Owner-Assessed Behaviours and Relationships to Gut Microbiota Composition in the Domestic Dog (*Canis familiaris*)

by

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ABSTRACT

Behavioural issues in dogs, such as anxiety and aggression, are a prominent reason for both the relinquishment and euthanasia of pet dogs. These issues can cause a break-down of the dog-owner bond, and negatively impact quality of life for both dog and human. One potential area of research, the gut microbiome, provides the opportunity for better understanding the physiological process underlying the occurrence of aggression and anxiety, and recent studies have shown promise that changes in canine gut microbiota composition are associated with behaviour in dogs. However, a precise link between gut microbiota and behaviour has not yet been established in pet dogs, and previous studies have used clinical or shelter populations, rather than a community sample. I first highlight the demographic and lifestyle factors that may be related to the behaviour of dogs in this study, including the presence of conspecifics and the dog's daily activities. In addition, I report on validity concerns for a subscale (familiar dog aggression) of a widely used, owner-reported dog behaviour survey, and present considerations for survey interpretation and improvement. Dogs cohabiting with other dogs in the same home, as well as singleton dogs whose owners unexpectedly completed questions about rivalry towards familiar (co-habiting) dogs, had different behavioural profiles compared to dogs living alone, whose owners did not respond to questions about dog rivalry. Finally, I investigate the links between the anxiety and aggression profiles and gut microbiota composition in dogs from the local area. Gut microbiota composition differed somewhat between dogs with higher and lower aggression scores, but most notably differed between those with differing anxiety scores, with the genus *Blautia* consistently associated with anxiety across multiple analyses. This thesis contributes to our growing understanding of gut microbiota composition in dogs with aggression or anxiety issues.

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"Experts say that the key to a well-behaved dog is the provision of treats,
frequently, and generously,
and in particular - cheese."

- Denzel & Leeloo; The Experts.

TABLE OF CONTENTS

CHAPTER 3: INVESTIGATION OF OWNERS' INTERPRETATION OF THE FAMILIAR DOG AGGRESSION SUBSCALE

CHAPTER 4: GUT MICROBIOME COMPOSITION IS ASSOCIATED WITH ANXIETY AND AGGRESSION IN DOMESTIC DOGS (*CANIS FAMILIARIS*)

CHAPTER 5: GENERAL DISCUSSION AND SUMMARY

LIST OF TABLES

LIST OF FIGURES

LIST OF SUPPLEMENTARY MATERIALS

ABBREVIATIONS & DEFINITIONS

AUC-ROC: Area under curve - receiver operating characteristic

AUPRC: Area under the precision-recall curve

C-BARQ: 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

CHAPTER 1: A REVIEW OF THE CURRENT UNDERSTANDING OF THE ROLE OF THE GUT MICROBIOME IN CANINE BEHAVIOUR

1.1 CURRENT LITERATURE

Behavioural issues, such as anxiety and aggression, are reported as the major reason for relinquishment of dogs to shelters (Kwan & Bain, 2013; Salman *et al*., 1998; Segurson *et al*., 2005), and are a considerable source of stress for owners that can result in the breakdown of the dog-human bond (Meyer & Forkman, 2014). For dog owners[1] who are experiencing behavioural issues with their dogs, there are typically three options: behaviour modification, rehoming or relinquishment to a shelter, or behavioural euthanasia. It is estimated that 3.2 million dogs were surrendered to shelters in the USA in 2023, of which 360,000 were euthanized (Shelter Animals Count, 2024). Previous studies investigating the reasons for relinquishment have suggested that behavioural issues are one of the primary reasons for surrendering a dog to a shelter (Eagan *et al*., 2022; Powell *et al*., 2021; Salman *et al*., 2000). The implementation of behaviour modification to treat problem behaviours provides the opportunity for dogs to stay in their current home, but does require considerable investment from dog owners in terms of time, finances, and resources. From my personal experience working as a dog trainer and behaviour consultant, there is a demand within the dog training industry to provide affordable, accessible solutions not only to reduce the rates of relinquishment and euthanasia, but also to improve education in canine behaviour to prevent the development of severe behavioural issues.

[1]*Animal welfare organizations, such as the ASPCA, advocate for the use of 'guardian' over 'owner' when referring to the human's involvement in their relationship with companion animals, as it depicts more clearly the responsibility associated with providing adequate care for animals. It can be argued that all dog guardians are dog owners, but not all dog owners are guardians. Due to the subjective nature of the nomenclature around the human-dog relationship, I have chosen to refer to humans in this thesis as 'dog owners' with the acknowledgement that not all people involved in canine studies, or this thesis, may consider themselves guardians – however, they are all the legal owners of their pets.*

The gut microbiome encompasses the microorganisms occupying the gut, and their "theatre of activity", including structural elements, metabolites/signal molecules, and surrounding environmental conditions, resulting in the formation of specific ecological niches (Berg *et al*., 2020), and its role in behavioural and neurological conditions has become increasingly apparent in recent years. There is mounting evidence that the composition of, and changes to, the gut microbiota (i.e. the collection of microorganisms that are present in the gut: Marchesi & Ravel, 2015, Berg *et al*., 2020) is correlated with behaviour and mental health (Bravo *et al*., 2011; Dinan & Cryan, 2017; Valles-Colomer *et al*., 2019); however, the majority of the current research details human and other mammalian systems (Cresci & Bawden, 2015). There is considerably less information available about the link between canine behaviour and changes in the gut microbiota composition, although recent studies have highlighted differences in the composition of the gut microbiota between domestic dogs of differing ages, behavioural tendencies (such as anxious or aggressive), and cognitive abilities (Kirchoff *et al*., 2019; Kubinyi *et al*., 2020; Mondo *et al*., 2020). The relationships between gut microbiota, behaviour and mental health are emerging topics of great interest for humans, and the canine gut has been suggested to be the most similar of the mammalian models to that of humans in terms of its response to diet (Coelho *et al*., 2018). The use of companion dogs in such studies allows a unique opportunity to further develop our knowledge of these relationships in a non-human animal that shares much of their environment with humans, while also having a direct application to dog health and welfare.

Given the current knowledge that dietary changes in dogs can alter both gut microbiota composition (Bresciani *et al*., 2018; Middelbos *et al*., 2010; Sandri *et al*., 2019) and behaviour (Kato *et al*., 2012; Landsberg *et al*., 2015), and that the gut microbiome is linked to behaviour (Kirchoff *et al*., 2019; Mondo *et al*., 2020; Puurunen *et al*., 2018), there is some promise that modifying the gut microbiota 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 relating to behaviour, any direct translation of this research for therapeutic treatment in dogs will first require a more thorough description and understanding of the core gut microbial populations that exist in domestic dogs, and what their relationships to behaviour might be.

Recent studies have demonstrated interesting patterns in the relationships between the gut microbiota and behaviour in some populations of dogs, and they provide a foundation to further expand this line of research towards dogs that live as family companions. Kirchoff *et al*. (2019) present an interesting comparison between aggressive and non-aggressive dogs in one breed type - "pitbull"; the dogs were all housed in a shelter environment after being seized from a potentially traumatic situation (fight operation) and were assessed based solely on conspecific aggression. They found 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 state in their discussion that these correlations should be further investigated with a larger sample size and better controls for diet and life history. Mondo *et al*. (2020) also utilized a cohort of shelter dogs in Bologna, Italy, for their comparison of gut microbiota between aggressive, anxious and "normal" dogs. Similar to Kirchoff *et al*. (2019), 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 welfare conditions, may introduce confounding effects of acute stress on the dog's behaviour (and potentially their gut microbiota), which could make accurately describing the relationships between behaviour and the gut microbiome difficult. Thus, it is important to further develop our knowledge of the gut microbiota composition in domestic dogs living as family pets in a relatively secure, stable environment, while also considering the extent to which a dog's behavioural phenotype is actually relevant to its life history. For example, while a dog may be classified as "dog-aggressive", we may question how relevant that is to its welfare and physiology if owner management practices prevent the dog from encountering unfamiliar dogs. Similarly, dogs suffering from separation anxiety may not experience particularly stressful or challenging situations if their owners opt to manage their condition by never leaving them alone.

Another challenge arises in how dogs are classified into behavioural phenotypes. Mondo *et al*. (2020) used a veterinary behaviourist to identify each dog's behavioural phenotype in a short behavioural assessment while the dogs lived in the shelter. While no information is provided on the length of time the dogs had been in the shelter, the stress of shelter housing could have prevented an accurate representation of the dog's typical behaviour. Alternative assessments are available, such as the Canine Behavioural Assessment Research Questionnaire (C-BARQ), a comprehensive behavioural assessment tool originally developed by Hsu and Serpell (2003) and frequently used in behavioural studies to develop a reliable profile for a dog with minimal owner bias (Jakuba *et* al., 2013; Clay *et al*., 2020). As an owner-based questionnaire, the C-BARQ can be used to provide detailed information about 'owned' companion dogs who have lived in a more consistent environment for a longer period of time than the aforementioned shelter dogs. In assessing a broader scope of behaviour, I explore relationships between gut microbiota composition and behaviour, while considering additional variables such as diet, daily routine and lifestyle choices that have been shown to impact the gut microbiota and behaviour.

1.2 RESEARCH OBJECTIVES

First, I explore the relationships between the behavioural profiles of the dogs in this study, with elements of their lifestyle that may also impact the gut microbiome. I explore the behavioural subscales provided by the C-BARQ, and additional information collected in the Diet & Lifestyle questionnaire developed for this thesis. In particular, I am interested to know whether the dog's time spent off the property either exercising or socializing impacts their behaviour, and if the presence of other dogs in the home affects the severity of their aggression or anxiety.

Next, I explore the composition of the gut microbiota in a sub-sample of dogs who have participated in both the Diet & Lifestyle questionnaire, and the C-BARQ. Using the behavioural data, the dogs from this community sample will be split based on the severity of their behavioural issues (higher and lower aggression or anxiety), then the dogs from each group will have the bacterial populations of their gut quantified. To achieve this, I will first identify the bacteria present in each sample by referring to the online taxonomic database, Silva, then quantify the relative abundance of the bacteria to allow a comparison between behavioural groups at multiple taxonomic levels. I will then use machine learning algorithms in R to highlight specific bacteria that may be associated with increased levels of aggression or anxiety, and investigate if gut microbiota composition can accurately predict which behavioural group dogs have been assigned to.

It is my aim in presenting this study to provide insight into the relationships between the gut microbiome and canine behaviour, and to make this information accessible to the entire dog community including owners, breeders, trainers and veterinary professionals via Open Access journals.

1.3 CO-AUTHORSHIP STATEMENT

The first two data chapters in this thesis, detailing my investigation into the impacts of lifestyle on behaviour (Chapter 2), and the data relating to the familiar dog aggression subscale in the C-BARQ (Canine Behaviour and Research Questionnaire) (Chapter 3), are currently being further developed into one manuscript and will be submitted (along with a scoping review of the current 'familiar dog aggression' literature) for peer review. Dr. Carolyn Walsh is a co-author on this manuscript, and has contributed significantly to the questionnaire development, study design, and data analysis. Dr. Dawn Bignell and Dr. Lourdes Peña-Castillo also contributed to the data analysis, reviewed the manuscript, and facilitated the funding for the Masters student support.

The second manuscript, "Gut Microbiota Composition is Related to Anxiety and Aggression Scores in Companion Dogs", is co-authored by Allan Zhang, Dr. Carolyn Walsh, Dr. Dawn Bignell and Dr. Lourdes Peña-Castillo. This manuscript is being prepared to be submitted for peer review. Dawn Bignell, Carolyn Walsh and Lourdes Peña-Castillo conceived the study. Dawn Bignell, Carolyn Walsh, Lourdes Peña-Castillo and Sarita Pellowe developed the research methodology, and acquired two sources of funding for the study: MITACS eAccelerate program, with East Coast Canine Dog Training; and the Memorial University Seed, Bridge & Multidisciplinary Fund. Sarita Pellowe and Carolyn Walsh recruited participants and conducted the behavioural data collection and analyses. Sarita Pellowe and Dawn Bignell prepared the DNA samples used for the bacterial 16S rRNA gene sequencing, and Sarita Pellowe, Allan Zhang and Lourdes Peña-Castillo analyzed and interpreted the metataxonomic data. Dawn Bignell, Carolyn Walsh, Lourdes Peña-Castillo and Sarita Pellowe collectively wrote the manuscript, with Sarita listed as the corresponding author. All authors read and approved the final manuscript.

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CHAPTER 2: LIFESTYLE FACTORS ARE RELATED TO BEHAVIOURAL SUBSCALE SCORES IN AN OWNER-REPORTED SURVEY

2.1 ABSTRACT

Behavioural problems in dogs involve interactions of genetics, physiology, and the environment. While the contributions of genetics and the early environment are typically the responsibility of the dog breeder or shelter, owners are responsible for daily decisions about the dog's activities, including exercise and socialization that may influence their behaviour. I collected information from 235 participants about the dogs' daily activities, socialization opportunities, home life, and behaviour using a Diet & Lifestyle questionnaire, and the online Canine Behaviour and Research Questionnaire (C-BARQ). A positive correlation was seen between C-BARQ trainability scores and the number of activities in which a dog participated; those participating in social activities also had significantly lower C-BARQ dog-directed aggression and excitability scores. Dog-directed aggression, and stranger-directed fear and aggression, were significantly higher in dogs living in multi-dog homes. For dogs that scored at or above the median for both aggression and anxiety composite scores created from C-BARQ subscales, barking and staring were the most common behaviours seen when dogs were presented with an unfamiliar person or dog. Dogs scoring below the medians for anxiety and aggression were reported more frequently to respond to unfamiliar people with a wagging tail. Dogs scoring more highly for anxiety, regardless of their aggression scores, were most commonly reported to move away when presented with an unfamiliar person. The data collected in the Diet & Lifestyle questionnaire provide support that dogs assigned to higher or lower aggression/anxiety groups based on composite C-BARQ scores are indeed behaviourally different, and the findings highlight the benefits of using additional questionnaires to supplement the C-BARQ findings.

2.2 INTRODUCTION

The development of behavioural issues in dogs is multi-faceted: genetics, early rearing environment, maternal care and socialization during a puppy's critical developmental window can all contribute to the likelihood and severity of problem behaviours in adult dogs (Dietz *et al*., 2018). After puppy socialization, dogs continue to have ongoing exercise, veterinary care, training, enrichment and social needs that need to be met to ensure adequate quality of life (Griffin *et al*., 2023). While the early life events occurring during a dog's time with a breeder or shelter would typically be outside of an average dog owner's control, ongoing lifestyle choices, such as types of regular activities that the dog is exposed to, can affect the dog's behaviour (Meyer *et al*., 2023; Yamada *et al*., 2019). However, the dog's behaviour itself may affect an owner's capacity or motivation to participate in certain types of activities with the dog. For example, an owner's anticipation of a stressful encounter for their dog, such as reacting negatively towards people or dogs while on leash, can reduce their willingness or motivation to walk their dog in public (Westgarth *et al*., 2017).

2.2.1 Interactions Between Daily Activity, Lifestyle, & Canine Behaviour

Owner-dog exercise and time spent time away from their home property can provide opportunities for a dog's enrichment, socialization, and physical and mental stimulation, as well as health benefits for the owner. A large literature focuses on the benefits of exercising with dogs from a human perspective - benefits can include improved physical health aspects such as reduced obesity (Christian *et al*., 2018), as well as improved cardiovascular (Levine *et al*., 2013) and mental health (Barcelos *et al*., 2020) in dog owners. In addition, participating in activities with one's dog is associated with improved feelings of happiness, wellbeing and connection with the animal (Barcelos *et al*., 2020). Owner satisfaction of their relationship with their dog significantly affects the time spent exercising with the dog (Potter & Sartore-Baldwin, 2019; Westgarth *et al*., 2015), and level of reported enjoyment (Westgarth *et al*., 2017).

A stable exercise routine is also associated with fewer aggressive or fearful behaviours in the dog (Gonzalez-Ramirez, 2019). In one study, exercise appeared to provide resilience to noise sensitivity, and lower anxiety levels were generally seen in dogs with higher activity levels (Tiira *et al*., 2016). Exercise is recommended for shelter dogs to improve welfare by reducing stress (Menor-Campos *et al*., 2011) and the occurrence of harmful or problematic behaviours (Protopopova *et al*., 2018). Given the co-morbidities often seen between fear and aggression (e.g., Tiira *et al*., 2016), it is possible that exercise could also reduce aggressive behaviours in dogs; however, it is important to consider the motivation for aggression in the individual dog and their capacity to deal with real-life situations, as aggression is typically quite context-specific (Siracusa, 2021). For example, a dog who behaves aggressively towards other dogs would likely experience the known benefits of exercise by going on a hike with human companions, but it may become more stressed and behave aggressively if regularly exercised in a social situation around unfamiliar dogs. Given that dog owners tend to prioritize their dog's enjoyment of the walk over their own (Westgarth *et al*., 2017), it is likely that a responsible owner of an aggressive dog would avoid potentially reactive or dangerous situations if their dog posed a risk to others, meaning the dog would be limited in the activities in which it could partake.

Trainability, a behavioural trait that is considered highly heritable (Asp *et al*., 2014; MacLean *et al*., 2019; Morrill *et al.,* 2022), may be related to the daily activities a dog experiences, since owning a highly trainable dog may make outings more manageable and enjoyable if the dog readily takes cues from its owner. This, in turn, could increase the motivation of owners to participate in further activities with their dog. Similarly, a dog's level of excitability may influence different potential scenarios in relation to daily activity. For example, dogs with higher levels of excitability may require more activity and, thus, may spend more time engaging in activities with the owner away from their home. However, the inverse is also plausible; that is, more excitable dogs may get fewer opportunities to exercise and socialize if owners feel unable to adequately control them.

2.2.2 Evaluating Behaviour Problems in Dogs

There are two primary approaches to evaluating canine behaviour problems in scientific studies: in-person behavioural testing, typically seen for specific clinical samples, which are conducted by a trained observer (typically a behaviourist, or a researcher trained to assess observed behaviours; e.g., Kirchoff *et al*., 2019; Mondo *et al*., 2020; Svartberg, 2021), and owner-based surveys, that are usually directed towards larger community samples (Duffy *et al*., 2014; Hsu & Serpell, 2003; Ley *et al*., 2009a). In-person evaluations can become difficult (and costly) when recruiting large samples, as they require more extensive resources and time for data collection. In addition, these tests involve observing the dog for a relatively short period of time – as such, the dog may not display their typical behaviour during the assessment period (Christensen *et al*., 2007; Mornement *et al*., 2009), as their behaviour may be modified (i.e., more or less severe) due to stress of a new environment, or they may refuse to participate or interact with the test subjects/objects due to neophobia.

Owner-reported surveys are arguably more accessible in terms of resources, and owners can report on the dog's behaviour over a longer period of time. Despite the risk of owner bias, for example, under- or over-reporting the severity of their dog's behavioural issues (as explored by Powell *et al*., 2021), the surveys most commonly used in large community samples, such as the Canine Behaviour and Research Questionnaire (C-BARQ) (Hsu & Serpell, 2003), and the

Monash Canine Personality Questionnaire – Revised (MCPQ-R) (Ley *et al*., 2009a), have demonstrated relatively high inter-rater reliability and external validity, with different assessments frequently correlating with each other, suggesting that they are validly testing similar behavioural constructs (e.g., MCPQ-R and the Dog Personality Questionnaire, Posluns *et al*., 2017). While these studies support the use of broader personality traits within the literature, there is still considerable variation in the personality dimensions adapted from the Five-Factor Model (FFM) of personality in humans: extraversion, openness, conscientiousness, neuroticism and agreeableness (Digman, 1990). In dogs, multiple studies have proposed different dimensions for canine personality, including: energy, affection, emotional reactivity and intelligence (Gosling *et al*., 2003); calmness, trainability, dog sociability and boldness (Kubinyi *et al*., 2009); and stranger-directed sociability, activity, aggressiveness and trainability (Mirkó *et al*., 2012). While there is undoubtedly value in the evaluation of canine personality traits, an alternate approach is to ask owners to report on their dog's behaviours around specific contexts (for example, in the home) and targets (such as towards the owner, or towards strangers), and then to provide an inventory of the dog's problem behaviours. This is the approach that the C-BARQ has taken (Hsu & Serpell, 2003).

The C-BARQ is an online survey developed to identify and quantify problem behaviours in dogs across a range of contexts (Hsu & Serpell, 2003). It consists of 100 questions in 7 main sections: training and obedience, aggression, fear & anxiety, separation-related behaviours, excitability, attachment & attention seeking, and miscellaneous behaviours. Participants rank their dog's behaviour on a Likert scale from 0 ("Never") to 4 ("Always"), with the exception of three trainability items (questions 5, 6 and 7 in section 1) which are scored inversely from 0 ("Always") to 4 ("Never"). The C-BARQ calculates a score for 14 main subscales of behaviour

(Table 2.1) based on the owner's responses: stranger-directed aggression, dog-directed aggression, owner-directed aggression, familiar dog aggression aka "dog rivalry", stranger-directed fear, dog-directed fear, nonsocial fear, separation related problems, excitability, attachment & attention seeking, trainability, chasing, energy level and touch sensitivity. Owners also receive scores for an additional 22 miscellaneous behaviour problems including coprophagia, spinning, and tail chasing.

The C-BARQ has been validated in multiple languages (Broseghini *et al*., 2023; Canejo-Teixeira *et al*., 2018; Nagasawa *et al*., 2011; Tamimi *et al*., 2015), and has been shown to be a robust tool in assessing the behaviour of not only pet dogs (De Meester *et al*., 2008; Duffy *et al*., 2008; Friedrich *et al*., 2019; Hsu & Sun, 2010; Serpell & Hsu, 2005; van den Berg *et al*., 2010), but also service and therapy dogs (Bray *et al*., 2019; Duffy & Serpell, 2012; Hunt *et al*., 2020; Sakurama *et al*., 2023), working dogs (Hare *et al*., 2018; Lazarowski *et al*., 2021), and dogs in shelter environments (Duffy *et al*., 2014; Segurson *et al*., 2005). Many studies have demonstrated considerable external validity of the C-BARQ subscales by comparing the subscale scores to in-person evaluations – for example, stranger-directed and dog-directed aggression were both positively correlated with the observed behaviours in an aggression test in Golden Retrievers (van den Berg *et al*., 2006), while stranger-directed fear was significantly correlated with "fear for strangers" in the Socially Acceptable Behavior (SAB) test (De Meester *et al*., 2008). C-BARQ scores have also been associated with training program outcomes for service and guide dogs, with successful dogs scoring more favourably on 27 C-BARQ traits - in particular, "pulls excessively hard on leash" (Duffy & Serpell, 2012).

Table 2.1. C-BARQ subscale definitions, and the number of questions associated with each subscale.

Subscale	Number of questions	Definition
Stranger-directed aggression	10	Threatening or hostile responses to strangers approaching or invading the dog's or owner's personal space, territory, or home range
Dog-directed aggression	4	Threatening or hostile responses when approached by unfamiliar dogs.
Owner-directed aggression	8	Threatening or hostile responses to the owner or other members of the household when challenged, manhandled, stared at, stepped over, or when approached while in possession of food or objects.
Familiar dog aggression	4	Threatening or hostile responses to other familiar dogs in the same household.
Stranger-directed fear	4	Fearful or wary responses when approached by strangers.
Dog-directed fear	4	Fearful or wary responses when approached by unfamiliar dogs.
Nonsocial fear	6	Fearful or wary responses to sudden or loud noises, traffic, and unfamiliar objects and situations.
Separation related problems	8	Vocalizing and/or destructiveness when separated from the owner, often accompanied or preceded by behavioral and autonomic signs of anxiety including restlessness, loss of appetite, trembling, and excessive salivation.
Excitability	6	Displaying strong reactions to potentially exciting or arousing events, such as going for walks or car trips, doorbells, arrival of visitors, and the owner arriving home; has difficulty settling down after such events.
Attachment & attention seeking	6	Maintaining close proximity to the owner or other members of the household, soliciting affection or attention, and displaying agitation when the owner gives attention to third parties.
Trainability	8	Willingness to attend to the owner, obey simple commands, learn quickly, fetch objects, respond positively to correction, and ignore distracting stimuli.
Chasing	4	Chasing cats, birds, and/or other small animals, given the opportunity.
Energy level	2	Energetic, “always on the go”, and/or playful.
Touch sensitivity	4	Fearful or wary responses to potentially painful procedures, including bathing, grooming, nail-clipping, and veterinary examinations.

In addition, the C-BARQ has demonstrated considerable behavioural differences between working dogs (search and rescue) and pet dogs, with search and rescue dogs scoring significantly higher for trainability and energy, and lower for both aggression and fear towards strangers and dogs (Hare *et al*., 2018). Finally, aggression and fear subscale scores have been shown to correlate with shelter behaviour evaluations, and stranger-directed aggression scores in particular successfully discriminated between dogs who were ultimately adopted versus euthanized (Duffy *et al*., 2014).

While the C-BARQ has been widely used and validated, it shares the limitations of all pet owner-reported surveys, and is limited also by the fact that it asks relatively few questions about the dog's lifestyle or environmental factors that may contribute to the behavioural subscale scores calculated. An additional concern is that it uses a Likert scale for measuring an owner's opinion on the severity of types of behaviours, rather than asking them to report the specific behaviours that they may observe in different contexts. Thus, studies must often create additional surveys or use behavioural tests to supplement the information provided by C-BARQ to allow a broader interpretation of the behavioural scores (e.g., Barnard *et al*., 2012; Rayment *et al*., 2016). Finally, more recent additions to the C-BARQ, such as the familiar-dog aggression (FDA) subscale, have not received as extensive validation as the original subscales (for example, stranger-directed or dog-directed aggression, as presented in the original C-BARQ by Hsu and Serpell, 2003).

2.2.3 Differences in Behavioural Profiles of Dogs Living Alone or With Conspecifics

There is evidence to suggest that the presence or absence of other dogs (conspecifics) in the home is correlated with a dog's behaviour. In one study, dogs living as singletons (i.e., without another dog in the household) exhibited higher levels of fearful behaviours (Tiira *et al*., 2016). Similarly, in a recent owner-reported survey of standard poodles and soft-coated Wheaten terriers, dogs living with conspecifics showed lower noise sensitivity than did singletons (Handegård *et al*., 2020). Dogs living with conspecifics are more likely to exhibit behaviours related to resource guarding or possession aggression (i.e. threatening or hostile responses when approached while in possession of a valued object, such as a toy, chew, food, resting place or person) towards other dogs (Jacobs *et al*., 2018). However, this may be a matter of opportunity, as dogs living alone would have less exposure to this specific context. Other studies have demonstrated that an increased number of conspecifics in the home is positively correlated with frequency of aggressive behaviours exhibited between individuals (Jacobs *et al*., 2018; McGreevy & Masters, 2008). It is unknown if this is due to increased perceived competition for resources by the dogs, or the increased probability of the owner observing an encounter when cohabiting with increasing numbers of dogs. While aggression between conspecifics can be addressed by owning fewer dogs or increasing management efforts around resources, the likelihood of a dog guarding resources from a conspecific may also be predictive of its motivation to display aggression towards the owner. Multiple studies have demonstrated a correlation between owner-directed and familiar dog aggression in relation to resources (Casey *et al*., 2014; Duffy *et al*., 2008; Jacobs *et al*., 2018; Rayment *et al*., 2016; van der Borg *et al*., 2017), suggesting that dogs who display aggressive behaviours towards other dogs who approach their resources also tend to guard these resources from people. Unfortunately, this can significantly impact quality of life for both dog and owner, and break down the owner-dog relationship (McGuire, 2019).

