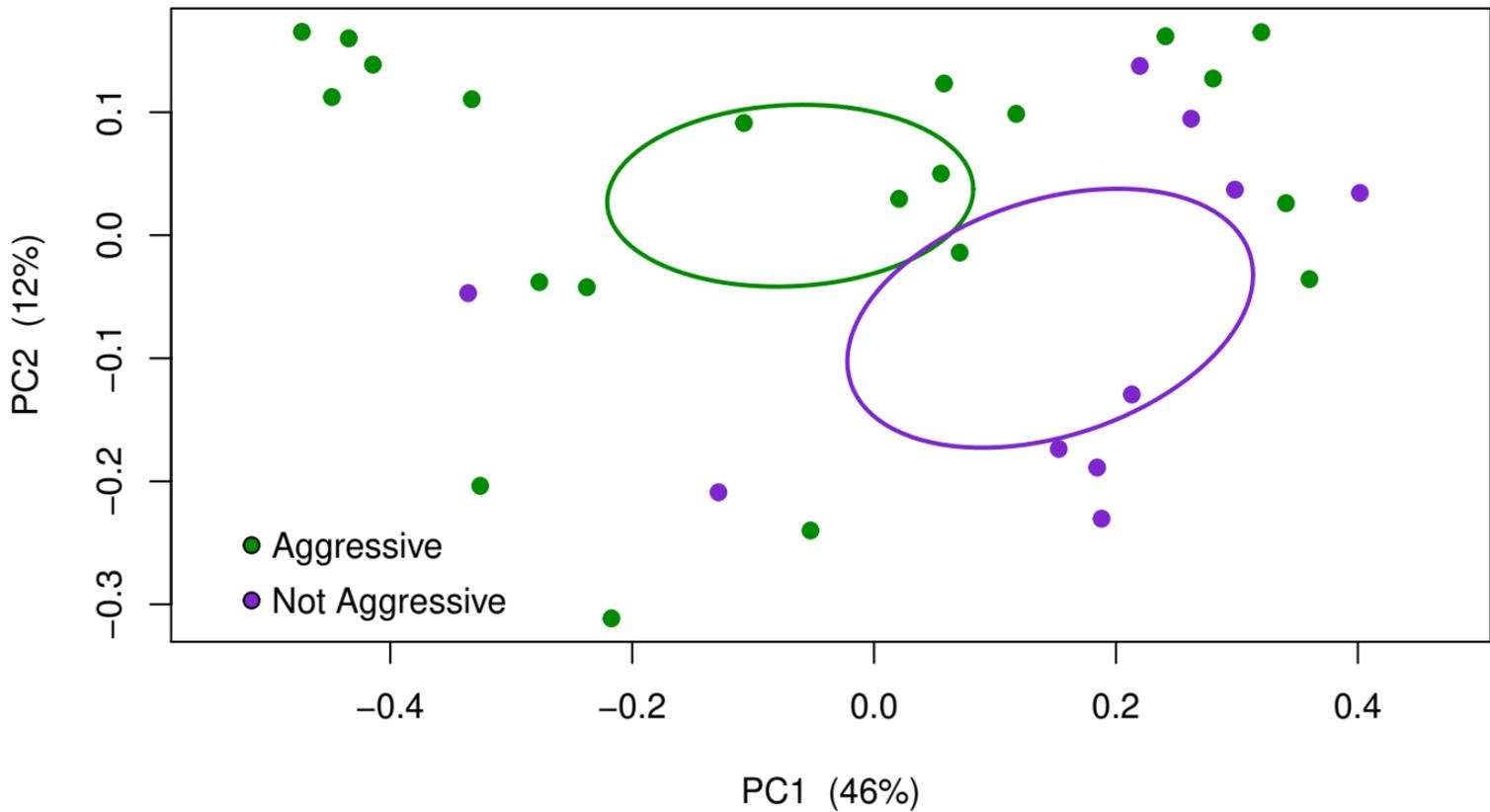


Figure 2(on next page)

Many of the most relatively abundant phylotypes in our dog fecal samples are significantly different across aggressive and non-aggressive dogs.

A metacoder (Foster et al., 2017) heattree illustrates the variation in microbiome phylotypes between the aggressive and non-aggressive dog populations. Nodes in the heattree correspond to phylotypes, as indicated by node labels, while edges link phylotypes in accordance to the taxonomic hierarchy. Node sizes correspond to the number of OTUs observed within a given phylotype. Colors represent the log fold difference of a given phylotype's median relative abundance in the aggressive dogs as compared to the non-aggressive dogs.

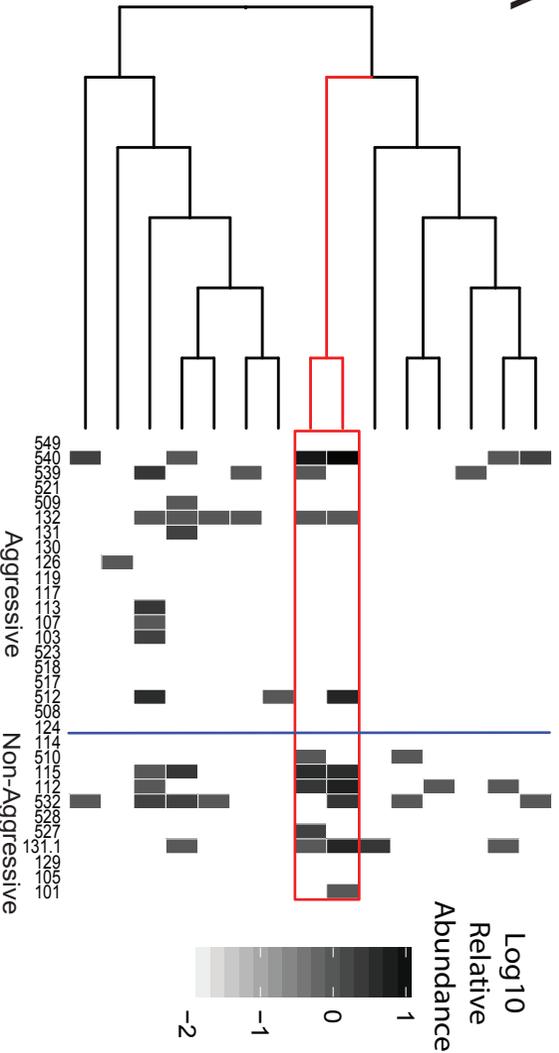
Figure 3(on next page)

The abundance of monophyletic clades within phylotypes stratify aggressive and non-aggressive dogs.

(A) Illustrating a subtree within the *Bacteroides* phylotype containing node 874 (red branches), which is a monophyletic clade that is both common to and relatively more abundant amongst the non-aggressive individuals than the aggressive individuals. The heat map adjacent to this subtree illustrates the log₁₀ relative abundance of each lineage in this subtree across the individuals subject to our investigation. The red rectangle highlights the relative abundance of the lineages within node 874. The vertical blue line separates aggressive and non-aggressive individuals. (B) illustrating a similar subtree, but in this case, it has been extracted from within the *Lactobacillus* phylotypes and highlights a monophyletic clade (node 3489) that is common to and relatively more abundant amongst the aggressive dogs.

A

Bacteroides (Node 874)



B

Lactobacillus (Node 3489)

