Gut microbiome composition is related to anxiety and aggression score in companion dogs

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Abstract

Background

There is mounting evidence for a link between behaviour and gut microbiome composition in several animal models and human health. However, the role of the gut microbiota in the development and severity of behavioural issues in companion dogs is not yet fully understood. In this work, we investigated the relationship between gut microbiome composition and aggression or anxiety in pet dogs. Pet dogs (n = 48) were assigned to higher or lower anxiety and aggression groups based on their owner's responses to the Canine Behavioral Assessment & Research Questionnaire (C-BARQ). Then the gut microbiome of each animal, sequenced from microbial DNA extracted from fecal samples, was assessed for association with the dog's assigned behavioural group using multiple approaches.

Results

While minimal differences in relative abundance were seen between behavioural groups, we were successful in predicting behavioural group based on gut microbiome composition using machine-learning based approaches and compositional balances. The generated models were particularly successful when distinguishing higher and lower anxiety dogs. The genus Blautia was identified across all our analyses, suggesting a strong link between this genus and anxiety in pet dogs.

Conclusions

This study builds on a growing area of research of great interest to dog owners, trainers, and behaviour professionals, and provides insight into specific bacteria that are linked to increased anxiety and aggression in pet dogs. Further research is required to identify bacteria to the species level, and to better understand the specific role of Blautia in the canine gut-brain axis.

Background

Dog behavioural issues, such as anxiety and aggression, are reported as the major reason for relinquishment of dogs to shelters [1]–[3], and are a considerable source of stress for dog owners or guardians that can result in the breakdown of the dog-human bond [4]. The role of the gut microbiota in behavioural conditions has become increasingly apparent in recent years, as there is mounting evidence that the composition of, and changes to, the gut microbiota is correlated with behaviour and mental health [5]–[7]. While most of the current literature focuses on mammalian models such as mice and humans [reviewed by [8]], recent studies have highlighted differences in the composition of the gut microbiome between domestic dogs of different behavioural tendencies (such as anxious or aggressive) [9]–[12]. The relationship between gut microbiota, behaviour and mental health in dogs provides a unique opportunity to further develop our knowledge of these relationships in a mammalian model that shares much of its environment with humans, while also having direct applications to dog health and welfare. The goal of this study was to expand the current knowledge on the relationships between the gut microbiome composition and owner-reported dog behaviour.

Recent studies have identified relationships between gut microbiota and behaviour in some populations of dogs. Kirchoff et al. [9] presented an interesting comparison between aggressive and non-aggressive dogs in pitbulls; the dogs were housed in a shelter environment after being seized from a potentially traumatic situation (fight operation) and were assessed based on conspecifc (dog-dog) aggression. There were differences in relative abundances of bacteria between aggressive and non-aggressive dogs, in particular increased amounts of Lactobacillus in aggressive dogs, and Firmicutes in non-aggressive dogs. The authors suggested that these correlations should be further investigated with a larger sample size and clearer controls for diet and life history. In a more recent study, Mondo et al. [10] examined a cohort of dogs from three shelters in Bologna, Italy, for their comparison of gut microbiota between aggressive, phobic and "normal" dogs. Similar to Kirchoff et al. [9], they found changes in relative abundances associated with aggression, characterized by increased abundance and diversity of typically sub-dominant genera (Catenibacterium and Megamonas), and increased abundance of Lactobacillus in anxious dogs. However, the use of dogs housed in a shelter and/or rescued from poor living conditions may introduce the confounding effects of acute stress on the dogs' behaviours (and potentially gut microbiota), which could impact the apparent relationship between behaviour and the gut microbiome. Our study aimed to build on the sparse literature by profiling gut microbiota in domestic dogs living as family pets (as per [12]) in a relatively secure and stable environment.

There are alternative approaches to determining a dog's behavioural profile. Mondo et al. [10] used a behavioural assessment performed by a veterinary behaviourist to identify each dog as either normal, phobic or aggressive while the dogs lived in the shelter. While no information is provided on the length of time the dogs had been in the shelter, such an assessment reports on observable behaviour in a potentially stressful situation. Alternative assessments are available, such as the Canine Behavioral Assessment Research Questionnaire (C-BARQ) [13]. This assessment tool is frequently used in behavioural studies to develop a reliable profile for a dog based on owner-reported behaviours. Owners are asked to rate their dog's reactions to an extensive range of scenarios and stimuli; based on the responses, C-BARQ produces a profile of continuous scores between 0–4 (0, "of little to no concern", 4, "of serious concern") across thirteen major behavioral traits or factors that describe much of the variation in canine temperament. These factors include aggression towards humans and/or dogs (both familiar and unfamiliar), and fearfulness in both social and non-social contexts. The C-BARQ has been validated in multiple studies (and languages) since its inception [14]–[18]. In this study, we opted to assess dog behaviour using the C-BARQ primarily for its robust profiling and ease of recruitment for larger sample sizes, and the additional benefit of owners being able to complete the questionnaire online during fluctuations in local health restrictions due to COVID-19. By assessing a broad scope of behaviour, we were able to explore relationships between gut microbiome composition and specific behaviours such as stranger-directed aggression, dog-directed aggression, and non-social fear.

