The Influence of Sample Processing and Storage Methods on the Feline Fecal Metabolome and Canine Fecal Microbiota

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ABSTRACT

THE INFLUENCE OF SAMPLE PROCESSING AND STORAGE METHODS ON THE FECAL METABOLOME AND MICROBIOTA

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The fecal microbiota and metabolome profiles provide insight into host health. However, standardization of methods for processing and storage of fecal samples prior to microbiota and metabolomic analyses are lacking. Therefore, the present thesis investigated: 1) the influence of room temperature exposure on the feline fecal metabolome; and 2) the influence of homogenization, the addition of RNA*later*, room temperature exposure, and storage temperature on the canine fecal microbiota. The first study found that 4 hours of room temperature exposure is acceptable for feline fecal metabolomic analyses, as metabolite concentrations (notably amino acids and volatile fatty acids) changed beyond that. The second study observed no impacts of homogenization, RNA*later*, 24-h room temperature exposure, or 24-h fridge storage on diversity, evenness, or richness, or bacterial community membership and structure; however, RNA*later* impacted the relative abundances of canine fecal phyla. These factors should be considered in future feline and canine fecal studies.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF TERMS AND ABBREVIATIONS

ANOVA — Analysis of variance

FISH — fluorescence in situ hybridization

GALT — gut-associated lymphoid tissue

GI — Gastrointestinal

IBD — Inflammatory bowel disease

NMR — Nuclear magnetic resonance

PCR — Polymerase chain reaction

rRNA — Ribosomal RNA

SCFA — Short-chain fatty acid

1 Literature Review

1.1 Introduction

Mammals have complex bacterial profiles, particularly in the gastrointestinal tract. Within the gut there is a diverse set of bacteria, including fungi, archaea, protozoa, and viruses (Barko et al., 2018). These microorganisms comprise the microbiota. Furthermore, the metabolites produced by the microbiota comprise the metabolome. The microbiota and metabolome contribute to many aspects of a host's health, including energy homeostasis, metabolism, gut epithelial health, and immune function (Barko et al., 2018). They are dynamic elements and are influenced by several factors such as the environment and diet (Barko et al., 2018). Aside from its link to a host's normal physiology, the gastrointestinal microbiota is associated with diseases developed because of intestinal microbial changes, such as a broad shift of microbial communities, a decrease of species diversity, or changes in relative abundance of organisms. These intestinal microbial changes that affect host health are known as dysbiosis (i.e., imbalance or disruption to the microbiota causing shifts in composition, function and activity) (Guard et al., 2015). Metabolomics is the study of metabolites found in cells, tissues, organisms and feces (Patti et al., 2012). The use of metabolomics has been a popular approach for metabolic profiling in both clinical and non-clinical contexts (Patti et al., 2012). Studying the metabolites produced by bacteria in the microbiota allows for a more detailed characterization of metabolic function and pathways in which bacteria are involved. Investigation of the metabolic activity of the microbiota can aid in uncovering disease etiology, monitor pathways of therapeutic intervention, and establish biomarkers of health and disease (Clish, 2015).

Microbiota and metabolome research has been conducted on a variety of species. Microbiomics is the study of the microbiota, wherein researchers use several different methods to analyze the microbiota (Barko et al., 2018). Advances in bioinformatics have allowed exploration of the microbial genome, interactions between the microbiota and the host, and the influence on disease pathology (Guard et al., 2015). The most common analyses involve extraction and amplification of DNA regions, then assessment of microbial identity and diversity based on similarity and a reference microbial genome database (Zoetendal et al., 2002). Metabolomics is the study of the metabolites produced by the bacteria that comprise the gut microbiota (Matysik et al., 2016), including organic acids, sugars, and amino acids (Karu et al., 2018). The gut metabolome is often observed using mass spectrometry and nuclear magnetic resonance spectrometry. These methods on their own provide measurements of a large range of metabolites, and provide even wider coverage when combined (Matysik et al., 2016).

While the importance of the gastrointestinal microbiota and metabolome in host health is well-known, the methods for sample collection, processing, storage, and analyses of the microbiota and metabolome are not standardized in the literature, making comparisons between current studies challenging. To help gain a stronger understanding of the considerations needed in standardizing these methods, this review will discuss the development and maintenance of the intestinal microbiota and metabolome, dysbiosis and disease of the microbiota and metabolome, and methods of sample collection, processing, storage, and analysis.

1.2 Development of the Gastrointestinal Microbiota

Previously, mammals were thought to have no existing microbiota profile prior to birth. In a few studies, there is evidence that the microbiota profile could be transferred vertically from mother to offspring before birth, but these claims are currently unreliable and warrant more research (Ardissone et al., 2014; Wassenaar & Panigrahi, 2014). A study in humans showed that the influence on the pace of microbial colonization was affected by the method of birth, diet and antibiotics; however, the overall maturation pattern was not affected long term (la Rosa et al., 2014). Additionally, in humans and mice, the formation of a symbiotic microbial-mucosal unit is influenced by the intricate interaction between immunological signaling pathways and bacteria, which guide the baseline immunological tolerance and bacterial colonization (Stockinger et al., 2011). Studies like these provide evidence on neonatal gut health, specifically on the microbial colonization present, and how the microbiota profile can be altered by post-parturient nutrient quality. These studies also demonstrate that the maturation of the gut microbiota is a complex process that requires complementary interactions between the microbiota and the host.

As the gastrointestinal microbiota is established, developmental programming determines an orderly function in which the microbial composition, function, and capacity can converge into a mature state (Palmer et al., 2007; Yatsunenko et al., 2012). This process can be observed through shifts in diet, especially in early childhood, where the diet transitions from liquid to solid during the weaning period (Palmer et al., 2007; Yatsunenko et al., 2012). Factors such as this subsequently affect how the gut microbiota evolves to extract energy from complex carbohydrates, metabolize other nutrients, and participate in vitamin biosynthesis, which are all processes of a mature microbiota (Nicholson et al., 2012). Although these are crucial processes, the final developed state

of the intestinal microbiota is characterized by individual variation and influenced by the diet and the geographical location in which the host resides (Yatsunenko et al., 2012).

The intestinal microbiota not only plays a role in adapting to nutrients but is also involved in critical developmental processes. In studies using laboratory animals, the intestinal microbiota seemed to point in the direction of immune system development, as well as development of the brain, gut epithelium, and other bodily systems (Martinez, 2014; Sommer & Bäckhed, 2013). As the gastrointestinal microbiota matures over developmental stages, it is affected by negative events. For example, maternal milk quality, health, premature birth, and malnutrition can be associated with persistent gut microbial immaturity (Arrieta et al., 2014). Although multiple studies have demonstrated evidence of abnormal microbiota development and associations with host health, the true cause of the disease states have not been fully elucidated.

Information on the development and acquisition of the microbiota in cats and dogs is limited. In humans and small animals, the pattern in which the microbiota profile matures seems to be a feature that is conserved across mammalian development (Barko et al., 2018). During the developmental period, the intestinal microbiota is subjected to alterations caused by environment, diet, age, and illness. In the published literature, however, there is a lack of long-term studies examining the fundamental question of how host physiology will be affected by the developing microbiota during maturation in cats and dogs. Additionally, there is lack of evidence of neonatal microbiota regarding the origin, function, characteristics, and structure. Further investigation is required in these areas.

1.3 Maintenance of the Gastrointestinal Microbiota

The adult human microbiota is stable and is defined by its essential functionality and adaptability over a host's lifetime (Lozupone et al., 2012). These features are reflected in adult cats and dogs, as a metagenomic analysis revealed the similarity between canines, feline, humans, and mice (Swanson et al., 2011). Many studies have described the microbiota profile of adult dogs and cats, including investigations of the impact of diet, genetics, and medical interventions.

In adult dogs and cats, the primary phyla found within the intestinal tract are *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria*. Studies have shown that the two major phyla found in canine feces are *Firmicutes* and *Bacteroidetes*, which are similar to human studies (Coelho et al., 2018; Garcia-Mazcorro et al., 2012; Handl et al., 2011a; Swanson et al., 2011). In comparison to humans, canine feces seem to have a higher abundance of *Fusobacteria*, and some studies have demonstrated that *Fusobacteria* was the most abundant phylum, whereas others found *Firmicutes* and *Bacteroidetes* were codominant (Hand et al., 2013; Middelbos et al., 2010). In canines, *Lactobacillus* is distributed uniformly throughout the entire intestinal tract but relative abundances of *Clostridiales*, *Fusobacteriales*, and *Enterobacteriales* varied through different segments of the intestines (Suchodolski et al., 2008). In particular, *Enterobacteriaceae* is abundant in the small intestine, and in the duodenum and jejunum, *Clostridiales* was predominant (Suchodolski et al., 2008). *Bacteroidales* and *Fusobacteriales* are the predominant bacterial order found in the ileum and colon segments of the gastrointestinal tract (Suchodolski et al., 2008). Similar to dogs, *Firmicutes* is the predominant phylum in cats, and within it, *Clostridium* is the most abundant genus (Handl et al., 2011b). However, in contrast with canines, *Proteobacteria* makes up 7.9-14% of the feline colonic microbiota (Ritchie et al., 2008). In cats, *Lactobacillus* is

distributed throughout the gut and is particularly abundant in the jejunum and colon (Ritchie et al., 2008). In further detail, *Bacteroidetes* and *Firmicutes* dominate the feline small intestine, whereas the ileum is mostly populated with *Actinobacteria* and *Proteobacteria*, and the colon is populated by *Proteobacteria, Fusobacteria* and *Firmicutes* (Ritchie et al., 2008). Variation in host genetics, diet, and sample collection methods have been shown to impact relative abundances of these phyla.

Regarding diet and its effect on the microbiota profile, the quantity, form, composition, and frequency of food play an important role in normal GI function (Nielsen et al., 2007). Dietary interventions such as prebiotics, probiotics, complex and simple carbohydrates, fats and protein all have potential to alter the gut microbiota in their own unique way. When the diets of cats and dogs are altered, the microbiota is also rapidly altered (David et al., 2014b; Mori et al., 2019). In particular, the differing amounts of protein, carbohydrate, and fat fed to cats and dogs will have significant impacts on the gastrointestinal microbiota (Mori et al., 2019). Dogs are facultative carnivores, meaning that they consume a diet that consists mostly of meat, but will also consume non-animal food as well (Pilla & Suchodolski, 2020). Like dogs, cats consume a diet with meat, however, they are obligate carnivores and require a diet that is higher in protein and lower in carbohydrates than that of dogs (Verbrugghe & Hesta, 2017). Humans, as omnivores, partake in short-term consumptions of either animal or plant diets, which is enough to alter the microbial community present within the gut and subsequently produce differences in the microbial gene expression (David et al., 2014b). Like humans, dogs consuming a diet high in plant ingredients or carbohydrate will decrease the relative abundance of *Fusobacteria* and *Proteobacteria* and increase the abundance of Firmicutes (Alexander et al., 2018; Middelbos et al., 2010). Although cats are obligate carnivores and do not have any particular requirements for carbohydrates, the

gastrointestinal microbiota is similar to other mammals including dogs. In cats, the predominate phylum is *Firmicutes* (36-92%), *Clostridiales* is the most abundant order, and in opposition to dogs, *Proteobacteria* makes up a large proportion of the feline gut microbiota at 7.9-14% (Barry et al., 2012; Ritchie et al., 2008), due to a diet that is primarily protein based.

Many cats and dogs are fed conventional dry food diets in Canada, USA, Australia, UK and New Zealand, and apart from these diets, 7% and 9% of owners will exclusively feed homemade or raw diets, respectively (Dodd et al., 2020). Some studies have investigated the effects of raw or homemade diets on the gastrointestinal microbiota in comparison to dogs fed a kibble diet. In studies examining these diets, it was noted that these diets had contrasting macronutrient content and contained more protein compared to the control diet of kibble (Bermingham et al., 2017; Schmidt et al., 2018). In addition, carbohydrate and fiber content were lower compared to the control diet. In turn, the gut microbiota was altered by increasing *Clostridiaceae* in dogs fed raw diets versus dogs fed a kibble diet, suggesting that *Clostridiaceae* may play a role in protein metabolism or elucidate its ability to survive in an environment with very little dietary carbohydrate content (Bermingham et al., 2017; Hooda et al., 2013; Schmidt et al., 2018). Cats consuming higher protein diets will also have higher amounts of *Clostridiaceae* as well as *Clostridium* (Hooda et al., 2013). In comparison, there is little research available on the effects of homemade diets on the fecal microbiota and metabolome, and this is likely due to the variability of contents that make up homemade diets. Although fresh pet food seems to be an increasing trend on the pet food market, research assessing the impact of these types of diets on the fecal microbiota and metabolome is certainly of interest.

Although individual variation of the microbiota is influenced by many factors, our understanding of their effects are unclear. For example, in a study examining canine gastrointestinal dysbiosis, there were no noticeable differences in bacterial gene families in dogs consuming a low-fiber diet compared to healthy dogs or dogs with intestinal dysbiosis (Guard et al., 2015; Guard & Suchodolski, 2016). These results may have been due to sequencing methods, or complications with sample size, where statistical analyses may be unable to detect subtle changes in profiles that would be important when noting changes in disease states or experimental conditions (Guard & Suchodolski, 2016).

In a human study, the fecal microbiota was affected by the antibiotic, ciprofloxacin, where both the taxonomic richness and diversity were altered (Dethlefsen et al., 2008). Administration of medication affected the existing microbiota, and once it was discontinued the microbiota only recovered partially and never returned to its original state (Dethlefsen & Relman, 2011). Similarly, in dogs, when the antibiotic tylosin was administered, the richness and diversity decreases (Suchodolski et al., 2009). In addition to antibiotics, other medications can also affect the microbiota and the bacterial population present within the gastrointestinal tract of canines. For example, omeprazole, a proton pump inhibitor, is used to treat gastric reflux and stomach ulcers and has been found to decrease the population of *Helicobacter* as well as increase the population of *Fusobacteria* and *Firmicutes* in the dog's stomach (Garcia-Mazcorro et al., 2012). Further down the gastrointestinal tract, changes in the bacterial population in the duodenum have been associated with administration of the omeprazole, and were illustrated by an increase in *Lactobacillus* but a decrease in *Bacteriodes and Faecalibacterium* (Garcia-Mazcorro et al., 2012).

The studies discussed above suggest that the long-term stability, compositional and functional diversity characterizes the intestinal microbiota of humans, cats and dogs. The symbiotic interaction between the host and the resident microbiota profile is vital in governing the physiological processes of the host and can be affected by several genetic and environmental factors. The next section will review and examine function of the gastrointestinal microbiota.

1.4 Gastrointestinal Function of the Microbiota and Metabolome

The gut microbiota participates in physiological and immunological processes that are vital for normal host function. These processes include homeostasis, metabolism, endocrine signaling, and regulation of immune function (Nicholson et al., 2012). Although the gut is associated with various systemic and gastrointestinal diseases, the microbiota also plays a role in disease pathogenesis itself (Kieler et al., 2017; Montoya-Alonso et al., 2017). There is evidence implicating the gut microbiota in mediating interactions between the host's immune system and metabolic processes. The fecal metabolites present depend on the available substrates and bacteria in the gut, which are affected by diet. For example, if a host consumes more carbohydrates than protein, less protein will be available for fermentation in the gut and carbohydrate fermentation will dominate. Conversely, if carbohydrate consumption is depleted, gut fermentation will shift to proteolytic fermentation, which is dominated by bacteria such as *Clostridium, Bacteroides, Fusobacterium, Propionibacterium, Lactobacillus,* and *Streptococcus* (Diether & Willing, 2019). The primary metabolites produced from proteolytic fermentation are amino acids, while the primary metabolites produced from carbohydrate fermentation are volatile fatty acids such as

butyrate and propionate (Aguirre et al., 2016). Hence, the relative abundances of metabolites within in a host's gut and luminal content and hence also feces will reflect the nutrient composition of their diet.

The synchronous nature between the host and the existing microbiota relies heavily on the intricate network of signals between each microbial community throughout the intestine. The communities of the microbiota changes through the different layers of the intestinal mucosa as well as the length and width of the intestine (Sommer & Bäckhed, 2015), leading to more immunologically active sites in the distal end of the digestive tract compared to the proximal end (i.e, the small intestine) (Sommer & Bäckhed, 2015). The complex interaction between host, microbe, and cell will differ from individual to individual (Olszak et al., 2012). Additionally, homeostasis is a difficult task, particularly in immune function, therefore discriminating between regular microbes and a pathogen or invader is an important process (Klose & Artis, 2016).

When a pathogen is recognized by the immune system, the innate immune system is activated in a defense mechanism known as the gut-associated lymphoid tissue (GALT). T cells, B cells, and dendritic cells start by initiating an inflammatory or anti-inflammatory response based on microbial signaling, then dendritic cells assess the pathogen (Klose & Artis, 2016). Intestinal microbes play an essential role in GALT, as demonstrated by a study showing germ-free animals have significantly less developed lymphoid follicles (Štěpánková et al., 1980).

1.5 Gastrointestinal Microbiota and Metabolome Dysbiosis and Disease

1.5.1 Dysbiosis

There is considerable evidence linking the microbiota to gut inflammation, and some studies on metabolomics show the microbiota plays an integrative role in metabolism. The microbiota also contributes to the modulation of immune response in the intestinal mucosa. Furthermore, host health is associated with the diversity of microbes present, and subsequent loss of diversity has been correlated with multiple diseases found in humans and other mammals (Frank et al., 2007; Tong et al., 2013; Turnbaugh et al., 2006). In a loose definition, intestinal dysbiosis refers to a microbial change in the intestine that subsequently affects the health of the host. A broad shift of microbial communities, a decrease of species diversity, or changes in relative abundance of organisms is known as dysbiosis (Kamada et al., 2013). It is important to note that dysbiosis is not exclusively caused by external pathogens, but instead may result from an absence of commensal microbes (Sellon et al., 1998). Dysbiosis can be caused by other extrinsic factors such as a change in diet intake and composition, pharmaceutical medications, viral or bacterial infections, or other gut modulating drugs/supplements such as prebiotics and probiotics (Singh et al., 2021).

Since a host's microbiota produces the metabolites that comprise the metabolome, the metabolome can contribute to disease as well. In human studies, fecal metabolomics mainly focus on chronic enteropathy diseases such as inflammatory bowel disease. Probert et al. (2004) used mass spectroscopy combined with gas chromatography to analyze the volatile organic compounds using fecal samples from health patients and those with gastroenteritis, and well over three hundred studies have used the same method in human studies (de Lacy Costello et al., 2014; Probert, 2004).

Marchesi et al. (2007) later used NMR spectroscopy, making it the first time that the non-volatile fecal metabolome was assessed, comparing patients with inflammatory bowel disease and healthy patients acting as controls (Marchesi et al., 2007). Since these initial studies, many more have used highly advanced equipment to analyze metabolomics that covered broader topics, such as cancer, dietary influences, and gut microflora activity (Karu et al., 2018).

Amines are metabolites produced from proteolytic fermentation (i.e., fermentation of proteins by gut bacteria). When there are changes in amines, the health of the host can be compromised. Studies have shown a threshold in which the amines are beneficial to the body, and when the threshold is exceeded, it can lead to detrimental function of the gut barrier. Amines are absorbed through the lumen of the intestine and act as critical regulators in mechanisms of cellular growth, proliferation, and metabolism (Bardócz et al., 1995). However, inflammation and gastrointestinal epithelial shedding (i.e., increased rate of epithelial cell apoptosis) has been observed when concentrations of amines are high, and, especially in young animals, can cause pancreatic tissue to become disorganized (Fusi et al., 2004). Changes in fecal volatile fatty acid profiles have been associated with diseases such as colorectal cancer, inflammatory bowel disease, and obesity, with potential use for biomarkers in detecting these diseases in humans (Vancamelbeke et al., 2017; McNabney and Henagan, 2017). Human patients with inflammatory bowel disease have exhibited different fecal amino acid profiles compared to healthy patients, again displaying the potential of fecal metabolomics in future studies (Diederan et al., 2017; Bosch et al., 2017). In dogs, Minamoto et al. (2019) found that dogs with chronic enteropathy had lower concentrations of acetate, butyrate, and propionate in the fecal matter compared to healthy dogs.

In cats, Summers et al. (2020) concluded that cats with chronic kidney disease had increased levels of isovaleric acid in fecal samples and were correlated with the severity of the disease.

1.5.2 Gastrointestinal dysbiosis in canines

In dogs, dysbiosis is defined by decreased diversity and relative abundance of bacterial species that produce short-chain fatty acids (SCFA) (Suchodolski et al., 2010; Vázquez-Baeza et al., 2016). In the past, overgrowth of bacterial populations in the small intestine was used as a marker to quantify changes within the intestinal microbiota. These tests were done using culture based analyses of mucosa of the duodenum and jejunum, where there are associations with the enteropathies (Rutgers et al., 1996). In more recent literature, dysbiosis has been used as a term representing alterations of the gut microbiota in relation to disease states and the abnormalities identified as part of the disease (Suchodolski et al., 2010). However, the studies performed have been small and there was no universal pattern of dysbiosis identified, like in human studies. In studies sequencing dysbiosis in dogs, there was overall a lower relative abundance of *Bacteroidetes* but a higher abundance of *Proteobacteria* (Vázquez-Baeza et al., 2016). Dogs with IBD revealed significant differences from that of healthy dogs. Dogs with IBD had lower alpha diversity; however, there was no noticeable association with a dog's body condition or diet, among other factors (Vázquez-Baeza et al., 2016). Interestingly, there is lower relative abundances of Fusobacteria in dogs that have IBD when comparing the relative abundance of healthy dogs (Vázquez-Baeza et al., 2016). In studies that observed a more diverse population of dogs that had chronic intestinal inflammation, it was found that the mucosa-adherent bacteria found in the small intestine increased, specifically *Proteobacteria* and *Escherichia-coli* bacteria (Xenoulis et al.,

2008). Additionally, in dogs with acute diarrhea, dysbiosis is exponentially increased and this can be observed through a decrease in SCFA producing bacteria, which therefore can be observed through the overall microbial diversity and communities of microbes found in the GI tract (Guard et al., 2019; Suchodolski et al., 2012).

