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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.

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Abstract

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.

Introduction

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

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

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

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

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

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

Materials and Methods

Sample Collection

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

Ethical Statement

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

Fecal DNA Extraction and 16S Sequencing

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

Bioinformatic and Statistical Analyses

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

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

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

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

Results

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

Conclusions

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

Ultimately, our results indicate that the composition of the gut microbiome associates with conspecific canine aggression in this group of dogs. These results pave the way for future 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
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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).

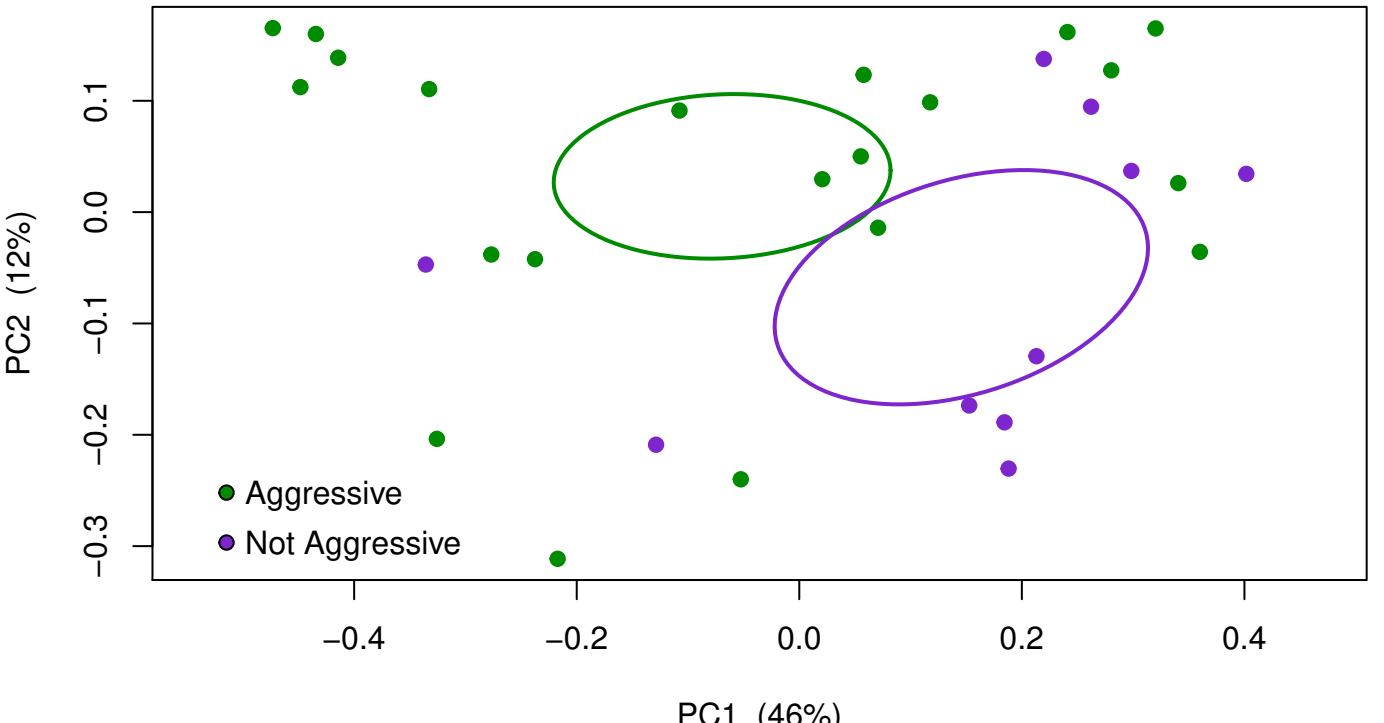

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Figure 2(on next page)

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.