While most of literature relating to conspecifics, i.e., dogs living in multi-dog homes, has focused on resource-based aggression between household dogs, it is less clear whether living with conspecifics also affects other manifestations of aggressive behaviour unrelated to resources, such as fear-based aggression. Fewer studies have expanded their analysis of conspecifics to include other behavioural issues; however, the C-BARQ offers the opportunity to collect information on a range of other behaviours. In addition to resource-based aggression (familiar dog aggression), the C-BARQ explores other aggressive or hostile responses, including towards unfamiliar people (stranger-directed aggression subscale), and dogs (dog-directed aggression subscale). Most notably, these subscales score aggression in dogs in contexts that are unrelated to the possession of resources. Fear-related aggression can be identified in the C-BARQ when the aggression and fear subscales are combined (for example, dog-directed fear and dog-directed aggression subscales) (Duffy *et al*., 2008), and studies have shown positive correlations between fear and aggression scores within the C-BARQ (Stellato *et al*., 2021; van der Borg *et al*., 2017) as well as in other behavioural questionnaires (e.g., Salonen *et al*., 2020; Tiira *et al*., 2016).

When a perceived threat or challenge appears in a dog's environment, it activates the stress response and dogs typically display one of four types of behaviours: aggression, escape, freeze, or fidget (Carlson & Birkett, 2017). Aggressive behaviour typically appears as an escalating attempt to intimidate a person, dog or situation that is perceived as a threat (Siracusa, 2021). However, other types of aggression unrelated to fear may also be present within the aggression subscales, such as leash reactivity caused by frustration (Siracusa, 2021). Similarly, not all fearful dogs will behave aggressively – some will present with calming signals or appeasement gestures, such as lip-licking, making themselves look smaller, or holding up their paw (Firnkes *et al*., 2017; Mariti *et al*., 2017), to diffuse tension and prevent the escalation of conflict (Carlson & Bickett, 2017), or will display avoidant behaviour (moving/turning away) to remove themselves from the perceived threat (Firnkes *et al*., 2017; Mariti *et al*., 2017; Siracusa, 2021). Given that the display of aggressive behaviour can be nuanced and context-specific, and there are other antecedents to aggressive behaviour outside of possession of resources, this study will investigate a range of aggression and fear subscales to explore these different contexts in dogs living with or without other dogs in the home.

2.2.4 Research Objectives & Hypotheses

This study explores the relationships between various aspects of lifestyle and living arrangements with reported problem behaviours in dogs living in pet homes. The data were collected via two questionnaires: the Diet & Lifestyle Questionnaire (Supplementary Material A), that was developed specifically for this study, and the C-BARQ (Supplementary Material B). The data in the Diet & Lifestyle Questionnaire were collected for two purposes: 1) to further explore any relationships between previously validated behavioural subscales of the C-BARQ (Duffy *et al*., 2008; Hsu & Serpell, 2003), and additional lifestyle factors that are not specifically addressed in the C-BARQ, and 2) to aid in the phenotype and lifestyle "matching" process during the selection of participants for the gut microbiome study reported in Chapter 4.

This chapter will focus on the relationships between C-BARQ behavioural subscales and lifestyle factors, primarily: the time the dog spends away from the home/off the property, the frequency of the dog's outings, a dog's participation in "social" activities (i.e., in the presence of other dogs), and the presence or absence of conspecifics in the home. In addition, C-BARQ subscale scores related to both aggression and fear/anxiety, will be separately combined in order to categorize dogs into meaningful groups of lower vs. higher aggression, and lower vs. higher anxiety, both for the gut microbiota analysis (Chapter 4), and to investigate whether some specific behaviours, assessed by the Diet & Lifestyle questionnaire, but not specifically characterized in the C-BARQ, differ among dogs in these groupings.

Based on the current literature, it is expected that dogs who participate in more frequent activities, spending more time off the property, and engaging in more social activities with other dogs, will have lower C-BARQ scores for the subscales related to anxiety and aggression, and will score more highly for trainability. Dogs living with conspecifics likely benefit from companionship in terms of resilience to social and nonsocial fears (Tiira *et al*, 2016; Handegård *et al*., 2020), and thus are expected to show lower scores for both stranger and dog-directed fear and aggression subscales, nonsocial fear, and separation-related issues. However, given the increased likelihood of resource-related problems in multi-dog households, it is expected that dogs living with conspecifics will also receive higher scores for owner-directed aggression. As well, familiar-dog aggression scores may be positively correlated with the number of dogs in the home due to the increased opportunity for conflict around resources.

Using composite scores, based on C-BARQ subscales for grouping dogs into higher and lower aggression and higher and lower anxiety categories, I explore whether dogs in these groups display different context-specific behaviours not specifically quantified in the C-BARQ. It is predicted that dogs who score more highly for both aggression and anxiety will exhibit more reactive behaviours (for example, lunging and barking), while lower aggression and anxiety scores will be associated with dogs displaying more affiliative or neutral behaviours, such as tail-wagging, or ignoring unfamiliar dogs or people. Dogs who score more highly for aggression, but lower for anxiety, may have different underlying motivations for their "aggressive" behaviours; it is expected that these dogs will display more excitable behaviours associated with frustration and a lack of training/socialization, such as barking while excited, rather than typically aggressive behaviours associated with intimidation of a perceived threat. Finally, dogs who appear in both the lower aggression and higher anxiety group should represent dogs who are fearful but engage in minimal conflict, and are expected to exhibit behaviours such as moving away and ignoring others.

2.3 METHODS

2.3.1 Recruitment of participants

Dog owners from the St. John's Metro area were recruited via word of mouth, online postings via email and social media, and postings in local vet clinics and pet care businesses. Participants first reviewed the informed consent form, and then were invited to complete two online questionnaires. First, they were directed to the Diet & Lifestyle questionnaire via a Qualtrics (www.qualtrics.com) survey link. Second, upon completion of the Diet & Lifestyle questionnaire, participants were directed to the online C-BARQ (Canine Behaviour and Research Questionnaire; Hsu & Serpell, 2003) website (https://vetapps.vet.upenn.edu/cbarq/), hosted by the University of Pennsylvania. They were required to create an account for their dog on the C-BARQ website, and enter a study code specific to the current study. Online questionnaires were open to public participation from May 6th, 2021 to July 5th, 2021. Participants who owned multiple dogs were asked to select one dog from their household on which to base their responses for both questionnaires. Ethical approval for all procedures was obtained from the ICEHR (Interdisciplinary Committee on Ethics in Human Research, File 20210935-SC).

2.3.2 Questionnaire Data

The Diet & Lifestyle questionnaire (Supplementary Material A) acquired important information related to diet, lifestyle, and medical history that could potentially impact either behaviour, the gut microbiome, or both. Owners were asked to report on their dog's living arrangements (if they live with other animals, and for how long), daily activity (frequency and duration of time spent off the property, participation in various activities), and were asked to report on the specific behaviours their dog displays when seeing unfamiliar dogs and people while walking on leash. Owners were asked questions about their dog's medical history (weight, spay/neuter status, current or prior medical issues) and the use of any medications, flea/dewormer, probiotics or other supplements. Participants were also asked to provide details about their dog's diet, including the brand and formula, how long they had been eating the particular formula, and any treats they had recently been given prior to collection of the fecal sample (as described in Chapter 4).

During the preliminary analysis of the C-BARQ sub-scales, it was noted that a considerable number of participants who reported that their dogs lived alone (in the Diet & Lifestyle Questionnaire) received a score for familiar dog aggression, which assesses threatening or hostile responses to other familiar dogs in the same household. This surprising finding lead to the creation of a follow-up questionnaire (Chapter 3) to better understand the participants' interpretation of the familiar dog aggression questions. For the purpose of the current chapter, the familiar dog aggression scores remain in analyses in order to make the behavioural data comparable to data from most other studies reporting these scores. However, it should be noted that the interpretation of any significant findings related to the familiar dog aggression subscale should be met with caution at this time due to a high rate of responses from participants of singleton dogs.

2.3.3 Data Organization & Analyses

Data from the Diet & Lifestyle Questionnaire were exported into Excel for coding of responses and organization of data. C-BARQ scores were manually combined with the Diet & Lifestyle data for each participant who had completed both questionnaires. For C-BARQ subscales, participants had to complete 75% or more of the relevant questions within a subscale to be given a score; otherwise, the subscale was reported as missing.

Voluntary participation in surveys can bias the data towards a certain demographic (Bautista, 2012); in this study it was expected that dog owners who are highly motivated or have a particular interest in canine behaviour and welfare would form the majority of the cohort. While this phenomenon is largely unavoidable in such studies, I wished to confirm that the participants who successfully completed both surveys were not significantly different from those who did not complete the study to help ensure the data was representative of the local dog-owning community and not subject to non-response bias (Hibberts *et al*., 2012). Participants were grouped into either “Completed” or “Did Not Complete” the C-BARQ, and the two groups were compared based on their responses to the Diet & Lifestyle questionnaire, using non-parametric statistical tests (Mann Whitney-U, Kruskal Wallis) and chi-square tests, to investigate any potential differences between the two populations in terms of important demographic factors and their responses to questions of particular interest in the proposed analysis. After completion of this initial check, the Diet & Lifestyle factors were compared to C-BARQ behavioural subscales in an exploratory analysis, guided by some specific research questions and hypotheses based on the current literature. As expected, the behavioural data in this chapter were not normally distributed, thus non-parametric tests were used in the statistical analyses.

2.3.4 Daily Activity and Behaviour Analysis

In the Diet & Lifestyle questionnaire, owners were asked to select any activities their dog has participated in over the previous 3 months. Their responses to this question were then analyzed on a point system from 0-10, calculating how many of the activities they had participated in. These 10 activity options were further categorized into “highly social” (e.g., dog park, doggie daycare, dog sports and group classes) and "non-social" (walking, running, hiking, fetch, garden/yard time, none). Participants were also asked how frequently their dog leaves their property (from "Never" to "Every Day"), and how much time their dog had spent off their property each week over the previous 3 months.

The first analysis for this dataset investigated the relationships between the number of activities (Q18), frequency of outings (Q19), time spent off the property (Q20), and participation in social activities, as they relate to C-BARQ subscales for fear (dog-directed, stranger-directed, and nonsocial fear), aggression (stranger-directed, dog-directed, owner-directed and familiar dog aggression), excitability, trainability, and separation related issues. First, lifestyle factors were analysed for a positive or negative relationship using a correlation matrix (Spearman's rho, r_s), with any significant relationships further investigated using Kruskal-Wallis (one-way ANOVA for nonparametric data) and Dwass-Steel-Critchlow-Fligner (DSCF) pairwise comparisons.

2.3.5 Presence of Conspecifics

In the Diet & Lifestyle questionnaire, owners were asked if their current dog lives with other dogs (conspecifics), and if yes, how many dogs were currently living in their household. In this analysis, the subscale scores for singleton dogs were compared to dogs from multi-dog households using Mann Whitney-U (student's T test for nonparametric data). Due to the low number of households with 3+ dogs, categories were combined, and I compared dogs that did live with other dogs to those that lived as singleton dogs in the home.

2.3.6 Composite Scores and Observable Behaviours

In this analysis, scores from the C-BARQ aggression and anxiety subscales were compared to the observed behaviours reported in the Diet & Lifestyle questionnaire to explore which behaviours were typical of dogs who scored highly for each subscale, and to support the use of the composite score in future analyses. Dogs were assigned to one of four behavioural groups based on subsets of their C-BARQ scores: Low anxiety, low aggression; low anxiety, high aggression; high anxiety, low aggression; high anxiety, high aggression. To achieve this, dogs were first assigned composite scores for aggression and anxiety based on their respective subscale scores. Composite scores for aggression were calculated as the mean of stranger-directed, dog-directed, and owner-directed aggression subscales. Familiar dog aggression (FDA) was not used in the composite aggression score for two reasons relating to the participant interpretation of the questions: first, there were a large number of missing values for dogs who lived alone as the questions are not applicable to their living arrangements, and second; there were unexpected responses highlighted by the Diet & Lifestyle questionnaire leading to the development of a follow-up study presented in Chapter 3. Composite anxiety scores were the mean of stranger-directed fear, dog-directed fear, nonsocial fear, and separation related issues.

To create the four behavioural groups, the composite scores were then split by the median for each measure, with dogs below the median score assigned to the lower category and those equal to or above the median assigned to the higher category. Although the category "higher" reflects that the dog scored the same or greater than half of the dogs in the cohort, it should not be interpreted as inherently "high anxiety" or "high aggression". Indeed, given the community-based sample, we did not expect to recruit dogs who were highly anxious or highly aggressive, as those dogs are likely rare, and owners of such dogs may be reluctant to involve the dog in a non-clinical research study. Specific behaviours reported in the Diet & Lifestyle questionnaire were counted and analysed using chi-square tests to determine if the assigned behavioural groups, based on composite score, could be differentiated by the owner-reported behaviours.

While each C-BARQ subscale used in the creation of the composite scores measures different aspects of aggression or anxiety, the calculation of a single score for the purposes of this study captures all relevant incidents of aggression or anxiety reported across the C-BARQ. The composite score is not intended to be inherently more meaningful than the individual subscales; rather, it allows a broader categorization of aggression or anxiety and provides a single score to be used as a proxy for evaluating this non-clinical cohort, which is anticipated to score relatively low for behavioural problems, across multiple analyses in this study.

2.3.7 Correction for Multiple Comparisons

Due to the exploratory nature of these analyses, and number of comparisons made per test, it was necessary to adjust the p-value accepted as statistically significant for test outcomes, as each analysis increases the likelihood of Type 1 error (Armstrong, 2014). For analyses in which I explored how specific factors related to 10 C-BARQ subscale scores, the Bonferroni method was used, and only p-values less than 0.005 were considered statistically significant, with values $0.005 < p < 0.05$ considered marginally significant. Similar adjustments to p-values were made in other analyses, depending on the number of comparisons made. Statistical analyses were completed in Jamovi (version 1.6.23.0).

2.4 RESULTS

2.4.1 Participant Demographics & Participation in C-BARQ

A total of 494 participants completed the Diet & Lifestyle questionnaire, with approximately half of those (N = 235) continuing to complete the full C-BARQ. The age range of dogs in this study were from 12 weeks to 18 years old, and there were no significant differences in lifestyle factors noted between those who did or did not complete the C-BARQ (Mann-

Whitney U). There were 96 female dogs (7 intact, 89 spayed), and 139 male dogs (20 intact, 119 neutered) whose owners completed both questionnaires. The distribution of C-BARQ scores for participants who completed both questionnaires is provided in Figure 2.1.

2.4.2 Daily Activity & Behaviour

Most (95.7%) respondents reported that their dog participated in between two to seven activities. The number of activities in which a dog participated was significantly correlated with the C-BARQ trainability score (Figure 2.2.A; $r_s=0.301$, $p<0.001$), and marginally significant relationships were seen with dog-directed aggression (Figure 2.2.B; $r_s=-0.153$, $p=0.020$) and familiar dog aggression (Figure 2.2.C; $r_s=-0.178$, $p=0.020$). Trainability scores of dogs participating in two activities (Mean ± SD, 2.27 ± 0.132) were marginally lower than those participating in six (2.89 ± 0.09, W=4.69, p=0.026) or seven activities (2.90 ± 0.10, W=4.39, p=0.050) (Kruskal Wallis with DSCF pairwise comparisons).

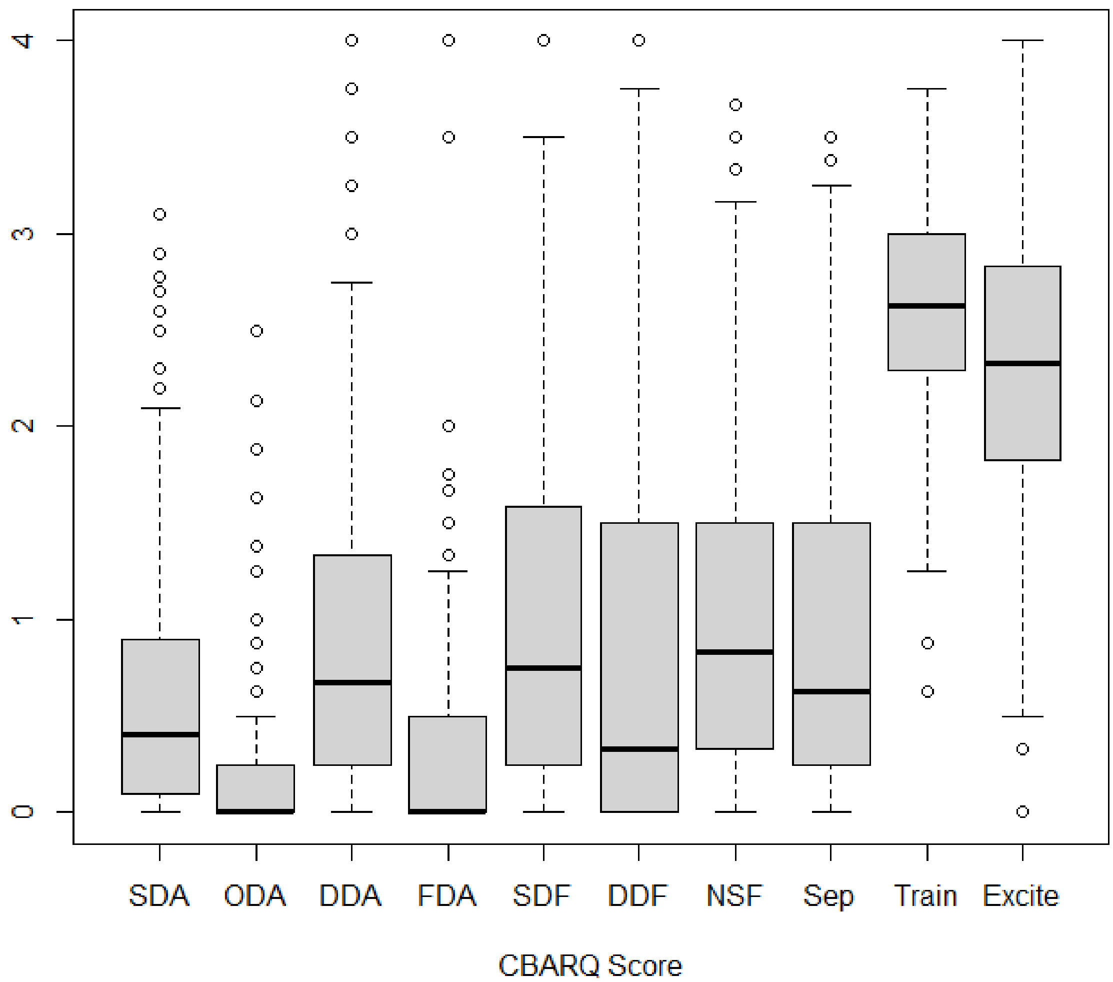

Figure 2.1. Distribution of C-BARQ subscale scores for 235 dogs who completed the full online C-BARQ. C-BARQ subscales included stranger-directed aggression (SDA), owner-directed aggression (ODA), dog-directed aggression (DDA), familiar dog aggression (FDA), stranger-directed fear (SDF), dog-directed fear (DDF), nonsocial fear (NSF), separation-related issues (Sep), trainability (Train) and excitability (Excite). Scores ranged from 0 (No concern) to 4 (Most concern) on a Likert scale, with the exception of trainability (0: Most concern to 4: no concern).

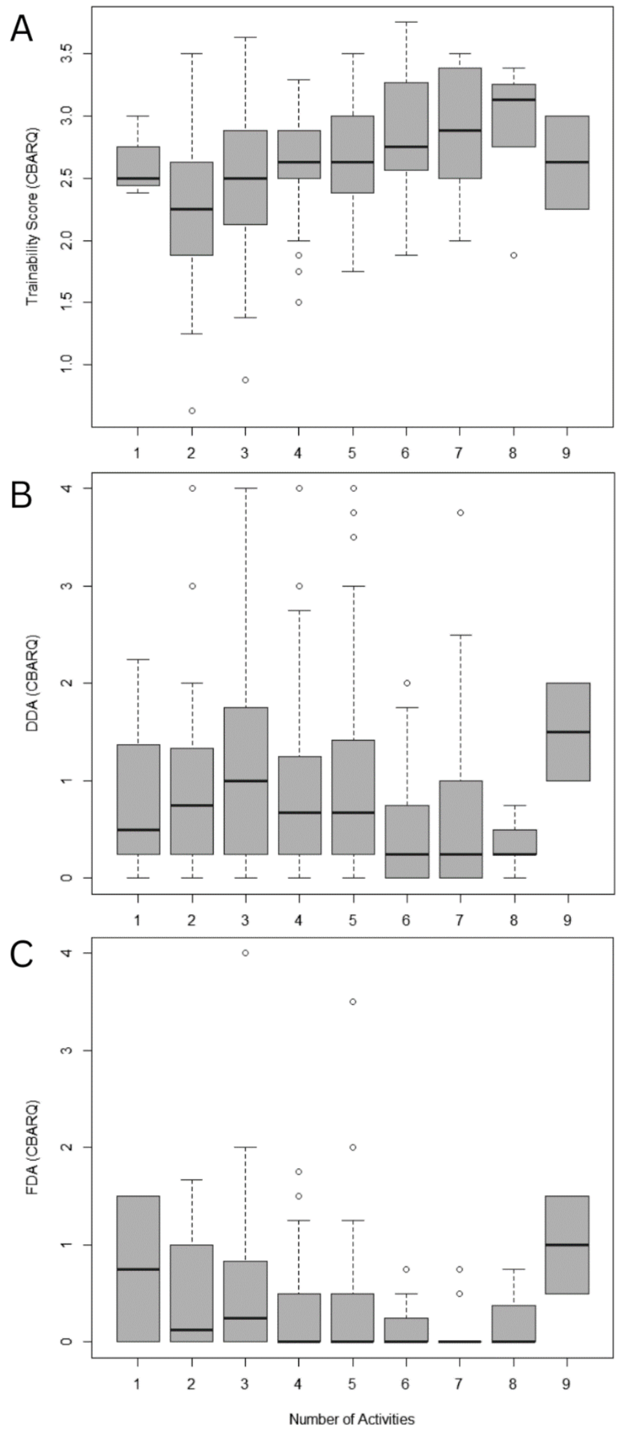

Figure 2.2. C-BARQ subscale distributions for (A) trainability, (B) dog-directed aggression (DDA) and (C) familiar dog aggression (FDA) in dogs participating in 1-9 different activities. Trainability was significantly correlated with number of activities (r_s=0.301, p<0.001), and marginally significant relationships were seen with dog-directed aggression (r_s=-0.153, p=0.020) and familiar dog aggression (r_s=-0.178, p=0.020).

When asked about the frequency of a dog's outings away from the property, most owners (43.8%) responded "Daily", while a similar number responded "More than once per week" (42.5%). Only two owners (0.8%) reported that their dog never left the home, and 12.7% responded with "Once per week or less". There was a weak but marginally significant negative correlation between the frequency of a dog's outings and both familiar dog aggression (r_s=-0.162, p=0.035) and excitability (r_s=-0.150, p=0.021) (Figure 2.3). Pairwise comparisons indicated that dogs leaving the property more than once per week had slightly higher excitability scores (2.45 ± 0.07) than those leaving the property daily (2.16 ± 0.07), although this difference was only marginally significant (W=-4.335, p=0.012; Kruskal Wallis). No significant differences were seen in pairwise comparisons between groups for familiar dog aggression. The amount of time spent off the property was not significantly correlated with any C-BARQ behavioural subscale.

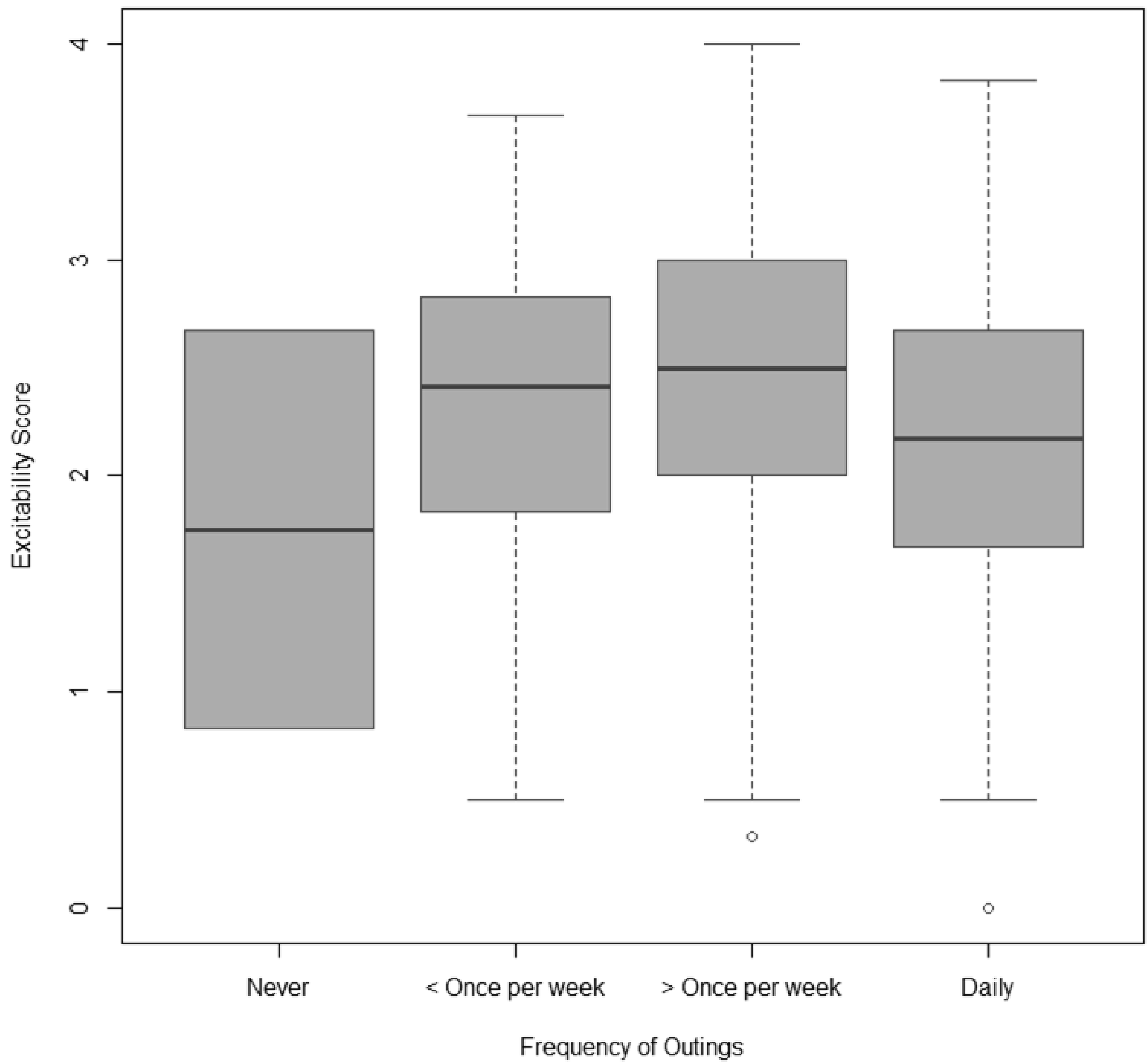

Figure 2.3. Excitability subscale scores for dogs who never left the home (N=2), left once per week or less (N=30), more than once per week (N=100) and daily (N=103). A marginally significant negative correlation was found (r_s=-0.150, p=0.021).

