AMINO ACIDS AND METABOLITES IN DOGS WITH GASTROINTESTINAL DISEASE

A Dissertation

by

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Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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May 2022

Major Subject: Biomedical Sciences

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ABSTRACT

Chronic and acute gastrointestinal diseases, characterized by vomiting, diarrhea, and/or weight loss, are common reasons why dogs are presented to veterinarians. Over the last decade, the microbiota has been well characterized in these disease states. However, less is known about the functional set of metabolites that interacts with the microbiota and host. Previous studies have identified alterations in amino acid metabolism in dogs and humans with gastrointestinal diseases. Our study aims were to, 1) validate amino acid measurement in dog serum and compare amino acid concentrations in whole blood, plasma, and serum, 2) measure serum and fecal amino acid concentrations in dogs with chronic enteropathy and compare them to healthy control dogs, and 3) measure tryptophan metabolites and relate them to microbiota alterations in dogs with chronic and acute gastrointestinal diseases at initial presentation and over time.

The assay for measurement of amino acids in dog serum was accurate and reproducible, and the majority of amino acids were found to be stable under frequently used storage conditions. Whole blood, plasma, and serum samples had distinct amino acid profiles, suggesting reference intervals for these sample types cannot be used interchangeably. Serum concentrations of valine and fecal concentrations of tryptophan were significantly higher in dogs with chronic enteropathy compared to healthy controls. Correlations between serum and fecal amino acid concentrations, and clinical activity and histopathological scores did not reach the level of significance in this study

population. The fecal microbiota composition was significantly different in dogs with acute diarrhea, acute hemorrhagic diarrhea, and inflammatory bowel disease when compared to healthy dogs. These differences often persisted at follow up timepoints within each group of diseased dogs. Additionally, dogs with acute hemorrhagic diarrhea and inflammatory bowel disease had higher concentrations of fecal indole-3-acetamide, whereas dogs with acute diarrhea had higher concentrations of fecal indole.

These results demonstrate that dogs with gastrointestinal disease have altered amino acid profiles and tryptophan metabolism in addition to altered microbial communities. Additional studies are needed to elucidate mechanisms behind these alterations, and to determine if altered amino acid absorption or digestion contribute to altered amino acid profiles.

DEDICATION

This dissertation is dedicated to all of the people in my life who helped foster my love of animals and research from a young age: my high school Ag and health science teachers, my aunt Jennifer Steakley, my grandfather Tom Gardiner, my grandmother Bonnie Gardiner, and my parents Greg and Cindy Blake.

Also to my cat, Little Bit. You weren't there to help me get through the writing this time, but you still inspire me to be a better researcher every day.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Suchodolski, for his mentorship and support throughout my time at the GI lab. His endless enthusiasm for research and motivation pep talks have helped foster and grow my love for research. I would also like to thank my committee co-chair, Dr. Steiner, and my committee members, Dr. Lidbury, Dr. Dangott, and Dr. ten Have, for their guidance and encouragement.

The GI lab has provided me with so many amazing opportunities that would not have been possible without the talented people keeping it running, past and present: Nancy Cangelose, Lori Kessler, Sarah Read, Rhonda Rosa, Robynne Gomez, and So Young Park. Thank you all for your unending support and guidance. And a special thank you to my fellow graduate students who have made this experience truly the best in my life. I have made lifelong friends with many of you as we celebrated our successes and supported each other through our failures. I could not have done it without you all (and our outside lunch breaks to see the sun and COVID walks). Thank you to Kyle Phillips and Dr. Patricia Ishii for helping to organize and execute the many blood drives we have done over the years. I think the shoulder cramps from pipetting for 8 hours were worth it now that the end is in sight.

Thank you to the many collaborators I have worked with, including the Integrated Metabolomics Analysis Core and Protein Chemistry Lab (Dr. Larry Dangott, Dr. Cory Klemashevich, Smriti Shankar, and Ivantha Nawaratna) and the Center for Translational Research in Aging and Longevity (Dr. Gabriella ten Have, Dr. Nicolaas

Deutz, Dr. John Thaden, and Dr. Sunday Simbo). Additionally, I would like to thank Jeff Line with Harvard Biosciences for being the best field service engineer to walk the Earth. Not many FSEs would spend their 30-minute layover between flights deciphering cell phone images to help troubleshoot an instrument.

Thank you also to the owners and veterinarians who collected and sent us samples to use for our research. I apologize to any spouses who found feces next to the milk in the fridge. Thank you, Folgers, for your "aroma-seal" technology—truly life changing! I also want to thank the numerous student workers who have helped me over the years. The freezer logs also thank you.