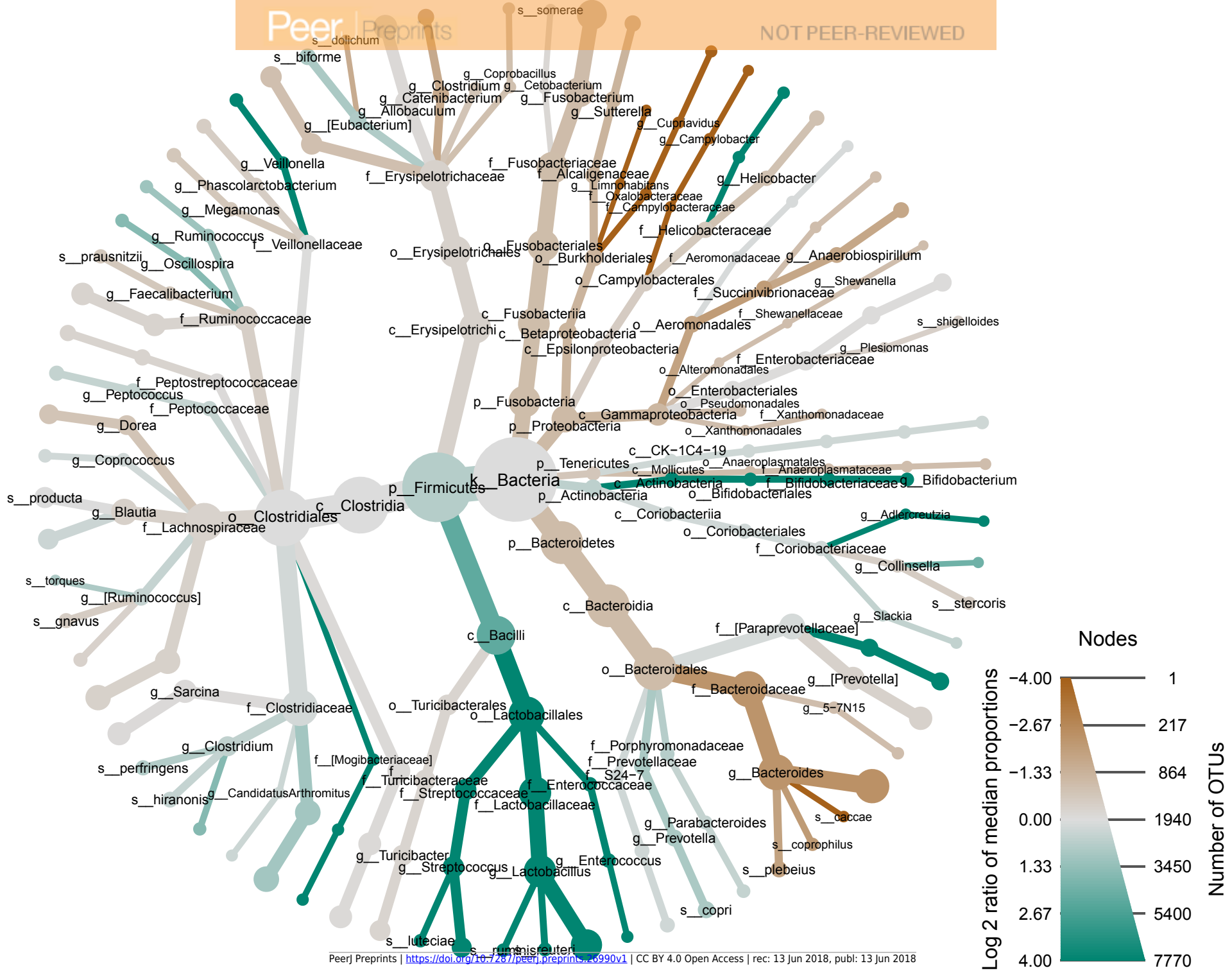

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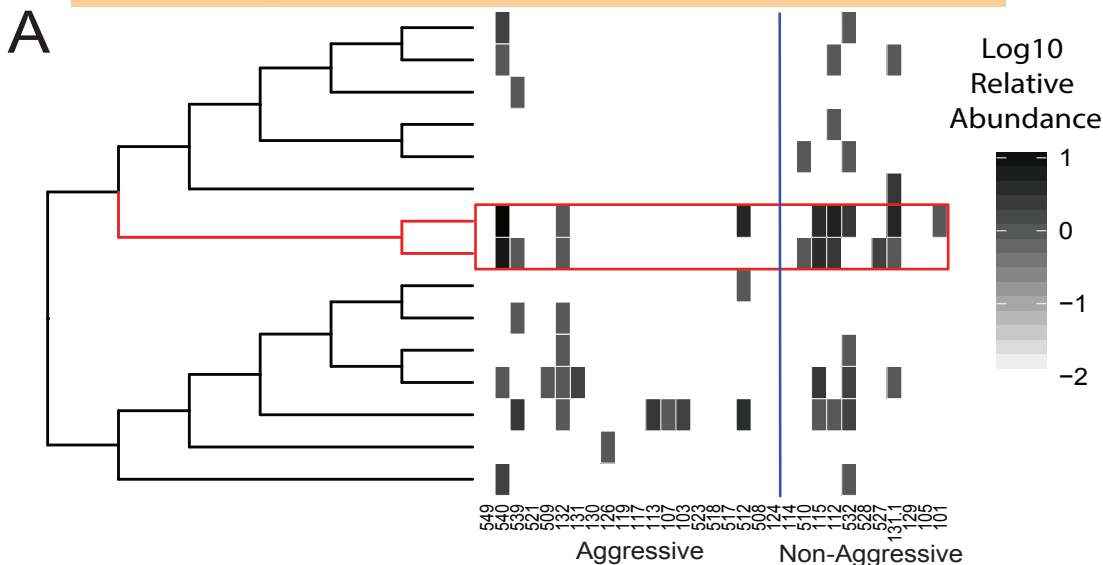
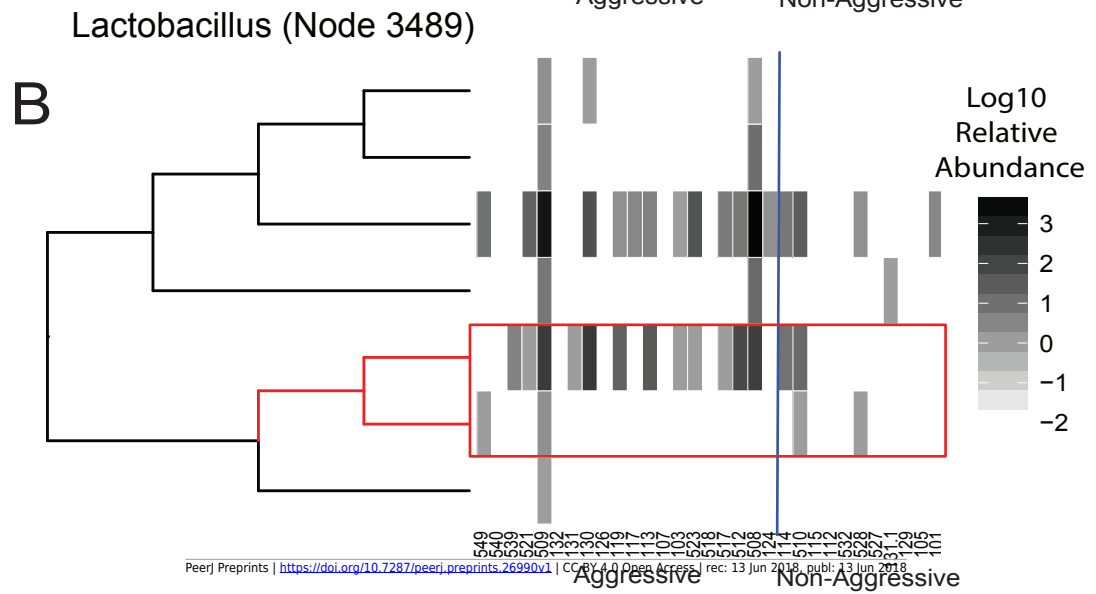
Figure 3(on next page)

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.





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