A majority of participants (53.6%) reported that their dog did not participate in any social activities, such as visits to the dog park, dog daycare, or walks with friends. Of the dogs involved in social activities, most participated in either one (31.4%) or two (12.3%) social activities. A significant negative correlation was seen between number of social activities and both excitability (r_s=-0.205, p=0.002) and dog-directed aggression (r_s=-0.202, p=0.002). Stranger-directed aggression was marginally negatively correlated (r_s=-0.152, p=0.020) with the number of social activities. Due to the differences in how many dogs were reported to participate in the different activity categories, this variable was dichotomized into "Did" or "Did Not" participate in social activities (Figure 2.4). Dogs participating in social activities had significantly lower scores for dog-directed aggression (0.769 ± 0.09) than those who did not (1.12 ± 0.09) (U=5101, p=0.003, Mann-Whitney U), and excitability scores were also significantly lower in those dogs participating in social activities (2.15 ± 0.07) compared to those who did not (2.43 ± 0.06) (U=5444, p=0.006, Mann-Whitney U).

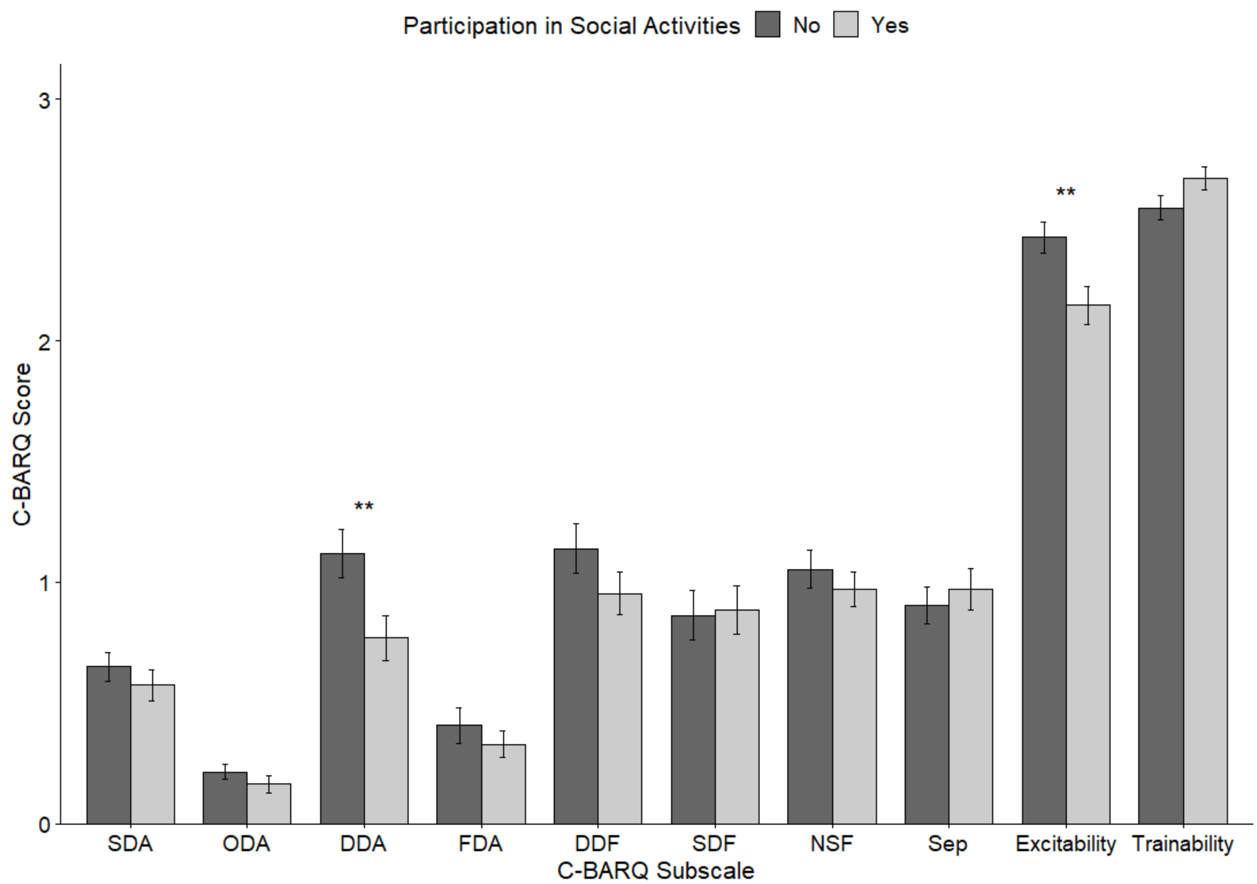

*Figure 2.4. C-BARQ subscale scores for dogs who participated in social activities (light grey, N=109) compared to those who did not participate in any social activities (dark grey, N=126). C-BARQ subscales included stranger-directed aggression (SDA), owner-directed aggression (ODA), dog-directed aggression (DDA), familiar dog aggression (FDA), stranger-directed fear (SDF), dog-directed fear (DDF), nonsocial fear (NSF), separation related issues (Sep), excitability and trainability. Significant differences (denoted with **) were seen for dog-directed aggression (p=0.003) and excitability (p=0.006, Mann-Whitney U).*

2.4.3 Presence of Conspecifics

Of the 235 dog owners who completed the C-BARQ, 33.2% reported that their dog lived with one or more conspecifics (other dogs), while 66.8% reported that their dog lived alone in their household. Dogs who lived alone had significantly lower scores for stranger-directed aggression scores (0.497 ± 0.04, n=157) than those living with other dogs (0.863 ± 0.08, n=78, U=4183, p<0.001, Mann-Whitney U), as well as lower stranger-directed fear scores (0.701 ± 0.07 vs. 1.23 ± 0.14; U=4615 p=0.004, Figure 2.5). Dog-directed aggression scores were also significantly lower in dogs living alone (0.814 ± 0.07, compared to 1.24 ± 0.131; U=4437, p=0.003, Figure 2.5). Dogs living alone also showed lower scores for dog-directed fear, but this difference was marginally significant (0.943 ± 0.07, compared to 1.27 ± 0.13; U=4832, p=0.036, Figure 2.5). Similarly, dogs living alone received marginally significant lower nonsocial fear scores (0.913 ± 0.05, compared to 1.22 ± 0.10; U=4951, p=0.029, Figure 2.5). While a pattern of lower scores in singleton dogs was seen across all other subscales, these differences were not statistically significant. Curiously, while the familiar dog aggression (FDA) score should not have been provided for those who did not live with conspecifics, 92 participants who owned only one dog completed the FDA questions to obtain a score. As with the other subscale scores reported, the FDA scores of these singleton dogs was marginally lower than those for dogs living in a multi-dog household (U=2925, p = 0.021). This finding prompted the deeper investigation into the FDA questions in Chapter 3.

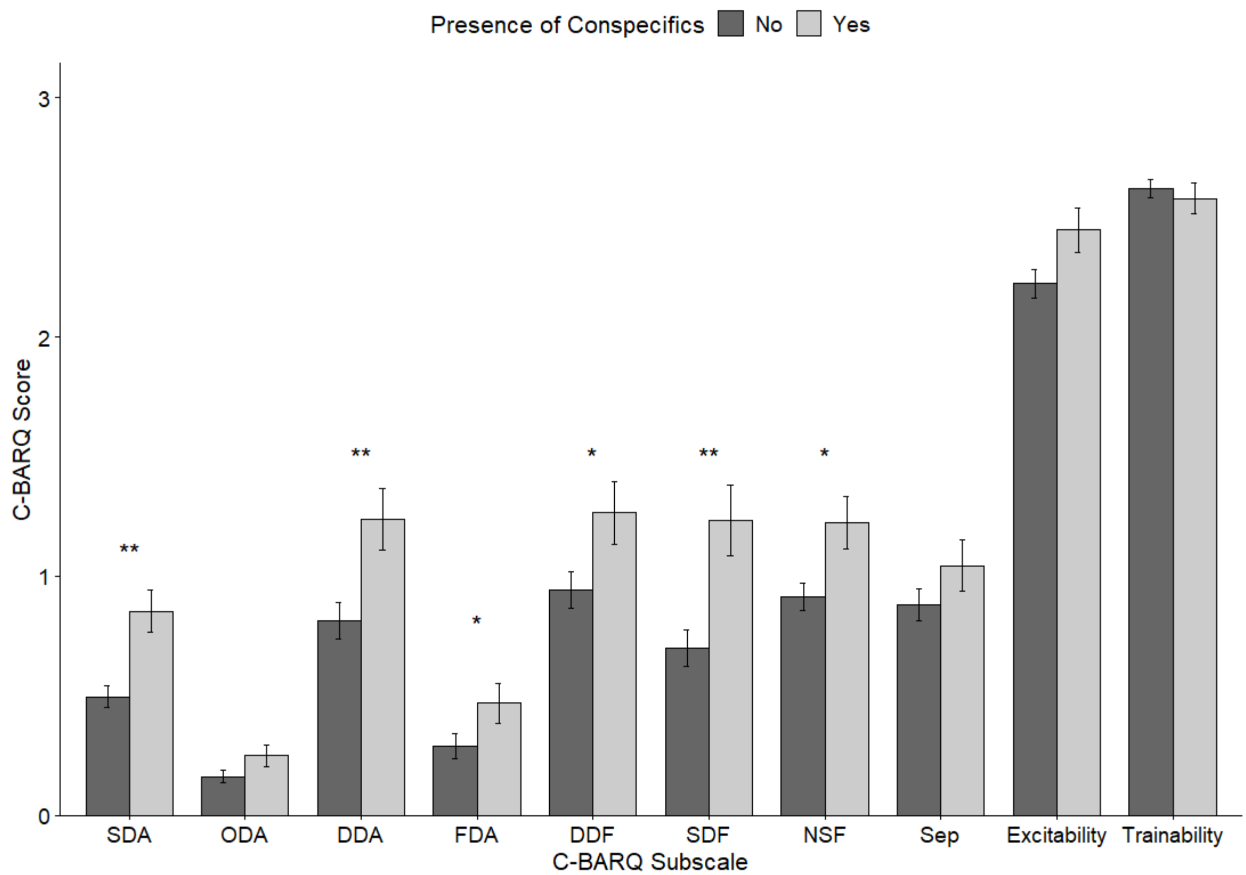

*Figure 2.5. C-BARQ subscale scores for dogs who either lived with (light grey bars, N=78) or did not live with (dark grey, N=157) conspecifics. C-BARQ subscales included stranger-directed aggression (SDA), owner-directed aggression (ODA), dog-directed aggression (DDA), familiar dog aggression (FDA), stranger-directed fear (SDF), dog-directed fear (DDF), nonsocial fear (NSF), separation-related issues (Sep), excitability and trainability. Groups denoted with ** were statistically different for stranger-directed aggression (p<0.001), dog-directed aggression (p=0.003) and stranger-directed fear (p=0.004), and marginally different (denoted with *) for dog-directed fear (p=0.036) and nonsocial fear (p=0.029) (Student's T-test, Mann-Whitney U). FDA scores were also marginally different (p=0.021); however, those living alone (light grey) should not have received a score for this subscale.*

2.4.4 Observable Behaviours Differ between Composite Anxiety/Aggression Subgroups

As described above, dogs were assigned to a higher or lower grouping based on the median split of composite anxiety and aggression scores. In this sample (n = 235), the median composite anxiety score was 0.835, while the median aggression score was 0.397. When aggression and anxiety groups were cross-tabulated for this analysis, a larger proportion of dogs were in the higher aggression/higher anxiety (n=85, 36.2%) and the lower aggression/lower anxiety groups (n=83, 35.3%) than expected by chance. Relatively fewer dogs were in the lower anxiety/higher aggression group (14.5%) or higher anxiety/lower aggression group (14.0%) (Table 2.2). This assortment of dogs indicated that anxiety and aggressions scores were not independent for individual dogs (chi-square test for goodness of fit test, $\chi^2(3) = 43.45$, $p<0.0001$).

Assignment of dogs to one of the four behavioural groups created by their anxiety and aggression score was strongly associated with differential proportions of behaviour towards unfamiliar people, specifically, lunging, barking, staring, wagging tail, excited behaviour and moving away (Table 2.2). Dogs in the high anxiety/high aggression group were reported to lunge (18.82%), bark (63.52%), and stare (37.64%) more than any other behavioural group, while dogs in the low anxiety/low aggression group were reported most frequently to display a wagging tail (81.92%). Dogs in the high anxiety/low aggression group were reported as more frequently moving away from unfamiliar people (39.39% of the dogs), and owners reported low occurrences of lunging (6.06%) and barking (18.18%) for these dogs. Dogs in the low anxiety/high aggression group were reported to appear excited (61.76%), with a wagging tail (64.70%), and a

Table 2.2. Behaviours reported by owners in the Diet & Lifestyle questionnaire when dogs observed either unfamiliar people or dogs. The percentages displayed represent the proportion of dogs within each behavioural group who displayed the behaviour, with chi-square values presented for each behaviour across aggression and anxiety groups. To adjust for multiple comparisons across 9 behaviours in each analysis, p-values of <0.006 are considered significant (bold & italics), while values between 0.006 – 0.05 are considered marginally significant (italics).

	Total (n=235)		Low Anx Low Agg (n=83)		Low Anx High Agg (n=34)		High Anx Low Agg (n=33)		High Anx High Agg (n=85)			
Towards People												
	n	%	n	%	n	%	n	%	n	%	χ2	p
Lunging	***24***	***10.21***	***4***	***4.81***	***2***	***5.88***	***2***	***6.06***	***16***	***18.82***	***16.40***	***0.0009***
Barking	***85***	***36.17***	***14***	***16.86***	***11***	***32.35***	***6***	***18.18***	***54***	***63.52***	***45.79***	***<0.0001***
Staring	***59***	***25.11***	***14***	***16.86***	***4***	***11.76***	***9***	***27.27***	***32***	***37.64***	***13.41***	***0.0038***
Pulling Towards	96	40.85	38	45.78	11	32.35	11	33.33	36	42.35	2.70	0.439
Wagging Tail	***152***	***64.68***	***68***	***81.92***	***22***	***64.70***	***21***	***63.63***	***41***	***48.23***	***20.88***	***0.0001***
Excited	*122*	*51.91*	*51*	*61.44*	*21*	*61.76*	*18*	*54.54*	*32*	*37.64*	*11.37*	*0.0099*
Interested/Calm	72	30.64	32	38.55	12	35.29	10	30.30	18	21.17	6.37	0.09
Move Away	***44***	***18.72***	***3***	***3.61***	***3***	***8.82***	***13***	***39.39***	***25***	***29.41***	***30.28***	***<0.0001***
Ignore	49	20.85	18	21.68	10	29.41	5	15.15	16	18.82	2.41	0.49
Towards Dogs												
	n	%	n	%	n	%	n	%	n	%	χ2	p
Lunging	***64***	***27.23***	***7***	***8.43***	***12***	***35.29***	***3***	***9.09***	***42***	***49.41***	***42.49***	***<0.0001***
Barking	***114***	***48.51***	***19***	***22.89***	***22***	***64.71***	***8***	***24.24***	***65***	***76.47***	***61.84***	***<0.0001***
Staring	***91***	***38.72***	***25***	***30.12***	***13***	***38.23***	***8***	***24.24***	***45***	***52.94***	***12.75***	***0.005***
Pulling Towards	150	63.83	49	59.03	24	70.58	18	54.54	59	69.41	3.87	0.275
Wagging Tail	134	57.02	51	61.44	16	47.05	23	69.69	44	51.76	5.16	0.160
Excited	*114*	*48.51*	*47*	*56.62*	*17*	*50.0*	*19*	*57.57*	*31*	*36.47*	*8.23*	*0.041*
Interested/Calm	*57*	*24.25*	*28*	*33.73*	*7*	*20.58*	*11*	*33.33*	*11*	*12.94*	*11.71*	*0.008*
Move Away	*24*	*10.21*	*2*	*2.41*	*2*	*5.88*	*6*	*18.18*	*14*	*16.47*	*12.12*	*0.006*
Ignore	44	18.72	16	19.28	8	23.52	7	21.21	13	15.29	1.324	0.723

considerable amount of barking (32.35%) upon meeting a stranger. The occurrence of pulling towards strangers, ignoring, and interested/calm behaviours were not significantly different between behavioural groups.

When reporting behaviours towards unfamiliar dogs, the proportion of dogs engaging in lunging, barking, staring, interested/calm behaviour and moving away from the dog were significantly different between groups (Table 2.2). Within the high anxiety/high aggression group, a high proportion of dogs were reported to lunge (49.41%), bark (76.47%) and stare (52.94%) at other dogs. These dogs also showed the lowest occurrence of interested/calm behaviours (12.94%). Dogs in both of the lower aggression groups (i.e., higher anxiety/lower aggression and lower anxiety/lower aggression) were reported to show interested/calm behaviours (33.33% and 33.73%, respectively), whereas dogs with higher anxiety were reported to more often to move away from unfamiliar dogs (18.18% for high anxiety/low aggression dogs; 16.47% for high anxiety/high aggression dogs).

2.5 DISCUSSION

The distribution of C-BARQ subscale scores for the 235 participants are characteristic of a community sample – there is clustering of lower scores, indicating low concern for problem behaviours, across all subscales, as is typical of "normal" pet dogs. However, there were some higher scoring dogs indicating that more severe behavioural issues were present within the population. As the possible range of either subscale or composite scores was 0-4, it is clear that this sample of dogs, on average, were neither highly anxious nor highly aggressive. The distribution of scores within this community sample is consistent with those of other studies that have used historical C-BARQ data with larger sample sizes (Duffy *et al*., 2008). The lack of significant differences in lifestyle or demographic factors between participants who completed only the Diet & Lifestyle questionnaire, or completed both questionnaires, suggests that the participants in the C-BARQ subscale analysis are representative of the larger cohort of participants. It should be noted that the use of an online modality for the questionnaires may have skewed the population towards those who had access to (and/or interest in using) the internet and/or a browsing device, which could be associated with a younger or more affluent demographic (Bautista, 2012; Hibberts *et al*., 2012). However, investigating this link is beyond the scope of the current study. In addition, given the considerable proportion of participants who did not continue on to complete the C-BARQ, future studies may consider the use of the shortened mini C-BARQ (42 question version, rather that the current 100 question version) to potentially improve retention of participants between questionnaires.

In this study, a positive correlation was seen between the number of activities in which dogs participated and their trainability score. This link between trainability and activities is likely somewhat circular. For example, it could be argued that a highly trainable dog can be readily exercised and socialized, providing ample opportunity for exposure and learning in real life scenarios, strengthening the owner-dog relationship, as well as increasing the likelihood of further participation in activities. On the other hand, a dog with a lower trainability score may be limited in their participation in such events as an owner may opt to avoid potentially challenging situations, leading to fewer training opportunities and reducing the owner's motivation to participate in activities with their dog. It could be suggested that the snowballing of these experiences is reflected in other subscales in this study – both dog-directed and familiar dog aggression scores were also lower in dogs participating in more activities, and although these findings were only marginally significant in the current study, it has been noted in other studies that infrequent participation in activities is associated with higher scores for fear and aggression

(Hakanen *et al*., 2020; Mikkola *et al*., 2021; Puurunen *et al*., 2020). I initially hypothesized that dogs who participated in more frequent activities and spent more time away from the property would have lower scores for subscales related to anxiety and aggression, and score more highly for trainability. While the data demonstrate higher trainability scores in dogs participating in more activities, there was little evidence that dogs who participated in more activities were significantly less anxious or aggressive. Of course, this could be the result of a floor effect, given that my sample of dogs did not include many dogs who could be considered highly anxious or aggressive. Similarly, there was some evidence to support the notion that participation in social activities is somewhat associated with lower C-BARQ subscale scores for problematic behaviours; however, the only significant differences were seen in dog-directed aggression and excitability scores. Dogs who participated in social activities had significantly lower dog-directed aggression scores, which is to be expected considering a highly dog-aggressive dog would pose a risk to the safety of other dogs in these contexts, and conscientious owners are unlikely to bring such a dog to social activities. However, it is unclear why dogs participating in social activities had significantly lower excitability scores. It could be suggested that, given social interactions are considered a welfare need for dogs (Griffin *et al*., 2023), these dogs may exhibit reduced excitability as a result of having these needs met. Alternatively, it is possible that these dogs are simply considered by their owners to be able to participate in social activities because they are less excitable, and thus easier to manage in social contexts.

It is possible that the events a dog experiences during exercise, rather than their total time spent exercising, would be more informative in terms of the impacts of their outings on their behaviour. For example, a dog who regularly experiences a perceived threat, such as an unfamiliar person or dog on a leash, will acquire more negative experiences in these situations than a dog who exercises in quieter locations. The upregulation of the nervous system associated with this stress response, in conjunction with the outcomes of these experiences, could contribute to the resulting development or absence of behavioural problems. On the problematic end of the spectrum, dogs who have negative experiences in these situations will learn from previous encounters and could be more likely to show aggressive behaviours in subsequent encounters, whereas dogs who have positive experiences around unfamiliar people and dogs would learn that there is no actual threat in these contexts, thus reducing the likelihood of displaying aggressive behaviour. Some research suggests that dogs with lower levels of serotonin are more likely to exhibit aggressive behaviours (Siracusa, 2021), and given that exercise is associated with increased levels of serotonin and its pre-cursor, tryptophan (Wilson & Marsden, 1996), physical exercise may be beneficial in the supportive treatment of behavioural issues, in particular aggression. The notion of exercise as an adjunct treatment for psychiatric disorders is widely supported in humans (Salmon, 2001; Muldoon *et al*., 2004), and multiple studies have demonstrated the effects of exercise on the neurochemistry of the brain in rats (Chaouloff *et al*., 1985; Gomez-Merino *et al*., 2001; Rueter & Jacobs, 1996; Wilson & Marsden, 1996). In dogs, a study of Labrador Retrievers noted less aggression, and less fear of humans and objects, in dogs who were exercised for longer periods (Lofgren *et al*., 2014). However, there are no studies directly addressing the impacts of exercise on the range of canine behaviour problems addressed in the C-BARQ. Thus, further study should quantify the use of exercise as a treatment for behaviour problems in dogs.

In this study, 33.2% of owners reported they lived with more than one dog, while 66.8% had one dog living alone in their home. Based on the current literature, it was expected that dogs who live with other dogs would receive lower scores in fear-related subscales, and both dog-directed and stranger-directed aggression. Interestingly, dogs living with conspecifics actually scored somewhat higher for all subscales, with the exception of trainability. Scores were significantly higher in dogs living with conspecifics for stranger-directed aggression, dog-directed aggression, and stranger-directed fear, and marginally higher for dog-directed fear and nonsocial fear. These findings are not completely in agreement with some other published research regarding dog-dog interactions. For example, a lack of regular exposure to conspecifics has previously been shown to be associated with increased aggression towards people (Mikkola *et al*., 2021), higher levels of nonsocial fear (Hakanen *et al*., 2020; Tiira *et al*., 2016), and increased noise sensitivity (Handegård *et al*., 2020). Interestingly, these aforementioned studies were all conducted in European countries, which raises the question whether differences between dog populations, management practices, and/or owner expectations may account for some portion of the apparent differential effects of conspecifics on aggression and fear-related behaviours. As well, working dogs housed alone as puppies had less successful training outcomes compared to those housed with other puppies (Serpell & Duffy, 2016). In this study, trainability did not differ significantly between dogs living with or without conspecifics, however it is possible that owners of singleton dogs enlisted in this study potentially invested more time in socializing and/or training their dogs than the aforementioned studies, resulting in less severe behavioural issues in comparison to the other published research.

Given the greater probability for interactions related to resources in multi-dog homes, it was expected that dogs living with conspecifics would score higher for both owner-directed, and familiar dog aggression. In fact, dogs living with conspecifics scored marginally higher only for familiar dog aggression. Previous studies have found increased levels of resource-related aggression in multi-dog homes (Jacobs *et al*., 2018; McGreevy & Masters, 2008), albeit not measured with the C-BARQ. It is possible that all the dogs in this study were simply less aggressive towards familiar people and dogs in comparison to other studies that have noted aggression issues in multi-dog homes (Jacobs *et al*., 2018; McGreevy & Masters, 2008). Indeed, a direct comparison with these studies is challenging as they did not use the C-BARQ to measure the severity of the issues. In the current study, both singleton and multi-dog home groups reported lower scores on average for owner-directed and familiar dog aggression in comparison to stranger-directed and dog-directed aggression, which appears to be a typical pattern in larger C-BARQ studies (e.g., Duffy *et al*., 2008). The lack of expected significant differences between the singleton vs. multi-dog home dogs in this community sample could simply reflect a lack of severe behavioural issues relating to resources for the dogs. It is also plausible that the multi-dog homes in this study implement management or training strategies to prevent the escalation of aggressive behaviours between dogs, such as feeding dogs separately and providing safe sleeping areas (Benoit, 2019), implementing training protocols to prevent the development of problem behaviours (Gonzalez-Martinez *et al*., 2019), or a selection of various methods used to address existing problems (Casey *et al*., 2014; Mehrkam *et al*., 2020). While these are possible explanations for the lack of differences in the current study between single-dog and multi-dog homes, these questions relating to management and training were not directly asked in the questionnaire. Thus, it is not possible to draw conclusions related to day-to-day management of behaviour in these participants. As correlations have been shown previously between familiar dog aggression and multiple other C-BARQ subscale scores (Casey *et al*., 2014; Jacobs *et al*., 2018; Rayment *et al*., 2016; van der Borg *et al*., 2017), future research should continue to consider all C-BARQ subscales when investigating the relationship between living arrangements and behavioural issues in dogs.