1.5.3 Gastrointestinal dysbiosis in felines

There are very few studies investigating feline intestinal dysbiosis using 16S rRNA sequencing, however other sequencing methods such as fluorescence in situ hybridization (FISH) have been used to suggest that dysbiosis in felines with chronic enteropathies significantly differ from healthy counterparts. A study found that there was a decrease in *Bacteroides* and *Bifidobacterium* in cats with chronic enteropathies such as IBD (Inness et al., 2007). Gastrointestinal diseases include chronic enteropathies which often occur in response to food and antibiotic administration (Suchodolski et al., 2004). Another study investigating the chronic enteropathies in felines found that there were an increase in the *Streptococcus* and *Escherichia-coli*, but a decrease in many bacterial populations such as *Bacteroides*, *Bifidobacterium*, and *Turicibacter* in cats with chronic enteropathies compared to a healthy counterparts (Sung et al., 2022). Dysbiosis of the host's microbiota can lead to deleterious effects, and subsequently a reduction in metabolites that may have anti-inflammatory effects and can be noted with accompanying intestinal inflammation (Honneffer, 2014). The findings of these studies appear to lack significance in a clinical setting, notably when studies compare intestinal and fecal samples, and feline studies on the fecal microbiota are few and far in between since dogs are often an easier model to conduct studies on. Although the data reviewed lacks transparency of the microbiota's

role in pathogenesis of disease, the link between integral host health and the gut microbiota is evident. The fecal microbiota is therefore a target to assess the microbiota using a variety of diagnostic methods.

1.5.4 Gastrointestinal Diseases

The involvement of the gastrointestinal microbiota is not limited to the gut and can be linked to other organs in the body (Björkholm et al., 2009). The mammalian brain is a complex organ that has been of high interest within the scientific community for its complexity and its ability to be influenced by internal and external environmental factors. In humans, microbial infections during the perinatal period have been associated with some of the most common neurological disorders, including autism (Finegold et al., 2002). In atopic diseases such as asthma, human infants with atopic skin disease had lower levels of *Bacteroides, Lactobacilli* and *Bifidobacteria* compared to those without atopic disease (Böttcher et al., 2000). Experimental studies have highlighted mechanisms associating the gut microbiota with obesity and type 2 diabetes in cats (Kieler et al., 2019), dogs (Jergens et al., 2019) and humans (Ahmad et al., 2019). Other studies have shown that metabolic disorders can be reversed with manipulation of the microbiota or the diet (Ly et al., 2011). Also, other conditions have been linked to microbial dysbiosis such as psychiatric illnesses and demonstrates a high comorbidity with bowel disorders (Neufeld & Foster, 2009). Via the gut-brain axis, dysbiosis of the gut microbiota can also contribute to hepatic encephalopathy (Schiano, 2010), cirrhosis and cognitive dysfunction (Bajaj et al., 2012). However, more research is needed to better understand the etiology of these associations and what other diseases are associated with the gut microbiota.

1.6 Methods of Analysis

Using feces to study health and disease dates to thousands of years ago where the use of colour and odour were used to assess the function of the liver, colon, as well as the diet of what one had consumed (Karu et al., 2018). Since then, this field has evolved to study microbial structure and composition as well as microbial function. With the major influx of the microbiota studies over the last decade, the microbiomics field has growing at an increasingly fast rate. Since the study of the microbiota encompasses many other fields such as metabolomics, metaproteomics, metatranscriptomics, ecology, and many more, this has led to an explosion of available data (Stephens et al., 2015). As a result, there is a wide variety of analytical methods to use, thus making interpreting, standardizing, and comparing results extremely difficult in a way that would be meaningful (Berg et al., 2020). In turn, this has also restricted the scientific community to translate the research into clinical settings (Kitsios et al., 2017).

Gene amplicon sequences have been used over the last two decades as the primary method to study the microbiota and its taxonomy and phylogeny, which were considered difficult to profile before (Weisburg et al., 1991). Many of the markers that are used to characterize the microbiota are conserved, and can be used to aid in determining any changes. 16S rRNA is the most common method for bacterial identification, and is considered the gold standard (Baker et al., 2003; Pel et al., 2018). The highly conserved areas of the 16S rRNA genes suggest there is a crucial role in cellular function, which is why it is used to identify unknown bacterial taxa based on precise genomic classification (Bharti & Grimm, 2021). The gene sequences used is approximately 1564 base pairs long and consists of nine variable regions and conserved sites in which primers may

bind to, and these are used to aid in identifying microbial profiles (Woo et al., 2008). In many microbiota studies, analysis involves parallel sequencing to match specific marker genes, which are then amplified and pooled. Once the DNA has been sequenced, the sequence reads can then be classified according to an existing database so that taxonomic groups and species can be identified. This analysis is highly advantageous since hundreds to thousands of samples can be sequenced at the same time, allowing this method to be cost effective (Minich et al., 2018). With microbial sequencing, there is low complexity and with its tractable computational abilities, it is acceptable for a wide range of research (Sims et al., 2014). Overall, the use of 16S rRNA technique has been successfully used to characterize microbial communities from various sources, and has aided in identifying changes within microbial communities associated with the alteration of community function (Bharti & Grimm, 2021).

Bacterial biomass composes the majority of fecal matter (Stephen & Cummings, 1980), and has subsequently pushed the focus characterizing the metabolic function of the bacteria present using methods such as DNA sequencing and other metagenomic techniques (Karu et al., 2018). In addition, fecal matter is made up of small and large molecules that demonstrate the processes of nutritional ingestion, digestion, and absorption from the gut bacteria and the gastrointestinal tract itself. Small molecules such as organic acids, amino acids, and sugars are what make up the fecal metabolome (Karu et al., 2018). These metabolites represent endogenous metabolism, and can provide insight into the molecular and chemical reactions occurring within the body and the gastrointestinal tract (Dayalan et al., 2019; Wishart et al., 2018). In addition to that, the use of fecal metabolomics can aid in the detection of health in humans, dogs and cats (Sebedio & Brennan, 2004). The metabolome can be used to understand the intricate and complex relationship between

the gut microbiota and the gastrointestinal tract. This will in turn display the association between the functionality of the gastrointestinal tract, metabolome and microbiota, and ultimately the overall health outcomes that can be seen as a result (Karu et al., 2018). With the advances in metabolomics over the last decade, there has been particular interest in exploring the metabolome using human feces, and has ultimately translated into using animal feces from dogs and cats for research. With the use of mass spectrometry, it is possible to efficiently measure thousands of metabolites within a sample using only a small amount of it (Smith et al., 2006). In targeted metabolomics, the focus leans towards one area of research, allowing for a more specified list of metabolites to be measured (Dudley et al., 2010). Nuclear magnetic resonance (NMR) is a form of mass spectrometry that allows for targeted metabolic studies, and because of its reproducibility and specificity there is an advantage of using it (Dirren et al., 1975).

1.7 Methods of Sample Collection and Storage

In companion animals, methods of collection, storage, and analysis have not been standardized yet even with the increasing popularity of microbiota studies. There are very few studies in which repeatability in methodology is noted. In published literature, sample storage temperature can range from being stored at -20ºC only, -20ºC followed by -80ºC, or only -80ºC for metabolome and microbiota studies alike (Bermingham et al., 2018; Hall et al., 2020; Hart et al., 2015; Kerr et al., 2013; Mathay et al., 2015; Tal et al., 2020). Fecal sample collection methods have even more variability than that of storage. In one study on dogs, fecal samples were collected within 5 hours of defecation (Kerr et al., 2013), leaving a large window of time between defecation and collection. Another study permitted a maximum of 20 minutes at ambient room temperature

prior to aliquots being stored (Mathay et al., 2015). In a multi-species study including zebrafish, mice, dogs, cats, and horses, fecal samples were collected immediately at defecation (Hart et al., 2015). The methods of sample collection and storage are subjective, often making one study on the canine fecal microbiota or metabolome different from the next, and making results more difficult to compare with the previous literature. Indeed, several studies have demonstrated the ability for room temperature exposure post-collection to impact the microbiota and metabolome.

While studies on felines (Tal et al., 2017) and humans (Carruthers et al., 2019; Lauber et al., 2010) observed minimal impacts on alpha diversity, beta diversity, and composition of the fecal microbiota with room temperature exposure (ranging from 32 hours to 14 days in those studies), a study on equines observed alterations to the alpha diversity and beta diversity of the fecal microbiota profile within 6 hours of exposure (Martin de Bustamante et al., 2021). Room temperature exposure has also been shown to alter concentrations of fecal metabolites (notably amino acids and volatile compounds) in humans as early as 5 hours post-collection (Gratton et al., 2016) and in 34-month-old children within 52 hours post-collection due to the persistence of microbial fermentation at room temperature (Liang et al., 2020). Regarding long-term storage temperature, no differences in the fecal microbiota profile were observed in humans between storage at 4°C for 24 hours prior to freezing at -80°C and direct storage at -80°C (Choo et al., 2015); however, in canines the same comparison showed differences in beta diversity and the relative abundances of phyla but may have depended on the time for room temperature exposure (Horng et al., 2018). Further research is needed combining factors of room temperature exposure post-collection with storage temperature to further clarify their effects on the fecal microbiota.

Another factor causing results to vary from one study to the next is RNA*later*, a stabilizing solution used in many studies, including those that involve fecal samples for microbiota or metabolomic analyses (Choo et al., 2015; Dominianni et al., 2014; Flores et al., 2015; Liang et al., 2020). Some studies noted that RNA*later* was a sufficient preservative to use for analyses of fecal samples (Nechvatal et al., 2008; Sinha et al., 2016). However, in other studies, researchers have found a decrease in alpha diversity (Voigt et al., 2015), and decreased purity or yield of DNA (Dominianni et al., 2014; Gorzelak et al., 2015a). A decrease in DNA stability was also found when the samples were exposed to room temperatures for long periods of time in comparison to samples that were frozen immediately after collection, but in the absence of RNA*later* (Baxter et al., 2016). Studies on humans have showed reduced alpha diversity of the human fecal microbiota with the addition of RNA*later* (Choo et al., 2015), and alterations to relative abundances of phyla and genera (Liang et al., 2020). And a study on canines showed that, among several different preservatives, RNA*later* resulted in the lowest values of diversity and richness in the fecal microbiota. Another study on canines found that the ability for RNA*later* to alter the fecal microbiota profile may depend on storage temperature (Horng et al., 2018). Altogether, the influence of RNA*later* on the microbiota profile, including when combined with variations in time of room temperature exposure and storage temperature, requires further investigation.

Another method that can vary between studies is homogenization, with some studies homogenizing fecal samples (Carruthers et al., 2019; Tal et al., 2020), and others leaving samples as-is (Liang et al., 2020; Martin de Bustamante et al., 2021). Homogenization is a process in which a sample, in this case a fecal sample, is blended with a liquid media (most often distilled water) to provide uniformity. There are different methods in homogenizing a sample such as using a blender,

pneumatic mixer, or manually homogenizing. Some studies have investigated the ability for homogenization to reduce intra-individual variability in microbiota composition between samples (Gorzelak et al., 2015b; Hsieh et al., 2016), however, the impact of homogenizing samples compared to leaving them as-is on the fecal microbiota profile is unclear. The main concern with homogenization of fecal samples is the oxygen exposure to internal layers of feces which are not normally exposed to such environmental gases, and therefore may impact the stability of metabolites in general.

Similarly, analysis of fecal samples in terms of DNA extraction and purification are variable between studies (Swanson et al., 2011). With a variety of DNA extraction kits available, there is freedom to use whichever one is most suited to your study. The protocol for each of the extraction kits are unique to their own manufacturer, and will all extract DNA in some form (Schiebelhut et al., 2017). However, there has been minimal research as to which DNA extraction kit is the most optimal to use. Additionally, it is unknown how different methods of DNA extraction, including PCR, will affect results, again making comparisons difficult between studies on the microbiota and metabolome.

1.8 Conclusion

The microbiota is affected by various factors such as age, diet, environment, medication, and disease. In affecting the gut microbiota, the metabolites produced by the bacteria present within the microbiota are affected, hence the metabolome will be impacted by external and internal factors, and influence host health as well. Despite the plethora of research on how the intestinal microbiota and metabolome affect host health, there is little standardization of the methods for

sample collection, storage, and analysis used in microbiota and metabolomic research, with a wide variety of methods currently used. This lack of standardization makes comparisons between studies in existing literature difficult. Therefore, a gap exists in the literature in developing appropriate protocols before further elucidating the complex interactions between the microbiota and metabolome, and health and disease. It follows that the research question for the present thesis is: how does exposure to room temperature post-collection, storage temperature, the addition of RNA*later*, and homogenization of samples impact the microbiota and metabolome profiles?

1.9 Thesis Hypotheses and Objectives

It is hypothesized that:

1. Exposure of samples to room temperature post-collection will increasingly change the composition of the fecal metabolome over time (Chapter 2).
2. Exposure of samples to room temperature over time post-collection, storage temperature, the addition of RNA*later*, and homogenization will all impact the alpha diversity, beta diversity, and relative abundances of phyla and genera in the fecal microbiota (Chapter 3).

To test the above hypotheses, the research objectives are as follows:

1. Analyze and compare the feline fecal metabolome after 2, 4, 6, 8, 12, and 24 hours of exposure to room temperature post-collection (Chapter 2).
2. Analyze the canine fecal microbiota profile after 0.5, 4, 8, and 24 hours of exposure to room temperature post-collection, with samples stored in the fridge (4ºC) or freezer (-20ºC), with or without the addition of RNA*later*, and with or without homogenization (Chapter 3).

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2 The Influence of Room Temperature Exposure on the Feline Fecal Metabolome

2.1 Abstract

Analyzing the metabolomic profile of feline fecal samples can provide insight into overall gastrointestinal and microbial health. Standardization of methods for handling, storing, and analyzing fecal samples for the purposes of metabolomics are variable, and therefore may impact results. The purpose of this study was to investigate the effect of room temperature exposure on microbial-derived metabolites of feline fecal samples using a metabolomics approach. Fecal samples were collected from 11 healthy cats from a local boarding facility. Samples were manually homogenized and aliquoted. The first aliquot was frozen at -80°C within 1 hour of collection, and remaining samples were exposed to room temperature for 2, 4, 6, 8, 12, and 24 h prior to freezing at -80°C. Fecal metabolites were quantified using ^{1}H NMR spectroscopy. Fifty metabolites were grouped into six categories (27 amino acids, 8 fatty acids, 6 sugars, 2 alcohols, 2 nitrogenous bases, 4 miscellaneous). Twenty out of fifty metabolites analyzed had significant differences in concentrations due to room temperature exposure (7 amino acids, 6 fatty acids, 1 sugar, 2 alcohols, 1 nitrogenous base, 3 miscellaneous). The earliest detected significant changes occurred 6 h post-collection for cadaverine and fumaric acid. Exposure to room temperature alters the composition of the feline fecal metabolome profile; however, not all metabolites were uniformly affected. Significant changes were not noted up to a minimum of 4 h post-collection; therefore, short-term exposure to such a temperature may prove to have little effect on the feline fecal metabolome profile.

2.2 Introduction

Fecal matter contains a variety of widely diverse chemical structures, including metabolites, downstream products of multiple genomic and proteomic interactions. These fecal metabolites can be used as a sensitive measure of phenotypes when studying environment-gene interactions (Bouatra et al., 2013) and reflect nutrient ingestion, digestion, and absorption within the gastrointestinal tract, as well as microbial fermentation within the large intestine (Karu et al., 2018). Fecal metabolomics, a rapidly expanding area of research, provides a "snapshot" of the entire fecal metabolome (Wood, 2021) and has been applied to assess the microbiota, to aid in understanding gastrointestinal physiology and to explore correlations between dysbiosis, fecal metabolic perturbations and disease pathogenesis (Gao et al., 2010; Jacobs et al., 2008). Moreover, this emerging technique generates information to discover specific metabolites that could potentially be used as biomarkers for disease (Jansson et al., 2009). Although there is rising popularity in the use of fecal metabolomics, methods in collecting, storing, and analyzing fecal samples have yet to be standardized, particularly in companion animal species. In studies collecting fecal samples in field locations, it may not be feasible to process and/or analyze samples immediately. This delay could alter a sample's metabolite composition, leading to equivocal conclusions. This is particularly challenging for studies requiring fecal collection from client-owned companion animals as the samples received are variable due to exposure to different storage temperatures for extended periods of time prior to handling and storage in the laboratory setting. In studies involving human fecal samples, significant differences in metabolic profiles are reported when fecal samples are stored at room temperature compared to samples either frozen immediately or freshly analyzed (Gratton et al., 2016; Saric et al., 2008). More specifically, volatile organic

compounds are higher in samples stored at room temperature in comparison to samples stored at 4°C and -20°C. The current study aimed to determine the effect of room temperature exposure over 24 h on metabolites in feline feces using a metabolomics approach. It was hypothesized that significant changes in the fecal metabolic profile would occur within the first 12 h of exposure to room temperature, especially for volatile organic compounds.

2.3 Methods

Animals

Eleven healthy adult cats, housed at a boarding facility in Guelph, Ontario were included in this study. The cats were deemed healthy based on information provided by cat owners prior to boarding. In consultation with the University of Guelph Animal Care and Use Committee, an animal use protocol was deemed unnecessary for a study performing no other procedures than collecting fecal samples from cat litter boxes.

Sample Collection

Samples were collected as part of a previously published microbiota study (Tal et al., 2017). Cats boarded at this facility were observed by facility personnel daily. Fecal samples were collected from each cat within 15 minutes of defecation. Samples were then taken immediately to the laboratory unrefrigerated before they were manually homogenized and aliquoted. A single aliquot was frozen at -80°C within the first hour after defecation (time point (T) 1). All other aliquots were kept at room temperature (20 to 23°C) in a biosafety cabinet[a] before freezing at -80°C at the specified time points; 2, 4, 6, 8, 12, and 24 h after sample collection. All samples were kept frozen until analysis. The time points designated for this study were chosen based on data

from previous studies completed with other species, indicating that metabolome changes mainly occurred in the first 12 h after sample collection (Nicolson et al., 1999; Jacobs et al., 2007).

Nuclear Magnetic Resonance (NMR) Analysis

Samples were sent frozen on dry ice to The Metabolomic Innovation Centre[b] for quantitative NMR spectroscopy. Briefly, samples were thawed then centrifuged, and a 200 mL aliquot of each sample was taken and placed in a 1.5 mL Eppendorf tube. Fifty mL of a standard buffer solution was then added (54% D2O: 46% 1.75 mM KH_2PO_4 pH 7.0 v/v containing 5.84 mM DSS (2,2-dimethyl-2-silcepentane-5-sulphonate), and 0.1% NaN_3 in H2O). The total sample, now containing 250 mL, was then transferred for spectral analysis using the 3mm SampleJet NMR tube. The ^{1}H-NMR spectrum was then collected on the 700 MHz Avance (Bruker) spectrometer, which was equipped with a 5 mm HCN Z-gradient pulsed-field gradient cryoprobe. The first transient of NOESY pre-saturation pulse sequence (noesy1dpr) was used to acquire the ^{1}H-NMR spectra at 25°C and was chosen due to the high degree of quantitative accuracy (Saude et al., 2006). Free induction decays were all zero-filled to 250 K data points, and the singlet produced from the DSS methyl groups was used as an internal standard for chemical referencing. This was set to zero parts per million (ppm) for quantification of all ^{1}H-NMR spectra. This was processed and analyzed using the Chenomx NMR Suite Professional software package version 8.4 (Chemomx Inc., Edmonton, AB). Two spectroscopists then inspected each spectrum to minimize misidentification and misquantification of compounds. All visible peaks were annotated with a compound name (Saude et al., 2006). The metabolites analyzed were categorized into six metabolite groups based on descriptions of each compound available via MetaboAnalyst[c] pathway analysis and the National Library of Medicine PubChem[d] platforms; (1) Amino acids and amines, (2) Fatty

acids, (3) Sugars and sugar metabolites, (4) Alcohols, (5) Nitrogenous bases, and (6) Other metabolites. Metabolite group classifications and compounds analyzed are described in Table 1.

Statistical Analysis

Statistical Analyses were completed using SAS (SAS Institute Inc.). If values for metabolites were not distributed normally (determined via a Shapiro-Wilk test), a logarithmic transformation was performed before further analysis. For metabolites with normal distribution and for transformed data, repeated measures analysis of variance (ANOVA) was used to detect differences over time for each metabolite, using a 5% significance level ($P < 0.05$). Following repeated measures ANOVA, post-hoc paired t-tests with Bonferroni corrections were used to detect differences between each time point. When multiple hypotheses are tested (e.g., whether concentrations differed between each time point in the present study), the probability of a type I error increases (Armstrong, 2014). The Bonferroni correction compensates for this by testing each individual hypothesis at an adjusted significance level of α divided by the total number of hypotheses (Armstrong, 2014). In this case, 21 total t-tests are being performed between the time points (1, 2, 4, 6, 8, 12, 24 h) for each metabolite; therefore, an adjusted significance level of $P < 0.002$ was used ($0.05 / 21 = 0.002$). If the values were still not normally distributed after logarithmic transformation, a Friedman's test was performed on the non-transformed values, using a 5% significance level ($P < 0.05$), followed by post-hoc Wilcoxon tests with Bonferroni corrections (adjusted significance level $P < 0.002$, as described above) to compare between each time point. Normally distributed untransformed values are reported as mean ± standard deviation, transformed values as back-transformed mean, and back-transformed lower and upper limits, while non-parametrically analyzed data is presented as median, minimum, and maximum values.

2.4 Results

Fifty metabolites were quantified: 27 amino acids and amines, eight fatty acids, six sugars and sugar metabolites, three alcohols, two nitrogenous bases, and four other metabolites (Table 1). Repeated measures ANOVA (normally distributed and log-transformed data) and Friedman's tests (non-normally distributed data) showed 20 of 50 (40%) metabolites had significant differences in concentration over time when exposed to room temperature (Tables 2-7). This included seven amino acids and amines (Table 2), six fatty acids (Table 3), one sugars and sugar metabolites (Table 4), two alcohols (Table 5), one nitrogenous base (Table 6), and three other metabolites (Table 7). Specifically, alanine, dimethylamine, putrescine, cadaverine, phenylacetate, trimethylamine, acetic acid, propionate, butyrate, isobutric acid, isovaleric acid, valerate, ethanol, methanol, uracil, acetoin, acetone, and choline all increased in concentration over time. In contrast, fecal creatine and fumaric acid concentrations decreased over time.

Post-hoc analyses showed significant differences between individual time points in 15 metabolites: putrescine, cadaverine, phenylacetate, trimethylamine, acetic acid, butyrate, isobutyric acid, isovaleric acid, propionate, valerate, fumaric acid, ethanol, methanol, acetoin, and acetone. The earliest detected changes occurred at 6 h after room temperature exposure with an increase in cadaverine (Figure 1) and a decrease in fumaric acid (Figure 2). At 8 h post-collection, phenylacetate, trimethylamine, putrescine, acetic acid, isobuytric acid, isovaleric acid, ethanol, methanol, and acetone began increasing (amino acids and volatile fatty acids shown in Figures 1 and 3, respectively). Acetoin started increasing at 12 h post-collection. Propionate, butyrate, and valerate concentrations increased at 24 h post-collection (Figure 3).

Table 2.1: Metabolite group classifications and compounds that were analyzed in feline fecal samples (n=11) using Nuclear Magnetic Resonance spectrometry.