Methods
Recruitment, Behavioural Assessment & Participant Selection

Dog guardians from the St John's Metro area in Newfoundland, Canada were recruited via word of mouth, online postings via email and social media, and postings in local vet clinics and pet care businesses. Participants were asked to complete two questionnaires: first, they completed a Diet, Lifestyle & Medical questionnaire online via Qualtrics (n = 494; www.qualtrics.com), and upon completion of this questionnaire, they were directed to complete the online C-BARQ (Canine Behaviour and Research Questionnaire; [13]) (n = 235) hosted by the University of Pennsylvania (https://vetapps.vet.upenn.edu/cbarq/). The online questionnaires were open to public participation from May 6th, 2021 to July 5th, 2021. The initial questionnaire (Additional File) acquired important information related to diet, lifestyle, and medical history that was not obtained via the C-BARQ and which could potentially impact either behaviour, gut microbiota, or both.

Dogs were assigned a composite score for aggression based on the mean of their C-BARQ scores for Stranger-Directed Aggression (SDA), Owner-Directed Aggression (ODA), and Dog-Directed Aggression (DDA). Familiar Dog Aggression (FDA), a score reporting the severity of aggression towards other family dogs living within the same home, was not utilized in calculating a composite aggression score, as a surprisingly large proportion of dogs living alone acquired a score for FDA, the reasoning for which is currently under further investigation. Dogs were assigned a composite anxiety score based on the mean of their results for Dog-Directed Fear (DDF), Stranger-Directed Fear (SDF), Nonsocial Fear (NSF) and Separation-Related Problems (SRP).

To select dogs for fecal sampling from the sample with C-BARQ scores (n = 235), we assessed the dogs based on criteria from the Diet & Lifestyle questionnaire. Dogs were required to be: (i) located within the St John's Metro Area for ease of sample collection, (ii) between 2–7 years old, (iii) eating a consistent diet/formula for more than 3 months, and (iv) living in a consistent environment (at the same address, with the current number of conspecifics) for more than 6 months. This matching process was designed to limit the effects of variability in diet and lifestyle factors within the population known to impact the gut and/or behaviour, and increase the likelihood that statistically significant effects of behavioural profiles would be detected from a relatively small sample size.

The population of dogs produced from this initial selection process (n = 72; Table 1) were then split by the median of their composite anxiety and aggression scores to create higher and lower anxiety and aggression groups, with those at the median assigned to the higher anxiety or aggression groups. Once their behavioural groups were assigned, dogs were then further matched as closely as possible on additional factors that have been reported to influence behaviour or microbiota in dogs or other mammalian taxa. These factors included their age, diet type (kibble, mixed or raw), breed group [19], body condition [from 1 (severely underweight) to 9 (severely overweight)], supplementation with probiotics, and use of deworming medications. Finally, 50 dogs that differed in behavioural scores (above/equal to, or below the median) were matched in pairs to each other, with a priority given to pairs who occupied opposite behavioural groups (i.e., high anxiety and high aggression dogs were matched to low anxiety and low aggression dogs) while maintaining similar or identical classifications within the diet and lifestyle criteria. We successfully assigned 20 pairs of dogs as a high anxiety/high aggression to low anxiety/low aggression match, with the remaining 5 pairs consisting of dogs with low anxiety/high aggression scores matched to dogs with high anxiety/low aggression scores.

Fecal Sample Collection

Following our matching process, we invited these 50 dog owners to provide a fecal sample from their dog. Participants were provided with a fecal swab collection and preservation device (Norgen Biotek Corp., Canada) with instructions for sample collection: the first bowel movement of the day was sampled immediately after evacuation by inserting the swab into the center of the feces while avoiding debris or potential contamination. The swab was sealed inside the collection device, and the device was collected that day by researchers via contactless pickup. Participants also repeated a shortened version of the Diet and Lifestyle questionnaire on collection day to give immediate information on the dog’s overall health and diet at the time the sample was provided, all of which indicated there had been no changes to any of the diet and lifestyle factors being considered in this study. Of the 50 sample kits provided, 48 were successfully returned with adequate quality of sample to run DNA extraction. Once brought to the lab, the samples were stored at -18°C until processed.

DNA Extraction, Library Preparation & Sequencing

DNA was extracted from the collected fecal samples using the Microbiome DNA Isolation kit (Norgen Biotek Corp., Canada) as per the manufacturer’s instructions. Extracted DNA was checked for quality and concentration using an Implen P300 nanophotometer (Implen, Inc., USA) before being sent to the Integrated Microbiome Resource (IMR) (https://imr.bio/index.html) at Dalhousie University (Halifax, NS, Canada) for amplification and sequencing. Briefly, PCR was performed using the primers 515FB (GTGYCAGCMGCCGCGGTAA) and 926R (CCGYCAATTYMTTTRAGTTT) [20], [21] to amplify the V4-V5 sub-region of the bacterial 16S rRNA gene. Library amplicons were then sequenced using a 2 × 300 bp paired-end run on an Illumina MiSeq machine.