In this study, dogs were grouped into higher and lower anxiety and aggression groups based on the median split of their composite scores in preparation for the gut study in Chapter 4. The different behaviours displayed by dogs in each group provides support that the groupings used in this study are meaningful. Some behaviours, such as “pulling towards” and “ignore”, did not differ between groups, suggesting these behaviours are not helpful in determining how anxious or aggressive a dog may be. Lunging, barking, and staring appeared to be quite polarized behaviours between groups – when the target of the behaviour was unfamiliar people, dogs in the higher aggression/ higher anxiety group were more often reported to exhibit these behaviours, whereas all three other behavioural groups were reported to display these behaviours more rarely. Interestingly, when the target of the behaviours was unfamiliar dogs, more dogs in both of the higher aggression groups displayed lunging, barking, and staring, while the two behavioural groups associated with lower aggression were reported less often to display these behaviours. This would suggest that when aggressive behaviours such as lunging, barking, and staring are directed towards humans, there is a high likelihood that fear may be motivating the aggression as these dogs are scoring higher for both aggression and anxiety. This is also supported by the evidence that, in this study and others, aggression and anxiety are not independent of each other (Tiira *et al*., 2016; Salonen *et al*., 2020; Stellato *et al*., 2021; van der Borg *et al*., 2017). However, when aggressive behaviours are directed towards other dogs, anxiety appears to play less of a role in the display of these behaviours. While most aggressive behaviour is a response to a perceived threat (Carlson & Birkett, 2017; Siracusa, 2021), it could be suggested that the dogs in this study are behaving aggressively towards unfamiliar dogs for other reasons, such as competition for resources, or perhaps predatory aggression directed towards smaller dogs. Given that the dogs in the lower anxiety/ higher aggression group were also displaying similar levels of lunging, barking, and staring, it could be argued that these lower anxiety dogs are, in fact, displaying signs of frustration or lack of training rather than intimidation tactics. However, it would also be expected that these dogs would also display some signs of more affiliative or excitable behaviour – "wagging tail" did not differ significantly between groups in relation to unfamiliar dogs, although it was reported significantly less often in the higher aggression/higher anxiety group when compared to all three other groups as a reaction around unfamiliar people. Interestingly, one hypothesis for the purpose of a wagging tail suggests it has been artificially selected for by humans due to our preferences for a rhythmic stimulus in communication (Leonetti *et al*., 2024), which may explain its significance here when dogs are presented with unfamiliar people, but not unfamiliar dogs. Similarly, the lack of significance of the wagging tail when presented with unfamiliar dogs could be related to the function of the tail wag in these contexts – both the speed of tail movement and height of carriage can be used to convey a range of signals when interacting with other dogs, such as asserting dominance or indicating submission (Borg *et al*., 2015), and inviting play (Horowitz, 2009). Thus, "wagging tail" may be too vague of a descriptor to elucidate any significant differences. The display of "excited" behaviour was also only marginally different between groups; thus, it cannot be stated what underlying motivators are driving these behaviours. Finally, there was some evidence that avoidant behaviours were representative of behavioural group, particularly in relation to anxiety. The dogs most likely to "move away" were those in the higher anxiety/lower aggression group, regardless of whether they were moving away from unfamiliar people or dogs. This provides evidence that dogs who score high for anxiety but low for aggression can, and do, perform avoidance behaviours to avoid potential conflict. As these findings comparing the behaviours indicated by the Diet & Lifestyle questionnaire to dog groupings for higher and lower anxiety and aggression based on C-BARQ subscales are preliminary, further research that attempts to predict dog behavioural phenotypes for aggression and anxiety, based on behaviours towards unfamiliar people and unfamiliar dogs, could be fruitful, particularly if a wider range of dogs (i.e., those with more problematic anxiety or aggression) could be sampled.

Interestingly, I did not detect any breed-related differences in C-BARQ subscale scores in this analysis. Breed has previously been shown to have significant associations with the trainability subscale score (Serpell & Hsu, 2005), along with stranger-directed, dog-directed, and owner-directed aggression scores (Duffy *et al*., 2008). In addition, both of these C-BARQ studies (Serpell & Hsu, 2005; Duffy *et al.*, 2008) found differences in subscale scores between show-line and working-line dogs, which suggests a genetic contribution to the underlying behaviours. In my study, it is possible that the use of a community sample may have limited the potential for a breed comparison, as many owners reported their dog's breed ambiguously (for example, "husky mix" or "terrier mix"). Breeds were assigned to one of 24 breed groups (based on Parker *et al*., 2017), but the behaviour analysis did not show any breed differences. Future study would benefit from recruiting dogs of a known genetic origin via organizations such as the Canadian Kennel Club in order to fully investigate the relationship between breed and C-BARQ subscale scores.

One the most interesting findings for this investigation was the number of owners who received a score for familiar dog aggression. The purpose of the familiar dog aggression questions is to derive a score reflecting the severity of "threatening or hostile responses to other familiar dogs in the same household", and as such, owners of single dogs should not receive a score as the questions are not relevant to their living situation. As reported, a large proportion of single-dog homes in this study (58.5%) received a score for familiar dog aggression. Thus, the follow-up questionnaire presented in the following chapter was developed to better understand why this occurred.

2.6 CONCLUSIONS

This chapter provides evidence that living arrangements (with or without conspecifics), and participation in social activities, are significantly associated with the severity of problem behaviours in companion dogs. There was surprisingly less evidence for the roles of exercise and time spent off the property in the reporting of problem behaviours; however, this study has highlighted the importance of collecting demographic data to establish robust inclusion criteria, and ensuring owner-reported surveys use precise language to facilitate accurate interpretation of the questions.

The majority of dogs in this study were representative of a community sample (showing low to moderate concern for problem behaviours), scoring lower for problem behaviours than would be expected in a clinical sample (i.e., they were average pet dogs, who have not been referred to a veterinarian or behaviourist for behavioural problems). However, participants with dogs who lived with conspecifics did report greater concern for aggression and fear-related behaviours than did owners of dogs who lived alone, and it is recommended that owners of multiple dogs pay particular attention to management within the home to reduce potential conflict around resources (quantified by owner-directed and familiar dog aggression). Owners of multi-dog homes should also ensure that the individual dogs within the home receive an adequate amount of training and socialization to prevent the escalation of problem behaviours both on and off the property, such as stranger-directed and dog-directed fear and aggression. While regular activity and excursions off the property are undoubtedly beneficial for the dog and owner, there was little evidence in this study to support a relationship to problem behaviours; however, based on the current literature, owners would likely benefit from exercise curated to the dog's needs and capabilities (for example, exercising a dog-aggressive dog away from busy locations) to manage behavioural issues and prevent the escalation of aggressive behaviours. In addition, dogs which show different combinations of reported aggression and anxiety, as categorized in this study using composite scores from appropriate C-BARQ subscales, appear to be more or less likely to respond to unfamiliar dogs and people with specific behaviours, such as staring, barking, lunging, wagging tail, and moving away. Further research into how these behaviours may help identify or differentiate motivational states underlying such responses could help owners and trainers more efficiently address any behavioural concerns around anxiety and aggression.

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CHAPTER 3: INVESTIGATION OF OWNERS' INTERPRETATION OF THE FAMILIAR DOG AGGRESSION SUBSCALE

3.1 ABSTRACT

The familiar dog aggression (FDA) subscale of the Canine Behaviour and Research Questionnaire (C-BARQ) consists of four questions relating to threatening or hostile responses towards familiar dogs in the same household. In my first study, 92 of the 157 participants who owned one dog responded to the questions for familiar dog aggression, thereby obtaining an unexpected FDA score. This finding prompted a follow-up study to investigate how these participants interpreted the FDA questions. An additional questionnaire, including the four C-BARQ FDA questions, was distributed to dog owners who had previously participated in the initial study. Singleton dogs belonging to owners who unexpectedly answered the FDA questions had marginally lower dog-directed, stranger-directed, and nonsocial fear scores compared to singleton dogs belonged to owners who (correctly) did not respond to the FDA questions, as well as lower nonsocial fear score compared to dogs living in multi-dog households. These dogs with an unexpected FDA score were reported to have regular participation in social activities with other dogs, but were not more likely to have previously lived with other dogs. The FDA subscale scores increased for the majority dogs who participated in the follow-up questionnaire, likely due to demand characteristics of the current study design in presenting the FDA questions out of the context of the complete C-BARQ. I discuss ways in which the C-BARQ could be modified in its use and delivery to clarify the interpretation of the FDA questions, and consider the potential limitations of the use of the FDA subscale scores in the current literature.

3.2 INTRODUCTION

The familiar dog aggression (FDA), or dog rivalry, subscale in the Canine Behaviour and Research Questionnaire (C-BARQ), is defined as “threatening or hostile responses to other familiar dogs in the same household” (Duffy & Serpell, 2008). This subscale consists of four questions that owners are posed pertaining to specific contexts in which a dog may show varying signs of aggression towards another dog, including: 1) Towards another (familiar) dog in your household (Q32); 2) When approached at a favourite resting/sleeping place by another (familiar) household dog (Q33); 3) When approached while eating by another (familiar) household dog (Q34); and 4) When approached while playing with/chewing a favourite toy, bone, object etc., by another (familiar) household dog (Q35). While the term “familiar dog” is not explicitly defined in the C-BARQ for respondents, note that the wording in each question includes “another (familiar) household dog”, and is intended to refer to another dog living with the dog being assessed by the C-BARQ.

Some studies using the C-BARQ have acknowledged the environmental requirement that a dog should be living with at least one conspecific to receive an FDA score, and note that these questions would be irrelevant for dogs living alone, resulting in non-responses to the FDA/dog rivalry questions for singleton dogs (e.g., Duffy *et al*., 2008; Friedrich *et al*., 2019; Lofgren *et al*., 2014). To improve the accuracy of subscale scores when averaged across multiple questions, researchers may define a reasonable threshold for missing values in their subscale calculation – for example, in one study by the creators of C-BARQ; if more than 20% of items within a subscale had missing values, the mean subscale score would be not be calculated excluded for that individual (Duffy & Serpell, 2012). In the above studies, similar screening processes flagged high numbers of missing values within the FDA subscale, highlighting that this subscale is not relevant to all dog owners. However, a considerable number of studies reporting significant findings for familiar dog aggression do not indicate that they have screened for missing values (Dodman *et al*., 2018; Hare *et al*., 2021b; McMillan *et al*., 2011, 2016), or provide an accurate definition of familiar dog aggression (Dodman *et al*., 2018; Hare *et al*., 2021b; McMillan *et al*., 2016; Plueckhahn *et al*., 2023; Rayment *et al*., 2016; Wauthier & Williams, 2018; Zapata *et al*., 2022a). In fact, none of these cited studies acknowledge how the interpretation/inclusion of dog rivalry scores may be problematic. Although precise methodological information is sometimes lacking in detail, studies that have reported significant findings for FDA scores indicate correlations with owner personality and gender (Dodman *et al*., 2018), negative outcomes in puppy prison programs for dogs with higher FDA scores (Hare *et al*., 2021a), increased FDA scores in dogs with atopic dermatitis (McAuliffe *et al*., 2022), and decreased scores in dogs from either hoarding situations (McMillan *et al*., 2016) or commercial breeding establishments (McMillan *et al*., 2011). One of the most consistent findings across the literature is that familiar dog aggression is reported to differ significantly between breeds (Duffy *et al*., 2008; Hare *et al*., 2021b; Shouldice *et al*., 2019; Sumridge *et al*., 2021; Zapata *et al*., 2022b). Unfortunately, if these findings are based on FDA scores that include singleton dogs, which in the absence of reported data screening protocols seems likely, how they should be understood is not clear.

Some researchers have both reported significant findings related to FDA scores and provided their quantitative data in available supplementary materials (Chen *et al*., 2023; Lopresti-Goodman & Bensmiller, 2022; Powell *et al*., 2021; Zapata *et al*., 2022b). Of these, it appears that only one paper collected/provided relevant information on living with conspecifics: Zapata *et al*. (2022b). In the study, 59.3% of singleton dogs received a score for FDA (Supplementary Materials for Zapata *et al*., 2022b). Interestingly, while this information is available, the discrepancy is not acknowledged or reported in the study itself, so it is unknown whether FDA scores for singleton dogs were kept or discarded in data analyses. Thus, it is somewhat unclear how the study's findings related to increased FDA scores, which included dog risk factors such as being male and competing in dog sports, should be interpreted.

As reported in Chapter 2, 92 of 157 (58.5%) participants in the current study who owned only one dog provided responses to the questions for familiar dog aggression and, therefore, their dog received a FDA subscale score. The fact that such a large proportion of participants with single-dog homes answered these subscale questions raises questions around how the C-BARQ FDA score should be interpreted, not only in this thesis, but also in other studies that report on FDA results. Given the unexpected FDA responses in the current study, and an apparent lack of consistency when reporting the FDA subscale in other published studies, it seems likely that many studies may have included FDA scores for singleton dogs that do not live with other dogs in the household. If this is the case, it warrants closer examination not only of these published results, but also of how dog owners are interpreting the FDA questions when completing the C-BARQ. For example, why do some owners of singleton dogs answer the questions and, thereby, apparently ignore the text that indicates they are being asked about their dog's behaviour towards "another familiar (household) dog"? Are their dogs different from other singleton dogs (who do not receive the FDA score) in terms of their behaviours or experiences that predispose their owners to interpret the FDA questions more broadly?

In this study, I explored whether there were differences in C-BARQ subscale scores for owners who correctly interpreted the FDA questions (i.e., received an "expected" score for their living situation - specifically, either a score if more than one dog lived in their home, or no score if they lived with a single dog only), compared to owners who appear to have incorrectly interpreted the FDA questions (i.e., those with single-dog homes who nonetheless completed the questions). A difference in C-BARQ subscale scores may indicate that owners who have incorrectly interpreted the FDA questions may also have interpreted other areas of the questionnaire differently, and this would be reflected by either under-reporting or, conversely, exaggerating, their dog's behavioural issues. Of course, these dogs actually may be different behaviourally from singleton dogs who did not receive any FDA score, and/or from dogs living with other dogs in the home. As well, I explored some social factors that may impact the owner's familiarity with dogs who do not live in their household, such as the frequency and location of social activities with other dogs. Since this second study was focussed and would draw the participants' attention to the familiar dog questions, it was expected that FDA scores will increase when the four FDA questions are repeated outside of the normal presentation of the complete C-BARQ. Finally, those participants who responded to FDA questions were asked directly which "familiar" dog(s) they were thinking about when answering the FDA subscale questions.

3.3 METHODS

3.3.1 Recruitment of participants for FDA follow-up questionnaire

Dog owners who had previously completed both the Diet & Lifestyle Questionnaire and C-BARQ (between May 6 – July 5, 2021) were contacted via email and invited to participate in a follow-up questionnaire to investigate the FDA section of the C-BARQ questionnaire. The questionnaire, delivered via Qualtrics (www.qualtrics.org), was available between May 4 and June 16, 2022 and was comprised of 14 questions relating to the dog's current living arrangements, social activities, and the 4 FDA questions from C-BARQ (Supplementary Material B).

The primary focus of the questionnaire was to establish why dogs from some single-dog households received an unexpected score for FDA, and to compare these dogs' behavioural profiles to those of dogs whose owners correctly interpreted the FDA questions. Participants were grouped into the following categories for the analyses:

- Group 1: Received an unexpected score for FDA; dogs were living alone with no conspecifics
- Group 2: Did not receive a score for FDA (expected); dogs were living alone with no conspecifics
- Group 3: Received a score for FDA (expected); dogs were living in multi-dog households

In addition to these groupings based on their receipt of the FDA score, dogs were then further grouped by their conspecific cohabitation history (i.e., either had, or had not, previously lived with other dogs), and frequency of socialization both on and off their property. At the end of the questionnaire, participants were specifically asked which dog they were thinking about if/when they answered the FDA questions to better understand their interpretation of the term "familiar dog".

3.3.2 Statistical Analyses

As the data in this study were non-normally distributed, non-parametric tests were selected for the statistical analyses. C-BARQ subscale scores for dogs who received an unexpected FDA score were compared with those who received an expected score, using Mann-Whitney U tests. Further analyses used a Kruskal Wallis and Dwass-Steel-Critchlow-Fligner pairwise comparisons between single dogs with expected FDA scores, single dogs with unexpected FDA scores, and multi-dog home dogs with expected FDA scores. These analyses explored 9 C-BARQ subscales; thus, p-values were considered significant below 0.0055 after Bonferroni correction (Armstrong, 2014). Social activity, both in and out of home, was investigated for each group using chi-square test for association with Fisher's exact test. Statistical analyses were completed in Jamovi (version 1.6.23.0).

3.4 RESULTS

Of the 235 respondents of the original C-BARQ and Diet & Lifestyle questionnaires in 2021, 76 participants were successfully recruited for the 2022 follow-up study. At the time the 2022 questionnaire was delivered, 51 dogs were living as single dogs while 25 were living in multi-dog households. When compared to the original questionnaire data (from 2021), a total of 5 dogs had changed living arrangements: one dog had been living with another dog in 2021 but was now a singleton, while 4 dogs were living alone in 2021 but now had a conspecific in the home. These 5 dogs were excluded from the analysis due to the potential impacts of the changes in living arrangements on their interpretation of "familiar dog". This left 50 single-dog and 21 multi-dog households in the study (total N = 71 dogs).

3.4.1 Behavioural Profiles Differ in Dogs with Unexpected FDA Scores

The portion of the first-study cohort (2021, n = 235) which received an FDA score (n = 170) was compared to those in the second-study cohort (2022, n = 71), based on their 2021 (n = 46) and 2022 (n = 57) responses to the FDA questions (Table 3.1). For those dogs who received a FDA score in both 2021 and 2022 (n = 42), the mean score increased significantly from 0.311 ± 0.08 to 0.649 ± 0.12 (W=381, $p<0.001$, Wilcoxon), and the median score increased from 0 to 0.5.

Table 3.1. Mean, median and range of familiar dog aggression (FDA) scores for dogs who participated in Study 1 (Original Cohort, 2021), and the follow-up questionnaire for 2022. The follow-up cohort's FDA scores are presented from the 1st study (2021) and from the 2022 questionnaire. The total number of participants for each study is presented, with the number of participants who received a score for FDA displayed in brackets.

FDA Scores	N	Mean ± SEM	Median	Range
Original Cohort (2021)	235 (170)	0.373 ± 0.05	0	0-4
FDA Study Cohort (2021)	71 (46)	0.343 ± 0.08	0	0-2
FDA Study Cohort (2022)	71 (57)	0.782 ± 0.11	0.5	0-2.75

Of the 50 single-dog household dogs that were reported on in the follow-up survey, 25/50 had received an unexpected FDA score in the original 2021 questionnaire. The remaining 25/50 singleton dogs had not received a score for FDA (which was an expected outcome). The other 21 (of the total 71) dogs in 2022 were living in multi-dog homes, and all had received an FDA score (as expected) in the original 2021 study. Based on this information, respondents were assigned to one of three groups: singleton dogs with unexpected FDA outcomes (Group 1, n = 25), singleton dogs with expected outcomes (Group 2, n = 25), and multi-dog homes with expected outcomes (Group 3, n = 21) (Table 3.2). There were no dogs from multi-dog homes who did not receive an FDA score. Pairwise comparisons of C-BARQ subscale scores indicated that the majority of significant differences between groups were for scores from single-dog homes with unexpected (Group 1) and expected (Group 2) FDA outcomes (Figure 3.1). Compared to Group 2, Group 1 dogs displayed marginally lower dog-directed fear (DDF; 0.907 ± 0.21 vs. 1.45 ± 0.18, W =

3.479, p = 0.037), stranger-directed fear (SDF; 0.410 ± 0.14 vs. 1.27 ± 0.23, W = 4.21, p = 0.008) and nonsocial fear (NSF scores; 0.762 ± 0.17 vs. 1.27 ± 0.15, W = 3.564, p = 0.032). Only one marginally significant difference was noted between the NSF scores for Group 1 (0.762 ± 0.17) and Group 3 dogs (1.39 ± 0.21, W = 3.646, p = 0.027), and no significant differences were seen between Group 2 (expected outcome singletons) and Group 3 (expected outcome multi-dog homes).

Table 3.2. Sample sizes and outcomes of each group used in the analysis of the current study. Participants were categorized based on the accuracy of their FDA score relative to their living arrangements at the time of the original study in 2021. "Expected" outcomes for FDA scores are either singleton dogs with no FDA score, or multi-dog homes with an FDA score. "Unexpected" outcomes are singleton dogs with an FDA score.

	Outcome	**N**	**Descriptives**
Group 1	Unexpected	25	Singleton dogs who received a score for FDA
Group 2	Expected	25	Singleton dogs who did not receive a score for FDA
Group 3	Expected	21	Multi-dog homes who received a score for FDA

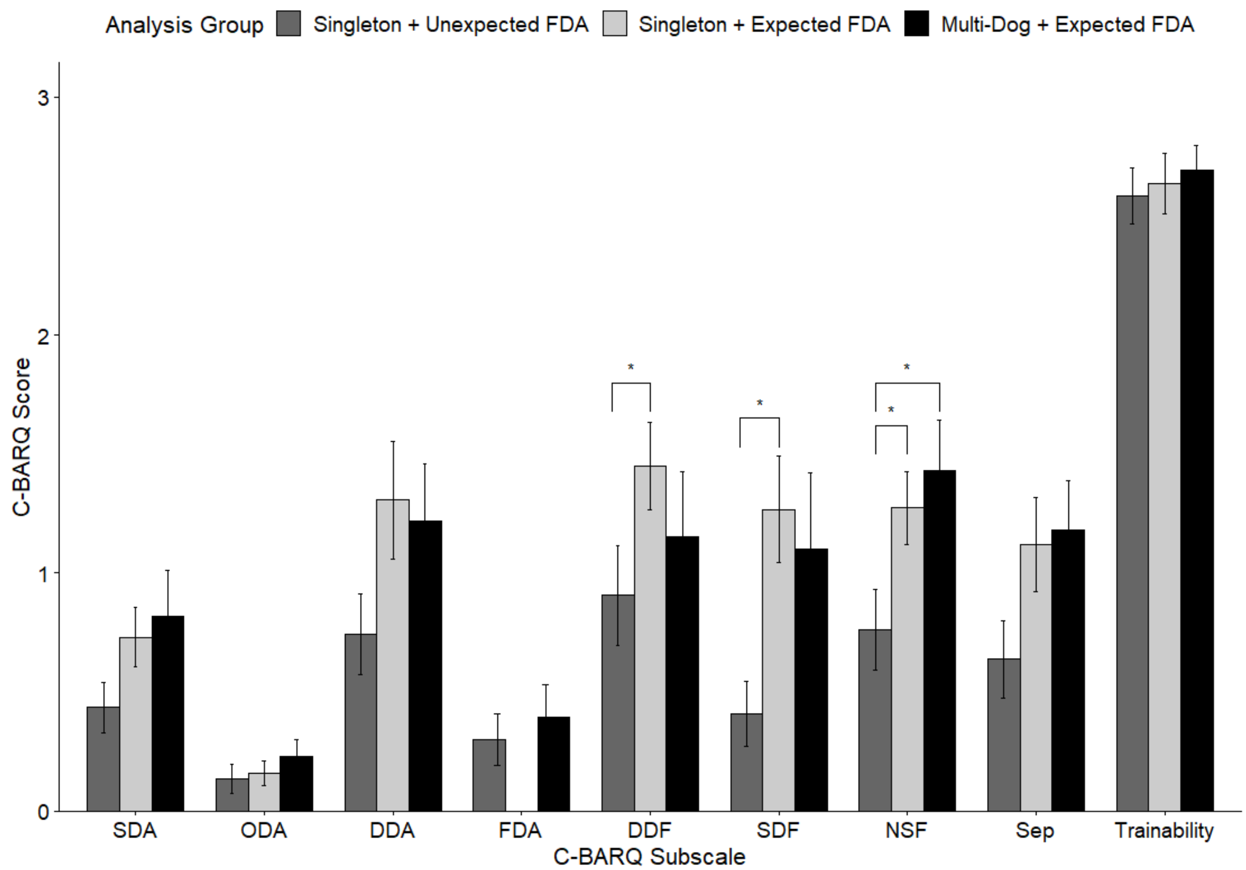

*Figure 3.1. Comparison of C-BARQ subscale scores from 2021 for dogs who lived alone and received an FDA score (Group 1 - Unexpected FDA, dark grey, n=25), dogs who lived alone and did not receive an FDA score (Group 2 – Expected FDA, light grey, n=24), and dogs who lived in multi-dog homes and received an FDA score (Group 3 – Expected FDA, black, n=22). C-BARQ subscales included stranger-directed aggression (SDA), owner-directed aggression (ODA), dog-directed aggression (DDA), familiar dog aggression (FDA), dog-directed fear (DDF), stranger-directed fear (SDF), nonsocial fear (NSF), separation related issues (Sep) and trainability. Marginally significant differences (denoted with * between analysis groups) were seen between Groups 1 and 2 for dog-directed fear (p=0.037), stranger-directed fear (p=0.008) and nonsocial fear (p=0.032), which was also marginally different between Groups 1 and 3 (p=0.027) (Kruskal Wallis with DSCF pairwise comparisons, after Bonferroni corrections for multiple comparisons p<0.0055 was considered significant).*

3.4.2 Reported Socialization Behaviour

Dogs living alone who received a score for FDA were reported to socialize in the home more than both singleton dogs without an FDA score, and dogs from multi-dog homes (Table 3.3, p = 0.026, Fisher's exact test). When owners were asked about the frequency of in-home socialization, singleton dogs with an unexpected FDA score were more often reported to socialize with other dogs in the home more than once a week (8/25 dogs, 32%) compared to dogs from the other two groups. Group 2 (singleton dogs without FDA scores) showed the highest proportion of never having other dogs in the home (9/25 dogs, 36%). A similar significant pattern was seen for out of home socialization (Table 3.3, p = 0.041, Fisher's exact test).

Table 3.3. Chi-squared tests for association with Fisher's exact test for study group, and participation in either in home or out of home socialization. Percentages displayed refer to the proportion of dogs within each group.

	Total (n=71)		Group 1 Singleton dogs unexpected (n=25)		Group 2 Singleton dogs expected (n=25)		Group 3 Multi-dog homes (n=21)			
	n	%	n	%	n	%	n	%	$\chi 2$	p
In Home Socialization									14.0	0.026
Never	16	22.5	3	12.0	9	36.0	4	19.1		
Once a week or less	43	60.5	13	52.0	15	60.0	16	76.2		
More than once a week	10	14.1	8	32.0	1	4.0	1	4.7		
Every day	2	2.8	1	4.0	0	0	1	4.7		
Out of Home Socialization									12.6	0.041
Never	14	19.7	1	4.0	7	28.0	6	28.6		
Once a week or less	48	67.6	17	68.0	17	68.0	14	66.7		
More than once a week	7	9.9	5	20.0	1	4.0	2	9.5		
Every day	2	2.8	2	8.0	0	0	0	0		

Singleton dogs with an unexpected FDA score were further separated based on their historical living arrangements – either they had lived with other dogs (Group 1A), or they had never lived with other dogs (Group 1B). These two subgroups were compared to singleton dogs with no FDA (as expected; Group 2) and dogs from multi-dog homes with FDA scores (Group 3) from the previous analysis (Table 3.4). Three singleton dogs who had received an unexpected score for FDA were excluded from this analysis as the owners did not respond to "Has your dog ever lived with another dog in the same household?". Of the singleton dogs in Group 2 who did not receive a score for FDA, 17 dogs were reported to have never lived with another dog, four had previously lived with another dog and there was no response to this question for the remaining four dogs.

Table 3.4. Further grouping of dogs based on FDA outcome in 2021, and historical living arrangements. In comparison to earlier groupings, the singleton dogs with unexpected FDA scores (Group 1) were split based on previous living arrangements with other dogs to better understand their life experiences with "familiar" dogs.