Metabolite Group	**Compounds**
Amino Acids and Amines	Asparagine, creatine, glutamine, methylamine, 4-aminobutyrate, alanine, aspartate, betaine, dimethylamine, glycine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, putrescine, taurine, threonine, tryptophan, tyrosine, valine, cadaverine, creatinine, phenylacetate, phenylalanine, trimethylamine
Fatty Acids	Acetic acid, propionate, 3-hydroxyisobutyrate, butyrate, 3-hydroxyisovaleric acid, isobutyric acid, isovaleric acid, valerate
Sugars and Sugar Metabolites	D-glucose, D-galactose, L-fucose, succinate, fumaric acid, L-lactic acid
Alcohols	Ethanol, methanol, glycerol

Nitrogenous Bases	Uracil, xanthine
Other Metabolites	Choline, acetoin, acetone, formate

Table 2.2: The fecal concentrations of amino acids, amines, and their metabolites in feline fecal samples (n=11) across time points over 24 h storage at room temperature.

Amino Acids, Amines, and their Metabolites								
Compound	Time post-collection exposed to room temperature							P-value
	1 h	2 h	4 h	6 h	8 h	12 h	24 h	
Untransformed data[1]								
Asparagine	0.85 ± 0.41[a]	0.73 ± 0.39[a]	0.83 ± 0.32[a]	0.80 ± 0.37[a]	0.77 ± 0.28[a]	0.76 ± 0.36[a]	0.67 ± 0.25[a]	0.340
Creatine	0.08 ± 0.03[a]	0.07 ± 0.03[a]	0.07 ±0.04[a]	0.04 ± 0.02[a]	0.04 ± 0.02[a]	0.04 ± 0.03[a]	0.05 ± 0.03[a]	0.009
Glutamine	0.89 ± 0.50[a]	0.80 ± 0.35[a]	0.84 ± 0.48[a]	0.95 ± 0.45[a]	1.07 ± 0.46[a]	0.98 ± 0.50[a]	0.93 ± 0.63[a]	0.127
Methylamine	0.33 ± 0.16[a]	0.35 ± 0.16[a]	0.35 ± 0.18[a]	0.37 ± 0.17[a]	0.39 ± 0.17[a]	0.36 ± 0.18[a]	0.39 ± 0.16[a]	0.126
Transformed data[2]								
4-Aminobutyrate	0.13 (0.10-0.16)[a]	0.20 (0.12-0.32)[a]	0.23 (0.13-0.39)[a]	0.25 (0.13-0.500[a]	0.28 (0.16-0.51)[a]	0.21 (0.09-0.49)[a]	0.26 (0.11-0.60)[a]	0.068
Alanine	2.28 (1.51, 3.43)[a]	2.58 (1.71, 3.89)[a]	3.03 (2.04, 4.51)[a]	3.35 (1.89, 5.94)[a]	3.64 (2.12, 6.25)[a]	3.43 (2.37, 4.96)[a]	4.58 (3.00, 6.97)[a]	0.004
Aspartate	1.26 (0.83-1.91)[a]	1.38 (1.03-1.85)[a]	1.47 (1.01-2.12)[a]	1.67 (1.09-2.56)[a]	1.51 (0.98-2.33)[a]	1.48 (1.09-1.99)[a]	1.37 (0.89-2.11)[a]	0.524
Betaine	0.02 (0.02-0.03)[a]	0.03 (0.02-0.04)[a]	0.03 (0.02-0.04)[a]	0.02 (0.02-0.03)[a]	0.04 (0.02-0.06)[a]	0.03 (0.02-0.04)[a]	0.03 (0.02-0.05)[a]	0.587
Dimethylamine	0.02 (0.02, 0.03)[a]	0.03 (0.02, 0.04)[a]	0.03 (0.02, 0.04)[a]	0.04 (0.02-0.05)[a]	0.04 (0.03, 0.05)[a]	0.03 (0.02, 0.05)[a]	0.04 (0.03, 0.06)[a]	0.003

Glycine	1.26 (0.88-1.80)[a]	1.19 (0.83-1.73)[a]	1.32 (0.92-1.87)[a]	1.43 (0.83-2.48)[a]	1.46 (0.89-2.39)[a]	1.46 (1.00-2.14)[a]	1.44 (0.84-2.48)[a]	0.415
Glutamic acid	2.43 (1.45-4.07)[a]	2.38 (1.62-3.48)[a]	2.60 (1.70-3.99)[a]	2.76 (1.62-4.71)[a]	2.79 (1.67-4.64)[a]	2.50 (1.76-3.55)[a]	2.57 (1.40-4.71)[a]	0.635
Histidine	0.22 (0.15-0.34)[a]	0.25 (0.15-0.41)[a]	0.24 (0.16-0.36)[a]	0.30 (0.20-0.46)[a]	0.27 (0.16-0.45)[a]	0.26 (0.16-0.45)[a]	0.21 (0.14-0.32)[a]	0.247
Isoleucine	0.47 (0.25-1.12)[a]	0.56 (0.29-1.08)[a]	0.44 (0.17-1.15)[a]	0.70 (0.33-1.48)[a]	0.50 (0.25-1.01)[a]	0.55 (0.25-1.23)[a]	0.65 (0.30-1.38)[a]	0.539
Leucine	1.32 (0.82-2.12)[a]	1.47 (0.90-2.41)[a]	1.69 (1.07-2.67)[a]	1.79 (0.96-3.34)[a]	1.82 (1.07-3.10)[a]	1.63 (1.03-2.58)[a]	1.85 (1.12-3.03)[a]	0.345
Lysine	1.51 (0.77-3.00)[a]	1.80 (1.14-2.84)[a]	1.97 (1.22-3.18)[a]	1.84 (0.99-3.42)[a]	2.03 (0.91-4.52)[a]	1.86 (0.83-2.89)[a]	1.81 (0.82-3.99)[a]	0.438
Methionine	0.50 (0.29-0.86)[a]	0.56 (0.35-0.89)[a]	0.61 (0.39-0.94)[a]	0.73 (0.45-1.18)[a]	0.64 (0.38-1.07)[a]	0.64 (0.42-0.96)[a]	0.73 (0.44-1.21)[a]	0.281
Putrescine	0.55 (0.35-0.86)[ab]	0.58 (0.41-0.83)[a]	0.66 (0.40-1.09)[abc]	0.92 (0.57-1.48)[abc]	1.04 (0.73-1.48)[c]	0.82 (0.55-1.22)[abc]	1.10 (0.75-1.61)[bc]	<0.001
Taurine	0.35 (0.10-1.18)[a]	0.39 (0.13-1.12)[a]	0.41 (0.14-1.20)[a]	0.39 (0.12-1.77)[a]	0.44 (0.14-1.41)[a]	0.30 (0.08-1.06)[a]	0.37 (0.10-1.35)[a]	0.901
Threonine	0.84 (0.56-1.26)[a]	0.91 (0.69-1.21)[a]	0.95 (0.72-1.25)[a]	1.01 (0.67-1.51)[a]	0.97 (0.72-1.30)[a]	0.84 (0.63-1.11)[a]	0.73 (0.5-1.07)[a]	0.260
Tryptophan	0.21 (0.15-0.28)[a]	0.22 (0.16-0.29)[a]	0.22 (0.16-0.31)[a]	0.28 (0.21-0.37)[a]	0.25 (0.18-0.35)[a]	0.22 (0.17-0.30)[a]	0.25 (0.16-0.39)[a]	0.270
Tyrosine	0.69 (0.42-1.13)[a]	0.77 (0.49-1.20)[a]	0.87 (0.55-1.38)[a]	1.03 (0.61-1.73)[a]	1.03 (0.62-1.70)[a]	0.91 (0.61-1.36)[a]	0.92 (0.55-1.53)[a]	0.192
Valine	1.13 (0.61-2.09)[a]	1.36 (0.83-2.21)[a]	1.73 (1.14-2.63)[a]	1.88 (0.99-3.58)[a]	2.12 (1.23-3.63)[a]	1.76 (1.12-2.78)[a]	2.26 (1.38-3.71)[a]	0.122
Non-parametrically analyzed data[3]								
Cadaverine	0.64 (0.25-3.77)[a]	1.13 (0.62-2.26)[ab]	0.98 (0.44-3.61)[ab]	1.25 (0.76-4.44)[b]	1.69 (0.94-5.07)[b]	1.13 (0.17-3.88)[ab]	1.79 (0.25-2.75)[b]	0.001
Creatinine	0.21 (0.16-0.73)[a]	0.22 (0.17-0.80)[a]	0.23 (0.19-0.72)[a]	0.22 (0.14-0.62)[a]	0.24 (0.17-0.48)[a]	0.24 (0.16-0.65)[a]	0.21 (0.16-0.54)[a]	0.882

Phenylacetate	1.49 (0.12-5.05)[a]	1.50 (0.16-3.71)[a]	1.60 (0.19-4.54)[ab]	2.11 (0.23-3.43)[ab]	2.26 (0.26-3.92)[b]	1.85 (0.20-3.81)[ab]	2.03 (0.21-3.91)[b]	<0.001
Phenylalanine	0.63 (0.30-2.45)[a]	0.67 (0.33-2.65)[a]	0.73 (0.52-3.25)[a]	0.92 (0.37-3.13)[a]	0.93 (0.43-3.42)[a]	0.78 (0.51-3.26)[a]	0.81 (0.38-5.04)[a]	0.254
Trimethylamine	0.08 (0.01-0.17)[a]	0.12 (0.02-0.19)[ab]	0.11 (0.03-0.23)[ab]	0.16 (0.02-0.19)[ab]	0.20 (0.02-0.33)[b]	0.14 (0.02-0.26)[ab]	0.20 (0.01-0.30)[b]	0.003

Letters denote significant difference between time points ($P < 0.05$), where any shared letters show no differences between timepoints and having no shared letters show differences between timepoints (e.g. a and abc have no significant difference, whereas a and bc do).
1: Untransformed values reported as mean ± standard deviation.
2: Transformed values reported as back-transformed mean, and back-transformed lower and upper limits.
3: Non-parametrically analyzed presented as median, minimum and maximum values.

Table 2.3: The fecal concentrations of fatty acids that significantly differed across time point in feline fecal samples (n=11) over 24 h storage at room temperature.

Fatty Acids								
Compound	Time post-collection exposed to room temperature							P-value
	1 h	2 h	4 h	6 h	8 h	12 h	24 h	
Untransformed[1]								
Acetic Acid	52.78 ± 12.68[a]	59.94 ± 18.89[a]	64.59 ± 15.49[ab]	69.16 ± 17.92[ab]	79.98 ± 17.81[bc]	74.58 ± 15.52[ab]	94.42 ± 20.22[b]	<0.001
Propionate	26.91 ± 8.41[ab]	27.97 ± 10.67[a]	28.91 ± 8.17[ab]	29.90 ± 12.38[a]	32.47 ± 11.70[ab]	32.10 ± 12.58[ab]	38.51 ± 15.23[b]	<0.001
Transformed[2]								
3-Hydroxyisobutyrate	0.55 (0.39-0.76)[a]	0.62 (0.42-0.92)[a]	0.84 (0.60-1.18)[a]	0.80 (0.52-1.22)[a]	0.60 (0.36-0.99)[a]	0.66 (0.42-1.03)[a]	0.83 (0.54-1.27)[a]	0.161
Butyrate	18.57 (13.99-24.65)[a]	20.41 (17.05-24.43)[ab]	22.82 (18.62-27.98)[abc]	23.49 (18.00-30.64)[abc]	27.10 (21.74-33.77)[ac]	24.28 (18.38-32.06)[abc]	29.61 (22.69-38.65)[c]	<0.001

Non-Parametrically Analyzed[3]								
3-Hydroxyisovaleric acid	0.03 (0.01-0.04)[a]	0.03 (0.01-0.04)[a]	0.03 (0.00-0.05)[a]	0.03 (0.00-0.04)[a]	0.03 (0.01-0.05)[a]	0.02 (0.01-0.06)[a]	0.02 (0.01-0.10)[a]	0.961
Isobutyric Acid	1.44 (0.11-7.25)[a]	2.12 (0.32-4.76)[ab]	2.18 (0.33-6.28)[abc]	2.74 (0.41-6.20)[abc]	3.24 (0.17-6.43)[bc]	2.64 (0.23-7.77)[bc]	3.35 (0.35-8.37)[c]	<0.001
Isovaleric Acid	2.36 (0.25-11.09)[a]	3.49 (0.28-7.51)[ab]	3.84 (0.31-10.26)[abc]	4.58 (0.21-10.06)[abc]	4.55 (0.30-10.00)[bc]	4.40 (0.35-12.47)[bc]	5.27 (0.32-12.28)[c]	<0.001
Valerate	6.67 (0.61-10.75)[ab]	7.24 (0.53-9.68)[a]	8.34 (0.69-10.52)[ab]	8.33 (0.59-11.28)[ab]	8.89 (0.91-11.76)[ab]	7.92 (0.83-10.41)[ab]	8.72 (1.56-12.02)[b]	0.007

Letters denote significant difference between time points ($P < 0.05$), where any shared letters show no differences between timepoints and having no shared letters show differences between timepoints.

1: Untransformed values report mean ± standard deviation.

2: Transformed values report back-transformed mean, and back-transformed lower and upper limits.

3: Non-Parametrically analyzed report median, minimum and maximum values.

Table 2.4: The fecal concentrations of sugars and sugar metabolites that significantly differed across time point in feline fecal samples (n=11) over 24 h storage at room temperature.

Sugars and Sugar Metabolites								
Compound	Time post-collection exposed to room temperature							P-value
	1 h	2 h	4 h	6 h	8 h	12 h	24 h	
Untransformed								
D-Glucose	2.82 ± 1.41[a]	3.28 ± 2.16[a]	3.12 ± 2.13[a]	3.04 ± 1.80[a]	3.21 ± 1.74[a]	2.78 ± 1.36[a]	2.58 ± 1.58[a]	0.679
Transformed								
D-Galactose	0.53 (0.37-0.77)[a]	0.36 (0.19-0.69)[a]	0.44 (0.31-0.64)[a]	0.55 (0.39-0.78)[a]	0.45 (0.30-0.69)[a]	0.45 (0.35-0.59)[a]	0.38 (0.22-0.65)[a]	0.415
L-Fucose	0.42 (0.29-0.61)[a]	0.39 (0.27-0.57)[a]	0.27 (0.17-0.42)[a]	0.33 (0.22-0.52)[a]	0.35 (0.23-0.52)[a]	0.28 (0.22-0.35)[a]	0.27 (0.16-0.46)[a]	0.270
Succinate	0.08 (0.04-0.16)[a]	0.06 (0.04-0.10)[a]	0.06 (0.03-0.10)[a]	0.05 (0.02-0.12)[a]	0.07 (0.04-0.12)[a]	0.06 (0.03-0.14)[a]	0.11 (0.04-0.28)[a]	0.220

Non-Parametrically Analyzed[3]								
Fumaric Acid	0.09 (0.05-0.19)[a]	0.06 (0.03-0.15)[ab]	0.08 (0.04-0.10)[abc]	0.05 (0.02-0.15)[bc]	0.05 (0.03-0.13)[bc]	0.06 (0.02-0.12)[bc]	0.05 (0.02-0.10)[c]	<0.001
L-Lactic Acid	0.26 (0.09-54.01)[a]	0.21 (0.10-57.96)[a]	0.26 (0.11-33.25)[a]	0.22 (0.11-67.31)[a]	0.33 (0.18-67.34)[a]	0.24 (0.09-61.55)[a]	0.38 (0.15-68.84)[a]	0.163

Letters denote significant difference between time points ($P < 0.05$), where any shared letters show no differences between timepoints and having no shared letters show differences between timepoints.
1: Untransformed values report mean ± standard deviation.
2: Transformed values report back-transformed mean, and back-transformed lower and upper limits.
3: Non-Parametrically analyzed report median, minimum and maximum values.

Table 2.5: The fecal concentrations of alcohols that significantly differed across time point in feline fecal samples (n=11) over 24 h storage at room temperature.

Alcohols								
Compound	Time post-collection exposed to room temperature							P-value
	1 h	2 h	4 h	6 h	8 h	12 h	24 h	
Transformed[2]								
Ethanol	0.67 (0.34-1.29)[ab]	0.93 (0.55-1.57)[a]	1.11 (0.68-1.82)[abc]	1.12 (0.53-2.35)[abd]	1.38 (0.72-2.62)[bcd]	1.56 (0.83-2.92)[ce]	1.81 (0.86-3.80)[de]	0.006
Methanol	0.32 (0.23-0.44)[a]	0.37 (0.25-0.57)[ab]	0.41 (0.27-0.61)[ab]	0.43 (0.28-0.66)[abc]	0.48 (0.31-0.76)[bc]	0.51 (0.34-0.77)[bc]	0.68 (0.38-1.20)[c]	0.001
Non-parametrically analyzed data[3]								
Glycerol	2.59 (1.37-3.95)[a]	2.86 (1.87-4.19)[a]	2.86 1.68-3.93)[a]	3.07 (1.94-3.52)[a]	3.01 (1.39-4.24)[a]	2.69 (1.39-3.52)[a]	2.38 (1.32-2.98)[a]	0.082

Letters denote significant difference between time points ($P < 0.05$), where any shared letters show no differences between timepoints and having no shared letters show differences between timepoints.
1: Untransformed values report mean ± standard deviation.

2: Transformed values report back-transformed mean, and back-transformed lower and upper limits.
3: Non-Parametrically analyzed report median, minimum and maximum values.

Table 2.6: The fecal concentrations of nitrogenous bases that significantly differed across time point in feline fecal samples (n=11) over 24 h storage at room temperature.

Nitrogenous Bases							
Time post-collection exposed to room temperature							P-value
1 h	2 h	4 h	6 h	8 h	12 h	24 h	
Non-Parametrically Analyzed[3]							
0.33 (0.14-1.25)[a]	0.41 (0.16-1.39)[a]	0.52 (0.28-1.46)[a]	0.54 (0.09-1.13)[a]	0.67 (0.21-1.84)[a]	0.52 (0.14-1.36)[a]	0.58 (0.19-1.80)[a]	0.011
0.03 (0.02-0.10)[a]	0.04 (0.02-0.06)[a]	0.04 (0.03-0.17)[a]	0.04 (0.03-0.06)[a]	0.04 (0.02-0.06)[a]	0.03 (0.02-0.07)[a]	0.04 (0.02-0.08)[a]	0.357

Letters denote significant difference between time points ($P < 0.05$), where any shared letters show no differences between timepoints and having no shared letters show differences between timepoints.
1: Untransformed values report mean ± standard deviation.
2: Transformed values report back-transformed mean, and back-transformed lower and upper limits.
3: Non-Parametrically analyzed report median, minimum and maximum values.

Table 2.7: The fecal concentrations of other metabolites that significantly differed across time point in feline fecal samples (n=11) over 24 h storage at room temperature.

Other Metabolites								
Compound	Time post-collection exposed to room temperature							P-value
	1 h	2 h	4 h	6 h	8 h	12 h	24 h	
Untransformed data[1]								
Choline	0.09 ± 0.03[a]	0.09 ± 0.04[a]	0.09 ±0.04[a]	0.10 ± 0.05[a]	0.11 ± 0.04[a]	0.09 ± 0.05[a]	0.11 ± 0.04[a]	0.045
Transformed[2]								

Acetoin	0.15 (0.10-0.23)[a]	0.21 (0.15-0.29)[ab]	0.21 (0.15-0.30)[abc]	0.28 (0.22-0.37)[abc]	0.30 (0.23-0.38)[abc]	0.26 (0.21-0.33)[bc]	0.31 (0.24-0.40)[c]	<0.001
Non-Parametrically Analyzed[3]								
Acetone	0.14 (0.06-0.29)[a]	0.23 (0.07-0.45)[a]	0.25 (0.07-0.51)[ab]	0.31 (0.07-0.44)[ab]	0.36 (0.04-0.70)[b]	0.27 (0.07-0.46)[ab]	0.43 (0.08-0.53)[b]	0.001
Formate	0.19 (0.13-2.14)[a]	0.16 (0.12-2.12)[a]	0.16 (0.05-0.10)[a]	0.19 (0.13-2.10)[a]	0.18 (0.12-2.47)[a]	0.36 (0.13-1.98)[a]	0.16 (0.13-2.06)[a]	0.131

Letters denote significant difference between time points ($P < 0.05$), where any shared letters show no differences between timepoints and having no shared letters show differences between timepoints.

1: Untransformed values reported as mean ± standard deviation.

2: Transformed values reported as back-transformed mean, and back-transformed lower and upper limits.

3: Non-parametrically analyzed presented as median, minimum and maximum values.

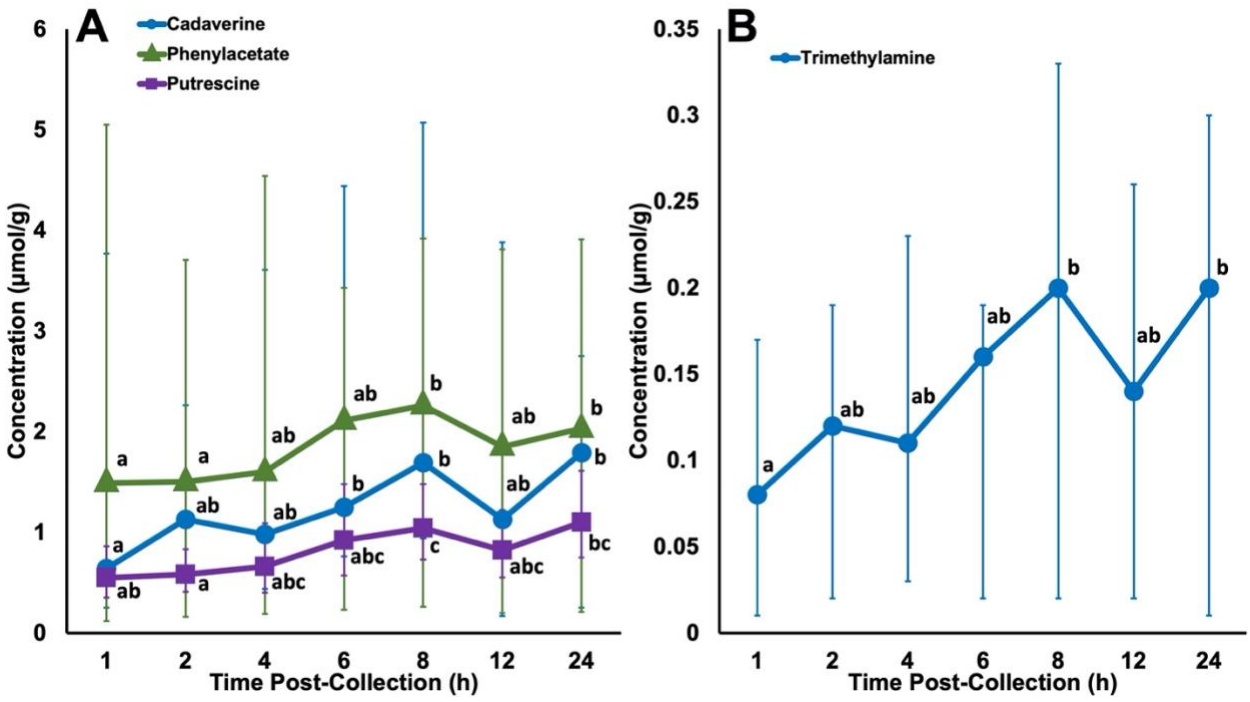

Figure 2.1: Changes over time to amine/amino acid concentrations following exposure of fecal samples to room temperature for up to 24 h after sample collection in healthy adult cats (n = 11). Letters denote a significant difference between time points ($P < 0.05$), where any shared letters show no differences between timepoints and having no shared letters show differences between timepoints. Putrescine is displayed as the back-transformed mean, with error bars representing the back-transformed upper and lower limits. Cadaverine, phenylacetate, and trimethylamine are displayed as the median, with error bars representing the maximum and minimum.