Bioinformatics Analysis

The quality of the Illumina raw reads was assessed using the FastQC software (version 0.11.9; [22]). Reads were trimmed using trimmomatic (version 0.39; [23]) with the parameters: PE, -phred33, and sliding window 4:20. Trimmed paired reads were then inputted to the Bioconductor package DADA2 (version 1.22; [24]) in R (version 4.1.2) to obtain a table of DNA sequences (sub-OTUs; operational taxonomic units) and counts of these different sequences per sample. Trimming and filtering within DADA2 was done using the parameters trunclen 250/190, maxN 0 and trucQ 2. The truncation length was set empirically to maximize the percentage of reads kept and the number of unique sequences identified. With 250/190 truncation length, the minimum percentage of reads kept per sample was 54.8% (average 63.5%) and roughly 6.5k sOTUs were identified. All other steps in the DADA2 pipeline, namely, dereplication, sample inference, merging of paired reads and chimera removal were performed using default parameters. Taxonomic assignment was done using the Silva database (version 138.1; [25]). We used DADA2 as this method is recommended in best practices for microbiome analysis [26].
Abundance and diversity of taxonomic groups present in each fecal sample were investigated using the Bioconductor packager MicrobiotaProcess (version 1.6.6; [27]) with alpha metrics ACE and Chao1 analyzed for both anxiety and aggression groups. Relative abundance of bacteria at the family level for individual dogs and behaviour groups were produced, and the major bacteria differing in relative abundance between behaviour groups were statistically represented by a linear discriminant analysis (Log$_{10}$(LDA)). As recommended in best practices [26], we used ‘balance’ approaches for microbiome compositional data to identify changes in log ratios between abundances in the microbial communities that differ between behaviour groups. The two balance approaches we used were: PhILR (phylogenetic isometric log-ratio transform) (version 1.20.1; [28]), which produced the top 5 nodes on the phylogenetic tree (balances) to distinguish between behavioural groups using a sparse logistic regression model, and Selbal [29], which used a forward-selection method to identify combinations of taxa whose balance was associated with behavioural group. Selbal analysis was run on both behavioural group classification (lower/higher aggression or anxiety) and as a regression based on the continuous C-BARQ scores. Finally, we separately used the PhILR transformed data and raw taxonomic abundances as input to train a Random Forest [30] to generate a behaviour group classifier. Four different Random Forest classifiers were assessed based on the probability of accurately predicting behavioural group using 10-fold cross-validation. In 10-fold cross-validation the data is divided into 10 partitions and iteratively a classifier is generated using nine partitions and tested in the one left out of the training process. The hyper-parameters (number of trees and number of features considered) for the Random Forest were selected to maximize the area under the precision recall curve (AUPRC) which approximates the average precision across recall levels. Finally, the most important features (raw abundances or balances) were identified by quantifying the mean decrease in accuracy resulting from randomly permuting each feature.

**Statistical Analysis**

Differences in the relative abundance of individual OTUs between higher and lower aggression or anxiety groups were tested for significance with Mann-Whitney U.

**Results**

**Cohort Metadata & Behavioural Scores**

A total of 494 dog owners completed the Diet & Lifestyle Questionnaire via Qualtrics, with 235 participants continuing to complete the C-BARQ. After filtering respondents based on age, location, and consistency of diet and living arrangements, 72 dogs remained for the matching process (described above). Before matching, the behavioural scores for these 72 dogs were evaluated; the summary is displayed in Table 1. The mean composite anxiety score was 0.955, with a median of 0.782. Dogs with a composite anxiety score less than 0.782 were assigned to the lower anxiety group, with those with an anxiety score equal to or greater than 0.782 were assigned to the higher anxiety group. Similarly, the dogs with a composite aggression score less than the 0.455 median were assigned to the lower aggression group, with those scoring equal to or greater than 0.455 placed in the higher aggression group. The median values were overall low scores with respect to the maximum possible score for the most extreme aggression and anxiety cases (C-BARQ is scored from ‘no concern’ values of 0, to ‘most concern’ values of 4), indicating a clustering of dogs scoring close to 0 for both behavioural scales.

While fewer dogs had more concerning scores of 3–4 on C-BARQ, values for the n = 72 group ranged from 0–3.25 for individual anxiety scores on scales for stranger-directed fear, dog-directed fear, and separation-related fear, 0–4 for dog-directed aggression, and 0–2.9 for stranger-directed aggression.

<table>
<thead>
<tr>
<th>Anxiety Score (C-BARQ)</th>
<th>N</th>
<th>72</th>
<th>72</th>
<th>72</th>
<th>72</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± S.E.</td>
<td></td>
<td>0.955 ± 0.07</td>
<td>0.82 ± 0.121</td>
<td>1.155 ± 0.119</td>
<td>1.05 ± 0.107</td>
<td>0.82 ± 0.101</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td>0.782</td>
<td>0.5</td>
<td>1</td>
<td>0.83</td>
<td>0.5</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>0.0425–2.625</td>
<td>0–3.25</td>
<td>0–3.25</td>
<td>0–3.17</td>
<td>0–3.25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aggression Score (C-BARQ)</th>
<th>N</th>
<th>72</th>
<th>72</th>
<th>70</th>
<th>72</th>
<th>55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± S.E.</td>
<td></td>
<td>0.565 ± 0.05</td>
<td>0.618 ± 0.08</td>
<td>1.061 ± 0.117</td>
<td>0.158 ± 0.03</td>
<td>0.406 ± 0.08</td>
</tr>
<tr>
<td>Median</td>
<td></td>
<td>0.455</td>
<td>0.4</td>
<td>0.875</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Range</td>
<td></td>
<td>0–1.8825</td>
<td>0–2.9</td>
<td>0–4</td>
<td>0–1.38</td>
<td>0–1.75</td>
</tr>
</tbody>
</table>
The mean age of the 48 dogs included in the microbiome analysis was 3.95 years (± 0.23 S.E.), and included 30 males and 18 females (Table 2). Of these dogs, the majority were spayed or neutered (n = 45) with 3 dogs remaining intact. Half of the cohort (n = 24) were regularly using a dewormer, while only 5 dogs were regularly supplemented with a commercial probiotic. Abundance analysis comparisons between dogs using probiotics and those not using probiotics were not found to be significant, so we left these in the dataset for further analyses.