Analysis Grouping	**FDA Score Outcome**	**N**	**Descriptives**
Group 1A	Unexpected	11	Singleton dogs who received a score for FDA (n = 25), previously lived with dogs
Group 1B	Unexpected	14	Singleton dogs who received a score for FDA (n = 25), had never lived with other dogs
Group 2	Expected	25	Singleton dogs who did not receive a score for FDA
Group 3	Expected	21	Multi-dog homes who received a score for FDA

When the unexpected FDA score groups were separated based on historical living arrangements, there were no significant differences between C-BARQ scores for Groups 1A & 1B. The only marginally significant finding in this analysis was between stranger-directed fear (SDF) scores for Groups 1A & 2 (0.364 ± 0.25 and 1.27 ± 0.23 respectively, W=3.721, p=0.042, Kruskal-Wallis with Dwass-Steel-Critchlow-Fligner pairwise comparisons). Interestingly, there were no significant differences in C-BARQ scores when dogs were grouped based solely on their historical living arrangements (i.e., had or had not lived with other dogs).

3.4.3 Changes in response to FDA questions between 2021 and 2022

Of the 25 single-dog homes who did not receive a score for FDA in the original 2021 questionnaire, 15 respondents answered a sufficient number of questions in the 2022 questionnaire to obtain a score for FDA, while the remaining 10 maintained no score (answering N/As, or skipping the question). Of the 25 single-dog homes who had previously received an unexpected score for FDA in 2021, 21 participants received an FDA score in the 2022 questionnaire, while 4 participants did not receive a score. Participants from multi-dog homes remained consistent in answering all of the FDA questions in the 2022 questionnaire.

The FDA scores in 2021 were compared to those in the 2022 questionnaire for both the unexpected and expected FDA categories. In this analysis, only respondents who answered both 2021 and the 2022 FDA questions were included. Mean FDA scores for both categories increased from the 2021 questionnaire to the 2022 questionnaire (Figure 3.2).

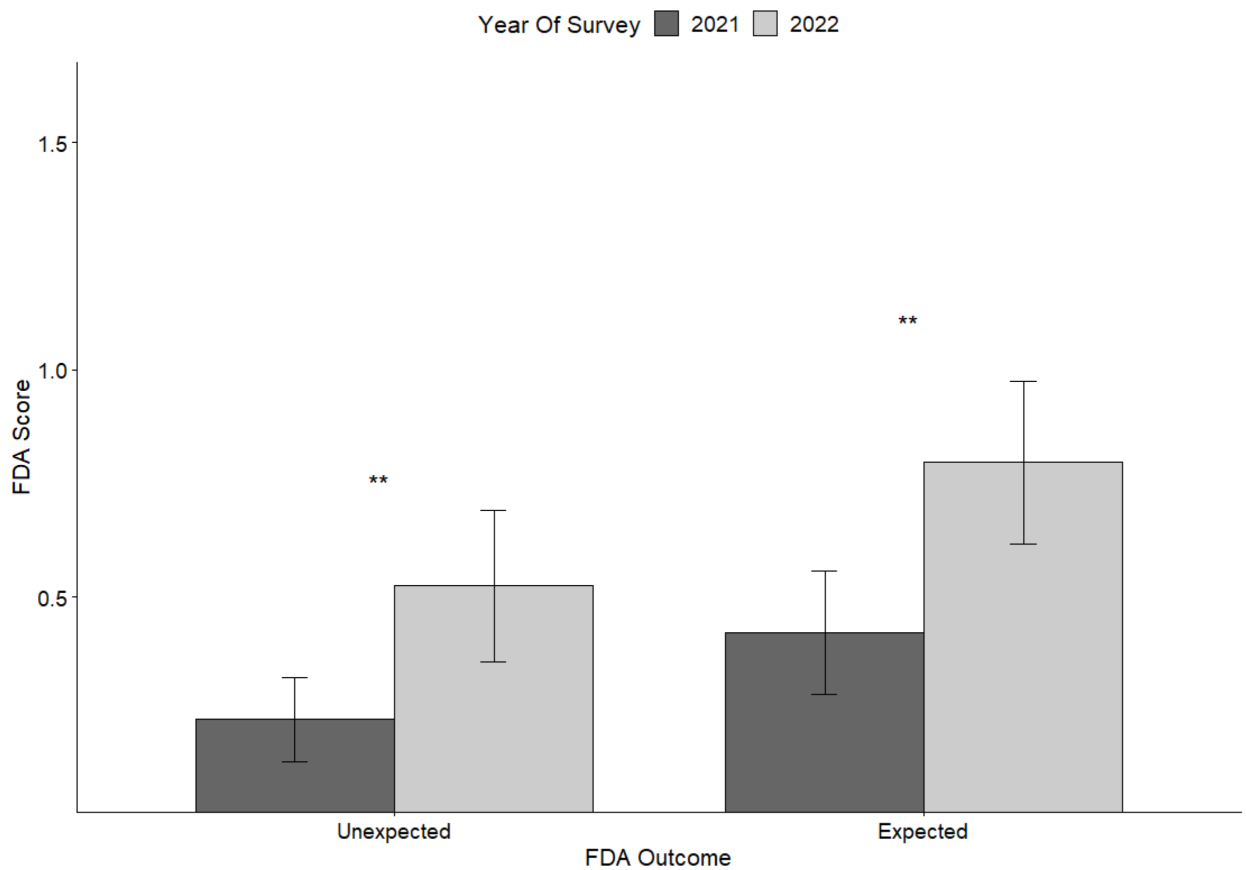

*Figure 3.2. Changes in FDA scores reported between 2021 (dark grey) and 2022 (light grey) for dogs who received either an unexpected (singleton dogs) or expected (multi-dog homes) FDA score. The increase in score was statistically significant (denoted with **) for both groups; unexpected (p=0.003), expected (p=0.012) (Wilcoxon).*

Dogs who received an unexpected score in both questionnaires (n=21) showed a significant increase in FDA score from 0.230 ± 0.09 to 0.524 ± 0.16 (W=8.0, p=0.009, Wilcoxon test) between the 2021 and 2022 questionnaire, while dogs in the expected score category (n=21) increased from 0.393 ± 0.14 in 2021 to 0.774 ± 0.19 (W=21.5, p=0.017) in the 2022 questionnaire. However, there were no significant differences in the proportion of changes in FDA score (increased, decreased, or no change), when considering individuals (Table 3.5).

Table 3.5. Proportion of respondents within the unexpected FDA group (singleton dogs with an FDA score, n=21) and expected FDA group (multi-dog homes with an FDA score, n=21) whose score increased, decreased, or did not change. A chi-square test for association indicated that the proportional changes were not statistically significant.

	Unexpected N = 21		**Expected N = 21**			
	n	%	n	%	$\chi 2$	p
FDA Score Change					1.06	0.668
Increase	11	52.4	13	61.9		
No Change	8	38.1	5	23.8		
Decrease	2	9.5	3	14.3		

3.4.4 Analysis of "Unexpected Score" Respondent's Reasoning

In this analysis, the respondents were asked specifically which dog they had been referring to when answering the familiar dog aggression questions. Participants with singleton dogs who had previously lived with other dogs showed the most diversity in their responses, with 3/11 (33%) participants referring to a friend or family member's dog spending time in their home, 2/11 (22%) referring to a dog outside the home, and 2/11 (22%) providing no response. The most common response for this group referred to previous ownership: 4/11 (44%) of owners selected a dog they previously owned, whereas neither the participants with dogs who had always lived alone nor those with singleton dogs and no FDA score selected this response. Participants with singleton dogs who had received an unexpected FDA score, but had not previously lived with other dogs, mostly selected responses relating to socialization: "A friend or family member's dog who spends time in your home" (9/14 participants; 64%), or "A friend or family member's dog who spends time away from your home" (3/14 participants, 21%), while 2/14 participants did not respond As expected, all participants with multi-dog homes selected "Another dog that you own" (21 participants).

3.5 DISCUSSION

This follow-up study provides evidence for marginal behavioural differences between dogs based on their owners' interpretations of the familiar dog aggression (FDA) subscale questions. Owners who did not interpret the FDA questions as intended in the C-BARQ (i.e., referring to their dog's aggressive behaviours towards another dog living in the household) tended to report overall lower scores for some other behavioural subscales, with marginally significant differences seen for dog-directed fear, stranger-directed fear, and nonsocial fear compared to other singleton dogs (with no FDA score). These singleton dogs with an unexpected FDA score also had marginally lower scores for nonsocial fear compared to that of dogs living in multidog homes. There were no differences in any C-BARQ subscale scores between singleton dogs without an FDA score (as expected) and dogs from multi-dog homes. Owners of singleton dogs with an expected FDA score were also more likely to report that their dog socializes with other dogs, both in and out of the home. However, there was no evidence that having lived with other dogs in the past impacted the owners' interpretations of the FDA questions.

More specifically, many of the singleton dogs who received an unexpected FDA score socialized with other dogs more frequently than other dogs in the study – 88% and 96% of dogs were reported to socialize with other dogs inside and outside of the home, respectively. This represents a greater proportion than the singletons who did not receive a score for FDA (i.e., those who interpreted the questions as intended) – only 64% of these dogs socialized with dogs inside the home, while 72% socialized with dogs away from the home. Singleton dogs who received an unexpected FDA score showed a trend towards lower scores (indicating low concern) across all subscales compared to those singleton dogs with no FDA score. This could be due to the under-reporting of behavioural issues by their owners, if, for example they wished to present their dog in a favourable manner, or simply had a different benchmark for what they considered "problematic" behaviours. However, given that these owners reported familiar dog aggression when they were not actually required to do so, the first explanation seems unlikely. It is possible that owners of dogs who experience frequent social interactions may be more conservative about what they are willing to call a behaviour problem vs. a normal interaction between dogs, given that their dogs probably appear to them to enjoy interactions with their familiar conspecifics (as, otherwise, they likely would not continue). What seems most plausible, however, is that these dogs, who spend time engaged in social activities with other dogs, actually do have fewer behavioural problems, as perceived by their owners and measured by the C-BARQ subscales. However, it is important to note that only three subscale scores were marginally significantly different between these groups, and, interestingly, all were related to fear behaviour: dog-directed fear, stranger-directed fear, and nonsocial fear. Given the relatively small sample size of the groups compared in this study, it would be worthwhile to evaluate these effects further. It does seem likely that most, if not all, other studies using C-BARQ collect participants with singleton dogs who complete the FDA subscale questions and thus receive an unexpected FDA score. The inclusion of these singleton dogs, who may have socialization experiences with other dogs (and people) that differs at least in quantity, if not in quality, compared to singleton dogs with no FDA score, in the evaluation of the FDA subscale score may skew not only the FDA subscale score evaluation, but potentially other C-BARQ subscale scores, particularly those related to fear.

Indeed, the amount of socialization a dog, and their owner, experiences may expose them to more opportunities for developing relationships with other dogs outside of their family; these dogs may then be considered ‘familiar’ by the owner. Studies have shown that early socialization experiences, such as participation in puppy classes, can prevent or reduce multiple problem behaviours (Hakanen *et al*., 2020; Harvey *et al*., 2016; Puurunen *et al*., 2020), although it cannot be confirmed in this study when in a dog’s life the socialization has occurred. While owner-reported surveys are typically reliable, in particular the C-BARQ which has undergone multiple validation studies (Broseghini *et al*., 2023; Duffy & Serpell, 2012; Hsu & Serpell, 2003), at least one study suggests that the owner’s age, location, and gender may bias an owner’s perception of problematic behaviours (Kimura *et al*., 2023). In addition to demographic factors, the type of behavioural issue is also associated with an owner’s perception of how problematic it may be, with fear/anxiety and aggression issues being perceived as particularly problematic and eliciting a desire for behavioural intervention (Pirrone *et al*., 2015). The C-BARQ subscale scores in this community sample were overall fairly low for all groups (ranging from low to moderate concern), indicating there were no severe or drastic differences in aggression between groups (i.e., the higher scoring dogs were not necessarily ‘clinical’ cases). Thus, I cannot rule out that the differences noted between groups may be, in part, driven by the owner’s perception of the behaviour, rather than solely the observable behaviour itself.

While the participants’ interpretation of ‘familiar dog’ as any dog that their dog socializes with is understandable, it does present issues for the relevance of the C-BARQ score for familiar dog aggression in particular, and the use or interpretation of the subscale in studies using the C-BARQ. First, the familiar dog aggression scores for dogs who technically “should not” receive a score may be removed from a study if the inclusion criteria requires that dogs cohabit with others. This would allow studies to remain consistent in the reporting of familiar dog aggression, as it refers solely to aggression shown towards other dogs living in the same home. However, valuable information regarding dogs who display resource-based aggression may be lost if they are excluded based strictly on living arrangements. If owners provide a score for the FDA subscale, based on their experiences of socializing with non-cohabiting dogs, the score may provide valuable information for the individual dog. Indeed, when comparing singleton dogs whose owners correctly interpreted the familiar dog aggression questions to those who did not, those who correctly interpreted the questions also had somewhat higher scores for all the other subscales. The marginally significant findings between these two singleton groups (expected, and unexpected FDA scores) suggests that for fear-related behaviours, these dogs may indeed be somewhat different, and this potentially relevant finding could be lost if the use of familiar dog aggression is solely limited to multi-dog households. Certainly, in cases where the owner is completing the C-BARQ to gain information about their dog's behaviour (versus participating in a research study using C-BARQ) omitting the FDA score for singleton dogs would not give the owner as much useful information. However, if these dogs are actually different from singleton dogs who do not get an FDA score, then failing to separate out these two groups in the reporting of C-BARQ to and by researchers conflates the groups and risks invalidating any findings pertaining to FDA scores.

Familiar dog aggression represents resource-based aggression (i.e., threatening or hostile responses to a familiar dog in the same household), and the four questions associated with the subscale ask owners to rank their dog's general aggression towards another familiar dog, when approached at a favourite resting place, while eating, and while chewing a toy/bone. The current study suggests that resource-based aggression is not exclusively seen in dogs who live with other dogs; however, by definition it is dependent on having a resource present (Benoit, 2019; Siracusa, 2021), which is most likely in the context of a home environment for both dogs who cohabit, and dogs who socialize in the home. There is not a general consensus on the definitions for terms related to this display of aggression, such as "possession aggression" and "resource guarding", within the clinical and consulting field (Jacobs *et al*., 2018b). While most appear to prefer the term "resource guarding", the definitions can be loose, and Jacobs suggests defining resource guarding as "the use of avoidance, threatening, or aggressive behaviors by a dog to retain control of food or non-food items in the presence of a person or other animal" (Jacobs *et al*., 2018b). The variability in the definitions for resource-based aggression used by professionals and academics highlights the importance of clarity when defining these behaviours in research questionnaires that enlist non-professionals (i.e., dog owners); it is the researcher's responsibility to be as precise as possible with the wording of questions and then to be vigilant in assuring that any questions that were "mis-answered" by respondents are either removed or evaluated separately.

It is evident from this study that the C-BARQ's questions relating to familiar dog aggression are not precise enough to prevent singleton dog owners from attempting to answer the questions, especially those who regularly spend time with dogs outside of their family unit. However, what their responses reveal is that singleton dogs who may engage in social activities in and out of the home are capable of behaving aggressively around resources, as queried by the FDA questions, and may be more similar behaviourally to dogs from multi-dog homes than to singletons who do not socialize. Thus, it may be beneficial for the C-BARQ to first accurately define the contexts and targets of familiar dog aggression, and for researchers to clearly outline what real-world measures they are attributing to familiar dog aggression in their study. The requirement that dogs live together to obtain a familiar dog aggression score could also have one of two implications which cannot be elucidated from the C-BARQ: a dog may be less likely to behave aggressively towards a familiar dog with whom they have a well-established relationship; however, while they have the ability to behave aggressively, they may not, perhaps due either to the positive relationship with the conspecific or to owner management. Alternatively, as previous studies have suggested, living with conspecifics increases the opportunity for practicing resource-based aggression (Jacobs *et al*., 2018a; McGreevy & Masters, 2008), and this would increase FDA scores for dogs in multi-dog homes. Thus, it may indeed be beneficial for the familiar dog aggression subscale to be redefined as "threatening or hostile responses towards a familiar dog (that you may or may not own) within your home", and researchers should pose additional questions to confirm not only the number of dogs in the home, but also the frequency of socialization with other dogs in the home.

For the most accurate and informative information about individual dogs, and the interpretation of the FDA behaviours, one solution may be to report the C-BARQ FDA subscale for dogs that live alone separately from those who live with other dogs in the household. This would allow researchers to evaluate dog rivalry behaviours among housemates who share access to resources, and are managed by the same owner, separately from those behaviours shown among familiar dogs who do not share resources or necessarily experience the same management practices. Whether FDA-related behaviours shown towards a housemate are the same as those shown towards a non-cohabiting familiar dog are the same in motivation and kind is a topic that warrants further consideration. Further research should also investigate the fear subscales in addition to the aggression subscales, as this study suggests that dogs who lived alone but socialized with others showed lower levels dog-directed, stranger-directed, and nonsocial fear.

While the dogs in this study may differ behaviourally, another potential reason for the differences seen in this study could be due to the human and their capacity for accurately answering the questionnaires. While the majority of C-BARQ subscales have been adequately validated in previous studies (Duffy *et al*., 2014, 2008; Duffy & Serpell, 2012; Hsu & Serpell, 2003), only a small number of specific questions can be omitted from the C-BARQ while still maintaining its validity. The recommendation to avoid omitting questions or “cherry-picking” relevant research questions from the C-BARQ is supported by the significant increases seen in familiar dog aggression scores for both the unexpected and expected groups in this study – when the four questions were delivered out of the context of C-BARQ and framed as a study specifically addressing familiar dog aggression, the mean scores for each group increased. While the dogs may have increased in their behavioural issues between the approximately 12 months between the questionnaires, it is more likely that the participants were simply more sensitive to the study question due to demand characteristics (Orne, 1996; Stalans, 2012). In surveys, an individual’s responses to questions can be affected by their fatigue during the survey or interview – when participating in lengthy questionnaires, some respondents may begin to choose the “easier” responses, such as the middle values on a Likert scale, N/A or skip, while others may select more extreme responses (Stalans, 2012). While neither of these responses to fatigue might have happened within this study, particularly for individuals who were motivated enough to return and complete the 2022 questionnaire, it is possible that the use of two substantial questionnaires in succession (the Diet & Lifestyle Questionnaire, followed by the full C-BARQ) could have contributed to a considerable rate of drop-out in the 2021 study (i.e., 259/494 participants who completed the Diet & Lifestyle questionnaire did not go on to complete the C-BARQ). This should be considered for future questionnaire development and provides justification for the use of the mini-CBARQ (Duffy *et al*., 2014), a condensed version of the C-BARQ that poses 42, rather than 100, questions. This would be of particular importance when an additional questionnaire is used in the study to reduce the demands placed on the participant and increase the reliability of their responses. However, it is also possible that the requirement to register their dog on the C-BARQ website once they had completed the Diet & Lifestyle questionnaire was also a barrier to continued participation, which would not be solved by using the shorter version of the C-BARQ.

3.6 CONCLUSIONS

This study has shown that the interpretation of the familiar dog aggression questions in the C-BARQ differs between dog owners, and is most likely influenced by their participation in social activities with other dogs that do not belong to the family. The reported differences in other C-BARQ subscale scores also suggests that dogs who live alone, but socialize often, are potentially behaviourally different from dogs who do not socialize regularly, and from those who live with other dogs. While Chapter 2 showed that dogs living alone reported less concerning behavioural issues, this finding may be influenced by the under-reporting of behaviours by owners of single dogs who interpreted the FDA questions in C-BARQ in a manner other than is intended by the instrument. Singleton dogs belonging to owners who provided a FDA score unexpectedly tended to be behaviourally different from other singleton dogs in this study, and many had exposure to other dogs both in and out of the home. Given the correlations shown in other studies between familiar dog aggression and other C-BARQ subscale scores, these issues may indeed permeate into other areas, and have the potential to be overlooked or under-reported. This ambiguity in the reporting of FDA scores could be addressed in two ways. First, the C-BARQ would benefit from a more precise definition of familiar dog aggression, i.e., threatening or hostile responses towards "a dog that you own and lives in the same home" or "a dog that your dog regularly spends time with in your home". While this is dependent upon the creators' intentions of what exactly familiar dog aggression should be quantifying, a more accurate definition is required to provide consistency across studies, and accurately report on the behaviours of dogs who do, or do not, spend time with other dogs. Second, researchers using the C-BARQ to evaluate dog behaviour should be aware of the validity (or possible lack thereof) of the FDA subscale within the scope of their research question. An additional questionnaire is recommended to confirm other factors that may not be addressed in C-BARQ alone (i.e., the presence of conspecifics, frequency of socialization), in order to draw clear conclusions from the subscale scores. Alternately, these questions could be integrated into the C-BARQ itself, with appropriate reporting to researchers of warnings for any "unexpected" FDA scores generated for singleton dogs.

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CHAPTER 4: GUT MICROBIOTA COMPOSITION IS RELATED TO ANXIETY AND AGGRESSION SCORES IN COMPANION DOGS (*CANIS FAMILIARIS*)

4.1 ABSTRACT

There is mounting evidence for a link between behaviour and the gut microbiome in several animal models and human health. However, the role of the gut microbiome 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 microbiota 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 microbiota composition 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. While minimal differences in relative abundance were seen between behavioural groups, we were successful in predicting behavioural group based on gut microbiota 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 link between this genus and anxiety in pet dogs. 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.

4.2 INTRODUCTION

Dog behavioural issues, such as anxiety and aggression, are reported as the major reason for the relinquishment of dogs to shelters (Kwan & Bain, 2013; Salman *et al*., 1998; Segurson *et al*., 2005), and are a considerable source of stress for dog owners or guardians that can result in the breakdown of the dog-human bond (Meyer & Forkman, 2014). The role of the gut microbiome 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 (Dinan & Cryan, 2017; Morais *et al*., 2021; Valles-Colomer *et al*., 2019). While most of the current literature focuses on mammalian models such as mice and humans (reviewed by Cresci & Bawden, 2015), recent studies have highlighted differences in the composition of the gut microbiota between domestic dogs of different behavioural tendencies (such as anxious or aggressive) (Craddock *et al*., 2022; Kirchoff *et al*., 2019; Kubinyi *et al*., 2020; Mondo *et al*., 2020). The relationship between the gut microbiome, 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 microbiota composition and owner-reported dog behaviour.

Recent studies have identified relationships between gut microbiota composition and behaviour in some populations of dogs. Kirchoff *et al*. (2019) 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 conspecific (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*. (2020) examined a cohort of dogs from three shelters in Bologna, Italy, and compared the gut microbiota composition between aggressive, phobic and “normal” dogs. Similar to Kirchoff *et al*. (2019), 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 Kubinyi *et al*., 2020) in a relatively secure and stable environment.

There are alternative approaches to determining a dog’s behavioural profile. Mondo *et al*. (2020) 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) (Hsu & Serpell, 2003). 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 (Canejo-Teixeira *et al*., 2018; Rosa *et al*., 2017; Serpell & Hsu, 2005; Tiira & Lohi, 2014; van den Berg *et al*., 2010). 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 microbiota composition and specific behaviours such as stranger-directed aggression, dog-directed aggression, and non-social fear.

4.3 METHODS

4.3.1 Recruitment, Behavioural Assessment & Participant Selection

Dog owners 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; (Hsu & Serpell, 2003)) (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 (Supplementary Material A) 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 (discussed in Chapter 3). Similarly, 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). The composite scores for both aggression and anxiety were used primarily as a way to group dogs who showed more or fewer aggressive and anxious behaviours within this community sample. That is, the composite score is an index of the expression of any aggressive or anxious behaviours by the dogs across a range of contexts, rather than the type or sub-category of those problem behaviours. Nevertheless, to assess the reliability of the composite scores we performed a factor analysis of the C-BARQ subscale scores and calculated the Spearman's correlation coefficients between the C-BARQ subscale scores and their corresponding composite score. Additionally, we calculated Cronbach's alpha and McDonald's omega as reliability estimates of the composite score using the alpha and omega functions available in the R package psych. Cronbach's alpha will underestimate reliability for few and multidimensional items (Tavakol & Dennick, 2011). These two characteristics (i.e., small number of items and multidimensionality) are present in our data and thus Cronbach's alpha should be taken as a lower bound of reliability. To account for these characteristics, we calculated McDonald's omega coefficient which is recommended for multidimensional measures (Widhiarso & Ravand, 2014). These analyses show that 1) all individual scores are positively correlated to their corresponding composite score (Spearman's correlation coefficients' range 0.47 – 0.83), 2) a single factor will only explain 0.486 and 0.311 of the total variance for aggression and anxiety, respectively, indicating that C-BARQ subscale scores are not unidimensional, 3) Cronbach's alpha is 0.6 and 0.57 for aggression-related and anxiety-related C-BARQ scores, respectively, and 4) McDonald's omega total is 0.73 for both aggression related and anxiety-related C-BARQ scores. These results indicate that the internal consistency of these C-BARQ scores is acceptable, and thus the composite scores are reliable.

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 inclusion criteria, along with the matching and selection process, are detailed in Supplementary Material C.

The population of dogs produced from this initial selection process (n=72; Table S1) were then split by the median of their composite anxiety and aggression scores to broadly categorize this community sample into higher and lower anxiety and aggression groups, with those at the median assigned to the higher anxiety or aggression groups. While the individual C-BARQ subscales have previously been validated in other studies (Canejo-Teixeira *et al*., 2018; Rosa *et* *al*., 2017; Serpell & Hsu, 2005; Tiira & Lohi, 2014; van den Berg *et al*., 2010), we also confirmed the validity of using a median split of the composite score in this community sample by comparing the owner-reported behaviours for each dog from the Diet & Lifestyle questionnaire to their assigned behaviour group, as detailed in Chapter 2 of this thesis (Table 2.2). The two questionnaires used different approaches for the questions about behaviour; in the Diet & Lifestyle questionnaire, owners were asked to select any behaviours that their dog displays when encountering unfamiliar people or dogs while on a leash, while the C-BARQ instructed owners to rank the severity of the dog's overall reaction to these contexts. A comparison of the reported behaviours to the dog's assigned behavioural group confirmed that dogs in the higher anxiety and aggression groups displayed some different behaviours than those in the lower groups (n=235; Table 2.2). Bioinformatics analyses were thus conducted using both categorical group assignments (higher versus lower), as well as the continuous C-BARQ subscale scores, as described below.

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 (Parker *et al*., 2017), 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., higher anxiety and higher aggression dogs were matched to lower anxiety and lower aggression dogs) while maintaining similar or identical classifications within the diet and lifestyle criteria. We successfully assigned 20 pairs of dogs as a higher anxiety/higher aggression to lower anxiety/lower aggression match, with the remaining 5 pairs consisting of dogs with lower anxiety/higher aggression scores matched to dogs with higher anxiety/lower aggression scores. As a result, there were 25 dogs per group (higher and lower anxiety or aggression). By design, there was high overlap in individuals rated as higher anxiety and higher aggression (and, likewise, lower anxiety and lower aggression), which reflects the positive correlations between measures of anxiety and aggression as demonstrated by C-BARQ (Duffy *et al*., 2014; Stellato *et al*., 2017), as well as other behavioural literature (Tiira *et al*., 2016). However, the current sample reflects that aggression and anxiety are not perfectly correlated (i.e., 10 dogs that grouped into opposite categories for aggression and anxiety were included). This pattern of association between composite anxiety and composite aggression scores is also reflected in the larger population (n=235) from which this sample was drawn (see Table 2.2, Chapter 2).