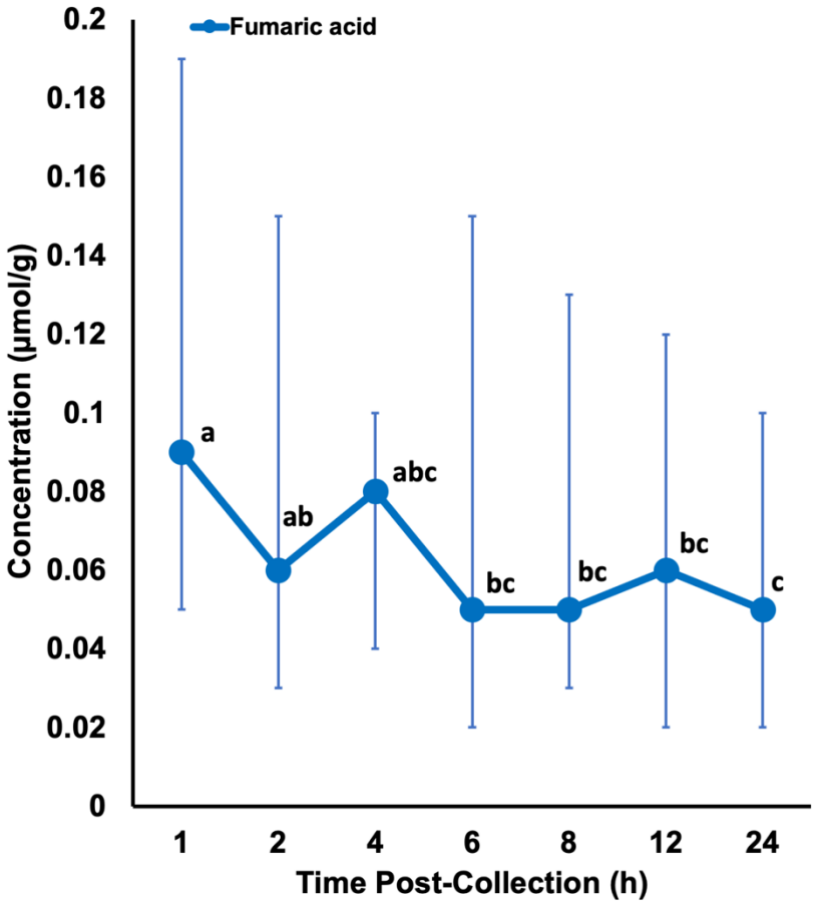

Figure 2.2: Changes over time for fecal fumaric acid concentrations following exposure of fecal samples to room temperature for up to 24 h after sample collection in healthy adult cats (n = 11). Letters denote a significant difference between time points ($P < 0.05$), where any shared letters show no differences between timepoints and having no shared letters show differences between timepoints. Values are displayed as the median, with error bars representing the maximum and minimum.

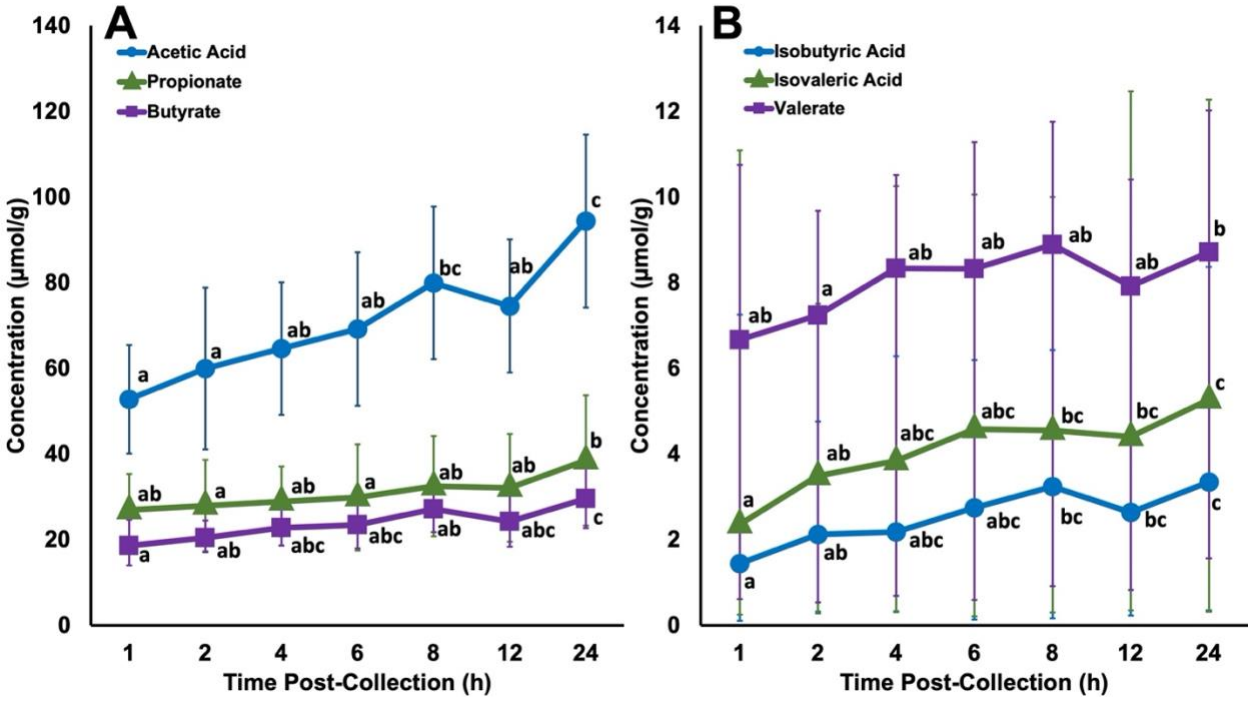

Figure 2.3: Changes over time for volatile fatty acid concentrations following exposure of fecal samples to room temperature for up to 24 h after sample collection in healthy adult cats (n = 11). Letters denote a significant difference between time points ($P < 0.05$), where any shared letters show no differences between timepoints and having no shared letters show differences between timepoints. Acetic acid and propionate displayed as the mean ± standard deviation. Butyrate is displayed as the back-transformed mean, with error bars representing the back-transformed upper and lower limits. Isobutyric acid, isovaleric acid, and valerate are displayed as the median, with error bars representing the maximum and minimum.

2.5 Discussion

Concentrations of more than half of the fecal metabolites remained stable with exposure to room temperature across the time points investigated. Some metabolites did change over time, however. Notably, fecal concentrations of the amine cadaverine (Figure 1) and the sugar fumaric acid (Figure 2) increased and decreased, respectively, 6 h post-collection. Other metabolites in the amine/amino acid group (i.e., putrescine, phenylacetate, and trimethylamine), and some in the

volatile fatty acid group (i.e., acetic acid, isobutyric acid, and isovaleric acid) increased 8 h post-collection. Three other metabolites in the volatile fatty acid group (i.e., propionate, butyrate, and valerate) increased 24 h post-collection. These findings are like those of Gratton et al. (2016) in humans, with changes in fecal metabolite concentrations occurring as early as 5 h into exposure to room temperature, particularly in amino acids and volatile compounds such as short-chain fatty acids and methanol. Another study on 34-month-old children observed differing concentrations of three fecal metabolites following exposure to room temperature for 52 h (Liang et al., 2020). Both studies determined that microbial fermentation was the main source of these time-related changes in metabolites.

The present study's findings suggest that in feline feces, metabolites categorized as amines/amino acids generally change with room temperature exposure earlier than those characterized as volatile fatty acids. This is likely a result of bacterial fermentation within the feces. Cats are historically obligate carnivores, consuming protein-rich diets. The diversity of microorganisms found in the feline gastrointestinal tract is thus regulated by carbon sources derived largely from protein fermentation (Macfarlane et al., 2007), with intestinal bacteria selecting for the associated substrates (Macfarlane et al., 1998). Amino acids are more readily available to the bacterial population present, and subsequently shift with exposure to oxygen at room temperature due to continued bacterial activity. Also, acetoin, a precursor in the synthesis of branched chain amino acids and bacterial growth (Xiao & Xu, 2007), increased 12 h post-collection. The fecal metabolites present depend on the available substrates and bacteria in the gut, which are affected by diet. If a host consumes more carbohydrates compared to protein, less protein will be available in the gut and carbohydrate fermentation will dominate. Conversely, if

carbohydrate consumption is depleted, a shift to proteolytic fermentation will occur. This coincides with shifts in relative abundances of microbial species, which can alter the gut microbiota and metabolome (Neis et al., 2015). Higher alpha diversity was found in cats consuming a higher protein diet compared to a lower protein and thus higher carbohydrate diet (Badri et al., 2021). Fecal metagenomics also revealed upregulation of amino acid and urea metabolism, and mucin foraging pathways and a positive association between amino acid catabolism and mucin degradation with a high protein diet. Moreover, cats fed the higher protein diet presented higher fecal concentrations of branched chain fatty acids and ammonia and a higher fecal pH, suggesting more proteolytic bacteria. Undigested peptides in the gut are broken down by bacteria with proteolytic properties such as *Clostridium, Bacteroides, Fusobacterium, Propionibacterium, Lactobacillus,* and *Streptococcus* (Diether & Willing, 2019). Although products such as acetate, butyrate and propionate (i.e., volatile fatty acids) are part of proteolytic fermentation, the production level is much lower compared to carbohydrate fermentation (Aguirre et al., 2016). Cats fed a lower protein diet and thus higher carbohydrate diet, were found to have higher fecal concentrations of carbohydrate-active enzymes, which are involved in the short chain fatty acid pathways and as such carbohydrate fermentation (Badri et al., 2021). The fermentation process to produce volatile fatty acids can also take longer, which may further explain why in the present study the volatile fatty acids shifts occurred later (Neis et al., 2015).

The lack of standardized fecal collection, storage, and analysis methods makes comparisons within existing literature difficult. The present study's findings suggest that exposure of feline fecal samples to room temperature should be limited to no more than 4 h, because beyond that the metabolome composition began to change. In further developing the use of fecal

metabolomics as novel diagnostic techniques, sample exposure to room temperatures must be considered when collection methods are out of laboratory settings, such as fecal collection in the field or in a home-setting. This may be further applied to areas where room temperatures in the southern hemisphere are warmer than temperatures in the northern hemisphere. Storage conditions post-collection must also be considered. As a fecal sample continues to be exposed to room temperature, the bacteria can continue to consume and produce metabolites, allowing the potential to continually alter the metabolomic profile. In a study on humans, fecal samples refrigerated and stored at 4°C demonstrated slowing down of the effects of exposure to room temperatures; although, many metabolic shifts still occurred 24 h post-collection (Gratton et al., 2016). Altogether, the time of exposure of fecal samples to room temperature prior to storage is an important factor to consider in future studies.

The present study also highlights some important considerations for further protocol development. The samples were not frozen immediately after defecation, but also required extra time for transportation from the boarding facility and for aliquoting samples, and were therefore exposed to room temperature for an additional hour. Potential metabolite changes during this time could not be detected. The samples in this study were also manually homogenized, which introduces oxygen into the sample that may have enhanced the effect of aerobic fermentation on the fecal metabolome. Future research should explore the effect of room temperature exposure on non-homogenized fecal samples, or in samples stored in an anaerobic environment. Freezing of samples before analysis may also impact the analyses, as changes could occur during the thawing process. Gratton et al. (2016) investigated the influence of the freeze-thaw process on human fecal metabolites and found that there were decreased levels of short chained fatty acids, and increased

levels of branched chain amino acids, concluding that when transporting samples, it is ideal to minimize the time to prevent thawing, and ideally ship on ice when possible. Finally, the method used for metabolite analysis, ^{1}H NMR spectroscopy, was an accurate method to quantify the metabolites of interest, allowing comparisons between several timepoints. However, this is a targeted methodology for detection of metabolites and the changes in metabolites that have yet to be profiled were undetected (Honneffer et al., 2017). Further research is warranted to narrow down collection and storage strategies to maintain the original metabolic profile in feline fecal samples.

2.6 Conclusion

This study demonstrated that storage of feline fecal samples at room temperature for the purpose of metabolic profiling using NMR spectrometry can be considered appropriate when these samples are stored for 4 h or less post-collection. Longer exposure to room temperature during collection and storage of fecal samples should be avoided to minimize effects on metabolite concentrations.

2.7 Acknowledgements

We would like to thank Amanda Santarossa for assistance with sample collection. The Metabolomic Innovation Centre (TMIC) performed ^{1}H NMR spectrometry to produce results used in this study.

2.8 Funding

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2.9 Endnotes

[a]Class II, Type A2 Biosafety Cabinet, Thermo Fischer Scientific, Waltham, Massachusetts, USA;

[b]University of Alberta Metabolomic Research Services Group, University of Alberta, Edmonton, Alberta, Canada

[c]MetaboAnalyst 5.0, 2021, www.metaboanalyst.ca

[d]PubChem, 2021, www.pubchem.nlm.nih.gov

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3 Impact of Sample Homogenization, RNA*later* Solution, Room Temperature Exposure, and Storage Temperature on the Canine Fecal Microbiota Profile

3.1 Abstract

Canine fecal microbiota profiling provides insight into host health and disease. Standardization of methods for collection, processing, and storage of fecal samples for microbiomics is currently inconclusive, however. This study investigated the effects of RNA*later*, homogenization, room temperature exposure, and storage in the fridge for 24 h prior to freezing on the canine fecal microbiota profile. We hypothesized these factors would alter the microbial alpha diversity, beta diversity, and relative abundances of phyla and genera. Fecal samples were collected from six intact healthy 1-year-old colony beagles. Within 15 minutes after voiding, samples were left non-homogenized or were homogenized and aliquoted, then kept at room temperature for 0.5, 4, 8, or 24 hours. Homogenized aliquots then had RNA*later* added or not. Following room temperature exposure, all aliquots were stored at 4°C for 24 hours prior to storage at -20°C, or directly stored at -20°C. DNA extraction, PCR amplification, then sequencing were completed on all samples. Alpha diversity (diversity, evenness, and richness), and beta diversity (community membership and structure), and relative abundances of phyla and genera were compared between groups. Homogenization, RNA*later*, 24 h of room temperature exposure, and 24 h of fridge storage did not alter any measures of alpha diversity or beta diversity. Bacterial relative abundances were affected by RNA*later*, but not homogenization, room temperature exposure, or 24 h of fridge storage. These findings suggest homogenization, 24 h of room temperature exposure and storage in the fridge for 24 h prior to freezing are acceptable for canine

fecal microbiota analyses, and the ability for preservation with RNA*later* to alter the fecal microbiota profile should be considered in future research.

3.2 Introduction

The microbiota, found in the gastrointestinal tract, is comprised of a variety of microorganisms, including bacteria, protozoa, viruses, and fungi (Honneffer, 2014). There are approximately 10^{10}-10^{14} microbial cells within the gastrointestinal microbiota, which is 10-fold more than normal host cells (Gill et al., 2006). The microbiota acts in a mutualistic relationship with the host, operating as a defense mechanism against pathogens, and thus can impact host health and the development of diseases (Gill et al., 2006). The gastrointestinal microorganisms are essential for digestion of carbohydrates and proteins to produce short chain fatty acids, which further develop the gastrointestinal microbiota and subsequently the immunity of the host (Honneffer, 2014). Additionally, the gastrointestinal microbiota plays major roles in metabolism, homeostasis, gut epithelial health, and neurological development (Cho & Blaser, 2012). The microbiota's dynamic capabilities allow adaptation to many variables such as the environment, diet, disease states, and medication interventions (Rowland et al., 2018).

Alterations to the gastrointestinal microbiota correlates to various gastrointestinal diseases and disorders (Queen et al., 2012; Suchodolski et al., 2009; Volkmann et al., 2017). Conditions such as oral health, allergies, body condition and weight management, diabetes mellitus, kidney disease and gastrointestinal disease are also associated with dysbiosis of the gastrointestinal microbiota (Wernimont et al., 2020). The direct characterization of the different compartments of the gastrointestinal tract is difficult and invasive, thus the microbiota of fecal samples is often used

as a proxy for the hind gut microbiota (Chen et al., 2019). Although the fecal microbiota has been widely investigated across various species and disease states (Barko et al., 2018), the methods for sample processing (e.g., homogenization), preservation (e.g., the addition of RNA*later*), and storage temperature vary between studies. Standardization of these methods is needed to make comparisons between studies easier, as external factors would be accounted for.

A previous study by our research team observed no changes to the diversity or relative abundances of the feline fecal microbiota with exposure to room temperature across a 4-day period; however, did not investigate the influence of homogenization or the use of RNA*later* as a preservative (Tal et al., 2017). To further build on these findings, the present study investigated the effect of homogenization, the addition of RNA*later*, room temperature exposure post-collection, and storage in the fridge for 24 h prior to freezing on the alpha diversity and bacterial membership and structure of the canine fecal microbiota.

3.3 Methods

Animals

Four intact female and two intact male healthy colony beagles, 1 year of age, housed at the existing dog colony from the Central Animal Facility at the University of Guelph were included in this study.

Housing

Dogs were group-housed in a free-living environment. Dogs had free access to various sources of environmental enrichment such as toys and beds. For 2 hours a day, 5 days a week, all dogs received human interaction with one or two familiar people. These interactions included petting, voluntary play, brushing, and general upkeep of the room. The housing space was environmentally controlled using a 12:12 h light:dark cycle, with lights turned on at 0800 h and turned off at 2000 h. Room temperature was maintained at 20.0°C and 40-60% relative humidity. The room surfaces were sanitized and cleaned daily. All dogs were provided *ad libitum* water access and fed the same commercial diet for adult maintenance daily.

Sample Collection

Dogs were observed daily, and fecal samples were collected immediately after defecation occurred. Samples were visually assessed using the Bristol Stool Form Scale (Langlois et al., 2020) and all fecal samples were given a score of 3 out of 7 (normal feces = 3 to 4). Samples were then immediately taken to the laboratory in a Styrofoam box at room temperature. Each sample was cut in half longitudinally (Figure 1). One half of the sample was manually homogenized and aliquoted (2 mL) into 36 Eppendorf tubes, and the other half was left non-homogenized and stored in 200-µg samples which included sections from the core and surface of the feces.

Experiment 1: Per dog, one aliquot of homogenized and one of non-homogenized samples were exposed to room temperature for 0.5 h then frozen at -20°C until further analysis was completed to investigate the effects of homogenization on the microbiota profile (Figure 1A).

Experiment 2: The effects of RNA*later* on the microbiota profile (Figure 1B) was assessed by exposing four aliquots of homogenized samples for each dog to room temperature for 0.5 h, then RNA*later* was added to two of the four samples at a 1:1 ratio of homogenized sample:RNA*later* (200 µL). One sample was immediately frozen at –20°C and the other sample was stored at 4°C for 24 h before freezing at -20°C until further analysis was completed. The other two samples were processed similarly but RNA*later* was not added.

Experiment 3: To investigate the effects of room temperature exposure on the microbiota profile (Figure 1C), for each dog, one aliquot of the homogenized samples was exposed to room temperature (20-22°C) for 0.5, 4, 8, and 24 h, then stored at 4°C for 24 h before freezing at -20°C. Additionally, per dog, one aliquot of the homogenized samples was exposed to room temperature (20-22°C) for 0.5, 4, 8, and 24 h then immediately frozen at -20°C for long term storage.

Experiment 4: The effects of storage in the fridge at 4°C for 24 hours prior to storing in the freezer on the at -20°C on the microbiota profile (Figure 1D) were also investigated. To do this, samples from Experiment 3 stored in the fridge at 4°C for 24 hours prior to storing in the freezer at -20°C were compared to samples immediately frozen at -20°C for long term storage at each room temperature exposure time point (0.5, 4, 8, and 24 h).

DNA extraction, amplification & sequencing

Using a commercial Kit[a], total DNA was extracted from 200 µg of wet weight fecal samples. Amplification of the 16S rRNA gene at the V4 region was done with forward and reverse primers (Klindworth et al., 2013). These primers were specifically designed to have overhanging adapters that allow for annealing to the sequencing adaptors used in Illumina universal index

sequencing when it is added for PCR later in the process. The amplification process of the V4 region was carried out using a reaction that consisted of 9.0 mL of molecular grade water, 12.5 mL of KAPA 2G Fas Hot Start ReadyMix 2X (KapaBiosystems), 2.5 mL template DNA, and 0.5 mL each of the reverse and forward 16S rRNA V4 primers (Slifierz et al., 2015). For the PCR reaction, the samples were processed at 94°C for 10 min, followed by another 27 cycles at 94°C for 45 sec each. After, the process is followed by 60 sec at 53°C and 90 sec at 72°C, which is completed by a period of 10 min at 72°C (Slifierz et al., 2015). Magnetic beads were then used to purify the PCR products[b]. The addition of Illumina adapters to the PCR purified 16S rRNA gene product. Using electrophoresis, the PCR products were then evaluated using agarose gel (1.5%) and purified. Once purification steps were completed, spectrometry was used for quantification of the PCR products. Samples were then finalized to a concentration of 2 nM and the library pool was submitted to the Genomics Facility at the University of the Guelph for sequencing with an Illumina MiSeq[c] for 250 cycles from each end.

Bioinformatic and statistical analysis

Using the Mothur software package[d], the bioinformatics analysis was performed. Samples were processed and aligned using the SILVA 16S rRNA reference data base (Quast et al., 2013) and paired-ends were merged to be completely overlapping. Any sequences that were misaligned with the target region of interest were removed. Irregular sequences with lengths greater than 270 bp and less than 240 bp were also removed. If the sequence had ambiguous bases or runs with homopolymers >8 bp, they were also removed. After these removals, identification of chimeras was completed using Uchime (Edgar et al., 2011), and these were subsequently removed. Using

a reference template, sequences that belonged to nonbacterial domains such as mitochondria, archaea, chloroplasts, and eukaryotes were removed from the sequences. The remaining sequences were then assigned groups based on operational taxonomic units (OTUs) by using a picking approach that permitted up to 0.03 difference, or 97% similarity. Subsampling was done to normalize sequence numbers by ensuring that random selection of the selected number of sequences corresponded to the lowest number of reads for a given sample. The coverage was then assessed using Good's coverage value, which is an estimate of what percentage of the total species is represented in the samples.