Sequence Data Quality
A total of 4,405,983 reads were obtained from Illumina sequencing (91,791 ± 4016 reads per sample ± SE). After filtering, denoising, merging and removal of chimeras using DADA2, a total of 1,737,507 reads remained for the analysis (36,198 ± 1448 reads per sample) (Table S1). These sequences were clustered into 5508 taxa by seven taxonomic ranks. The most reads per genus identified across the cohort were Bacteroides, Fusobacterium, Prevotella,9, Megamonas and Alloprevotella (Fig. 1). Some genera such as Bacteroides and Fusobacterium have relatively low variance among the 48 samples, while others such as Prevotella,9 and Alloprevotella have a wider range across the samples (Fig. 1).

Figure 1. The top 20 most abundant genera, as per total number of reads, identified across the entire cohort of dogs (n = 48). The horizontal line within each box indicates the median and the diamond indicates the mean of the log2 of the number of reads.

Descriptive Statistics for Relative Abundance and Diversity Across Taxonomic Levels
The most abundant phyla detected across the entire cohort were Bacteroidota (relative abundance ± SE, 53.6 ± 2.3%), Firmicutes (23.9 ± 1.7%), Fusobacteriota (18.5 ± 1.8%) and Proteobacteria (3.7 ± 0.3%), with all other subdominant phyla having a relative abundance below 1%. At the class level, Bacteroidia were most abundant across the entire cohort (53.6 ± 2.3%), followed by Clostridia (31.8 ± 1.6%), Bacteroidiales (21.8 ± 1.2%), Gammaproteobacteria (3.7 ± 0.3%) and Bacillales (21.8 ± 1.2%). Of these Microorganisms, the relative abundances of phylum Firmicutes, class Clostridia, order Erysipelotrichales and order Oscillospirales were highlighted as important differences across behaviour groups (Fig. 1). When comparing higher and lower anxiety, the class Bacillales had a greater relative abundance in higher anxiety dogs (2.9 ± 0.4%) than in lower anxiety dogs (2.4 ± 0.4%, p = 0.043), as did the order Erysipelotrichales (2.7 ± 0.3% in higher anxiety dogs, 2.2 ± 0.6% in lower anxiety dogs, p = 0.039). The most notable differences in relative abundance between aggression groups were the order Oscillospirales (4.8 ± 0.5% in higher aggression dogs, 3.0 ± 0.5% in lower aggression dogs; p = 0.05) and the family Ruminococcaceae (4.5 ± 0.5% in higher aggression dogs, 2.7 ± 0.5% in lower aggression dogs, p = 0.055).

The linear discriminant analysis (LDA) highlighted a difference in relative abundance of the genus Faecalibacterium as an important distinction between both higher and lower anxiety and aggression groups, with the genus Blautia also differing between anxiety groups (Table 3). In addition to Faecalibacterium and Blautia, the relative abundances of phylum Firmicutes, class Clostridia, order Erysipelotrichales and order Oscillospirales were highlighted as important distinctions between anxiety groups, while the order Oscillospirales and family Ruminococcaceae showed differing abundances between higher and lower aggression groups (Figure S1). While these were all considered interesting findings for the abundance LDA, only those indicated as different across two or more analyses are displayed in Table 3.

Microbiome balances associated with behaviour groups
Microbiome data are compositional due to the total number of reads being constrained by the sequencing technology. This constraint introduces strong dependencies among the abundances of different microbes: when the proportion of one microorganism increases, the proportion of others must decrease in the data for the total number of reads to remain within the limit. Note, however, that those microbes whose abundance decrease might not be related to the trait or treatment of interest. Thus, considering the abundances independently can lead to the discovery of false associations. Balance approaches are aware of the compositionality of microbiome data and test for differences in the log ratios between microbial abundances (called balances). Balance approaches

<table>
<thead>
<tr>
<th>Anxiety Group</th>
<th>Aggression Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Dogs (n = 48)</td>
<td>Lower (n = 25)</td>
</tr>
<tr>
<td>Age (years) ± S.E.M.</td>
<td>3.95 ± 0.23</td>
</tr>
<tr>
<td>Male</td>
<td>30</td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
</tr>
<tr>
<td>Lower (n = 23)</td>
<td>3.70 ± 0.33</td>
</tr>
<tr>
<td>Male</td>
<td>15</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
</tr>
<tr>
<td>Higher (n = 23)</td>
<td>4.22 ± 0.30</td>
</tr>
<tr>
<td>Male</td>
<td>15</td>
</tr>
<tr>
<td>Female</td>
<td>8</td>
</tr>
<tr>
<td>Higher (n = 25)</td>
<td>3.41 ± 0.33a</td>
</tr>
<tr>
<td>Male</td>
<td>14</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
</tr>
<tr>
<td>Lower (n = 25)</td>
<td>4.44 ± 0.28a</td>
</tr>
<tr>
<td>Male</td>
<td>16</td>
</tr>
<tr>
<td>Female</td>
<td>9</td>
</tr>
</tbody>
</table>
vary in how balances are calculated and how testing for differences in the balances is performed. We used two balance approaches: PhILR [28], which applies evolutionary models to guide the calculation of the log ratios, and Selbal [29]. Selbal searches for the two OTUs whose balance is most associated with the trait of interest, then adds other OTUs to this best balance to see if the new balance is better associated to the trait of interest in terms of the area under the receiver operating characteristic curve (AUC-ROC) for classification or the mean squared error (MSE) for regression.