4.3.2 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 (Fecal Swab Collection & Preservation System, product 45670; 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, which preserves DNA samples for 2 years at ambient temperatures (www.norgenbiotek.com), and the device was collected that day by researchers via contactless pickup due to public health restrictions for isolation and social distancing during the COVID-19 pandemic. 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. All samples were brought to the lab within 72 hours, where the samples were stored at -20°C until processed.

4.3.3 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) (Parada *et al*., 2016; Walters *et al*., 2016) 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.

4.3.4 Bioinformatics Analysis

The quality of the Illumina raw reads was assessed using the FastQC software (version 0.11.9; Andrews, 2010). Reads were trimmed using trimmomatic (version 0.39; Bolger *et al*., 2014) with the parameters: PE, -phred33, and sliding window 4:20. Trimmed paired reads were then inputted to the Bioconductor package DADA2 (version 1.22; Callahan *et al*., 2016) 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. Taxonomical assignment was done using the Silva database (version 138.1; Quast *et al*., 2012). We used DADA2 as this method is recommended in best practices for microbiome analysis (Knight *et al*., 2018).

Abundance and diversity of taxonomic groups present in each fecal sample were investigated using the Bioconductor package MicrobiotaProcess (version 1.6.6; Xu *et al*., 2023) 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 (Knight *et al*., 2018), we used 'balance' approaches for microbiota 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; Silverman *et al*., 2017), which produced the top 5 nodes on the phylogenetic tree (balances) to distinguish between behavioural groups using a sparse logistic regression model, and Selbal (Rivera-Pinto *et al*., 2018), 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 (Breiman, 2001) 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 are 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.

Differences in the C-BARQ subscale scores, and relative abundance of individual OTUs, between higher and lower aggression or anxiety groups, were tested for significance with Mann-Whitney U, using the false discovery rate (FDR-adjusted) approach to correct the p-values for multiple comparisons.

4.4 RESULTS

4.4.1 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 mean composite anxiety score was 0.955, with a median of 0.782 (Supplementary Material C). 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. The mean and range of C-BARQ subscale scores for dogs in the higher and lower anxiety and aggression groups are displayed in Table 4.1, with significant differences between higher and lower groups across all subscales ($p \leq 0.001$, Mann-Whitney U).

Table 4.1 Mean and range of C-BARQ subscale scores for the higher and lower aggression and anxiety groups used in the microbiome analysis. Anxiety subscales displayed are stranger-directed fear (SDF), dog-directed fear (DDF), nonsocial fear (NSF) and separation related issues (Separation). Aggression subscales are stranger-directed aggression (SDA), dog-directed aggression (DDA) and owner-directed aggression (ODA). Mean group scores were significantly different across all subscales (p≤0.001, Mann Whitney U).

	SDF		**DDF**		**NSF**		**Separation**	
Anxiety Group	Lower	Higher	Lower	Higher	Lower	Higher	Lower	Higher
N	25	23	25	23	25	23	25	23
Mean +/- SE	0.11 ± 0.05	1.55 ± 0.23	0.637 ± 0.14	1.64 ± 0.23	0.609 ± 0.10	1.42 ± 0.19	0.392 ± 0.09	1.19 ± 0.18
Range	0-1.0	0-4.0	0-3.0	0-3.5	0-1.83	0-3.0	0-1.38	0-2.88

	SDA		**DDA**		**ODA**	
Aggression Group	Lower	Higher	Lower	Higher	Lower	Higher
N	23	25	23	25	23	25
Mean ± SE	0.160 ± 0.05	1.10 ± 0.172	0.417 ± 0.08	1.85 ± 0.20	0.05 ± 0.02	0.349 ± 0.07
Range	0-0.90	0-2.90	0-1.50	0-4.0	0-0.38	0-1.38

The mean age of the 48 dogs included in the gut microbiota analysis was 3.95 years (±0.23 S.E.), and the cohort included 30 males and 18 females (Table 4.2). 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.

Table 4.2 Metadata for 48 pet dogs used in microbiome analysis. [a] denotes statistical difference between groups (p = 0.020, Mann-Whitney U).

		Anxiety Group		**Aggression Group**	
	All Dogs (n=48)	Lower (n=25)	Higher (n=23)	Lower (n=23)	Higher (n=25)
Age (years) ± S.E.	3.95 ± 0.23	3.70 ± 0.33	4.22 ± 0.30	3.41 ± 0.33[a]	4.44 ± 0.28[a]
Male	30	15	15	14	16
Female	18	10	8	9	9

4.4.2 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 S2).

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* (Figure 4.1). 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 (Figure 4.1).

4.4.3 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 Fusobacteria (18.5 ± 1.8%), Negativicutes (11.8 ± 1.6%), Clostridia (9.3 ± 0.6%), Gammaproteobacteria (3.7 ± 0.3%) and Bacilli (2.7 ± 0.4%). The order Bacteroidales was most abundant across the cohort (53.6 ± 2.3%), followed by Fusobacteriales (18.5 ± 1.8%), Veillonellales-Selenomonadales (11 ± 1.6%), Lachnospirales (4.4 ± 0.5%), Oscillospirales (3.9 ± 0.4%), Burkholderiales (3.1 ± 0.3%) and Erysipelotrichales (2.4 ± 0.3%). The seven most abundant families identified across all samples in this study were Bacteroidaceae (29.9 ± 2.5%), Prevotellaceae (23.5 ± 3.1%), Fusobacteriaceae (18.5 ± 1.8%), Selonomondaceae (11 ± 1.6%), Lachnospiraceae (4.4 ± 0.5%), Ruminococcaceae (3.6 ± 0.4%) and Sutterellaceae (3.1 ± 0.3%).

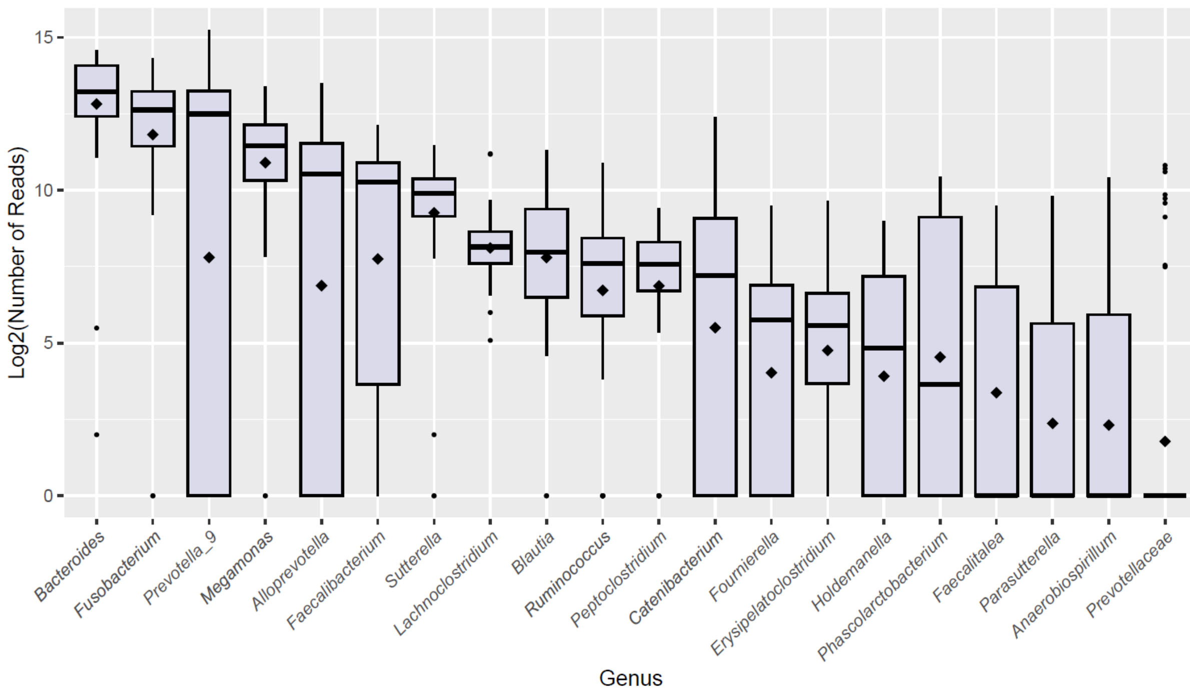

Figure 4.1 The top 20 most abundant genera, as per total number of reads, identified across the entire cohort of dogs (n = 48). The horizontal line within each box indicates the median and the diamond indicates the mean of the log2 of the number of reads.

Both higher anxiety and higher aggression groups showed a greater number of reads in alpha diversity metrics ACE and Chao1 (Figures 4.2A and 4.3A, respectively), with the aggression groups showing a greater distinction between the two curves. Overall, after multiple-testing correction, none of the FDR-corrected p-values were below 0.05 (Table S3), with both anxiety and aggression groups displaying similar profiles at the family level (Figures 4.2B and 4.3B).

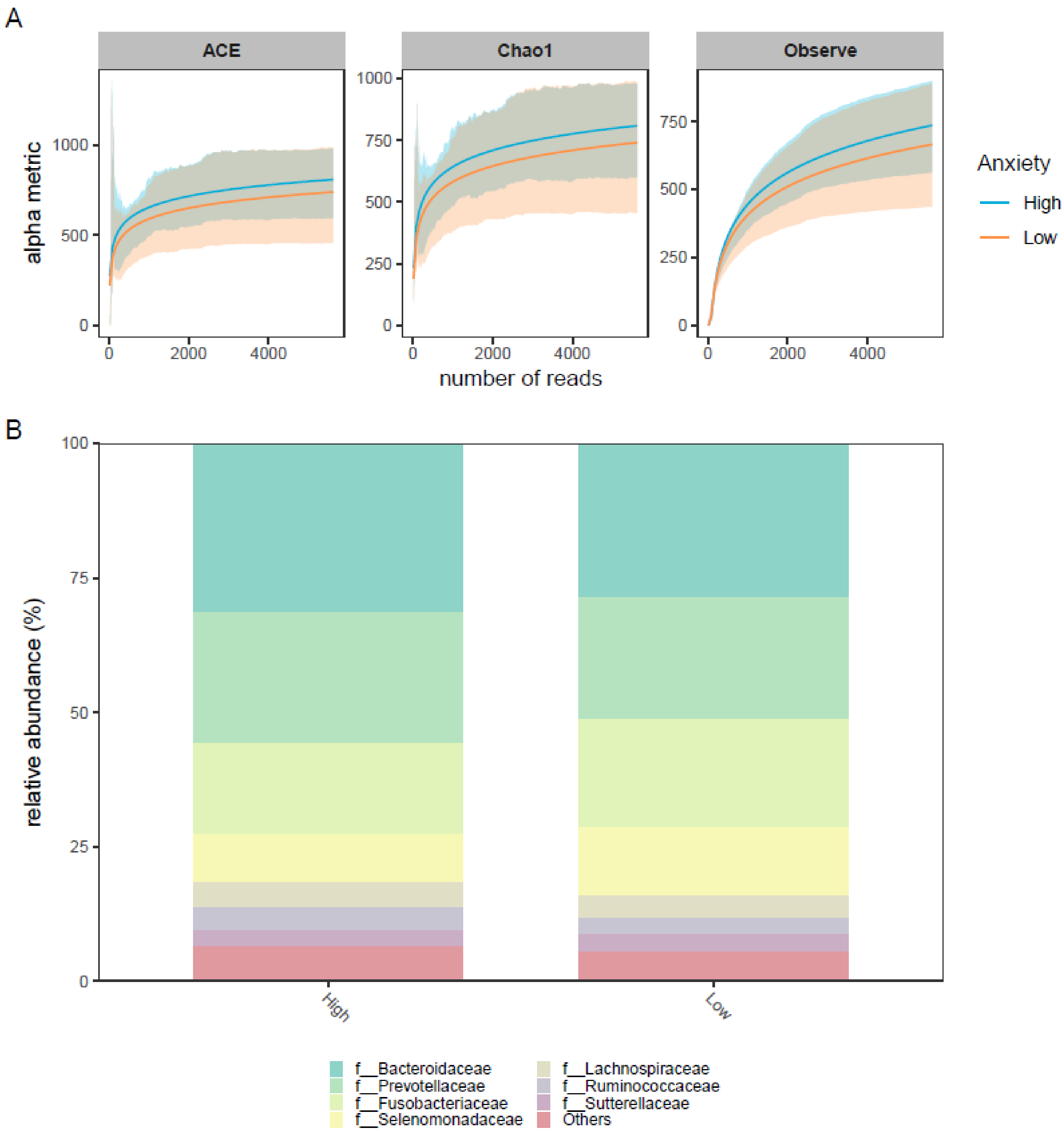

Figure 4.2. (A) Alpha metrics ACE, Chao1 and Observed in higher and lower anxiety groups. (B) Mean relative abundance (%) of the top 7 most abundant families identified for higher and lower anxiety groups.

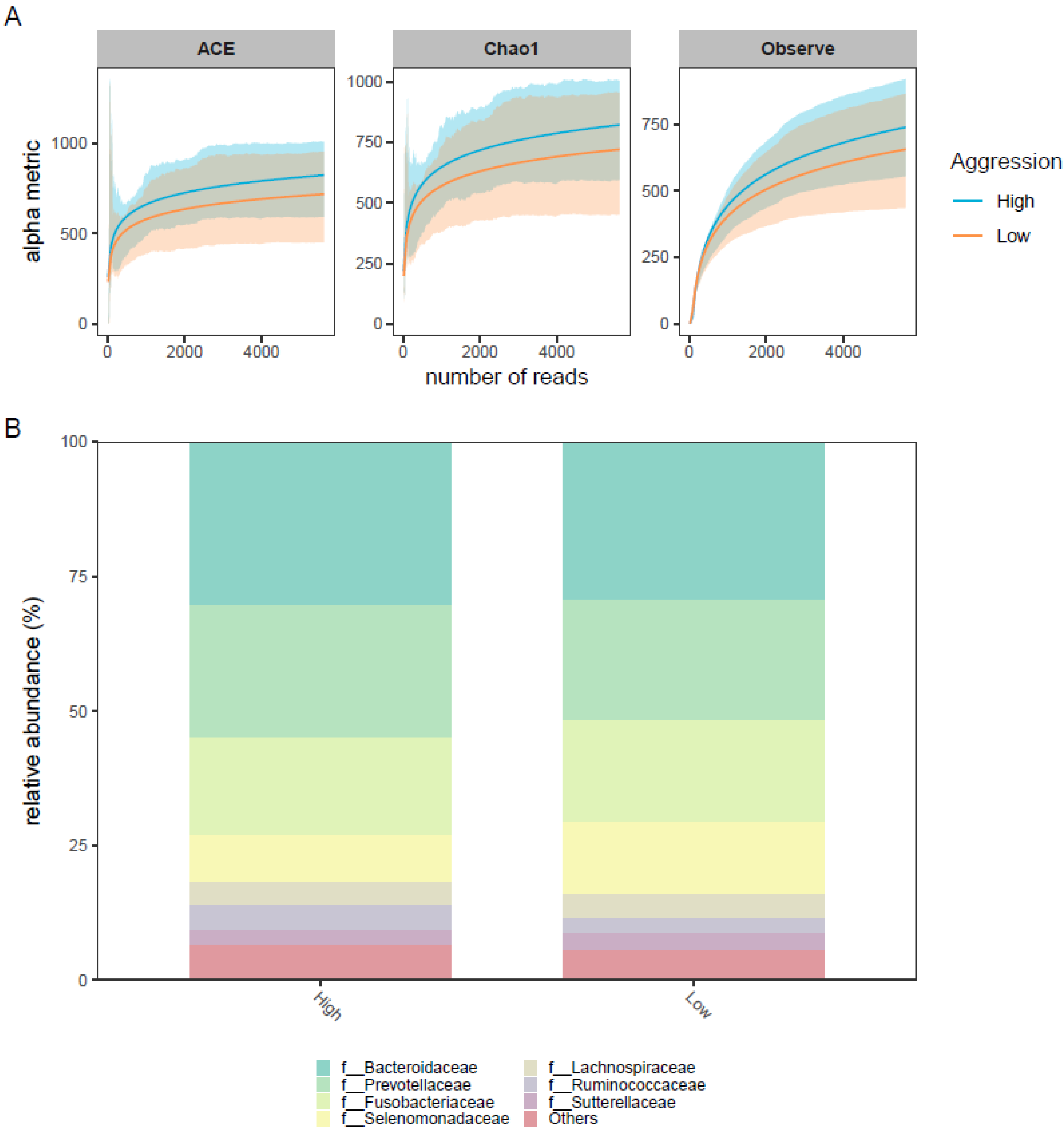

Figure 4.3. (A) Alpha metrics ACE, Chao1 and Observed in higher and lower aggression groups. (B) Mean relative abundance (%) of the top 7 most abundant families identified for higher and lower aggression groups.

The linear discriminant analysis (LDA) highlighted a difference in the 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 4.3). 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 4.3.

4.4.4 Microbial balances associated with behaviour groups

Metataxonomic 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 metataxonomic data and test for differences in the log ratios between microbial abundances (called balances). Balance approaches vary in how balances are calculated and how testing for differences in the balances is performed. We used two balance approaches: PhILR (Silverman *et al*., 2017), which applies evolutionary models to guide the calculation of the log ratios, and Selbal (Rivera-Pinto *et al*., 2018). 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 with 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 4.3. The genus *Blautia* was identified by all but one of the analyses, indicating 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 indicated in the PhILR and Random Forest analysis.

Table 4.3 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)).

		AGGRESSION						**ANXIETY**					
Taxonomic Level	Bacteria	LDA	PhILR	Selbal (Class)	Selbal (Regr)	Random Forest (Ab+FS)	Random Forest (ILR+FS)	LDA	PhILR	Selbal (Class)	Selbal (Regr)	Random Forest (Ab+FS)	Random Forest (ILR+FS)
Phylum	Firmicutes		*				*		*				*
Order	Burkholderiales		*				*		*				
Family	Oscillospiraceae									*	*		
Family	Peptostreptococcaceae		*				*		*				*
Genus	*Bacteroides*						*		*				*
Genus	*Blautia*		*			*		*	*	*	*	*	
Genus	*Faecalibacterium*	*				*		*				*	
Genus	*Faecalitalea*		*				*				*		*
Genus	*Parasutterella*		*				*		*				
Genus	*Turicibacter*			*	*							*	

Based on the balance between *Blautia* and the mean of Oscillospiraceae and Negativicutes (Figure 4.4), 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 higher-anxiety dog will be considered by the classifier more likely to belong to the higher anxiety group than a random lower-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.

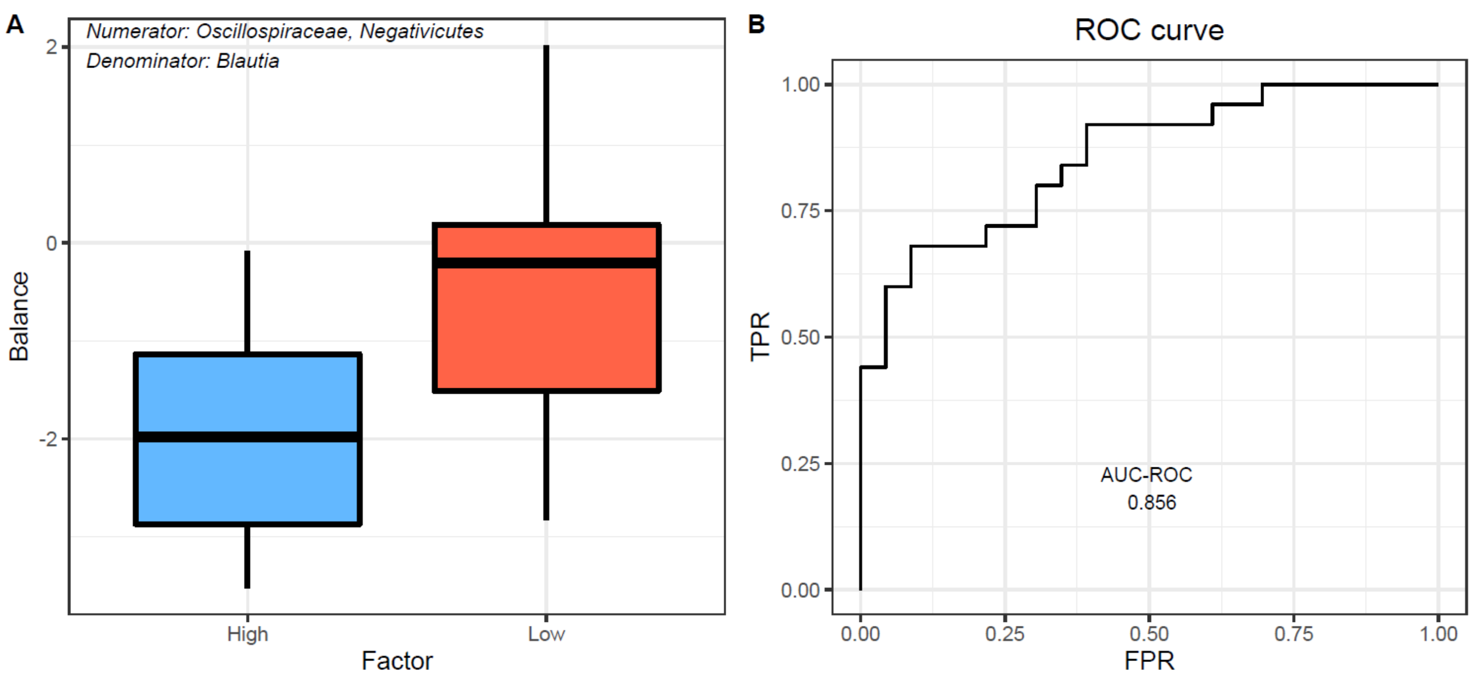

Figure 4.4 (A) Selbal analysis identified the balance between Oscillospiraceae and Negativicutes (numerator) and Blautia (denominator) as an important distinguishing factor between higher and lower anxiety dogs. (B) The balance between these bacteria could predict the assigned behavioural group (higher 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 4.3).

Using Selbal, further investigation into individual anxiety-related C-BARQ scores for dog-directed, stranger-directed, and nonsocial fear (Table 4.4) 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 nonsocial fear at the genus level, and interestingly, *Blautia* was further identified at 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.

Table 4.4 Further Selbal analyses based on continuous anxiety-related C-BARQ sub-scale scores dog directed fear, stranger directed fear, and nonsocial fear.

Further Selbal Results (Anxiety)				
Taxonomic Level	Bacteria	Dog Directed Fear	Stranger Directed Fear	Nonsocial Fear
Phylum	Campylobacterota	*		
Family	Oscillospiraceae		*	
Genus	*Faecalitalea*		*	
Genus	*Clostridium sensu stricto 1*	*		
Genus	*Parasutterella*			*
Genus	*Blautia*			*
Species	*Blautia hansenii*		*	
Species	*Phascolarctobacterium succinatens*		*	

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 uninformative. When comparing the 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) (Figure 4.5). 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% (Figure 4.5). Genera

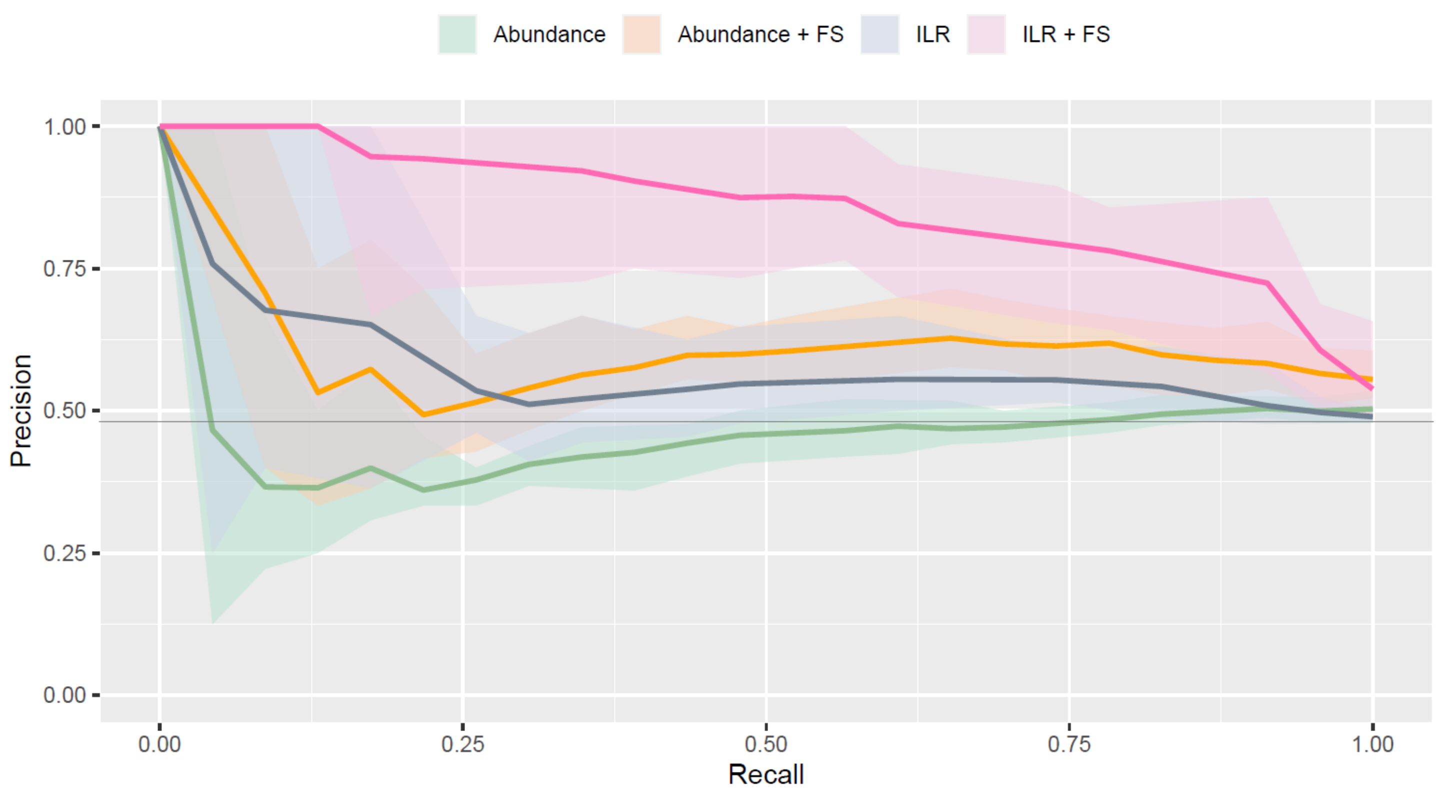

Figure 4.5 Precision-Recall Curves for Random Forest models predicting assignment of dogs based on their fecal microbiota to higher anxiety group, generated using abundance, abundance + feature selection (FS), PhILR log ratios (ILR), and PhILR log ratios with feature selection (ILR + FS). The solid line indicates the average cross-validation Precision-Recall curve and the shaded area indicates the performance range per model observed during cross-validation.

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% (Figure 4.6). 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.