Finally, thank you to my family and Logan McKinley, for your love, patience, and support throughout my PhD program.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Professors Jan S. Suchodolski [advisor], Jörg M. Steiner [co-advisor], and Jonathan A. Lidbury of the Department of Small Animal Clinical Sciences, Larry Dangott of the Department of Biochemistry and Biophysics, and Gabriella M. ten Have of the Department of Health and Kinesiology, Texas A&M University.

The samples utilized in Chapter 1 were collected in part by Kyle Phillips and Dr. Patricia Ishii (Texas A&M University), with help from other graduate students from the GI Lab. Samples utilized in Chapter 2 and the patients' clinical history were collected by Dr. Patricia Ishii and Dr. Melanie Werner (Ludwig-Maximilians-University). Fecal samples were lyophilized in part by Dr. Chi-Hsuan Sung (Texas A&M University), and fecal amino acid analysis conducted in part by Ivantha Nawaratna (Protein Chemistry Lab, Texas A&M University). The samples utilized in Chapter 3 were collected by Dr. Hannah Klein (Texas A&M University), Dr. Anna-Lena Ziese (Ludwig-Maximilians-University), Dr. Melanie Werner, and Dr. Linda Toresson (Helsinki University) and organized by Dr. Blake Guard (Texas A&M University). The data analyzed was provided by Dr. Rachel Pilla (Texas A&M University) and Dr. Cory Klemashevich (Integrated Metabolomics Analysis Core, Texas A&M University). Analysis was conducted in part by Dr. Rachel Pilla and Johnathan Shih (Texas A&M University). Samples utilized in Chapter 4 were collected in part by Kyle Phillips and Dr. Patricia

Ishii, with help from other graduate students from the GI Lab. Isotope preparation was conducted in part by Dr. Gabriella ten Have (Texas A&M University), and data collection and analysis were conducted in part by Dr. John Thaden and Dr. Sunday Simbo (Texas A&M University).

All other work conducted for this dissertation was completed by the student independently.

Funding Sources

Graduate study was supported by a travel fellowship from the University of Alabama at Birmingham Metabolomics Workshop.

NOMENCLATURE

AA	amino acid
AD	acute diarrhea
ADMA	asymmetric dimethylarginine
AHDS	acute hemorrhagic diarrhea syndrome
AhR	aryl hydrocarbon recptor
ANOSIM	analysis of similarity
ARE	antibiotic-responsive enteropathy
ASV	amplicon sequence variant
BCAA	branched-chain amino acid
BCFA	branched-chain fatty acid
CBC	complete blood count
CCECAI	canine CE clinical activity index
CE	chronic enteropathy
CRP	C-reactive protein
CV%	coefficient of variation
DI	dysbiosis index
EAA	essential amino acid
FDR	false discovery rate
FMOC	9-fluorenylmethyl chloroformate
FRE	food-responsive enteropathy

GC	gas chromatography
GI	gastrointestinal
HC	healthy control
HPLC	high-performance liquid chromatography
HS	hemolysis score
IBD	inflammatory bowel disease
IEC	ion exchange chromatography
LC	liquid chromatography
LLOQ	lower limit of quantitation
MS	mass spectrometry
NEAA	nonessential amino acid
OE%	observed to expected ratio
OPA	o-phthalaldehyde
PCoA	principal coordinate analysis
PLE	protein-losing enteropathy
PLI	pancreatic lipase immunoreactivity
QIIME	quantitative insights into microbial ecology
qPCR	quantitative polymerase-chain reaction
SCFA	short chain fatty acid
SDMA	symmetric dimethylarginine
SRE	steroid-responsive enteropathy
TLC	thin-layer chromatography

TLI	trypsin-like immunoreactivity
UV-Vis	ultraviolet-visible
WMS	whole metagenome sequencing
WSAVA	World Small Animal Veterinary Association
%R	recovery percentage

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1. INTRODUCTION

1.1. Overview of canine gastrointestinal diseases

The prevalence of gastrointestinal (GI) disease in pet dogs has been reported to be up to 17.8% making it an important and common disease in small animal veterinary medicine [1]. Numerous studies have examined the fecal microbiota of these patients, and recent studies evaluated the fecal metabolome in dogs with acute and chronic GI disease [2-4]. However, little is known about how chronic GI diseases develop and progress in dogs, especially prior to the onset of clinical signs. Non-invasive biomarkers have the potential to show changes that can lead to chronicity before the manifestation of clinical signs. This is why it is important to assess these biomarkers in acute as well as chronic disease states.