We then compared Random Forest models generated with either the log ratios calculated by PhILR or the raw abundances to assess the benefits of using balances for classification and identify the most informative features. To further reduce the likelihood of discovering false associations, we only consider as likely true associations those taxa identified as associated with the behaviour group in two or more analyses (abundance LDA, PhILR, Selbal-classification, Selbal-regression and the two best Random Forest models) as displayed in Table 3. The genus *Blautia* was identified by all but one of the analyses, indicating a strong support for an association between this genus and anxiety level in dogs. The family Oscillospiraceae was associated with anxiety score in both Selbal analyses, and the phylum Firmicutes and family Peptostreptococcaceae were also identified in the PhILR and Random Forest analysis.

Table 3 Summary of bacteria identified across two or more analyses. Analyses were linear discriminant analysis (LDA), phylogenetic isometric log ratio transform (PhILR), Selbal classification (Class), Selbal regression (Regr) & Random Forest (Abundance + Feature Selection (Ab+FS); Isometric Log Ratio Transform + Feature Selection (ILR+FS)).

<table>
<thead>
<tr>
<th>Taxonomic Level</th>
<th>AGGRESSION</th>
<th>ANXIETY</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDA</td>
<td>PhILR</td>
</tr>
<tr>
<td>Phylum</td>
<td>Firmicutes</td>
<td>*</td>
</tr>
<tr>
<td>Order</td>
<td>Burkholderiales</td>
<td>*</td>
</tr>
<tr>
<td>Family</td>
<td>Oscillospiraceae</td>
<td>*</td>
</tr>
<tr>
<td>Family</td>
<td>Peptostreptococcaceae</td>
<td>*</td>
</tr>
<tr>
<td>Genus</td>
<td>Bacteroides</td>
<td>*</td>
</tr>
<tr>
<td>Genus</td>
<td>Blautia</td>
<td>*</td>
</tr>
<tr>
<td>Genus</td>
<td>Faecalibacterium</td>
<td>*</td>
</tr>
<tr>
<td>Genus</td>
<td>Faecalitalea</td>
<td>*</td>
</tr>
<tr>
<td>Genus</td>
<td>Parasutterella</td>
<td>*</td>
</tr>
<tr>
<td>Genus</td>
<td>Turicibacter</td>
<td>*</td>
</tr>
</tbody>
</table>

Based on the balance between *Blautia* and the mean of Oscillospiraceae and Negativicutes (Fig. 4), Selbal was able to assign a dog based on the bacteria found in its fecal sample to the higher or lower anxiety group with AUC-ROC of 0.856. The AUC-ROC indicates the probability that a random high-anxiety dog will be considered by the classifier more likely to belong to the higher anxiety group than a random low-anxiety dog. A perfect classifier has an AUC-ROC of 1 and a random classifier has an AUC-ROC of 0.5. According to Selbal, higher anxiety dogs typically had greater amounts of *Blautia* with respect to Oscillospiraceae and Negativicutes than lower anxiety dogs.

Figure 4. (A) Selbal analysis identified the balance between Oscillospiraceae and Negativicutes (numerator) and Blautia (denominator) as an important distinguishing factor between higher and lower anxiety dogs. (B) The balance between these bacteria could predict the assigned behavioural group (higher or lower anxiety) with an AUC-ROC of 0.856.

The genus *Turicibacter* was associated with aggression score in both classification and regression Selbal analyses, and the phylum Firmicutes was an important distinguishing factor between higher and lower aggression groups in PhILR and Random Forest. However, there are fewer taxa associated with higher and lower aggression groups overall when compared to the anxiety analysis (Table 3).

Using Selbal, further investigation into individual anxiety-related C-BARQ scores for dog-directed, stranger-directed, and non-social fear (Table 4) indicated Oscillospiraceae as the family most closely associated with stranger-directed fear; along with the genus *Faecalitalea* and *Phascolarctobacterium succinatens*. *Blautia* and *Parasutterella* were associated with non-social fear at the genus level, and interestingly, *Blautia* was further identified to the species level as *Blautia hansenii* when associated with the stranger-directed fear analysis. Phylum Campylobacterota, and genus *Clostridium sensu stricto 1* were associated with dog-directed fear.
Based on observable behaviours within the shelter environment. It is possible that the Mondo study dogs experienced acute stress in the shelter which may groups between the Mondo study and ours are due to differences in categorizing those groups. In their study, dogs were assigned to a behavioural group there was no difference in alpha diversity between normal and phobic dogs in the Mondo study, it could be suggested that the differences seen in behavioural typically associated with healthy animals [$] to justify such a large difference in the core bacterial communities; however, it is important to highlight for future studies that core populations can vary disorders should closely consider the individual dog's core microbial population in the gut.

We generated four Random Forest models per trait: two using as input PhILR log ratios and two using as input the abundances. In addition, for two of the models we removed all those features whose permutation did not cause a decrease in classification accuracy (this process is called feature selection), as these features presumably are un-informative. When comparing higher and lower anxiety groups, the models with the highest classification performance were those generated using the PhILR log ratios (ILR) and feature selection (FS) (Fig. 5). This ILR + FS model for anxiety achieved an AUPRC of 0.82 and AUC-ROC of 0.87. Using this model one can identify half of higher-anxiety dogs with a precision of around 87% (Fig. 5). Genera Lachnoclostridium, Bacteroides, Butyricicoccus, Escherichia-Shigella, Catenibacterium, and Faecalitalea, family Peptostreptococcaceae, and phylum Firmicutes were associated by this model with anxiety levels. The ILR + FS model for aggression achieved an AUPRC of 0.74 and AUC-ROC of 0.73. Using this model, one can identify half of higher-aggression dogs with a precision of around 75% (Fig. 6). Genera Bacteroides, Prevotella_9, Faecalitalea, Blautia and Parasutterella, family Peptostreptococcaceae and Lachnospiraceae, order Burkholderiales, and phylum Firmicutes, were associated by this model with aggression levels.