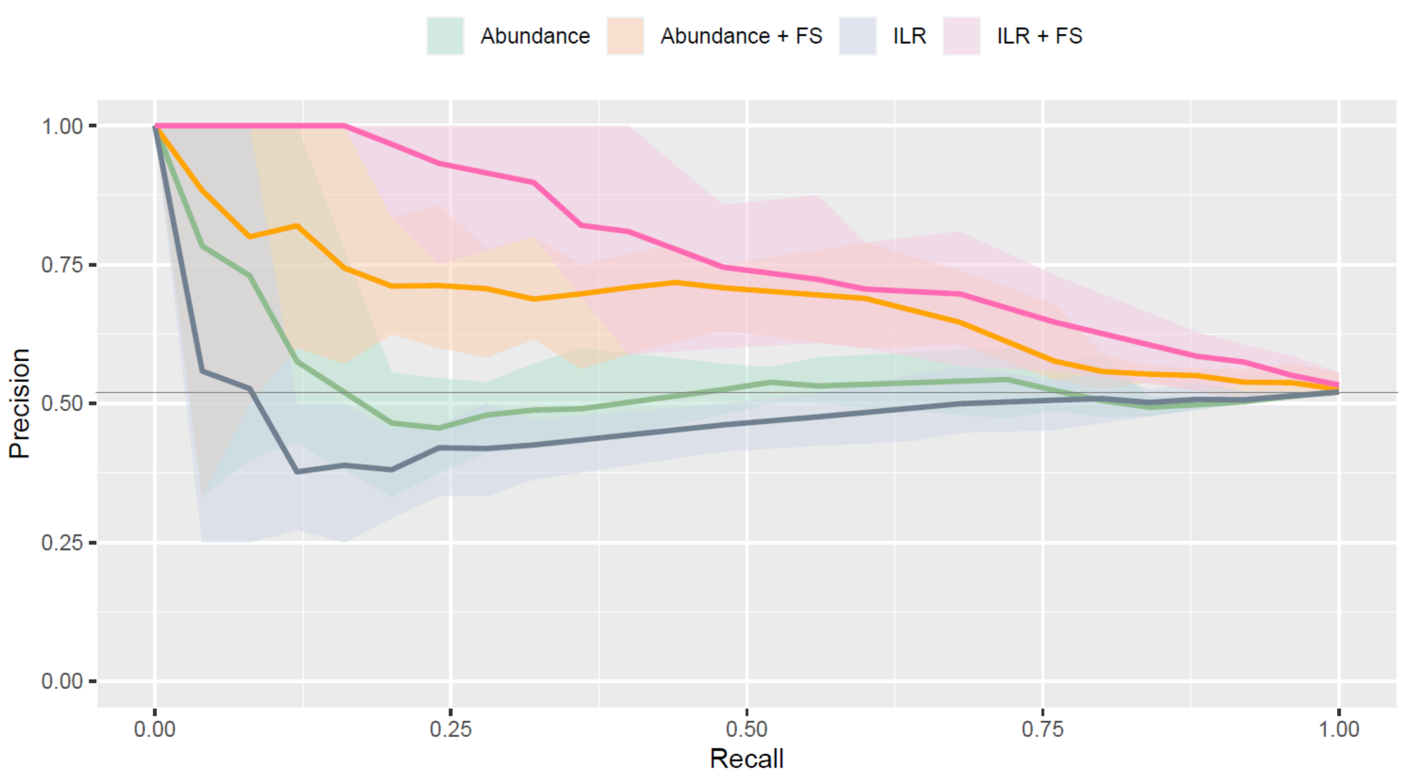

Figure 4.6 Precision-Recall Curves for Random Forest models predicting assignment of dogs based on their fecal microbiota to higher aggression group, generated using abundance, abundance + feature selection (FS), PhILR log ratios (ILR), and PhILR log ratios with feature selection (ILR + FS). The solid line indicates the average cross-validation Precision-Recall curve and the shaded area indicates the performance range per model observed during cross-validation.

4.5 DISCUSSION

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

In our cohort, the dominant phyla Bacteroidota, Firmicutes and Fusobacteria comprised approximately 95% of the gut microbiota, which is in line with other studies of healthy canines (Hand *et al*., 2013; Jha *et al*., 2020; Middelbos *et al*., 2010; Morelli *et al*., 2022), although considerable variation in percentages can be seen across the literature for specific taxa (Pilla and Suchodolski, 2020). In comparison to Mondo *et al*. (2020), the fecal microbiota 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/Bacteroidetes 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 (Jha *et al*., 2020). 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 (Alexander *et al*., 2018; Middelbos *et al*., 2010), and a higher protein content (as seen in raw-fed or BARF diets) is associated with decreased abundance of Firmicutes (Bermingham *et al*., 2017; Schmidt *et al*., 2018). Increased abundance of the genus *Fusobacterium* is generally associated with healthy control dogs

(Vázquez-Baeza *et al*., 2016) and increased access to the outdoors (Song *et al*., 2013), 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*. (2020) 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 the treatment of behavioural disorders should closely consider the individual dog's core microbial population in the gut, and the methodology used to collect samples and extract microbial DNA.

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 (Mondo *et al*., 2020), 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 (Craddock *et al*., 2022). In clinical studies of gastrointestinal disease, increased richness of gut microbiota is typically associated with healthy animals (Pilla & Suchodolski, 2020); 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 (highly anxious) dogs in the Mondo study, it is possible that this is due to how the dogs were categorized in their study compared to the current one. Mondo *et al.* (2020) assigned dogs to a behavioural group based on observable behaviours within the shelter environment. It is possible that these 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*.

(2020) 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 microbiota 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 (Tiira *et al*., 2016), and the clear association between aggression and anxiety C-BARQ scores in many of our dogs suggests that 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 (Liu *et al*., 2021) and is currently being investigated as a potential avenue for treatment of anxiety-like behaviours in autism spectrum disorder (ASD) in humans (Sen *et al*., 2022). In addition, improved sleep quality was associated with an increase in abundance of *Blautia* after exercise in patients suffering from sleep disorders (Qiu *et al*., 2022). Conversely, some studies have associated an increased abundance of *Blautia* with gastrointestinal disease (Nishino *et al*., 2018; Rajilić–Stojanović *et al*., 2011), increased risks of breast cancer (Luu *et al*., 2017), and acetic acid-producing *Blautia* species are considered to contribute to non-alcoholic fatty liver disease (Shen *et al*., 2017). An elevated relative abundance of *Blautia* has also been seen in humans with major depressive disorder (Chung et al., 2019).

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 (Suchodolski *et al*., 2012), and has been used as an indicator for gut dysbiosis in mathematical modelling (AlShawaqfeh *et al*., 2017). 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 (Xu *et al*., 2019). 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, Selbal analyses and Random Forest), and given the increased proportions of the genus *Blautia* in higher anxiety dogs in this study, it is likely that either the individual species we have detected do not possess the aforementioned protective effects, or they are present in such abnormally large amounts that the gut has become dysregulated. This notion is supported by the human literature which demonstrates that the elevated abundance of typically beneficial bacteria can be associated with increased risk of gastrointestinal disease (Nojoomi & Ghasemian, 2016), depression and anxiety (Capuco *et al*., 2020), however more information is needed about *Blautia*'s metabolomic role in the canine gut to determine a causal link. 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 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 (Panasevich *et al*., 2015) and probiotics (Xu *et al*., 2019), 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 (Peirce & Alviña, 2019). While the complexities of the gut-brain axis are still being investigated in multiple species, it has been shown that increased plasma glutamine and γ-glutamyl glutamine are also associated with fearfulness in dogs (Puurunen *et al*., 2018), 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 (Cortese & Phan, 2005). The Lachnospiraceae family, to which *Blautia* belongs, has been correlated with behavioural changes induced by stress in other mammalian models, including humans (Wang *et al*., 2022) and mice (Bangsgaard Bendtsen *et al*., 2012). Based on the human literature, there is evidence to suggest the Lachnospiraceae family is involved in the inflammatory pathway, as an increase in the abundance of this family promotes a decrease in SCFA concentration (Duncan *et al*., 2007), leading to intestinal wall dysfunction (Jiang *et al*., 2015). Similarly, *Turicibacter* (associated with aggression in this study) has been linked with inflammation and cancer in mice (Zackular *et al*., 2013). Along with *Blautia* species, a decrease in the abundance of *Turicibacter* is also an indication of gut dysbiosis in gastrointestinal disease in dogs (AlShawaqfeh *et al*., 2017). 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.

4.6 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 the gut microbiome 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 (Bresciani *et al*., 2018; Middelbos *et al*., 2010; Sandri *et al*., 2019) and behaviour (Kato *et al*., 2012; Landsberg *et al*., 2015), and that the composition of the gut microbiota is linked to behaviour (Kirchoff *et al*., 2019; Mondo *et al*., 2020; Puurunen *et al*., 2018), there is an 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 a 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 the gut microbiota, such a complex system should be respected as such, and great care taken before inferring a causational or directional relationship.

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CHAPTER 5: GENERAL DISCUSSION & FUTURE STUDY

5.1 GENERAL DISCUSSION

In this thesis, I have not only provided evidence in support of the link between the gut microbiome and behaviour in a community sample of domestic dogs, but I have also highlighted important elements in the research and treatment of canine behaviour that should be taken into consideration in both the clinical and scientific communities. As a dog training professional who frequently consults with dog owners on aggression and anxiety cases and develops behaviour modification protocols for these dogs, the completion of this thesis has not only expanded my knowledge and understanding of canine behaviour, but also shaped my view of 'best practices' with which to proceed in my professional career. While it is imperative for the progression of the dog training industry to incorporate the scientific literature, individuals working as trainers or behaviour consultants are not required to have a scientific background, and it can be challenging for many to access and/or interpret the scientific literature to better inform their practices. In addition to accessibility, it is equally as important that the information published in the scientific literature is accurate, valid, and replicable.

Unlike other studies to date, which have evaluated the relationship between behaviour and gut microbiota in clinical or shelter populations of dogs, the current study has leveraged companion dogs from the local community who are more likely to represent average "pet" dogs not experiencing extreme behavioural issues or environments. The use of a community sample in this thesis has provided valuable insight into the lifestyle factors that may impact canine behaviour in a broader context. Further, the use of online, owner-reported questionnaires has allowed for a deeper understanding of their use (and potentially mis-use, e.g., in the case of the C-BARQ FDA subscale) in the canine literature. By augmenting the C-BARQ with additional questions in a supplemental questionnaire (the Diet & Lifestyle Questionnaire), it is apparent that dogs who live with conspecifics, or those who participate regularly in social activities, are somewhat behaviourally different from those who do not. However, it is not possible to establish a causational relationship between the dog's behaviour and environment: does interacting regularly with other dogs alter a dog's behaviours, or do a dog's behaviours, particularly those related to aggression and fear, make it less likely that the dog will be given opportunities to interact with other dogs? Second, while the use of owner-based questionnaires provides an excellent opportunity to collect larger data sets and overcome limitations in resources when conducting research, this thesis has provided strong evidence for the importance of using clear language when delivering instructions and questions. The C-BARQ familiar dog aggression (FDA) subscale was often "misinterpreted" by dog owners, at least in relation to its stated purpose of evaluating aggression towards familiar dogs who share a household. In the original study reported here, a large number of dog owners with a single dog in the household responded to the FDA questions. In my follow-up study designed to specifically address this occurrence, it was discovered that these dogs with an "unexpected" FDA score also differed across other C-BARQ subscales compared to singleton dogs whose owners did not complete the FDA questions. Importantly, the former were found to have more frequent exposures to other dogs that they did not live with, but nevertheless, were considered familiar by their owners. In its strictest definition, the FDA subscale does not apply to these dogs who do not live with at least one other dog.

Yet, the question can be raised whether this FDA subscale should be expanded to incorporate these dogs who frequently spend time with other dogs either within or outside of their home environment. It is clear that these dogs are capable of exhibiting aggression towards other dogs, and the contexts of aggression (around resources such as food, toys, resting places) may be relevant to dogs who socialize with dogs owned by other people. Some consideration should be given to modifying the manner in which the FDA scores are reported by C-BARQ and/or are interpreted by other researchers using the C-BARQ. First, more clarity around the definition of familiar dog aggression could be provided in the questionnaire itself, in an attempt to prevent owners of singleton dogs from answering the questions, if the goal of the FDA subscale is to solely evaluate a dog's behaviour to another dog with which they live. However, even if this is the case, a proportion of participants are likely to respond to the questions regardless. Because of this, it would be useful if additional questions regarding the dog's living arrangement were included and reported in the C-BARQ results- i.e., if dogs were reported to live alone but the FDA questions were answered, this could be flagged in the C-BARQ output as a potentially invalid score, or indicative of a singleton dog that might have different social experiences. Alternatively, researchers using the C-BARQ could administer an additional questionnaire in order to confirm inclusion criteria of participants for their study, which should be specific to their research question.

The gut microbiome study (Chapter 4) has provided evidence that, even within a community sample where dogs are not severely polarized in their aggression or anxiety scores, the gut microbiota composition does differ in association with the dog's behaviour. While there was less evidence in support of differences in aggression, I showed consistently across different bioinformatics analyses that the genus *Blautia* has a close relationship with anxiety in this population of dogs. Further study would benefit from targeting more severe aggression and anxiety cases that were not captured in this community sample, and incorporate a matching process as used in this study to minimize the variability of factors other than behaviour that may impact gut microbiota composition.

5.2 CLINICAL RELEVANCE FOR THE TREATMENT OF BEHAVIOURAL ISSUES IN DOGS

The process of behaviour modification for issues such as aggression and anxiety involves not only the implementation of desensitization, counter-conditioning and response substitution (Orihel & Fraser, 2008; Overall & Dunham, 2002), but also supplementary approaches such as environmental management and lifestyle or routine changes to better meet the social, physiological and cognitive needs of the dog. Maslow's Hierarchy of Needs has been adapted to reflect the needs of companion dogs - essential needs such as veterinary care, safe shelter, and access to food & water form a baseline for owners to meet the minimum welfare needs (Griffin *et al*., 2023). However, a commitment to meeting as many dog-specific needs as possible is often one of the first changes owners will make when embarking on a behaviour modification plan with their dog. For example, a dog who has been struggling with reactivity towards other dogs will likely have been experiencing elevated stress during exercise, in particular those living in urban environments. For these dogs, my first "training" recommendation would be to facilitate exercise in a quieter environment by modifying the location or time of the walk to reduce the likelihood of repeated reactions towards other dogs. In addition to modifying the location or timing of the walk, owners would also be instructed to encourage natural dog behaviours such as sniffing and exploring their environment – commonly called "decompression walks" by trainers and behaviour consultants, these outings are intended to decrease stress, and build confidence in the individual, and are helpful in establishing a calmer baseline of behaviour ahead of the systematic re-introduction of the dog's triggers. The activities in which dog and owner participate can contribute to the development, maintenance and treatment of behaviour, and this relationship can be beneficial or detrimental to the dog's behaviour. For example, young dogs and puppies who are provided with safe socialization opportunities in life typically respond better to those contexts in adulthood, but dogs who are not appropriately socialized or have traumatic experiences are more likely to develop behavioural issues (Cutler *et al*., 2017; Howell *et al*., 2015). This study has provided evidence that dogs who participate in social activities, or live with conspecifics, have somewhat different behavioural profiles, particularly with respect to fear-related behaviours. However, the directionality of any relationship between environmental experiences and behaviour cannot be determined here, and is likely dynamic and complex. While a better understanding of the nature of this relationship would be helpful to determine best practices for the prevention of behavioural issues, for dog owners currently experiencing issues with aggression and anxiety, the implementation of lifestyle changes specific to their dog's needs, such as increased mental and physical stimulation, are likely beneficial as a supportive treatment for behavioural issues.

In addition to management strategies and lifestyle choices, dog owners are often in search of pharmaceutical or nutraceutical interventions to assist in the management or treatment of behavioural issues. Arguably, the best supported treatments for canine aggression and anxiety are behavioural medications, such as SSRIs (selective serotonin reuptake inhibitors, e.g. fluoxetine; Chutter *et al*., 2019; Landsberg *et al*., 2008), TCAs (tri-cyclic antidepressants, e.g. clomipramine; King *et al*., 2000), and SARIs (serotonin receptor antagonists & reuptake inhibitors, e.g. trazadone Gruen & Sherman, 2008; King *et al*., 2000; Landsberg *et al*., 2008). Both the mechanisms of action and impacts on behaviour in dogs are well-understood for these medications. Many other products on the market claim to treat behavioural issues, but often have inconsistent scientific support for their use. For example, pheromone-based products such as Adaptil collars and plug-ins are advertised as anxiolytic products, but few studies have provided strong evidence for their efficacy (e.g., Santos *et al*., 2020; Taylor *et al*., 2020). Anti-inflammatory supplements such as omega-3 oils are also recommended by veterinary behaviourists to treat cellular inflammation associated with chronic stress, and studies have shown some positive outcomes for the treatment of anxiety (Rahimi Niyyat *et al*., 2018). However, a causal relationship between omega-3 and behaviour has not yet been established (Re *et al*., 2008); it could be argued that this line of treatment is effective because the dog is experiencing underlying pain (e.g. from arthritis or soft tissue damage), which is often difficult to diagnose without extensive testing and imaging, but could be a contributor to aggressive behaviour.

Probiotics have been recommended more recently as a potential treatment for behavioural issues – since 2018, Purina (Nestlé Purina PetCare Company) has released a probiotic Calming Care™, which differs from their previous probiotic, Fortiflora™, in both its marketing and advertised effects. Fortiflora™ is comprised of *Enterococcus faecium*, a bacterial species known to assist in relieving symptoms of gastrointestinal issues, whereas Calming Care™ contains a more recently discovered bacterial strain, *Bifidobacterium longum 999*. An abstract was presented by Purina at the American College of Veterinary Behaviour Symposium in 2018 apparently confirming the efficacy of Calming Care in the treatment of anxiety in dogs. However, the publicly available information is severely lacking in detailed methods compared to other clinical probiotic studies. Thus, whether probiotics are actually helpful in influencing problematic behaviours in dogs remains largely unknown.

While human-derived probiotics are widely used in canine health, for gastroenteritis, IBD and allergy symptoms (Grzeskowiak *et al*., 2015), there is mounting evidence that developing host-specific probiotics may be beneficial. One illustration of this is that Kirchoff *et al*. (2019) showed that in their cohort of pitbulls, there were increased amounts of *Lactobacillus* species in the aggressive dogs; *Lactobacillus* is a commonly-used probiotic (Panja *et al*., 2023). While *Lactobacillus* is beneficial in the treatment of gastrointestinal disease, it is unclear if it would be beneficial in dogs who have pre-existing behavioural issues, or if supplementation of *Lactobacillus* may risk increased aggressiveness in dogs treated with this probiotic. Thus, any clinical research should closely monitor not only behavioural effects of probiotics, but also metabolites associated with changes in the gut microbiome composition and any physical symptoms of gastrointestinal dysfunction. Similarly, clinical studies investigating host-specific probiotics should also be aware of potential behavioural side effects within their treatment groups.

5.3 LIMITATIONS & FUTURE CONSIDERATIONS

The sample sizes in this study were adequate for the research objectives; however, a considerable number of people did not continue to complete the C-BARQ after completing the Diet & Lifestyle questionnaire. Of the 494 participants who completed the first questionnaire, only 235 continued to the C-BARQ. If the number of questions in the C-BARQ (101) contributed to this drop-out rate, adopting the mini C-BARQ, a 42 question version of the original questionnaire, which has been shown to have predictive validity in other studies (Duffy *et al*., 2014; Duffy & Serpell, 2012), might be useful. However, if the barrier to further participation in the current study was the requirement to register an account for the dog on the C-BARQ website after answering the first online questionnaire (Diet & Lifestyle), this might not help much. Any future studies using the Diet & Lifestyle questionnaire could condense it to the most relevant questions, and potentially improve participant retention between questionnaires.

The gut microbiome study would benefit from a follow-up study to include a dietary control, as multiple studies have established the effects of diet on the gut microbiota composition (Bresciani *et al*., 2018; Coelho *et al*., 2018; Kim *et al*., 2017; Middelbos *et al*., 2010). In addition, there are aspects of the gut microbiome aside from gut microbiota composition that could provide more detailed insight into the relationships between the gut microbiome and behaviour. For example, a transcriptomics analysis could investigate which microbial genes are expressed in dogs with differing behavioural profiles, while a metabolomics approach (combined with metagenomic sequencing) would allow characterization of the molecules produced by the microbes in the gut, and identify potential differences that could be associated with behaviour. While it was my intention to present a sample that was representative of “normal” owned dogs in the community, further study could establish if the association of *Blautia* with higher levels of anxiety persists in the face of dietary interventions of treatment with probiotics. Further study could also adopt a longitudinal approach to sampling – for example, taking fecal samples prior to dietary intervention, at 1 and 4 weeks after changing diet, and approximately 4 weeks after returning to their conventional diet. This would establish both the dog’s native population, and their response to diet, both of which would be necessary before administering any therapeutic interventions such as probiotics. Behavioural assessments can also be performed on a smaller sample size as part of a longitudinal study – while the C-BARQ (or similar) would be used to establish inclusion criteria, a longitudinal study would best be supported by in-person evaluations or interviews conducted at the time of sampling to track the dog’s behaviour and any changes associated with dietary changes or administration of probiotics.

5.4 FUTURE STUDY

This thesis has provided substantial evidence that both the dog’s external environment, and their gut microbiome, are strongly associated with their behaviour. In particular, dogs who spend time with other dogs (whether owned by the same family, or not) display different behavioural profiles than singleton or non-socialized dogs. The gut microbiota of dogs with higher levels of anxiety was consistently associated with different proportions of the genus *Blautia* compared to those with less anxiety; however, there was little evidence for differences in the gut microbiota between dogs with higher and lower aggression scores. This study adds to the ever-growing field of gut microbiome research, and provides novel insight upon which future studies can work towards better understanding the links between the canine gut microbiome and behaviour.

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5.6 SUPPLEMENTARY TABLES

Table S1 C-BARQ behavioural scores from 72 pet dogs used to calculate median split for pair-wise matching of dogs in higher vs lower anxiety or aggression groups. The cohort displayed was selected from questionnaire respondents based on location, age (2-7 years), consistent diet and living arrangements for 3+ months.

	Anxiety Score (C-BARQ)				
	Composite	Stranger Directed	Dog Directed	Nonsocial Fear	Separation Related
N	72	72	72	72	72
Mean ± S.E.	0.955 ± 0.07	0.82 ± 0.121	1.155 ± 0.119	1.05 ± 0.107	0.82 ± 0.101
Median	0.782	0.5	1	0.83	0.5
Range	0.0425 – 2.625	0 – 3.25	0 – 3.25	0 – 3.17	0 – 3.25
	Aggression Score (C-BARQ)				
	Composite	Stranger Directed	Dog Directed	Owner Directed	Familiar Dog
N	72	72	70	72	55
Mean ± S.E.	0.565 ± 0.05	0.618 ± 0.08	1.061 ± 0.117	0.158 ± 0.03	0.406 ± 0.08
Median	0.455	0.4	0.875	0	0
Range	0 – 1.8825	0 – 2.9	0 – 4	0 – 1.38	0 – 1.75

Table S2 Sequence quality (number of reads) through DADA2 pipeline

	input	filtered	denoisedF	denoisedR	merged	nonchim
Sample1	91680	58959	58518	58476	56700	41849
Sample10	101679	65325	64983	64838	63208	39221
Sample11	109479	70993	70525	69996	67737	43041
Sample12	103804	62069	61376	61131	57506	37529
Sample13	127183	82115	81020	80645	76219	51366
Sample14	102527	62778	62179	62121	60070	42915
Sample15	118395	75400	74444	74402	70866	43994
Sample16	88338	57516	56851	56688	53544	35764
Sample17	100166	62633	62388	62255	61032	40246
Sample18	68249	43298	43006	42583	40919	31059
Sample19	81879	51263	50986	50850	48839	32473
Sample2	77487	49679	49164	49036	46760	34589
Sample20	133346	83360	82726	82658	79540	56121
Sample21	87496	57685	57200	57165	55139	38436
Sample22	143556	89860	88729	88757	83430	49888
Sample23	98498	62450	61997	62015	59214	28162
Sample24	75542	48108	47617	47324	45075	31541
Sample25	80664	52271	51614	51494	48690	32046
Sample26	92974	61056	60423	60405	57602	38776
Sample27	85762	56523	56268	56165	55297	31875
Sample28	90459	56124	55361	55137	51832	33092
Sample29	101713	65482	64904	64930	61217	39266
Sample3	111738	73425	72880	72700	70367	47186
Sample30	76248	46079	45813	45708	44454	32357
Sample31	59746	38072	37821	37740	36770	27590
Sample32	38855	24916	24713	24555	23793	17037
Sample33	79521	52174	51800	51656	49872	35075
Sample34	65658	41701	41213	41164	39664	28225
Sample35	69883	46212	46060	45954	44806	32779
Sample36	92074	55182	54413	54358	51083	34128
Sample37	77995	49542	48955	48801	46295	30637
Sample38	89120	55900	55215	54975	52669	31185
Sample39	87416	54440	53982	53975	52420	30489
Sample4	71428	44610	44203	43912	41890	29779
Sample40	14772	8095	7988	7978	7743	5621
Sample41	80585	51042	50525	50370	47688	34919
Sample42	58685	38810	38576	38517	37901	26316
Sample43	76585	49123	48268	48023	44191	29251
Sample44	97094	59709	59290	59101	57274	37343
Sample45	115931	74594	73724	73413	69157	47160

Table S3 Relative abundance across taxonomic levels for the entire cohort (All Dogs, n=48), higher and lower anxiety and aggression groups. Values are displayed as percentages ± standard error, with p-values before adjustment for false discovery (Mann-Whitney U). After correction for false discovery, all p values were > 0.05.