The alpha diversity of samples was assessed using the Inverse Simpson's (diversity), Shannon's evenness (evenness), and Chao1 (richness) indices (Chao, 1984; Shannon, 1948; Simpson, 1949). Steel-Dwass tests (JMP statistical analysis software) were used to compare alpha diversity among the groups. Steel-Dwass tests were used because the data was non-normally distributed and control the overall experiment-wise error rate when many comparisons are being made.

The Jaccard and Yue & Clayton indices were calculated and used to assess beta diversity. The Jaccard index is a measure of the memberships within a community, where it only considers the number of genera shared, but not abundance (Smith & van Belle, 1984). The Yue and Clayton index, which measures the structure of the community, considers the shared genera and the relative abundances of each (Yue & Clayton, 2005). Using a Mothur software package, analysis of molecular variance (AMOVA) was used to compare the Jaccard and the Yue and Clayton indices between treatments. Principal coordinate analyses plots were built using JMP statistical analysis software to visualize the clustering of treatments based on Jaccard and Yue and Clayton indices.

Comparisons of relative abundances between treatments were completed using the nonparametric paired Wilcoxon rank sum tests (JMP statistical analysis software) for pairwise comparisons. False discovery rate corrections (Seed-based *d* Mapping software) were then applied to each relative abundance comparison to reduce the chance of a Type 1 error. Phyla and genera were included in the analyses based on a minimum of 1% relative abundance. Significance was set at $P < 0.05$.

3.4 Results

A total of 15,023,735 raw sequences were obtained, and 11,843,607 sequences were available for analysis after initial filtering and cleaning (mean: 154,557 ± 35,479; median:142,351; and range: 105,028 to 287,142). Per sample, subsampling was performed at 82,000 sequences, and was considered adequate based on a Good's coverage index value of 99.9%. Overall, 23 phyla, 141 families, and 343 genera were identified in all samples analyzed.

Experiment 1: Effect of homogenization on fecal microbiota profile

Alpha diversity **–** Homogenization did not affect alpha diversity, with the Inverse Simpson's ($P = 0.94$), Shannon's evenness ($P = 0.47$), and Chao1 ($P = 0.13$) indices not differing between homogenized and non-homogenized samples (Figure 2).

Beta diversity **–** AMOVA did not show any differences between homogenized and non-homogenized samples for bacterial community membership (Jaccard index, $P = 0.09$) or structure (Yue and Clayton index, $P = 0.60$). Correspondingly, the PCoA plots showed no evident clustering between treatments (Figure 3).

Relative abundances – Comparisons of relative abundance profiles are depicted visually in Figures 4 and 5. No phyla (Table 1) or genera (Table 2) significantly differed between homogenized and non-homogenized samples.

Experiment 2: Effect of RNAlater on fecal microbiota profile

Alpha diversity –Addition of RNA*later* to homogenized samples exposed to room temperature for 0.5 h and then stored in the fridge at 4°C for an additional 24 h before freezing at -20°C did not affect alpha diversity indices, with Inverse Simpson's (P = 0.58), Shannon's evenness (P = 0.30), and Chao1 (P = 0.69) being not significantly different between samples with and without RNA*later* (Figure 6). Addition of RNA*later* to homogenized samples exposed to room temperature for 0.5 h then frozen at -20°C immediately also did not affect alpha diversity indices, with Inverse Simpson's (P = 0.47), Shannon's evenness (P = 0.23), and Chao1 (P = 1.00) being not significantly different between samples with and without RNA*later* (Figure 6).

Beta diversity – There were not any significant differences in beta diversity between samples with and without RNA*later* exposed to room temperature for 0.5 h and then placed in the fridge at 4°C for 24 h before storing in the freezer at -20°C for bacterial community membership (Jaccard index, AMOVA P = 0.32) or structure (Yue & Clayton index, AMOVA P = 0.21). Correspondingly, the PCoA plots showed no evident clustering between treatments (Figure 7A and B). There were not differences between samples with and without RNA*later* exposed to room temperature for 0.5 h and then placed in the freezer at -20°C immediately for bacterial community membership (Jaccard index, AMOVA P = 0.20) or structure (Yue & Clayton index, P = 0.14).

Correspondingly, the PCoA plots showed no evident clustering between treatments (Figure 7C and D).

Relative abundances of phyla and genera – Comparisons of relative abundance profiles are depicted visually in Figures 8 and 9. In samples with RNA*later* exposed to room temperature for 0.5 h and then stored in the fridge at 4°C for 24 h prior to freezing at -20°C, the relative abundance of *Firmicutes* was lower (P = 0.02) and *Bacteroidetes* (P = 0.03), *Proteobacteria* (P = 0.03), and *Campilobacterota* (P = 0.02) were greater than samples without RNA*later* (Table 3). At the genus level, no relative abundances differed between samples with and without RNA*later* (Table 4). In samples with RNA*later* exposed to room temperature for 0.5 h and then frozen at -20°C immediately, the relative abundance of *Firmicutes (*P = 0.01) was lower and the relative abundances of *Bacteroidetes* (P = 0.02), *Proteobacteria* (P = 0.03), and *Campylobacteria* (P = 0.01) were greater than samples without RNA*later* (Table 5). At the genus level, no relative abundances differed between samples with and without RNA*later* (Table 6).

Experiment 3: Effect of Room temperature exposure on the fecal microbiota profile

Alpha diversity – Alpha diversity did not differ at 4, 8, or 24 h compared to 0.5 h of room temperature exposure in samples stored in the fridge at 4°C for an additional 24 h prior to freezing at -20°C, with the Inverse Simpson's (4 h: P = 0.89; 8 h: P = 0.73; 24 h: P = 0.52), Shannon's evenness (4 h: P = 0.94; 8 h: P = 0.73; 24 h: P = 0.33), and Chao1 (4 h: P = 1.00; 8 h: P = 1.00; 24 h: P = 1.00 for freezer, P = 0.33 for fridge) indices not differing between time points (Figure 10). Alpha diversity also did not differ at 4, 8, or 24 h compared to 0.5 h of room temperature exposure in samples frozen at -20°C immediately, with the Inverse Simpson's (4 h: P = 1.00; 8 h:

P = 1.00; 24 h: P = 0.52), Shannon's evenness (4 h: P = 1.00; 8 h: P = 0.94; 24 h: P = 0.52), and Chao1 (4 h: P = 1.00; 8 h: P = 1.00; 24 h: P = 1.00) indices not differing between time points (Figure 10).

Beta diversity – There were no differences across room temperature exposure time points for samples stored in the fridge at 4°C for 24 h prior to storing in the freezer at -20°C for bacterial community membership (Jaccard index, AMOVA P = 0.74) or structure (Yue and Clayton index, AMOVA P = 0.63). Correspondingly, the PCoA plots show no evident clustering between treatments (Figure 11A and B). There were no differences across room temperature exposure time points in samples that were frozen at -20°C immediately for bacterial community membership (Jaccard index, AMOVA P = 0.74) or structure (Yue and Clayton index, AMOVA P = 0.66). Correspondingly, the PCoA plots show no evident clustering between treatments (Figure 11C and D).

Relative abundances – Comparisons of relative abundance profiles are depicted visually in Figures 12 and 13. In samples exposed to room temperature for 0.5, 4, 8, and 24 h then stored in the fridge at 4°C for an additional 24 h prior to freezing at -20°C, there were no differences in the relative abundances of any phyla (Table 7) or genera (Table 8) between time points. In samples exposed to room temperature for 0.5, 4, 8, and 24 then frozen at -20°C immediately, there were also no differences in the relative abundances of any phyla (Table 9) or genera (Table 10) between time points.

Experiment 4: Effect of storage in the fridge for 24 h prior to freezing on the fecal microbiota profile

Alpha diversity – In homogenized samples exposed to room temperature for 0.5, 4 , 8, and 24 h, Inverse Simpson's (0.5 h: $P = 0.81$; 4 h: $P = 0.69$; 8 h: $P = 0.38$; 24 h: $P = 0.81$), Shannon's evenness (0.5 h: $P = 0.81$; 4 h: $P = 0.81$; 8 h: $P = 0.94$; 24 h: $P = 0.81$), and the Chao1 index (0.5 h: $P = 0.94$; 4 h: $P = 0.47$; 8 h: $P = 0.81$; 24 h: $P = 0.69$) did not differ between samples stored in the fridge at 4°C for 24 h prior to storing in the freezer at -20°C and samples frozen immediately at -20°C (Figure 10).

Beta diversity –There were no differences between homogenized samples stored in the fridge at 4°C for 24 h prior to storing in the freezer at -20°C and homogenized samples frozen immediately at -20°C for bacterial community membership or structure following 0.5 (Jaccard index, AMOVA $P = 0.90$; Yue and Clayton index, AMOVA $P = 0.93$), 4 (Jaccard index, $P = 0.95$; Yue and Clayton index, $P = 0.91$), 8 (Jaccard index, $P = 0.93$; Yue and Clayton index, $P = 0.91$), and 24 h (Jaccard index, $P = 0.92$; Yue and Clayton index, $P = 0.95$) of room temperature exposure. Correspondingly, the PCoA plots show no evident clustering between treatments (Figure 11C and D).

Relative abundances – Comparisons of relative abundance profiles are depicted visually in Figures 12 and 13. In homogenized samples exposed to room temperature for 0.5, 4, 8, and 24 h, the relative abundances of no phyla (for median, maximum, and minimum values see Tables 8 and 10) or genera (for median, maximum, and minimum values see Tables 7 and 9) differed between samples stored in the fridge at 4°C for 24 h prior to storing in the freezer at -20°C and samples frozen immediately at -20°C.

Table 3.1: Relative abundance of the eight most abundant canine fecal phyla between homogenized and non-homogenized samples stored in the freezer after exposure to room temperature for 0.5 (n = 6 per treatment)

Phylum	Non-homogenized	Homogenized
Firmicutes	66.31[a] [30.95-83.48]	71.54[a] [57.69-79.05]
Bacteroidetes	10.19[a] [1.95-40.02]	17.29[a] [7.74-23.03]
Fusobacteria	14.63[a] [2.07-21.42]	9.14[a] [3.23-14.88]
Proteobacteria	1.46[a] [0.38-9.14]	2.55[a] [0.98-6.09]
Actinobacteria	4.39[a] [2.41-6.85]	1.87[a] [0.51-5.43]
Campilobacterota	0.01[a] [0.00-0.07]	0.00[a] [0.00-0.01]
Deinococcus-thermus	0.00[a] [0.00-0.00]	0.00[a] [0.00-0.00]
Bacteria_unclassified	0.01[a]	0.01[a]

	[0.00-0.02]	[0.01-0.02]

Taxa showing different letters indicate a significant difference ($P < 0.05$), and those that share letters are not significantly different. Values are displayed as median [minimum - maximum].

Table 3.2: Relative abundance of the 20 most abundant canine fecal genera between homogenized and non-homogenized samples stored in the freezer after exposure to room temperature for 0.5 h (n = 6 per treatment)

Genus	Non-homogenized	Homogenized
Fusobacterium	12.39[a] [2.00-21.13]	8.22[a] [3.19-14.49]
Prevotella	3.76[a] [0.27-17.56]	9.22[a] [2.84-12.52]
Phocaeicola	4.62[a] [0.65-24.46]	4.27[a] [3.04-9.36]
Lactobacillus	1.01[a] [0.02-42.54]	0.45[a] [0.01-19.16]
Erysipelotrichaceae_unclassified	3.87[a] [1.02-10.07]	6.81[a] [0.59-16.31]
Megamonas	0.74[a] [0.06-3.42]	4.49[a] [0.51-18.09]

Turicibacter	3.45[a] [1.06-17.04]	4.95[a] [2.10-14.08]
Allobaculum	1.38[a] [0.25-18.86]	5.95[a] [0.70-13.12]
Peptacetobacter	6.62[a] [0.31-611.17]	4.66[a] [1.32-5.43]
Lachnospiraceae_unclassified	2.76[a] [1.15-9.99]	3.64[a] [2.47-6.22]
Blautia	3.91[a] [1.02-10.23]	4.22[a] [1.84-7.20
Romoutsia	2.38[a] [1.19-17.73]	4.17[a] [1.16-6.88]
Streptococcus	1.31[a] [0.46-11.44]	2.03[a] [1.43-10.01]
Faecalibacterium	2.79[a] [0.07-4.66]	4.12[a] [0.47-5.61]
Veillonella	1.33[a] [0.00-3.57]	1.53[a] [0.00-4.62]
Enterococcus	0.99[a]	1.97[a]

	[0.03-5.40]	[0.02-5.31]
Bifidobacterium	2.56[a]	0.98[a]
	[0.07-3.33]	[0.11-5.01]
Parasutterella	0.32[a]	1.45[a]
	[0.04-3.98]	[0.29-3.75]
Catenibacterium	0.29[a]	0.48[a]
	[0.01-4.96]	[0.00-10.70]
Ligilactobacillus	0.50[a]	0.39[a]
	[0.06-2.63]	[0.14-6.35]

Taxa showing different letters indicate a significant difference ($P < 0.05$), and those that share letters are not significantly different. Values are displayed as median [minimum-maximum].

Table 3.3: Relative abundance of the 20 most abundant canine fecal phyla between samples with and without RNA*later* after exposure to room temperature for 0.5 h then storage in the fridge for 24 h prior to freezing (n = 6 per treatment)

Phylum	No RNA*later*	RNA*later*
Firmicutes	67.11[a]	44.77[b]
	[54.44-79.84]	[39.36-58.51]
Bacteroidetes	19.14[a]	34.69[b]
	[8.65-26.83]	[19.43-38.03]

Fusobacteria	8.45[a] [2.04-15.72]	13.72[a] [10.02-18.37]
Proteobacteria	2.69[a] [0.70-5.77]	5.77[b] [5.09-8.56]
Actinobacteria	1.99[a] [0.72-5.42]	1.09[a] [0.21-2.45]
Campilobacterota	0.01[a] [0.00-0.02]	0.05[b] [0.03-0.10]
Bacteria_unclassified	0.01[a] [0.00-0.04]	0.07[b] [0.03-0.17]
Deinococcus-thermus	0.00[a] [0.00-0.00]	0.00[a] [0.00-0.00]

Taxa showing different letters indicate a significant difference ($P < 0.05$), and those that share letters are not significantly different. Values are displayed as median [minimum-maximum].

Table 3.4: Relative abundance of the 20 most abundant canine fecal genera between samples with and without RNA*later* after exposure to room temperature for 0.5 h then storage in the fridge for 24 h prior to freezing (n = 6 per treatment)

Genus	No RNA*later*	RNA*later*
Prevotella	11.95[a]	16.78[a]

	[3.61-14.75]	[7.48-20.07]
Fusobacterium	7.34[a]	11.99[a]
	[2.01-14.99]	[9.36-16.08]
Phocaeicola	5.35[a]	10.32[a]
	[2.34-10.31]	[8.61-13.59]
Megamonas	4.25[a]	4.28[a]
	[0.87-20.08]	[0.72-9.76]
Erysipelotrichaceae_unclassified	7.07[a]	4.72[a]
	[0.63-15.89]	[0.32-11.76]
Allobaculum	6.03[a]	3.74[a]
	[0.94-14.49]	[0.35-9.85]
Lachnospiraceae_unclassified	2.95[a]	3.71[a]
	[1.69-5.24]	[3.14-4.51]
Turicibacter	4.82[a]	2.23[a]
	[2.08-15.21]	[1.13-5.55]
Blautia	3.49[a]	2.76[a]
	[1.69-6.85]	[1.66-3.85]
Faecalibacterium	2.66[a]	2.64[a]
	[0.17-4.08]	[1.48-5.19]

Parasutterella	1.76[a] [0.31-3.91]	1.79[a] [0.43-3.99]
Lactobacilus	0.45[a] [0.01-18.26]	0.23[a] [0.01-11.48]
Bacteroidales_unclassified	1.26[a] [0.03-2.48]	3.39[a] [0.15-4.41]
Steptococcus	2.36[a] [1.47-9.79]	0.95[a] [0.75-4.82]
Peptacetobacter	4.76[a] [1.55-7.17]	1.74[a] [0.99-3.13]
Romboutsia	4.09[a] [1.23-6.51]	1.91[a] [0.57-3.78]
Veillonella	1.38[a] [0.00-4.92]	0.73[a] [0.00-1.87]
Catenibacterium	0.48[a] [0.01-10.62]	0.75[a] [0.01-6.29]
Escherichia/Snigella	0.02[a] [0.01-0.10]	0.02[a] [0.01-0.04]
Phascolarctobacterium	0.45[a]	1.86[a]

	[0.03-1.29]	[0.95-2.41]

Taxa showing different letters indicate a significant difference ($P < 0.05$), and those that share letters are not significantly different. Values are displayed as median [minimum-maximum].

Table 3.5: Relative abundance of the 20 most abundant canine fecal phyla between samples with and without RNA*later* after exposure to room temperature for 0.5 h then freezing immediately (n = 6 per treatment)

Phylum	No RNA*later*	RNA*later*
Firmicutes	71.53[a]	44.79[b]
	[57.68-79.05]	[43.90-54.97]
Bacteroidetes	17.29[a]	32.38[b]
	[7.73-23.02]	[21.10-34.77]
Fusobacteria	9.14[a]	13.59[a]
	[3.22-14.88]	[9.79-18.47]
Proteobacteria	2.55[a]	6.31[b]
	[0.97-6.08]	[5.33-7.71]
Actinobacteria	1.86[a]	1.00[a]
	[0.51-5.42]	[0.31-2.76]
Campilobacterota	0.00[a]	0.05[b]
	[0.00-0.01]	[0.03-0.09]

Bacteria_unclassified	0.01[a] [0.00-0.02]	0.07[b] [0.03-0.20]
Deinococcus-thermus	0.00[a] [0.00-0.00]	0.00[a] [0.00-0.00]

Taxa showing different letters indicate a significant difference ($P < 0.05$), and those that share letters are not significantly different. Values are displayed as median [minimum-maximum].

Table 3.6: Relative abundance of the 20 most abundant canine fecal genera between samples with and without RNA*later* after exposure to room temperature for 0.5 h then freezing immediately (n = 6 per treatment)

Genus	No RNA*later*	RNA*later*
Prevotella	9.22[a] [2.84-12.51]	15.32[a] [7.70-17.94]
Fusobacterium	8.22[a] [3.19-14.49]	11.83[a] [9.14-16.03]
Phocaeicola	4.28[a] [3.04-9.36]	10.01[a] [8.83-13.26]
Megamonas	4.49[a] [0.51-18.09]	4.15[a] [0.85-10.14]
Erysipelotrichaceae_unclassified	6.81[a]	5.41[a]

	[0.59-16.31]	[0.34-11.69]
Allobaculum	5.94[a]	4.19[a]
	[0.70-13.12]	[0.54-9.53]
Lachnospiraceae_unclassified	3.64[a]	3.81[a]
	[2.47-6.22]	[2.71-4.73]
Turicibacter	3.39[a]	2.79[a]
	[2.10-14.08]	[1.08-5.66]
Blautia	4.21[a]	3.13[a]
	[1.84-7.20]	[1.69-4.33]
Faecalibacterium	4.11[a]	3.07[a]
	[0.46-5.61]	[1.50-5.45]
Parasutterella	1.45[a]	1.84[a]
	[0.29-3.74]	[0.47-4.27]
Lactobacilus	0.44[a]	0.20[a]
	[0.01-19.16]	[0.01-8.76]
Bacteroidales_unclassified	1.37[a]	3.22[a]
	[0.01-3.08]	[0.13-3.83]
Steptococcus	2.02[a]	1.18[a]
	[1.42-10.01]	[0.67-4.62]

Peptacetobacter	4.66[a] [1.32-5.43]	2.15[a] [0.76-3.33]
Romboutsia	4.16[a] [1.16-6.87]	1.62[a] [0.58-3.85]
Veillonella	1.53[a] [0.00-4.62]	0.74[a] [0.00-1.97]
Catenibacterium	0.48[a] [0.00-10.70]	0.44[a] [0.00-6.20]
Escherichia/Snigella	0.02[a] [0.01-0.09]	0.02[a] [0.01-0.05]
Phascolarctobacterium	0.95[a] [0.04-1.24]	1.79[a] [1.04-2.28]

Taxa showing different letters indicate a significant difference ($P < 0.05$), and those that share letters are not significantly different. Values are displayed as median [minimum-maximum].

Table 3.7: Relative abundance of the 20 most abundant canine fecal phyla after exposure to room temperature for 0.5, 4, 8, and 24 h then storage in the fridge for 24 h prior to freezing (n = 6 per treatment)

Phylum	0.5 h	4 h	8 h	24 h
Firmicutes	67.11[a]	47.68[a]	54.98[a]	47.56[a]

	[54.43-79.84]	[32.71-69.67]	[35.86-76.59]	[34.35-63.56]
Bacteroidetes	19.14[a] [8.66-26.83]	30.66[a] [15.94-45.47]	24.85[a] [13.32-42.23]	27.89[a] [9.45-42.95]
Fusobacteria	8.45[a] [2.04-15.72]	11.72[a] [2.79-25.39]	11.44[a] [2.25-25.24]	9.57[a] [1.24-18.37]
Proteobacteria	2.69[a] [0.70-5.77]	5.16[a] [1.59-9.67]	4.10[a] [0.66-8.51]	12.63[a] [8.01-22.40]
Actinobacteria	1.99[a] [0.71-5.42]	1.10[a] [0.13-2.40]	1.67[a] [0.46-2.71]	1.08[a] [0.20-2.04]
Campilobacterota	0.01[a] [0.00-0.02]	0.01[a] [0.00-0.10]	0.01[a] [0.00-0.01]	0.01[a] [0.00-0.01]
Bacteria_unclassified	0.01[a] [0.00-0.03]	0.01[a] [0.00-0.03]	0.02[a] [0.00-0.03]	0.03[a] [0.01-0.06]
Deinococcus-thermus	0.00[a] [0.00-0.00]	0.00[a] [0.00-0.00]	0.00[a] [0.00-0.00]	0.00[a] [0.00-0.00]

Taxa showing different letters indicate a significant difference ($P < 0.05$), and those that share letters are not significantly different. Values are displayed as median [minimum-maximum].