**Table 4** Further Selbal analyses based on continuous anxiety-related C-BARQ scores dog directed fear, stranger directed fear, and nonsocial fear.

<table>
<thead>
<tr>
<th>Further Selbal Results (Anxiety)</th>
<th>Phylum</th>
<th>Bacteria</th>
<th>Dog Directed Fear</th>
<th>Stranger Directed Fear</th>
<th>Nonsocial Fear</th>
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</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Campylobacterota</td>
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<tr>
<td>Family</td>
<td>Oscillospiraceae</td>
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<tr>
<td>Genus</td>
<td>Faecalitalea</td>
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<tr>
<td>Genus</td>
<td>Clostridium sensu stricto 1</td>
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<tr>
<td>Genus</td>
<td>Parasutterella</td>
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<tr>
<td>Genus</td>
<td>Blautia</td>
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<tr>
<td>Species</td>
<td>Blautia hansenii</td>
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<tr>
<td>Species</td>
<td>Phascolarctobacterium succinatens</td>
<td>*</td>
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</table>

We generated four Random Forest models per trait: two using as input PhILR log ratios and two using as input the abundances. In addition, for two of the models we removed all those features whose permutation did not cause a decrease in classification accuracy (this process is called feature selection), as these features presumably are un-informative. When comparing higher and lower anxiety groups, the models with the highest classification performance were those generated using the PhILR log ratios (ILR) and feature selection (FS) (Fig. 5). This ILR + FS model for anxiety achieved an AUPRC of 0.82 and AUC-ROC of 0.87. Using this model one can identify half of higher-anxiety dogs with a precision of around 87% (Fig. 5). Genera Lachnoclostridium, Fusobacterium, Bacteroides, Butyricicoccus, Escherichia-Shigella, Catenibacterium, and Faecalitalea, family Peptostreptococcaceae, and phylum Firmicutes were associated by this model with anxiety levels. The ILR + FS model for aggression achieved an AUPRC of 0.74 and AUC-ROC of 0.73. Using this model, one can identify half of higher-aggression dogs with a precision of around 75% (Fig. 6). Genera Bacteroides, Prevotella_9, Faecalitalea, Blautia and Parasutterella, family Peptostreptococcaceae and Lachnospiraceae, order Burkholderiales, and phylum Firmicutes, were associated by this model with aggression levels.

**Figure 6.** Precision-Recall Curves for Random Forest models predicting assignment of dogs based on their fecal microbiota to higher aggression group, generated using abundance, abundance + feature selection (FS), PhILR log ratios (ILR), and PhILR log ratios with feature selection (ILR + FS). The solid line indicates the average cross-validation Precision-Recall curve and the shaded area indicates the performance range per model observed during cross-validation.

**Discussion**

This study provides further evidence that the canine gut microbiome differs in relation to behaviour, with the majority of evidence supporting a link between the gut microbiome composition and anxiety in family pet dogs.

In our cohort, the dominant phyla Bacteroidota, Firmicutes and Fusobacteria comprised approximately 95% of the gut microbiome, which is in line with other studies of healthy canines [31]–[34], although considerable variation in percentages can be seen across the literature for specific taxa [35]. In comparison to the recent study by Mondo [10], the fecal microbiome of our cohort showed considerable differences. Their "normal" (non-phobic, non-aggressive) dog profile was comprised of mostly Firmicutes (68%), Bacteroidetes (13.7%), Actinobacteria (9.9%), Fusobacteria (4.8%) and Proteobacteria (2.1%), whereas the top phyla identified in lower anxiety dogs in our study were Bacteroidota (51.5%) (synonymous with Bacteroidetes), Firmicutes (24.5%), Fusobacteriota (20%) and Proteobacteria (3.9%), with the remaining phyla having < 1% relative abundances. The considerable difference in abundance of Firmicutes and Bacteroidota/Fusobacteria could be explained by geographical location, which has been shown to have an appreciable impact on both alpha and beta diversity of canine gut microbiomes across the United States [34]. A greater abundance of Firmicutes could be due to different diet compositions between the two populations, as greater vegetable fiber content in the diet is associated with a greater abundance of Firmicutes [32], [36], and a higher protein content (as seen in raw-fed or BARF diets) is associated with decreased abundance of Firmicutes [37], [38]. Increased abundance of the genus *Fusobacterium* is generally associated with healthy control dogs [39] and increased access to the outdoors [40], which is supported by the demographic background of the dogs in our study (pet dogs, versus shelter dogs). There is little evidence to suggest that the diets were so significantly different between our study and that of Mondo et al. [10] to justify such a large difference in the core bacterial communities; however, it is important to highlight for future studies that core populations can vary greatly between individuals and studies. Thus, any clinical studies attempting to manipulate or adjust the gut microbiome in treatment of behavioural disorders should closely consider the individual dog's core microbial population in the gut.