			Anxiety Group			**Aggression Group**		
		All Dogs	Lower	Higher	p	Lower	Higher	p
Phylum	Bacteroidota	53.6 ± 2.3	51.5 ± 4.1	55.9 ± 2.1	0.483	51.8 ± 3.5	55.2 ± 3.25	0.307
	Firmicutes	23.9 ± 1.7	24.5 ± 2.9	23.3 ± 1.6	0.539	25.2 ± 3.1	22.8 ± 1.61	0.870
	Fusobacteriota	18.5 ± 1.8	20.0 ± 2.9	17 ± 2.2	0.550	18.9 ± 2.4	18.2 ± 2.9	0.628
Class	Bacteroidia	53.6 ± 2.3	51.5 ± 4.1	55.9 ± 2.1	0.483	51.8 ± 3.5	55.2 ± 3.2	0.307
	Fusobacteria	18.5 ± 1.8	20 ± 2.9	17 ± 2.2	0.550	18.9 ± 2.4	18.2 ± 2.9	0.628
	Negativicutes	11.8 ± 1.6	13.6 ± 2.9	9.9 ± 1.2	0.733	14.0 ± 3.0	9.8 ± 1.4	0.244
	Clostridia	9.3 ± 0.6	8.4 ± 0.9	10.4 ± 0.7	0.081	8.3 ± 0.9	10.3 ± 0.8	0.129
	Gammaproteobacteria	3.7 ± 0.3	3.8 ± 0.5	3.5 ± 0.3	0.918	3.8 ± 0.6	3.6 ± 0.3	0.741
	Bacilli	2.7 ± 0.4	2.4 ± 0.7	2.9 ± 0.4	0.043	2.7 ± 0.7	2.6 ± 0.4	0.392
Order	Bacteroidales	53.6 ± 2.3	51.5 ± 4.1	55.9 ± 2.1	0.483	51.8 ± 3.5	55.2 ± 3.2	0.307
	Fusobacteriales	18.5 ± 1.8	20.0 ± 2.9	17.0 ± 2.2	0.550	18.9 ± 2.4	18.2 ± 2.9	0.628
	Veillonellales-Selenomonadales	11 ± 1.6	12.9 ± 3	8.9 ± 1.3	0.628	13.5 ± 3.0	8.7 ± 1.5	0.19
	Lachnospirales	4.4 ± 0.5	4.1 ± 0.8	4.6 ± 0.6	0.110	4.4 ± 0.7	4.3 ± 0.6	0.584
	Oscillospirales	3.9 ± 0.4	3.2 ± 0.6	4.7 ± 0.5	0.081	3.0 ± 0.5	4.8 ± 0.5	0.05
	Burkholderiales	3.1 ± 0.3	3.2 ± 0.5	3.0 ± 0.3	0.934	3.3 ± 0.5	2.9 ± 0.3	0.687
	Erysipelotrichales	2.4 ± 0.3	2.2 ± 0.6	2.7 ± 0.3	0.039	2.5 ± 0.7	2.4 ± 0.3	0.297
Family	Bacteroidaceae	29.9 ± 2.5	28.7 ± 3.7	31.3 ± 3.4	0.563	29.3 ± 3.6	30.5 ± 3.6	0.877
	Prevotellaceae	23.5 ± 3.1	22.7 ± 4.7	24.4 ± 4.3	0.621	22.5 ± 4.9	24.5 ± 4.1	0.521
	Fusobacteriaceae	18.5 ± 1.8	20.0 ± 2.9	17.0 ± 2.2	0.550	18.9 ± 2.4	18.2 ± 2.9	0.628
	Selonomondaceae	11 ± 1.6	12.9 ± 3.0	8.9 ± 1.3	0.650	13.5 ± 3.0	8.6 ± 1.5	0.176
	Lachnospiraceae	4.4 ± 0.5	4.1 ± 0.8	4.6 ± 0.6	0.110	4.4 ± 0.7	4.9 ± 0.6	0.584
	Ruminococcaceae	3.6 ± 0.4	3.0 ± 0.6	4.3 ± 0.5	0.137	2.7 ± 0.5	4.5 ± 0.5	0.055
	Sutterellacae	3.1 ± 0.3	3.2 ± 0.5	2.9 ± 0.3	0.934	3.3 ± 0.5	2.9 ± 0.3	0.703

5.7 SUPPLEMENTARY FIGURES

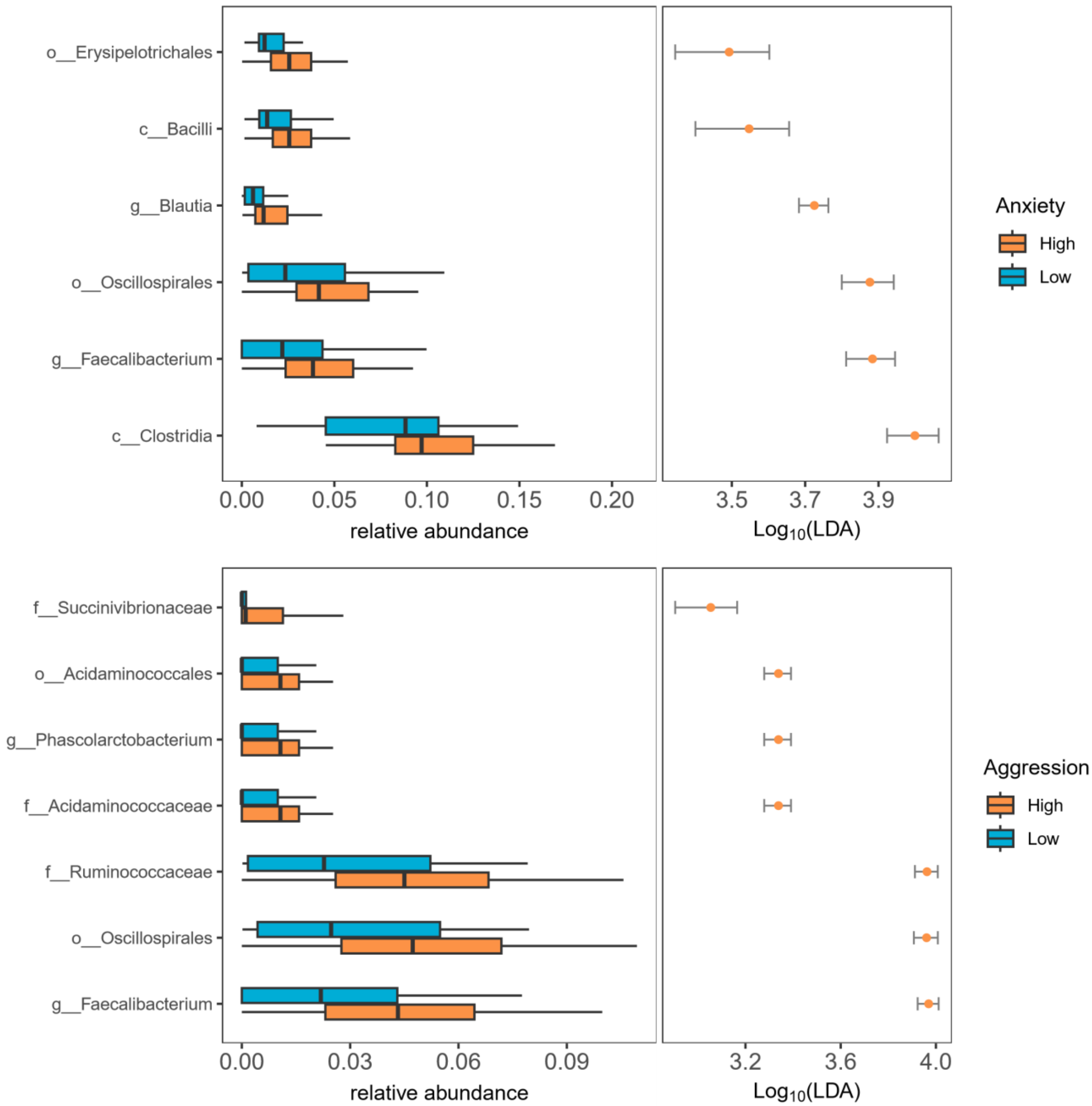


Figure S1 Linear discriminant analysis for higher and lower aggression and anxiety groups.

5.8 SUPPLEMENTARY MATERIALS

Supplementary Material A. The Diet & Lifestyle Questionnaire, delivered online via Qualtrics.

Lifestyle & Routine

How many dogs live in your home?

1

2

3

4+

What would you consider to be your dog's main source of exercise:

Walking (ie neighbourhood walks)

Running

Hiking

Fetch/Frisbee

Garden/Yard

Doggie Daycare

None/Not Applicable

On average, how frequently does your dog exercise away from your property (eg. Walks, hiking etc) each week?

Never

Less than once per week

Once per week

2-4 times per week

Daily

On average, how much time does your dog spend exercising away from your property (eg. Walks, hiking etc) each week?

Less than one hour per week

1-2 hours per week

2-4 hours per week

4-6 hours per week

6+ hours per week

How frequently does your dog encounter people outside your family?

Never

Less than once per week

Once per week

2-4 times per week

Daily

How frequently does your dog encounter dogs outside your family?

Never

Less than once per week

Once per week

2-4 times per week

Daily

Diet

Does your dog have scheduled meal times, or are they free-fed?

Scheduled meals

Free-fed

If your dog has scheduled meal times, how many times per day does your dog eat?

One

Two

Three

Four or more

How long has your dog been fed on this schedule?

Less than a month

1-3 months

3 – 6 months

6+ months

What type of food forms the majority of your dog's diet? (select all that apply)

Kibble

Canned/Wet food

Raw

Home-cooked

What brand of food do you feed your dog? (eg. Purina, Acana, Orijen, Royal Canin)

____

What formula of food do you feed your dog? (eg. Kirkland Salmon & Sweet Potato, Acana Wild Coast, Go! Sensitivity Salmon Formula) Please provide as much information as possible so we can search the ingredients and nutritional information – you can also email a photo of the food packaging to sdp575@mun.ca

____

How long has your dog been eating this food?

Less than a month

1-3 months

3 – 6 months

6+ months

Do you supplement your dog's diet regularly (ie. more than once per week) with any of the following foods (select all that apply):

Green vegetables (broccoli, spinach, kale, peas, beans)

Salad vegetables (tomato, peppers, cucumber)

Fruits (blueberries, banana, strawberries, apple)

Poultry

Red meats

Fish

Deli meats (hot dogs, ham, bologna)

Starchy vegetables (potato, sweet potato)

Yogurt

Peanut butter

From the following graphic, how would you rate your dog's body condition?

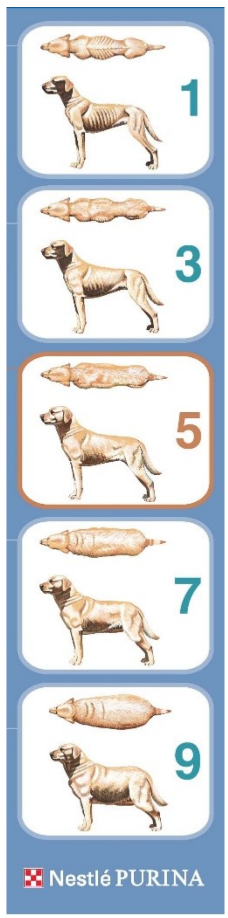
1

3

5

7

9



Medical

Does your dog currently eat a veterinary-prescribed diet?

Yes

No

If yes, please specify the brand, formula and reason for the particular diet (eg. Purina ProPlan Neurocare kibble, for epilepsy):

____

Has your dog ever taken a dewormer, such as Sentinel or Strongid?

Yes

No

On average, how frequently does your dog take a dewormer?

Every month

Every 3 months

Every 6 months

Every year

Never

When did your dog last receive their dewormer?

____

Has your dog ever taken flea medication, such as Advantage Multi?

Yes

No

On average, how frequently does your dog take flea medication?

Every month

Every 3 months

Every 6 months

Every year

Never

When did your dog last receive their flea medication?

____

Has your dog been vaccinated against parvovirus, distemper and parainfluenza? (hint – this is the vaccination your puppy gets at 8, 12 and 16 weeks, then as a booster in adulthood)

Yes

No

On average, how frequently has your dog been vaccinated since their puppy boosters?

Every year

Every 2 years

Every 3 years

Less than every 3 years

Never

When did your dog last receive their vaccinations for parvovirus, distemper and parainfluenza?

____

Does your dog currently taking any medication? If so, please specify:

____

Does your dog receive any of the following supplements:

Omega 3/6 oils

CBD products

Fortiflora/Probiotics

Other: __________________

On average, how many times does your dog defecate each day?

One

Two

Three

More than three

On average, which of these movements most accurately represent your dog's bowel movements?

1

2

3

4

5

6

7

8

Supplementary Material B. The Canine Behaviour and Research Questionnaire (C-BARQ) as presented to participants via https://vetapps.vet.upenn.edu/cbarq/

Section 1: Training and obedience

Some dogs are more obedient and trainable than others. By clicking on the appropriate choices, please indicate how trainable or obedient your dog has been in each of the following situations in the recent past.

Selected dog: **Study**	NEVER	SELDOM	SOMETIMES	USUALLY	ALWAYS	NOT OBSERVED/ NOT APPLICABLE
1. When off the leash, returns immediately when called.						
2. Obeys the "sit" command immediately.						
3. Obeys the "stay" command immediately.						
4. Seems to attend/listen closely to everything you say or do.						
5. Slow to respond to correction or punishment; "thick-skinned".						
6. Slow to learn new tricks or tasks.						
7. Easily distracted by interesting sights, sounds, or smells.						
8. Will "fetch" or attempt to fetch sticks, balls, or objects.						

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Section 2: Aggression

Some dogs display aggressive behavior from time to time. Typical signs of moderate aggression in dogs include barking, growling and baring teeth. More serious aggression generally includes snapping, lunging, biting, or attempting to bite. By clicking on the following scales, please indicate your own dog's recent tendency to display aggressive behavior in each of the following contexts:

Selected dog: **Study**	NO AGGRESSION No visible signs of aggression		MODERATE AGGRESSION Growling/barking, baring teeth		SERIOUS AGGRESSION Snaps, bites, or attempts to bite	NOT OBSERVED/ NOT APPLICABLE
9. When verbally corrected or punished (scolded, shouted at, etc.) by you or a household member.						
10. When approached directly by an unfamiliar **adult** while being walked/exercised on a leash.						
11. When approached directly by an unfamiliar **child** while being walked/exercised on a leash.						
12. Toward unfamiliar persons approaching the dog while s/he is in your car (at the gas station, for example).						
13. When toys, bones or other objects are taken away by a household member.						
14. When bathed or groomed by a household member.						
15. When an unfamiliar person approaches you or another member of your family at home.						
16. When unfamiliar persons approach you or another member of your family away from your home.						

17. When approached directly by a household member while s/he is eating.

18. When mailmen or other delivery workers approach your home.

19. When his/her food is taken away by a household member.

20. When strangers walk past your home while your dog is outside or in the yard.

21. When an unfamiliar person tries to touch or pet the dog.

22. When joggers, cyclists, rollerbladers or skateboarders pass your home while your dog is outside or in the yard.

NO AGGRESSION	MODERATE AGGRESSION	SERIOUS AGGRESSION	NOT OBSERVED/ NOT APPLICABLE
No visible signs of aggression	Growling/barking, baring teeth	Snaps, bites, or attempts to bite	

23. When approached directly by an unfamiliar **male** dog while being walked/exercised on a leash.

24. When approached directly by an unfamiliar **female** dog while being walked/exercised on a leash.

25. When stared at directly by a member of the household.

26. Toward unfamiliar dogs visiting your home.

27. Toward cats, squirrels or other animals entering your yard.

28. Toward unfamiliar persons visiting your home.

29. When barked, growled, or lunged at by another (unfamiliar) dog.

30. When stepped over by a member of the household.

31. When you or a household member retrieves food or objects stolen by the dog.

32. Towards another (familiar) dog in your household.

33. When approached at a favorite resting/sleeping place by another (familiar) household dog.

34. When approached while eating by another (familiar) household dog.

35. When approached while playing with/chewing a favorite toy, bone, object, etc., by another (familiar) household dog.

Are there any other situations in which your dog is sometimes aggressive? If so, please describe briefly:

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Section 3: Fear and anxiety

Dogs sometimes show signs of anxiety or fear when exposed to particular sounds, objects, persons or situations. Typical signs of mild to moderate fear include: avoiding eye contact, avoidance of the feared object, crouching or cringing with tail lowered or tucked between the legs, whimpering and whining, freezing, and shaking and trembling. Extreme fear is characterized by exaggerated cowering, and/or vigorous attempts to escape, retreat or hide from the feared object, person or situation.By clicking on the following scales, please indicate your own dog's recent tendency to display fearful behavior in each of the following contexts:

Selected dog: **Study**	NO FEAR OR ANXIETY (No visible signs of fear)		MILD – MODERATE FEAR/ANXIETY		EXTREME FEAR (Cowers, retreats, hides, etc.)	NOT OBSERVED/ NOT APPLICABLE
36. When approached directly by an unfamiliar **adult** while away from your home.	○	○	○	○	○	○
37. When approached directly by an unfamiliar **child** while away from your home.	○	○	○	○	○	○
38. In response to sudden or loud noises (e.g. vacuum cleaner, car backfire, road drills, objects being dropped, etc.).	○	○	○	○	○	○
39. When unfamiliar persons visit your home.	○	○	○	○	○	○
40. When an unfamiliar person tries to touch or pet the dog.	○	○	○	○	○	○
41. In heavy traffic.	○	○	○	○	○	○
42. In response to strange or unfamiliar objects on or near the sidewalk (e.g. plastic trash bags, leaves, litter, flags flapping, etc.).	○	○	○	○	○	○
43. When examined/treated by a veterinarian.	○	○	○	○	○	○
44. During thunderstorms, firework displays, or similar events.	○	○	○	○	○	○
45. When approached directly by an unfamiliar dog of the same or larger size.	○	○	○	○	○	○

	NO FEAR OR ANXIETY (No visible signs of fear)		MILD – MODERATE FEAR/ANXIETY		EXTREME FEAR (Cowers, retreats, hides, etc.)	NOT OBSERVED/ NOT APPLICABLE
46. When approached directly by an unfamiliar dog of smaller size.	○	○	○	○	○	○
47. When first exposed to unfamiliar situations (e.g. first car trip, first time in elevator, first visit to veterinarian, etc.).	○	○	○	○	○	○
48. In response to wind or wind-blown objects.	○	○	○	○	○	○
49. When having nails clipped by a household member.	○	○	○	○	○	○
50. When groomed or bathed by a household member.	○	○	○	○	○	○
51. When having his/her feet toweled by a member of the household.	○	○	○	○	○	○
52. When unfamiliar dogs visit your home.	○	○	○	○	○	○
53. When barked, growled, or lunged at by an unfamiliar dog.	○	○	○	○	○	○

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Section 4: Separation-related behavior

Some dogs show signs of anxiety or abnormal behavior when left alone, even for relatively short periods of time. Thinking back over the recent past, how often has your dog shown each of the following signs of separation-related behavior when left, or about to be left, on its own:

Selected dog: **Study**	NEVER	SELDOM	SOMETIMES	USUALLY	ALWAYS	NOT OBSERVED/ NOT APPLICABLE
54. Shaking, shivering, or trembling.	○	○	○	○	○	○
55. Excessive salivation.	○	○	○	○	○	○
56. Restlessness, agitation, or pacing.	○	○	○	○	○	○
57. Whining.	○	○	○	○	○	○
58. Barking.	○	○	○	○	○	○
59. Howling.	○	○	○	○	○	○
60. Chewing or scratching at doors, floor, windows, curtains, etc.	○	○	○	○	○	○
61. Loss of appetite.	○	○	○	○	○	○

Are there any other situations in which your dog is fearful or anxious? If so, please describe briefly:

[<-- Go Back] [Reset Scores] [Continue -->] [Save and Quit]

Section 5: Excitability

Some dogs show relatively little reaction to sudden or potentially exciting events and disturbances in their environment, while others become highly excited at the slightest novelty. Signs of mild to moderate excitability include increased alertness, movement toward the source of novelty, and brief episodes of barking. Extreme excitability is characterized by a general tendency to over-react. The excitable dog barks or yelps hysterically at the slightest disturbance, rushes toward and around any source of excitement, and is difficult to calm down.By clicking on the following scales, please indicate your own dog's recent tendency to become excitable in each of the following contexts:

Selected dog: **Study**	CALM (Little or no special reaction)		MILD – MODERATE EXCITABILITY		EXTREMELY EXCITABLE (Over-reacts, hard to calm down)	NOT OBSERVED/ NOT APPLICABLE
62. When you or other members of the household come home after a brief absence.	○	○	○	○	○	○
63. When playing with you or other members of your household.	○	○	○	○	○	○
64. When doorbell rings.	○	○	○	○	○	○
65. Just before being taken for a walk.	○	○	○	○	○	○
66. Just before being taken on a car trip.	○	○	○	○	○	○
67. When visitors arrive at your home.	○	○	○	○	○	○

Are there any other situations in which your dog sometimes becomes over-excited? If so, please describe briefly:

[<-- Go Back] [Reset Scores] [Continue -->] [Save and Quit]

Section 6: Attachment and attention-seeking

Most dogs are strongly attached to their people, and some demand a great deal of attention and affection from them. Thinking back over the recent past, how often has your dog shown each of the following signs of attachment or attention-seeking:

Selected dog: **Study**	NEVER	SELDOM	SOMETIMES	USUALLY	ALWAYS	NOT OBSERVED/ NOT APPLICABLE
68. Displays a strong attachment for one particular member of the household.	○	○	○	○	○	○
69. Tends to follow you (or other members of the household) about the house, from room to room.	○	○	○	○	○	○
70. Tends to sit close to, or in contact with, you (or others) when you are sitting down.	○	○	○	○	○	○
71. Tends to nudge, nuzzle or paw you (or others) for attention when you are sitting down.	○	○	○	○	○	○
72. Becomes agitated (whines, jumps up, tries to intervene) when you (or others) show affection for another person.	○	○	○	○	○	○
73. Becomes agitated (whines, jumps up, tries to intervene) when you (or others) show affection for another dog or animal.	○	○	○	○	○	○

<-- Go Back | Reset Scores | Continue --> | Save and Quit

Section 7: Miscellaneous

Dogs display a wide range of miscellaneous behavior problems in addition to those already covered by this questionnaire. Thinking back over the recent past, please indicate how often your dog has shown any of the following behaviors:

Selected dog: **Study**	NEVER	SELDOM	SOMETIMES	USUALLY	ALWAYS	NOT OBSERVED/ NOT APPLICABLE
74. Chases or would chase cats given the opportunity.	○	○	○	○	○	○
75. Chases or would chase birds given the opportunity.	○	○	○	○	○	○
76. Chases or would chase squirrels, rabbits and other small animals given the opportunity.	○	○	○	○	○	○
77. Escapes or would escape from home or yard given the chance.	○	○	○	○	○	○
78. Rolls in animal droppings or other "smelly" substances.	○	○	○	○	○	○
79. Eats own or other animals' droppings or feces.	○	○	○	○	○	○
80. Chews inappropriate objects.	○	○	○	○	○	○
81. "Mounts" objects, furniture, or people.	○	○	○	○	○	○
82. Begs persistently for food when people are eating.	○	○	○	○	○	○
83. Steals food.	○	○	○	○	○	○
84. Nervous or frightened on stairs.	○	○	○	○	○	○
85. Pulls excessively hard when on the leash.	○	○	○	○	○	○
86. Urinates against objects/furnishings in your home.	○	○	○	○	○	○
87. Urinates when approached, petted, handled or picked up.	○	○	○	○	○	○

	NEVER	SELDOM	SOMETIMES	USUALLY	ALWAYS	NOT OBSERVED/ NOT APPLICABLE
88. Urinates when left alone at night, or during the daytime.						
89. Defecates when left alone at night, or during the daytime.						
90. Hyperactive, restless, has trouble settling down.						
91. Playful, puppyish, boisterous.						
92. Active, energetic, always on the go.						
93. Stares intently at nothing visible.						
94. Snaps at (invisible) flies.						
95. Chases own tail/hind end.						
96. Chases/follows shadows, light spots, etc.						
97. Barks persistently when alarmed or excited.						
98. Licks him/herself excessively.						
99. Licks people or objects excessively.						
100. Displays other bizarre, strange, or repetitive behavior(s)*.						

*Please describe briefly:

<-- Go Back | Reset Scores | Continue --> | Save and Quit

C-BARQ site main page

Welcome to the main page of the C-BARQ site. To add a new dog for evaluation, update your profile, or log out, select from the options below.

Add a Dog | Update Profile | Log Out

Your Dogs

	Name	Sex	Breed	ID No/Tattoo	Type/Group	Initial	Follow-up	Reassess
1.	Study		Beagle		pet dog	- -	SCORES	- -

Supplementary Material C. Flowchart detailing the inclusion criteria, and matching process, used to assign dogs to higher or lower behavioural groups, and the participants selected for fecal sampling.

Selection Criteria	Quantitative Data
Completed BOTH C-BARQ and Diet & Lifestyle questionnaires	n=235
Age (2-7 years) Consistent Diet (3+ months) Consistent Living Arrangements (6+ months)	n=72
Composite Scores Calculated Aggression: (SDA+DDA+ODA)/3 Anxiety: (SDF+DDF+NSF+Sep)/4	Median Aggression Score: 0.455 Median Anxiety Score: 0.782
Dogs assigned to behavioural group (Lower = below median, Higher = equal to/above) 1: Lower aggression, lower anxiety 2. Lower aggression, higher anxiety 3. Higher aggression, lower anxiety 4. Higher aggression, higher anxiety	1: n=27 2: n=9 3: n=8 4: n=28
Dogs flagged & excluded for medical conditions & medications	1: n=24 2: n=6 3: n=6 4: n=23
Dogs grouped in order of: A: Diet type B. Deworming status C. Body condition	A: Kibble only (n=32), kibble + wet (n=14), raw (n=2) B: Used dewormer (n=24), did not use (n=24) C: Body condition 1 (n=3), 3 (n=6), 5 (n=33), 7 (n=6)
Priority matches were dogs from behavioural group 1 (lower/lower) with dogs from group 4 (higher/higher), who had the same diet type, deworming status, body condition. Once all eligible dogs were matched between groups 1 & 4, dogs from groups 2 & 3 (lower/higher) were then included.	Low/Low-High/High: n=36, 18 matched pairs Low/High Combinations: n=12, 6 matched pairs 2 pairs could not be matched for deworming status, 8 pairs could not be matched for body condition (bold in table)

Matched Pair	Behavioural Group	Diet Type	Deworming Status	Body Condition
1	1	Kibble Only	None	3
1	4	Kibble Only	None	3
2	1	Kibble Only	None	5
2	4	Kibble Only	None	5
3	1	Kibble Only	None	5
3	4	Kibble Only	None	5
4	2	Kibble Only	None	5
4	3	Kibble Only	None	5
5	1	Kibble Only	Yes	**1**
5	4	Kibble Only	Yes	**3**
6	1	Kibble Only	Yes	**1**
6	3	Kibble Only	Yes	**3**
7	1	Kibble Only	Yes	5
7	4	Kibble Only	Yes	5
8	1	Kibble Only	Yes	5
8	4	Kibble Only	Yes	5
9	1	Kibble Only	Yes	5
9	4	Kibble Only	Yes	5
10	1	Kibble Only	Yes	5
10	4	Kibble Only	Yes	5
11	1	Kibble Only	Yes	5
11	4	Kibble Only	Yes	5
12	2	Kibble Only	None	7
12	1	Kibble Only	None	7
13	1	Kibble Mix	Yes	**5**
13	4	Kibble Mix	Yes	**7**
14	4	Kibble Mix	None	**3**
14	1	Kibble Mix	None	**5**
15	1	Kibble Mix	Yes	5
15	4	Kibble Mix	Yes	5
16	1	Kibble Mix	Yes	5
16	2	Kibble Mix	Yes	5
17	4	Kibble Mix	None	5
17	3	Kibble Mix	None	5
18	1	Kibble Mix	Yes	5
18	4	Kibble Mix	Yes	5
19	1	Raw	**None**	5
19	4	Raw	**Yes**	5
20	4	Kibble Only	None	**5**
20	1	Kibble Only	None	**7**
21	4	Kibble Only	None	**3**
21	1	Kibble Only	None	**5**
22	2	Kibble Only	None	**1**
22	1	Kibble Only	None	**7**
23	4	Kibble Mix	**None**	5
23	1	Kibble Mix	**Yes**	5
24	4	Kibble Mix	None	**7**
24	1	Kibble Mix	None	**5**