Table 3.8: Relative abundance of the 20 most abundant canine fecal genera after exposure to room temperature for 0.5, 4, 8, and 24 h then storage in the fridge for 24 h prior to freezing (n = 6 per treatment)

Genus	0.5 h	4 h	8 h	24 h
Prevotella	11.95[a] [3.61-14.74]	18.99[a] [5.73-24.63]	15.45[a] [3.91-19.17]	16.50[a] [3.00-24.09]
Fusobacterium	7.33[a] [2.01-14.99]	9.78[a] [2.73-24.47]	9.78[a] [2.21-24.61]	8.17[a] [1.20-17.96]
Phocaeicola	5.35[a] [2.33-10.31]	7.58[a] [4.19-17.55]	7.98[a] [3.51-17.47]	9.44[a] [3.60-16.33]
Megamonas	4.24[a] [0.87-20.08]	5.90[a] [1.69-25.74]	8.19[a] [1.78-31.63]	5.93[a] [1.47-27.21]
Erysipelotrichaceae_unclassified	7.06[a] [0.63-15.89]	3.85[a] [0.47-10.24]	4.37[a] [0.39-12.16]	3.14[a] [0.42-11.26]
Allobaculum	6.03[a] [0.93-14.49]	3.18[a] [0.40-10.83]	3.85[a] [0.47-9.30]	2.98[a] [0.45-8.23]
Lachnospiraceae_unclassified	2.95[a] [1.68-5.24]	3.05[a] [2.55-3.56]	2.65[a] [2.27-4.27]	2.38[a] [1.71-3.37]

Turicibacter	4.81[a] [2.08-15.20]	1.90[a] [1.32-9.43]	3.17[a] [1.00-10.03]	2.32[a] [0.80-7.01]
Blautia	3.48[a] [1.68-6.84]	2.34[a] [1.23-3.53]	2.21[a] [0.82-3.64]	1.93[a] [1.01-2.36]
Faecalibacterium	2.65[a] [0.16-4.08]	4.17[a] [0.52-5.19]	2.31[a] [0.19-3.19]	2.11[a] [0.14-3.45]
Parasutterella	1.75[a] [0.31-3.91]	3.11[a] [0.44-6.32]	2.92[a] [0.36-7.02]	2.04[a] [0.37-7.46]
Lactobacillus	0.45[a] [0.01-18.26]	0.23[a] [0.03-12.51]	0.37[a] [0.01-14.27]	0.35[a] [0.02-10.17]
Bacteroidales_unclasified	1.26[a] [0.02-2.48]	2.32[a] [0.07-5.62]	1.77[a] [0.08-4.39]	1.85[a] [0.09-4.74]
Streptococcus	2.35[a] [1.46-9.78]	1.21[a] [0.77-5.69]	1.39[a] [0.87-7.40]	1.07[a] [0.93-7.29]
Peptacetobacter	4.75[a] [1.55-7.17]	1.78[a] [0.65-3.85]	2.06[a] [0.51-3.32]	1.67[a] [0.52-2.59]
Romboutsia	4.09[a] [1.23-6.51]	1.49[a] [0.66-3.14]	2.25[a] [0.65-3.35]	1.84[a] [0.53-2.40]

Veillonella	1.38[a] [0.00-4.91]	1.49[a] [0.00-5.59]	2.32[a] [0.00-11.27]	1.77[a] [0.00-10.29]
Catenibacterium	0.47[a] [0.01-10.62]	0.31[a] [0.00-3.66]	0.38[a] [0.00-6.67]	4.20[a] [0.00-4.20]
Escherichia/Shigella	0.02[a] [0.01-0.10]	0.03[a] [0.00-0.20]	0.01[a] [0.00-0.58]	10.00[a] [0.13-18.95]
Phascolarctobacterium	0.44[a] [0.02-1.28]	1.16[a] [0.17-3.41]	0.77[a] [0.11-3.32]	1.93[a] [0.10 -4.04]

Taxa showing different letters indicate a significant difference ($P < 0.05$), and those that share letters are not significantly different. Values are displayed as median [minimum-maximum].

Table 3.9: Relative abundance of the 20 most abundant canine fecal phyla after exposure to room temperature for 0.5, 4, 8, and 24 h then immediately freezing (n = 6 per treatment)

Phylum	0.5 h	4 h	8 h	24 h
Firmicutes	71.53[a] [57.68-79.05]	52.13[a] [31.30-72.07]	60.37[a] [39.89-83.53]	49.23[a] [37.59-68.59]
Bacteroidetes	17.29[a] [7.73-23.02]	26.33[a] [10.13-45.47]	19.02[a] [8.89-39.12]	26.03[a] [11.95-42.11]
Fusobacteria	9.14[a]	12.66[a]	10.90[a]	8.93[a]

	[3.22-14.88]	[3.94-25.36]	[2.53-22.52]	[1.32-20.28]
Proteobacteria	2.55[a]	4.71[a]	3.74[a]	11.03[a]
	[0.97-6.08]	[1.34-10.81]	[0.87-8.26]	[6.97-21.04]
Actinobacteria	1.86[a]	0.75[a]	1.81[a]	1.23[a]
	[0.51-5.42]	[0.12-2.50]	[0.49-2.56]	[0.28-2.03]
Campilobacterota	0.00[a]	0.01[a]	0.01[a]	0.01[a]
	[0.00-0.01]	[0.00-0.02]	[0.00-0.01]	[0.00-0.01]
Bacteria_unclassified	0.01[a]	0.01[a]	0.01[a]	0.02[a]
	[0.00-0.02]	[0.00-0.03]	[0.00-0.03]	[0.00-0.03]
Deinococcus-thermus	0.00[a]	0.00[a]	0.00[a]	0.00[a]
	[0.00-0.00]	[0.00-0.00]	[0.00-0.00]	[0.00-0.00]

Taxa showing different letters indicate a significant difference ($P < 0.05$), and those that share letters are not significantly different. Values are displayed as median [minimum-maximum].

Table 3.10: Relative abundance of the 20 most abundant canine fecal genera after exposure to room temperature for 0.5, 4, 8, and 24 h then immediately freezing (n = 6 per treatment)

Genus	0.5 h	4 h	8 h	24 h
Prevotella	9.22[a]	16.68[a]	10.26[a]	14.95[a]
	[2.84-12.51]	[3.04-23.35]	[2.30-16.76]	[3.66-21.77]

Fusobacterium	9.22[a] [2.84-12.51]	10.82[a] [3.89-24.68]	9.44[a] [2.48-22.06]	7.40[a] [1.27-19.39]
Phocaeicola	3.53[a] [3.04-9.36]	6.70[a] [3.87-17.87]	7.30[a] [2.47-16.86]	9.13[a] [2.58-16.35]
Megamonas	4.49[a] [0.51-18.09]	5.40[a] [1.81-22.91]	7.25[a] [1.13-43.43]	5.77[a] [1.50-27.36]
Erysipelotrichaceae_unclassified	2.04[a] [0.59-16.31]	3.98[a] [0.52-10.91]	4.84[a] [0.49-13.50]	3.79[a] [0.42-9.80]
Allobaculum	5.94[a] [0.70-13.12]	2.73[a] [0.40-11.83]	4.28[a] [0.41-10.12]	3.84[a] [0.55-7.28]
Lachnospiraceae_unclassified	3.01[a] [2.47-6.22]	3.35[a] [2.38-3.94]	2.93[a] [2.27-4.57]	2.05[a] [1.91-3.38]
Turicibacter	4.95[a] [2.10-14.08]	1.79[a] [1.41-9.62]	3.71[a] [1.23-6.22]	2.82[a] [0.83-8.23]
Blautia	2.38[a] [1.84-7.20]	2.40[a] [1.07-4.22]	1.96[a] [1.09-4.30]	1.76[a] [0.96-2.74]
Faecalibacterium	4.11[a]	5.66[a]	2.49[a]	2.01[a]

	[0.46-5.61]	[0.94-6.05]	[0.33-3.10]	[0.30-3.25]
Parasutterella	1.45[a]	2.80[a]	2.27[a]	2.32[a]
	[0.29-3.74]	[0.43-6.95]	[0.34-5.90]	[0.30-7.18]
Lactobacillus	0.44[a]	0.27[a]	0.42[a]	0.29[a]
	[0.01-19.16]	[0.01-14.77]	[0.02-14.69]	[0.01-13.82]
Bacteroidales_unclasified	1.37[a]	2.25[a]	1.57[a]	1.75[a]
	[0.01-3.08]	[0.07-6.12]	[0.07-4.74]	[0.10-4.53]
Streptococcus	2.02[a]	1.21[a]	1.31[a]	1.33[a]
	[1.42-10.01]	[0.61-5.94]	[0.73-8.41]	[0.94-6.85]
Peptacetobacter	4.66[a]	1.92[a]	1.99[a]	1.93[a]
	[1.32-5.43]	[0.48-3.43]	[0.70-3.08]	[0.50-2.32]
Romboutsia	4.16[a]	1.61[a]	4.21[a]	2.21[a]
	[1.16-6.87]	[0.79-2.89]	[0.69-4.21]	[0.53-3.10]
Veillonella	1.54[a]	1.63[a]	2.29[a]	1.69[a]
	[0.00-4.62]	[0.00-6.19]	[0.00-14.88]	[0.00-12.33]
Catenibacterium	0.48[a]	0.34[a]	0.44[a]	0.28[a]
	[0.00-10.70]	[0.00-3.72]	[0.01-7.65]	[0.00-5.06]
Escherichia/Shigella	0.02[a]	0.01[a]	0.02[a]	8.84[a]

	[0.00-0.09]	[0.00-0.20]	[0.00-0.82]	[0.10-17.85]
Phascolarctobacterium	0.95[a]	1.27[a]	0.62[a]	1.41[a]
	[0.04-1.24]	[0.18-3.31]	[0.09-3.31]	[0.25 -3.96]

Taxa showing different letters indicate a significant difference ($P < 0.05$), and those that share letters are not significantly different. Values are displayed as median [minimum-maximum].

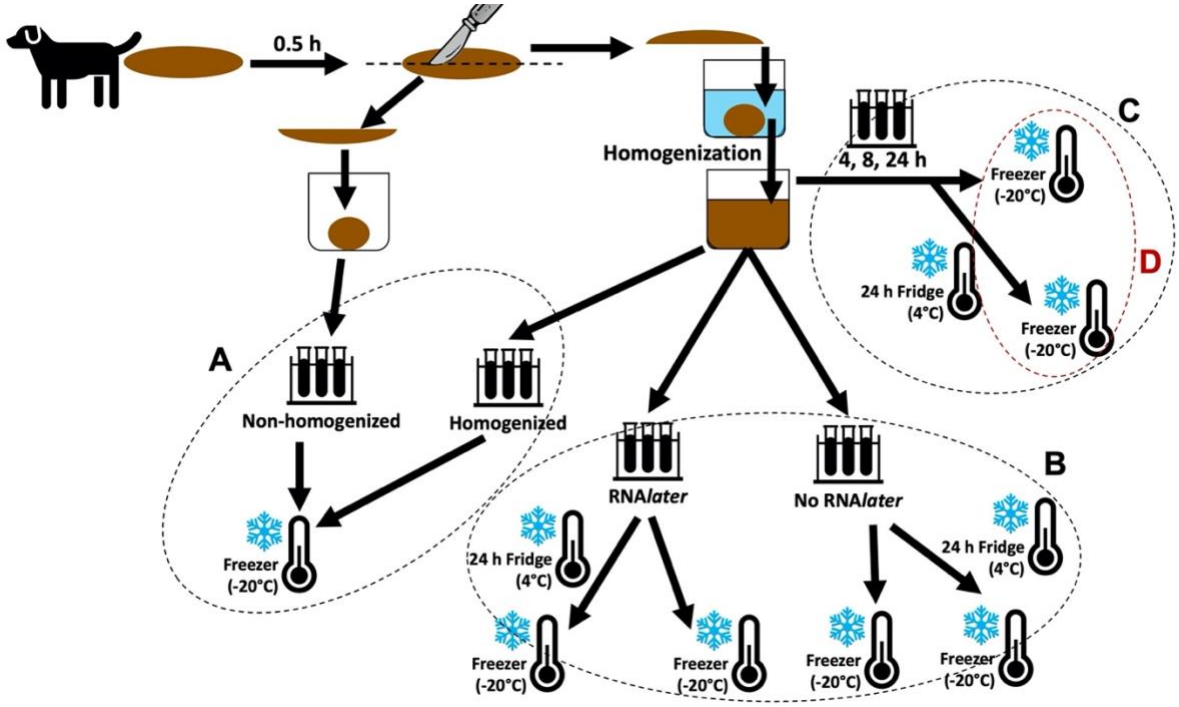

Figure 3.1: Experimental design, showing samples used for comparison of homogenized and non-homogenized samples (A), comparison of samples with and without RNA*later*, stored in the fridge for 24 h prior to freezing, or immediately frozen (B), comparison of samples exposed to room temperature for 4, 8, and 24 h, stored in the fridge for 24 h prior to freezing, or immediately frozen (C), and comparison between samples stored in the fridge for 24 h prior to freezing and samples immediately frozen, at each room temperature exposure time point (D).

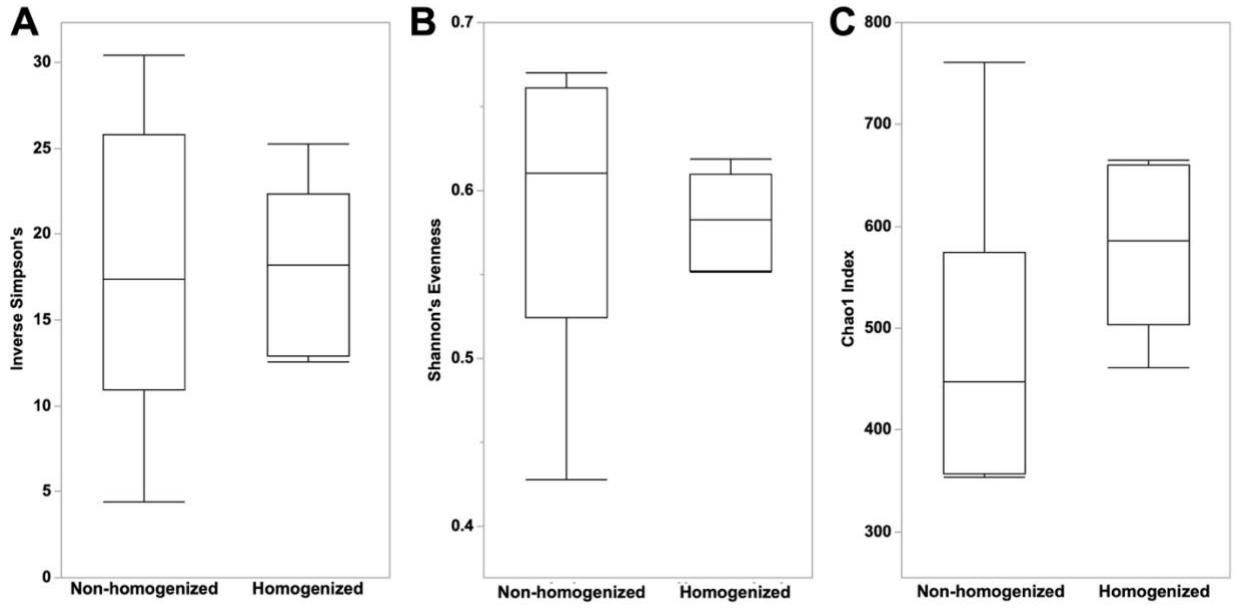

Figure 3.2: Inverse Simpson's (A), Shannon's evenness (B), and the Chao1 index (C) of the canine fecal microbiota in homogenized and non-homogenized samples. There were no differences between homogenized and non-homogenized ($P > 0.05$). Data are presented as the median with the minimum and maximum (n = 6 per treatment).

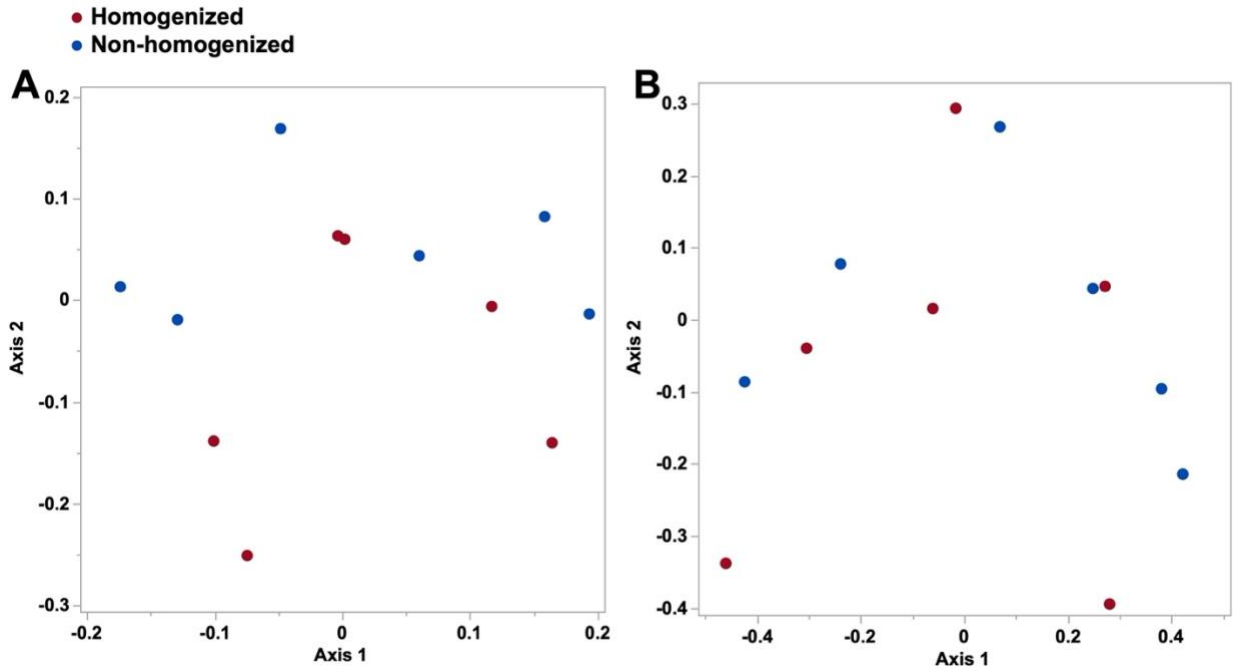

Figure 3.3: Principal Coordinate Analysis (PCoA) of the Jaccard (A) and Yue and Clayton (B) indices of the canine fecal microbiota of in homogenized (red) and non-homogenized (blue) samples.

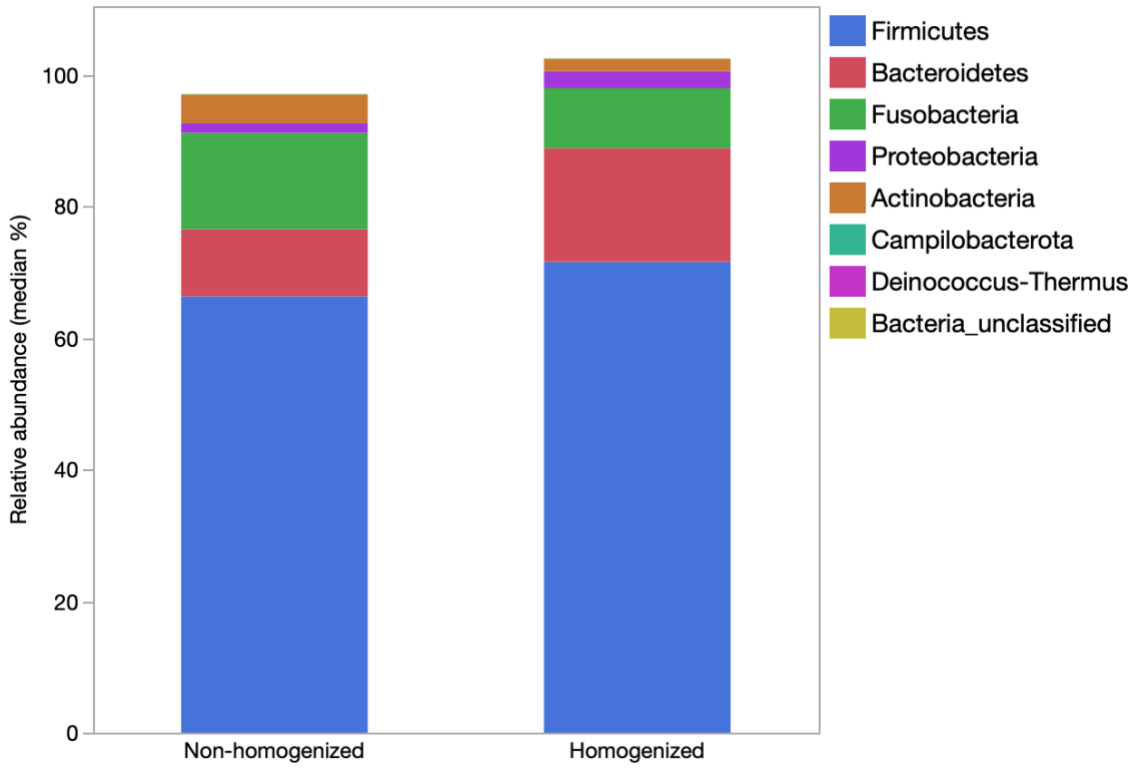

Figure 3.4: Comparison of median relative abundances of the most abundant phyla in the canine fecal microbiota between homogenized and non-homogenized samples (n = 6 per treatment).