Our study found a greater number of sOTUs in both the higher aggression and higher anxiety groups. This finding is consistent with Mondo's observation of an increased number of OTUs in aggressive dogs [10], and more recently it was found that in a population of working dogs, higher aggression scores were also associated with increased richness and Shannon diversity [11]. In clinical studies of gastrointestinal disease, increased richness of gut microbiota is typically associated with healthy animals [35]; thus, the explanation for the link between increased aggression and increased richness in dogs is unclear. While there was no difference in alpha diversity between normal and phobic dogs in the Mondo study, it could be suggested that the differences seen in behavioural groups between the Mondo study and ours are due to differences in categorizing those groups. In their study, dogs were assigned to a behavioural group based on observable behaviours within the shelter environment. It is possible that the Mondo study dogs experienced acute stress in the shelter which may...
have resulted in different observable behaviours and/or short-term changes to the gut microbiome when compared to the family pets in our study. Two key differences in our study compared to Mondo et al. [10] was our use of C-BARQ for determining behavioural group, and the participation of dogs from family homes who had not experienced any recent changes in living arrangements. The C-BARQ allowed owners to report information from observing the dog in many scenarios over a long period of time (versus an encounter with an unfamiliar dog in a stressful environment). Also, the dogs’ consistent living arrangements likely reduced fluctuations in behaviour or gut microbiome composition that may be caused by acute stress. Co-morbidities between anxiety and aggression also should be taken into consideration – fearful dogs are significantly more aggressive than non-fearful dogs [41], and the prevalence of dogs in our study exhibiting both higher aggression and anxiety C-BARQ scores suggests that in many of our dogs, aggressive behaviours may be expressed as a symptom of underlying anxiety. It is not clear if the Mondo study dogs exhibited the same co-morbidities between aggression and anxiety; indeed, dogs were assigned to discrete groups (i.e., phobic or aggressive), which appears to exclude this possibility. Future research should incorporate non-anxious dogs scoring highly for aggression, and non-aggressive dogs scoring highly for anxiety to more clearly address potential cross-over between behavioural groups.

The taxa most commonly identified across anxiety analyses were the family Oscillospiraceae and the genus Blautia, with B. hansenii associated with stranger-directed fear in particular. Blautia as a genus has divergent associations with human health in the literature. On the one hand, it is associated with protective and probiotic effects [42] and is currently being investigated as a potential avenue for treatment of anxiety-like behaviours in autism spectrum disorder (ASD) in humans [43]. In addition, improved sleep quality was associated with an increase in abundance of Blautia after exercise in patients suffering from sleep disorders [44]. Conversely, some studies have associated an increased abundance of Blautia with gastrointestinal disease [45], [46], increased risks of breast cancer [47], and acetic acid-producing Blautia species are considered to contribute to non-alcoholic fatty liver disease [48].

In dogs, Blautia is one of multiple short chain fatty acid (SCFA)-producing bacterial genera whose abundance is greatly decreased during bouts of acute diarrhea [49], and has been used as an indicator for gut dysbiosis in mathematical modelling [50]. However, in a recent study investigating the effects of probiotics on the gut microbiome in dogs, multiple Blautia species (including B. hansenii) were significantly lower in dogs supplemented with probiotics after 60 days when compared to control dogs, with the most significant effects seen in elderly dogs [51]. Thus, it appears that Blautia species in the canine gut microbiome may have as wide a range of implications as in the human and other mammalian literature. When comparing behavioural groups, our study identified Blautia to the genus level in all major analyses (abundance LDA, PhILR, Selanalyses and Random Forest), and given the increased proportions of the genus Blautia in higher anxiety dogs in this study, it is likely that the individual species we have detected do not possess the aforementioned protective effects. Similar to this study, the majority of the literature associating Blautia with host health only identifies it to the genus level; thus, it is necessary to identify to the species level before drawing specific conclusions about the effects of Blautia on health and behaviour. Nonetheless, Blautia presents an interesting finding for the clinical community due to its sensitivity to dietary changes [52] and probiotics [53], making treatment through dietary changes or supplements a convenient prospect.

The exact mechanisms by which long-term stress associated with behavioural disorders affects canine physiology are still unclear; however, in humans it has been proposed that long term stress increases intestinal permeability, resulting in increased release of endotoxins from the gut lumen into the bloodstream and initiating peripheral inflammation, which impacts mental health once the inflammation begins to affect the central nervous system [54]. While the complexities of the gut-brain axis are still being investigated in multiple species, it has been shown that increased plasma glutamine and y-glutamyl glutamine is also associated with fearfulness in dogs [55], and these metabolites have previously been associated with several psychiatric disorders in humans such as anxiety, schizophrenia, depression and PTSD due to the major role of glutamate in fear conditioning [56]. The Lachnospiraceae family, to which Blautia belongs, has been correlated with behavioural changes induced by stress in other mammalian models including humans [57] and mice [58]. Based on the human literature, there is evidence to suggest the Lachnospiraceae family is involved in the inflammatory pathway, as an increase in abundance of this family promotes a decrease in SCFA concentration [59], leading to intestinal wall dysfunction [60]. Similarly, Turibacter (associated with aggression in this study) has been linked with inflammation and cancer in mice [61]. Along with Blautia species, a decrease in abundance of Turibacter is also an indication of gut dysbiosis in gastrointestinal disease in dogs [50]. Thus, while the bacteria identified in our study could indeed be linked with stress and the inflammatory response in dogs, identification to the species level is required before further conclusions can be drawn from this information.