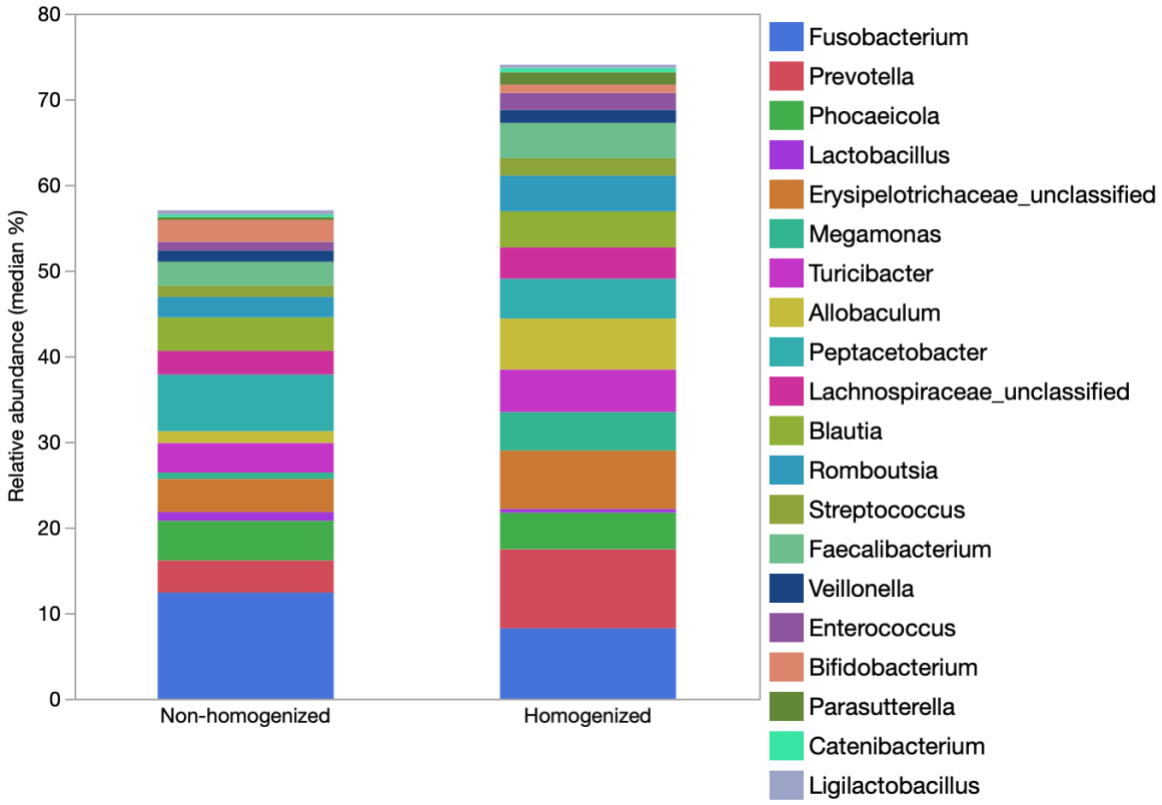

Figure 3.5: Comparison of median relative abundances of the most abundant genera in the canine fecal microbiota between homogenized and non-homogenized samples (n = 6 per treatment).

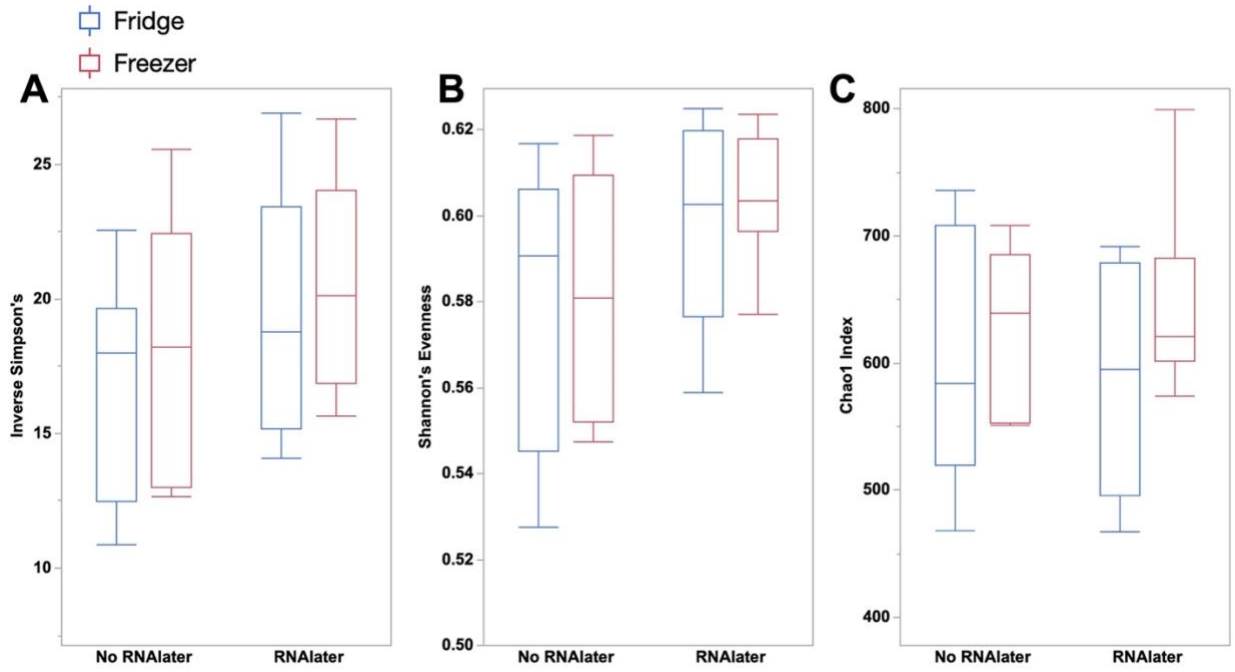

Figure 3.6: Inverse Simpson's (A), Shannon's evenness (B), and the Chao1 index (C) of the canine fecal microbiota in samples with and without RNA*later*, stored in the fridge for 24 h prior to storing in the freezer (blue) and stored directly in the freezer (red). There were no differences between RNA*later* and no RNA*later* for either storage method ($P > 0.05$). Data are presented as the median with the minimum and maximum (n = 6 per treatment).

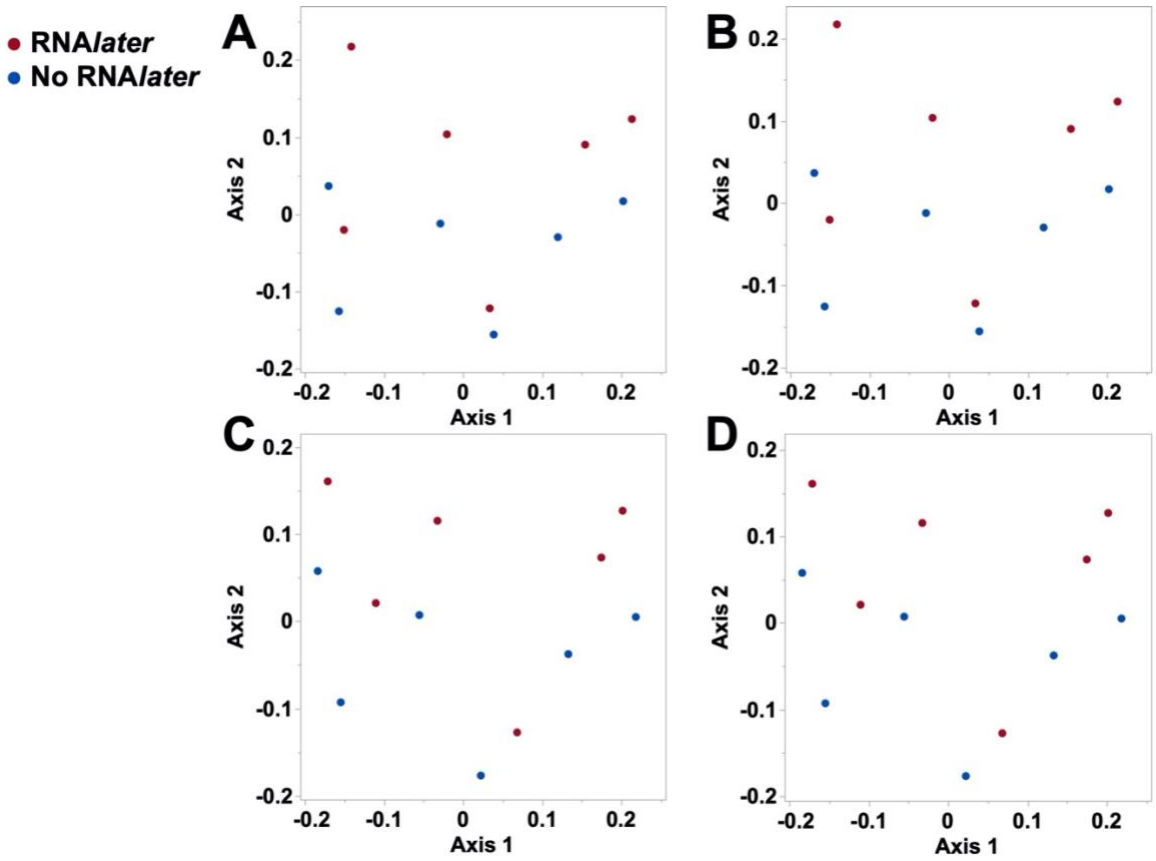

Figure 3.7: Principal Coordinate Analysis (PCoA) of the Jaccard (A and C) and Yue and Clayton (B and D) indices of the canine fecal microbiota between samples with (red) and without RNA*later* (blue), stored in the fridge for 24 h prior to storing in the freezer (A and B) and stored directly in the freezer (C and D).

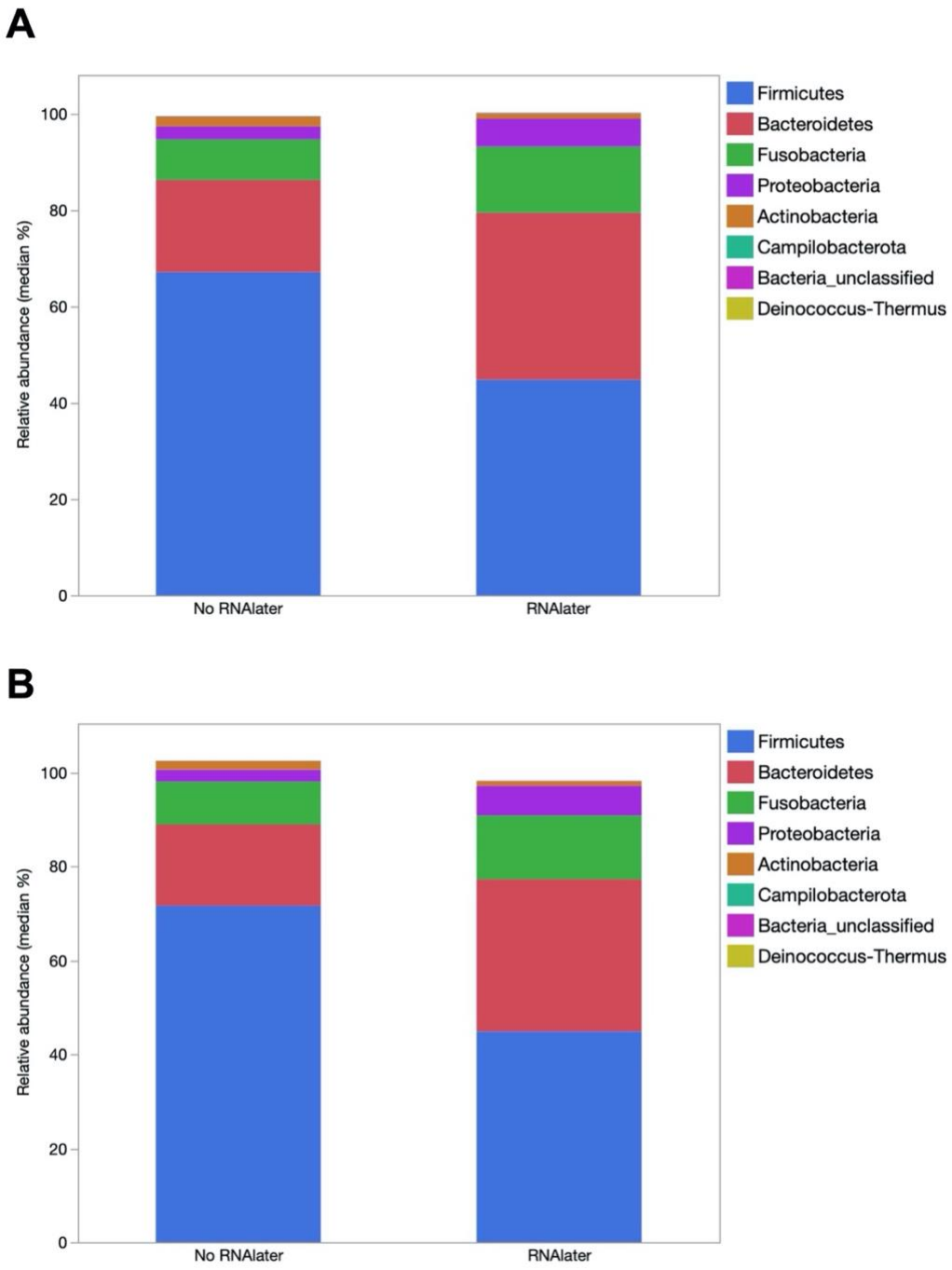

Figure 3.8: Comparison of median relative abundances of the most abundant phyla in the canine fecal microbiota between samples with and without RNA*later*, stored in the fridge for 24 h prior to storing in the freezer (A) and stored directly in the freezer (B) (n = 6 per treatment).

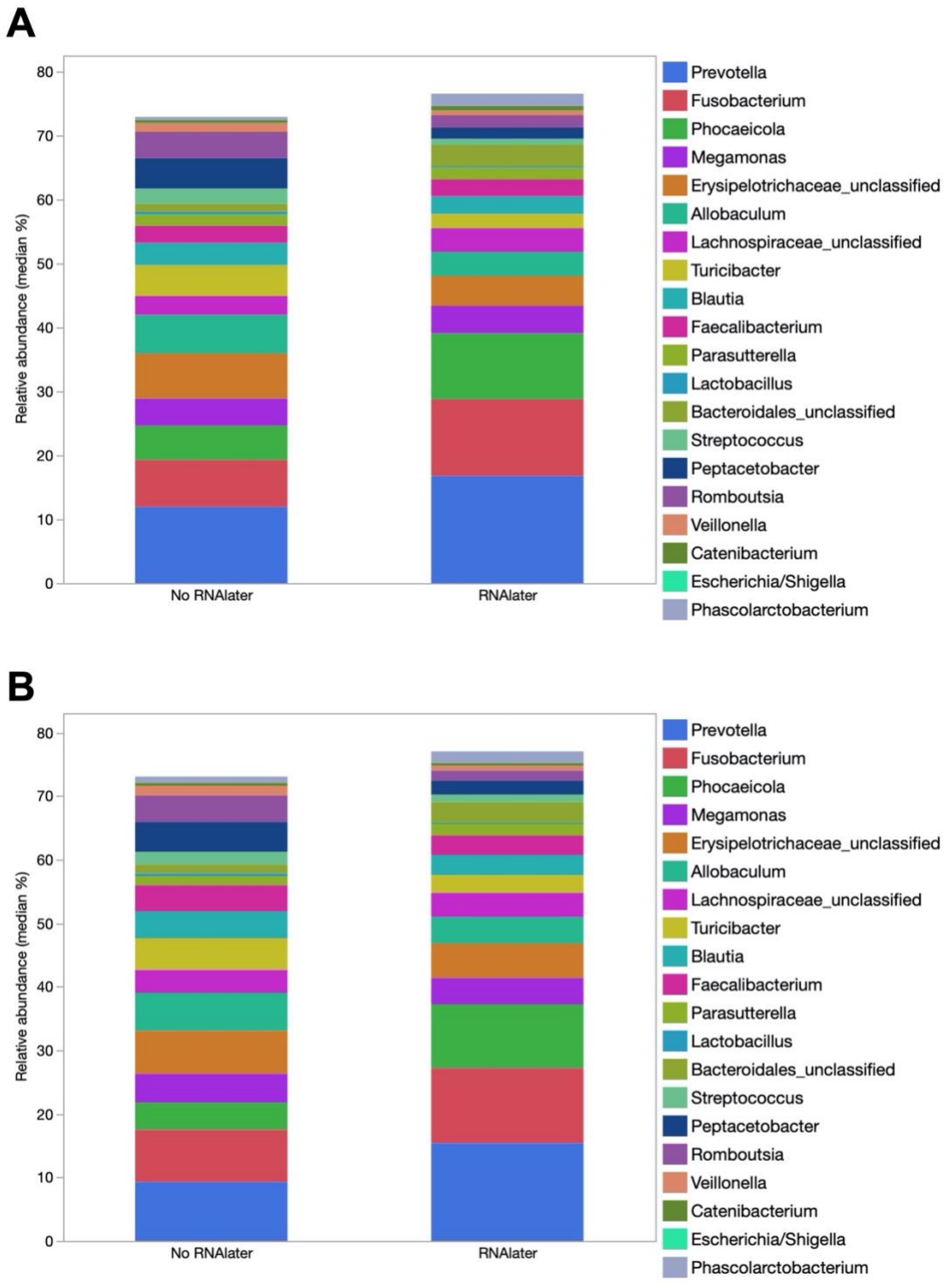

Figure 3.9: Comparison of median relative abundances of the most abundant genera in the canine fecal microbiota between samples with and without RNA*later*, stored in the fridge

for 24 h prior to storing in the freezer (A) and stored directly in the freezer (B) (n = 6 per treatment).

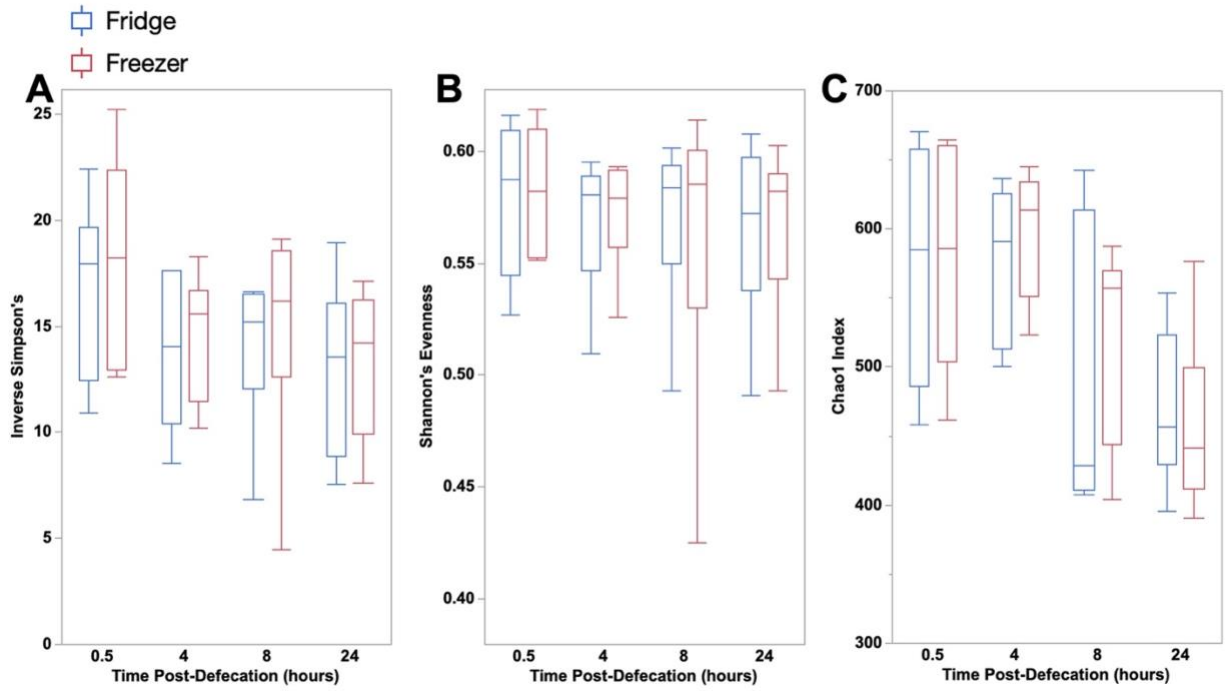

Figure 3.10: Inverse Simpson's (A), Shannon's evenness (B), and the Chao1 index (C) of the canine fecal microbiota with 0.5, 4, 8, and 24 h of room temperature exposure, stored in the fridge for 24 h prior to storing in the freezer (blue) and stored directly in the freezer (red). No differences were detected at 4, 8, or 24 h compared to 0.5 h ($P > 0.05$), and no differences were detected between samples stored in the fridge for 24 h prior to freezing and samples that were immediately frozen. Data are presented as the median with the minimum and maximum (n = 6 per treatment).

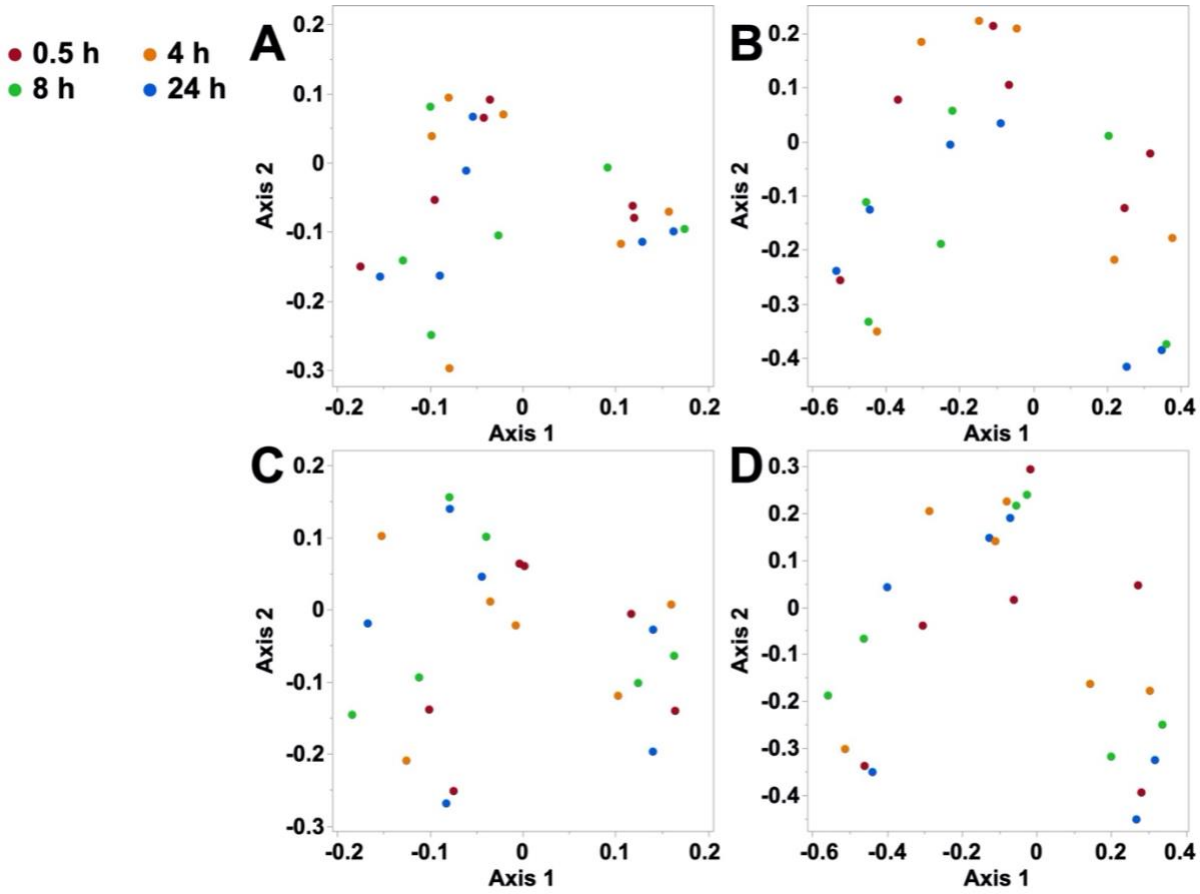

Figure 3.11: Principal Coordinate Analysis (PCoA) of the Jaccard (A and C) and Yue and Clayton (B and D) indices of the canine fecal microbiota with 0.5, 4, 8, and 24 h of room temperature exposure, stored in the fridge for 24 h prior to storing in the freezer (A and B) and stored directly in the freezer (C and D).

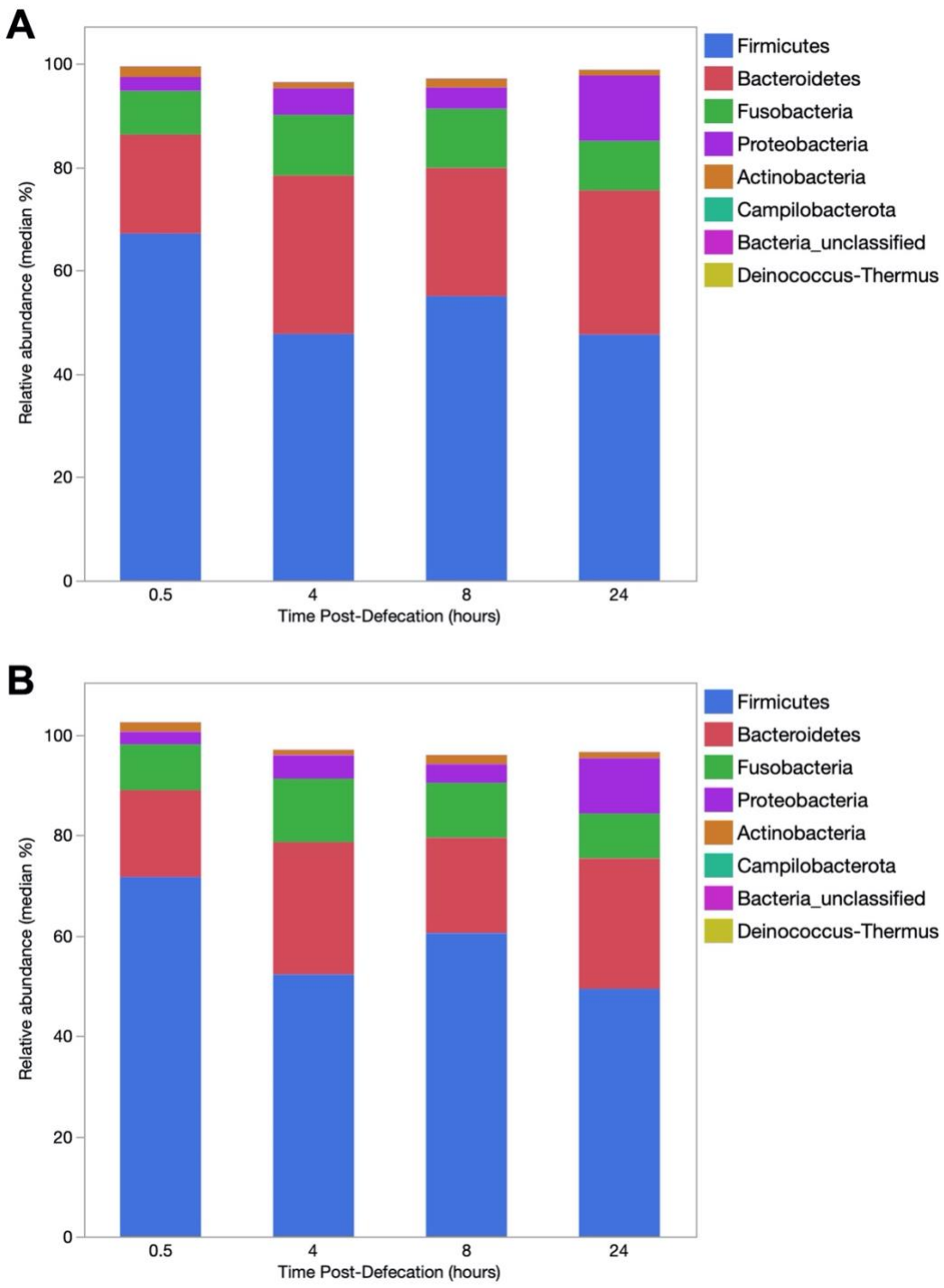

Figure 3.12: Comparison of median relative abundances of the most abundant phyla in the canine fecal microbiota with 0.5, 4, 8, and 24 h of room temperature exposure, stored in the fridge for 24 h prior to storing in the freezer (A) and stored directly in the freezer (B) (n = 6 per treatment).

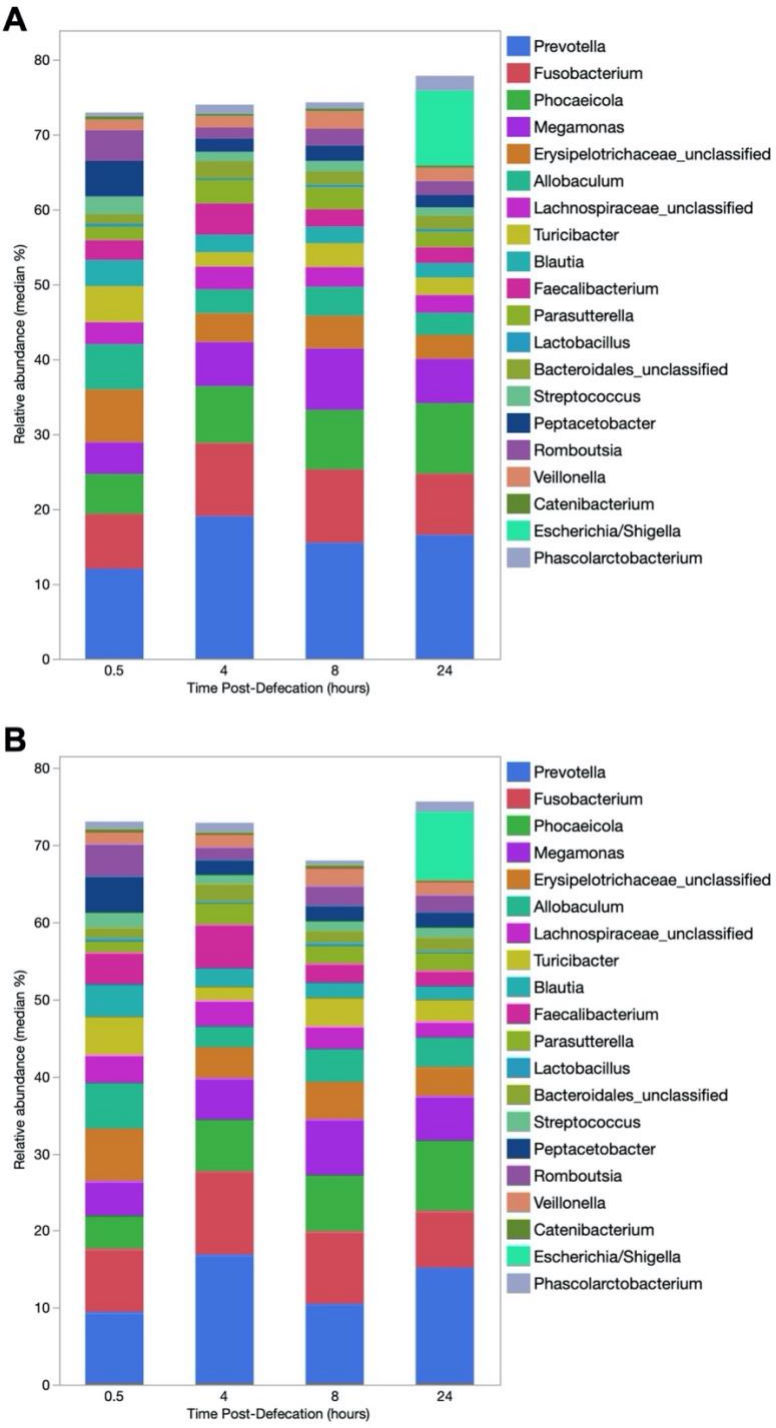

Figure 3.13: Comparison of median relative abundances of the most abundant genera in the canine fecal microbiota with 0.5, 4, 8, and 24 h of room temperature exposure, stored in the fridge for 24 h prior to storing in the freezer (A) and stored directly in the freezer (B) (n = 6 per treatment).

3.5 Discussion

This study aimed to assess the effects of homogenization, the addition of RNA*later*, room temperature exposure, and storage in the fridge at 4°C prior to storing in the freezer at -20°C on the canine fecal microbiota profile. It was hypothesized that these treatments would impact the microbiota's alpha diversity, beta diversity, and the relative abundances of the main phyla and genera. Homogenization, RNA*later*, 24 h of room temperature exposure, and storage in the fridge for 24 h at 4°C prior to storing in the freezer at -20°C did not alter any measures of alpha diversity (diversity, evenness, richness) or beta diversity (community membership and structure). Some differences in bacterial relative abundances were detected with the addition of RNA*later*, but not with homogenization, 24 h of room temperature exposure, or storage in the fridge for 4°C prior to storing in the freezer at -20°C.

Effect of Homogenization of Samples on the Fecal Microbiota Profile

Homogenization did not alter the diversity, richness, or evenness, nor the community membership and structure of the canine fecal microbiota profile. Relative abundances of bacterial phyla also did not differ between homogenized and non-homogenized samples. However, homogenization appears to reduce variability between dogs for measures of fecal alpha diversity. In agreement with this observation, Gorzelak et al. (2015) found that homogenization of human fecal samples (albeit in liquid nitrogen) prior to subsampling reduced the variability of bacterial taxa detected by qPCR. Similarly, Hsieh et al. (2016) found that homogenization can reduce the intra-individual variation in alpha diversity of human fecal samples compared to non-homogenized samples. It should be noted that many have also theorized that homogenization may introduce

more oxygen into a sample, potentially impacting the bacteria present as they carryout aerobic fermentation (Liang et al., 2020). However, as no aspects of the fecal microbiota profile differed between homogenized and non-homogenized samples in the present study, this does not seem to be a concern for canine fecal microbiota analyses.

Effect of RNAlater on the Fecal Microbiota Profile

The addition of RNA*later* did not alter the diversity, richness, or evenness, nor the community membership and structure of the canine fecal microbiota profile whether samples were stored in the fridge for 24 h prior to freezing or stored directly in the freezer. In contrast to the present study, Choo et al. (2015) observed reductions in diversity, evenness, and richness of the human fecal microbiota when RNA*later* was added to the samples. The dissimilarities between studies may be explained by the differences in the microbiota and characteristics of the feces between humans and dogs. Similarly, storage temperature could explain the differences in the results between human studies and our study: in the human study fecal samples were stored at -80°C while fecal samples in our study were stored at -20°C. Horng et al. (2018) compared the effect of several preservatives on the canine fecal microbiota and showed that, regardless of storage temperature, RNA*later* resulted in the lowest values of diversity and richness but did not alter evenness. Horng et al. (2018) also found that RNA*later* altered beta diversity compared to fresh samples more than other buffers (70% ethanol and 50:50 glycerol:PBS). The latter results may differ from the present study's because authors collected one fecal sample from one dog (compared to the present study's six), and a larger sample size introduces more inter-individual variation.

The addition of RNA*later* altered the relative abundances of multiple phyla, including the two most prominent phyla, *Firmicutes* (decreased) and *Bacteroidetes* (increased). Similar to the present study's findings, a study on humans observed that fecal samples with RNA*later* had increased relative abundances of *Bacteroidetes* and decreases in *Firmicutes* and *Actinobacteria* (Choo et al., 2015). Effects of RNA*later* on the relative abundances of various phyla were also observed by Liang et al. (2020) in the fecal microbiota of children. Another study on the canine fecal microbiota observed decreased relative abundances of *Steptococcus* and *Megamonas* and an increased relative abundance of *Prevotella* with the addition of RNA*later* (Horng et al., 2018). RNA*later* is one of many buffers used for DNA preservation in microbiota research, however, as demonstrated by the present study and others (Choo et al., 2015; Liang et al., 2020; Horng et al., 2018), it appears that in acting to preserve a sample's DNA, it causes alterations to the relative abundances of the fecal microbiota profile. Others have theorized that changes to bacterial relative abundances arise because RNA*later* reduces DNA purity and decreases DNA yield during DNA extraction, leading to a reduction in highly abundant bacteria and potentially loss of bacteria with a normally low abundance (Liang et al., 2020). These findings should be considered in future interpretations of microbiota data from fecal samples in which RNA*later* was used during storage.

Effect of Room temperature Exposure on the Fecal Microbiota Profile

The diversity, evenness, and richness, and community membership and structure of the canine fecal microbiota did not differ across 24 h of room temperature exposure in the present study. The present study also observed no alterations to relative abundances of phyla or genera across 24 h of ambient temperature exposure, whether samples were stored in the fridge at 4 °C

prior to freezing at -20°C or stored directly in the freezer at -20°C. These results are similar to a study on cats, in which there was no overall effect of time within a 96-h period on the fecal microbiota profile's diversity, richness, evenness, community membership and structure, or the relative abundances at any taxonomic level (Tal et al., 2017). A study on children also observed similar microbial composition in fecal samples over a 32-h period regardless of the preservation method used (Carruthers et al., 2019). In another study on humans, leaving fecal samples at room temperature for 14 days had minimal effect on relative abundances and community membership and structure (Lauber et al., 2010). By contrast, an equine study observed effects of 6 h of room temperature exposure on the alpha diversity and beta diversity of the fecal microbiota profile (Martin de Bustamante et al., 2021). Altogether, 24 h of room temperature exposure did not impact the canine fecal microbiota profile, like previous reports on humans and felines.

Effect of Storage in the Fridge for 24 h prior to Freezing on the Fecal Microbiota Profile

No outcome measures of alpha diversity (diversity, evenness, richness) or beta diversity (community membership and structure) differed between fecal samples stored in the fridge at 4°C for 24 h prior to storage in the freezer at -20°C and samples stored directly in the freezer at -20°C, regardless of the duration of room temperature exposure (0.5, 4, 8, and 24 h). The relative abundances of all phyla and genera also did not differ between these two storage treatments at any room temperature exposure time points. There is limited literature comparing the effect of short-term storage in the fridge versus direct freezer storage on the fecal microbiota profile. Somewhat similar to the present study, previous studies on humans (Tedjo et al., 2015) and felines (Tal et al.,

2017) observed no differences between fecal samples left at ambient temperature for 24 h and samples that were directly frozen. Choo et al. (2015) also observed no differences in alpha diversity, beta diversity, or relative abundances of phyla between human fecal samples refrigerated at 4°C for 72 hours prior to storage at -80°C and samples directly stored at -80°C. By contrast, a previous study on canines showed that with long-term (7-56 days) storage at 4°C, differences arise in beta diversity and relative abundances compared to storage immediately at -80°C (Horng et al., 2018). The present study's findings suggest storage of canine fecal samples in the fridge at 4°C for 24 h prior to freezing at -20°C for long-term storage has minimal impact on the fecal microbiota profile. These results may be important in circumstances when immediate storage in the freezer is not possible, such as field research.

Limitations

Though this study's convenience sample of six dogs was greater than previous studies, it is possible that different results would have been observed (including more noticeable differences between treatments) with a greater sample size. These are also research dogs living in the same environment and consuming the same diet, so variability was likely smaller than in the overall population. Additionally, the present study only assessed four time points (0.5, 4, 8, and 24 h post-collection). Using smaller intervals between time points (e.g., 0.5, 2, 4, 6, 8 h, etc.) or a longer period such as 96 h (Tal et al., 2017) would have provided more detailed information regarding variations in the fecal microbiota profile over time with room temperature exposure. This study also only assessed one type of preservative (RNA*later*), limiting applicability of the results to other types of preservatives such as 70% ethanol and 50:50 glycerol:PBS, which have also been found

to impact the fecal microbiota profile over time (Horng et al., 2018). Future research can explore the gaps left by these limitations to build on the present study's findings and provide further clarity to optimize methods used in the study of the canine fecal microbiota.

3.6 Conclusion

Homogenization, RNA*later*, 24 h of room temperature exposure, and storage in the fridge for 24 h prior to freezing did not alter any measures of alpha diversity (diversity, evenness, richness) or beta diversity (community membership and structure). RNA*later* altered relative abundances of phyla, but not genera, and homogenization, room temperature exposure, and storage in the fridge for 24 h prior to freezing did not alter relative abundances of phyla or genera. Further investigation is required to determine if the ability for RNA*later* to alter microbial relative abundances is related to deleterious effects on DNA purity (Liang et al., 2020). Our findings will aid in protocol development for future studies; however, more research is needed to further standardize sample collection and storage protocols in microbiota research.

3.7 Endnotes

[a] EZNA Stool DNA Kit, Omega Bio-Tek, Doraville, GA

[b] Agencourt, AMPure XP, Beckman Coulter, ON

[c] Illumina, San Diego, CA

[d] Mothur software package (v.1.44.3), Michigan State University, East Lansing, MI

[e] JMP 12, SAS Institue Inc., Cary, NC

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4 General Discussion

Analysis of the microbiota and metabolome are often done on fecal samples to gain an understanding of the bacteria residing in the gastrointestinal tract, and the metabolites they produce. These types of analyses have allowed researchers to link gut bacteria to host metabolism, immune function, and disease pathology (Barko et al., 2018; Guard et al., 2015). A prominent shortcoming of fecal microbiota and metabolome research is a lack of standardization of methods for sample collection, processing, and storage, as this makes between-study comparisons difficult. The time that samples are exposed to room temperature can vary, especially when fecal samples are collected in the field (Martin de Bustamante et al., 2021), and this may affect the compositions of the fecal metabolome and microbiota by allowing microbial fermentation. Some studies also homogenize samples (Gorzelak et al., 2015; Hsieh et al., 2016; Tal et al., 2020), while others leave samples as-is (Martin de Bustamante et al., 2021), and while homogenization makes a sample more uniform for subsequent analyses, it introduces more oxygen into the sample, which can be conducive to further microbial aerobic fermentation (Gangadoo et al., 2021). Furthermore, the use of preservatives such as RNA*later* varies among studies (Choo et al., 2015; Horng et al., 2018; Liang et al., 2020), and its effect on the microbiota is unclear. To contribute to the standardization of these methods, this thesis investigated: the influence of time of exposure to room temperature post-collection on the feline fecal metabolome and canine fecal microbiota; and the influence of homogenization and RNA*later* on the canine fecal microbiota.

In Chapter 2, I investigated how the feline fecal metabolome is affected by exposure to room temperature at time points 2, 4, 6, 8, 12, and 24 h post-collection prior to being stored at -80°C. The concentrations of most fecal metabolites did not change at the time points investigated,

however, there were notable increases in some amino acids and amines at 6 (cadaverine) and 8 h (putrescine, phenylacetate, and trimethylamine) post-collection, and some volatile fatty acids 8 h post-collection (propionate, butyrate, isobutyric acid, and isovaleric acid). As these are products of proteolytic and carbohydrate microbial fermentation, respectively, their concentrations being as close as possible to their concentrations at the time of defecation would be important for studies investigating, for example, the influence of different diets on the fecal metabolomic profile (Badri et al., 2021). Our findings were similar to previous investigations of the influence of room temperature exposure on the fecal metabolome in adult humans (Gratton et al., 2016) and 34-month-old children (Liang et al., 2020). From the present study's findings, it is recommended that fecal samples are exposed to room temperature for no longer than 4 h for investigation of the feline fecal metabolome. These findings are especially relevant for sample collection in a field or home setting. From the findings of Gratton et al. (2016), it appears that storage at 4°C can slow down the effects of room temperature exposure on the fecal metabolome (delaying the earliest changes to 24 h post-collection), but future research is required to ensure this applies to feline fecal samples as well.

Chapter 3 explored the influence of homogenization, the addition of RNA*later*, and time for room temperature exposure (at 4, 8, and 24 h post-collection) on the fecal microbiota profile of canines, specifically regarding alpha diversity, beta diversity, and the relative abundances of bacterial phyla and genera. I found that room temperature exposure, homogenization, and storage in the fridge for 24 h prior to freezing as compared to freeing immediately did not affect the alpha diversity (diversity, evenness, and richness), beta diversity (bacterial community membership and

structure), or bacterial relative abundances. The addition of RNA*later* also did not affect alpha diversity or beta diversity, however, did alter the relative abundances of some phyla.

While homogenization did not appear to impact the microbiota profile, the interindividual variability in alpha diversity measures were lower in homogenized compared to non-homogenized samples (Figure 3.2), which is consistent with previous studies (Gorzelak et al., 2015; Hsieh et al., 2016). By contrast, Adding RNA*later* altered relative abundances of phyla, including *Firmicutes* (decreased) and *Bacteroidetes* (increased). The influences on *Firmicutes* and *Bacteroidetes* are important to note, as the *Firmicutes:Bacteroidetes* ratio is a common outcome measure in studies investigating the influence of diet on the microbiota profile (Magne et al., 2020; Mariat et al., 2009; You & Kim, 2021). These changes may be caused by deleterious effects on DNA purity associated with adding RNA*later* (Liang et al., 2020) and requires further investigation. Lastly, exposure of samples to room temperature within a 24 h timeframe did not alter any outcome measures, nor did storage in the fridge at 4°C for 24 h prior to storing in the freezer at -20°C as compared to freezing at -20°C immediately. These results are similar to previous studies on felines (Tal et al., 2017) and human fecal samples (Carruthers et al., 2019; Choo et al., 2015; Lauber et al., 2010; Tedjo et al., 2015), and add to the literature by showing 24 h of room temperature exposure and a subsequent 24 h of storage in the fridge prior to freezing for long-term storage are acceptable for canine fecal microbiota analyses.

Altogether, exposure to room temperature appears to affect the feline fecal metabolome as early as 6 h post-collection. Hence, room temperature exposure for less than 6 h is acceptable for fecal sample collection studies. While the use of RNA*later* is desirable for its ability to preserve samples, its ability to alter the relative abundances of bacteria (including the widely studied

Firmicutes and Bacteroidetes) in canine fecal samples should be considered in studies using it as a preservative.

As fecal metabolites are produced by the bacteria present in the feces, factors affecting fecal microbiota relative abundances (i.e., RNA*later*) may impact the fecal metabolome as well. In 34-month-old children, Liang et al. (2020) concluded that RNA*later* impacted the fecal metabolome to a similar extent as the fecal microbiota. Future research can build on the present thesis's findings by corroborating the results of Liang et al. (2020) in feline and canine fecal samples. Furthermore, as the present studies investigated a limited number of time points post-collection (2, 4, 6, 8, 12, 24 h for metabolome and 4, 8, and 24 h for microbiota), future studies may provide more precise investigations of the time course of changes in the fecal metabolome and microbiota for standardization of storage methods. Lastly, as the ability for homogenization to introduce oxygen into a fecal sample is not fully understood, but appears to at least have no impact on the fecal microbiota, future research should explore whether homogenization can impact fecal metabolite concentrations.

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