**Conclusions**

This study adds to the growing area of microbiome research as it relates to animal behaviour, and provides novel insight into the links between behaviour and gut microbiome composition in family dogs. Despite a relatively small sample size, we were able to consistently identify differences between behavioural groups using various approaches. In particular, the genus Blautia was consistently identified by our analyses as having a close relationship with anxiety in pet dogs.

Given the current knowledge that dietary changes in dogs can alter both gut microbiota [32], [62], [63] and behaviour [64], [65], and that the composition of the gut microbiome is linked to behaviour [9], [10], [55], there is early promise that modifying the gut microbiome via dietary changes or supplementation with probiotics may be beneficial in the treatment of behavioural issues in dogs. However, given the limited basic information available to date, any direct translation of this research for therapeutic treatment in dogs will first require more thorough description and understanding of the core microbiome populations that exist in domestic dogs, and what their relationships with behaviour might be. Further investigation could focus on assessing the differences between gut microbiota in dogs supplemented with commercial probiotic supplements, and should attempt to target more dogs in the upper reaches of C-BARQ scores exhibiting higher levels of anxiety and aggression to further tease apart the links between behaviour and the gut microbiome. While we may identify correlations between behavioural phenotype and relative abundances of microbiota, such a complex system should be respected as such and great care taken before inferring a causational or directional relationship.

**Abbreviations**
AUC-ROC: area under curve - receiver operating characteristic
AUPRC: area under the precision-recall curve
CBARQ: Canine Behavioral Assessment & Research Questionnaire
DDA: Dog-Directed Aggression
DDF: Dog-Directed Fear
FDA: Familiar Dog Aggression
FS: Feature Selection
ILR: Isometric log ratio
LDA: linear discriminant analysis
NSF: Nonsocial Fear
ODA: Owner-Directed Aggression
OTU: operational taxonomic unit
PhILR: phylogenetic isometric log-ratio transform
SCFA: Short-chain fatty acid
SDA: Stranger-Directed Aggression
SDF: Stranger-Directed Fear
SRP: Separation-Related Problems

**Declarations**

**Ethics approval and consent to participate**
This study was reviewed by the Interdisciplinary Committee on Ethics in Human Research (ICEHR) and was found to be in compliance with Memorial University's ethics policy (ICEHR File: 20210935-SC).

**Consent for publication**
Participants in this study have consented to publication of data in aggregate form during the informed consent process approved by the Interdisciplinary Committee on Ethics in Human Research (ICEHR) (ICEHR File: 20210935-SC).

**Availability of data and material**
Sequence read archive (SRA) data are available via the NCBI repository (https://www.ncbi.nlm.nih.gov/sra) under BioProject PRJNA1020865. Companion Rscripts for this manuscript can be viewed at https://github.com/BioinformaticsLabAtMUN/CanineGutMicrobiomeStudy

**Competing interests**
The authors declare that they have no competing interests.

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**Authors' contributions**
DB, CW and LPC conceived the study. DB, CW, LPC and SP acquired the study funding and developed the research methodology. SP and CW recruited participants and conducted the behavioural data collection and analyses. SP and DB prepared the DNA samples used for the microbiome sequencing. SP, AZ and LPC analyzed and interpreted the microbiome data. SP, DB, CW and LPC collectively wrote the manuscript. All authors read and approved the final manuscript.

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References


37. Bermingham EN, Maclean P, Thomas DG, Cave NJ, Young W. Key bacterial families (Clostridiaceae, Erysipelotrichaceae and Bacteroidaceae) are related to the digestion of protein and energy in dogs. PeerJ. 2017;5:e3019.


Figure 1

The top 20 most abundant genera, as per total number of reads, identified across the entire cohort of dogs (n = 48). The horizontal line within each box indicates the median and the diamond indicates the mean of the log2 of the number of reads.
Figure 2

(A) Alpha metrics ACE, Chao1 and Observed in higher and lower anxiety groups. (B) Mean relative abundance (%) of the top 7 most abundant families identified for higher and lower anxiety groups.
Figure 3

(A) Alpha metrics ACE, Chao1 and Observed in higher and lower aggression groups. (B) Mean relative abundance (%) of the top 7 most abundant families identified for higher and lower aggression groups.
(A) Selbal analysis identified the balance between Oscillospiraceae and Negativicutes (numerator) and Blautia (denominator) as an important distinguishing factor between higher and lower anxiety dogs. (B) The balance between these bacteria could predict the assigned behavioural group (higher or lower anxiety) with an AUC-ROC of 0.856.

**Figure 4**

Precision-Recall curves for models predicting higher anxiety

**Figure 5**

Precision-Recall Curves for Random Forest models predicting assignment of dogs based on their fecal microbiota to higher anxiety group, generated using abundance, abundance + feature selection (FS), PhILR log ratios (ILR), and PhILR log ratios with feature selection (ILR + FS). The solid line indicates the average cross-validation Precision-Recall curve and the shaded area indicates the performance range per model observed during cross-validation.
Figure 6

Precision-Recall Curves for Random Forest models predicting assignment of dogs based on their fecal microbiota to higher aggression group, generated using abundance, abundance + feature selection (FS), PhiLR log ratios (ILR), and PhiLR log ratios with feature selection (ILR + FS). The solid line indicates the average cross-validation Precision-Recall curve and the shaded area indicates the performance range per model observed during cross-validation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- 08FigureS1LDA.pdf
- AdditionalFileDietLifestyleQuestionnaire.pdf
- SupplementaryTablesSequenceQualityRelativeAbundance.pdf