Acute uncomplicated diarrhea (AD) in dogs is characterized by an increase in frequency, fluidity, or volume of feces lasting less than 14 days without the need for hospitalization [5]. Acute hemorrhagic diarrhea syndrome (AHDS) in dogs is characterized by acute onset of bloody diarrhea, and patients often require supportive care at a veterinary hospital. Chronic enteropathy (CE) is characterized by persistent diarrhea and/or vomiting for at least two weeks and is diagnosed by excluding other causes of disease (extra-intestinal disease, enteropathogens, parasites, foreign bodies, or tumors) [6, 7]. Chronic enteropathy can be classified as food-responsive (FRE), antibiotic-responsive (ARE), steroid-responsive (SRE), or non-responsive based on clinical response to treatment. Dogs that fail to respond to diet and antibiotics and have

inflammation present in the GI tract upon histological examination are typically classified as having inflammatory bowel disease (IBD). Dogs with CE can also have protein losing enteropathy (PLE), in which there is increased loss of protein through the GI tract ultimately leading to hypoproteinemia and possible ascites and peripheral edema.

Common therapies for canine enteropathies include dietary change (i.e., limited ingredient diet, hydrolyzed protein diet), antibiotics, or steroids. However, there is growing interest in alternative and/or adjunctive therapies, such as probiotics, fecal microbiota transplant, or bile acids sequestrants [8-11]. These novel therapeutics may target changes in the fecal metabolome, or the entire set of small bioactive molecules in the feces of dogs with chronic or acute GI diseases [2, 3]. Thus, evaluating the metabolic changes that occur in canine enteropathies in more detail may provide new approaches to diagnosis and/or therapy [12].

1.2. Microbiota in health and disease

The intestinal microbiota refers to the collection of microorganisms residing within the GI tract, including bacteria, fungi, and viruses. These organisms have dynamic interactions with each other and with the host cells serving important functions, such as defense against pathogenic bacteria and development of the immune system and epithelial cells. Some bacteria even provide nutrients for the host cells, such as short-chain fatty acids (SCFA), through fermentation and metabolism of dietary nutrients. Methods of characterizing the microbiota have evolved over the years from traditional

culture methods to next generation high-throughput molecular methods. Characterization in feces is a method commonly employed, due to the non-invasive nature of fecal collection. Sequencing of 16S rRNA genes has allowed the identification of the major bacterial taxa present in feces of dogs. Firmicutes is commonly identified as the most abundant phylum in healthy dogs with the class Clostridia contributing a large portion to that abundance [4, 13]. Other bacterial taxa that are present in lower abundance might require quantitative polymerase-chain reaction (qPCR) for quantitative comparison between groups. qPCR allows the amplification and quantification of targeted bacterial DNA sequences, providing identification down to strain level. In contrast, 16S rRNA gene sequencing does not often provide accurate identification to species or strain level. Whole metagenome sequencing (WMS) evaluates additional genes within a sample instead of only the 16S rRNA gene and therefore can give more precise information about species level taxa. However, cost is a significant obstacle to using WMS routinely in veterinary medicine today.

Alterations in the fecal microbiota have commonly been identified in dogs with gastrointestinal disease. Dogs with AD have lower abundance of *Faecalibacterium*, *Blautia*, and Bacteroidetes and increased abundance of *Clostridium* when compared to healthy dogs [2, 4, 10]. Dogs with AHDS have decreased abundance of *Faecalibacterium*, *Turicibacter*, and Ruminococcaceae, and increased abundance of *Clostridium perfringens* compared to healthy dogs [4, 14]. Dogs with more chronic disease like IBD or CE have decreased abundance of *Faecalibacterium*, *Turicibacter*, Ruminococcaceae, *Blautia*, *Fusobacterium*, and *Clostridium hiranonis*, and increased

abundance of *Escherichia coli* and *Streptococcus* compared to healthy dogs [4, 8, 14, 15]. These alterations have been recently used to develop a qPCR-based algorithm called the Dysbiosis Index (DI) that uses abundance of 8 key bacterial groups to quantify dysbiosis [15]. The Dysbiosis Index is a useful tool that allows one to monitor how the microbiota responds to treatment in a simple, easy-to-understand way, with a short turnaround time from sample submission to release of results.

In addition to disease state, diet, body condition, and medications, such as antibiotics, have been shown to affect the intestinal microbiota. Pilla et al., recently provided a summary of the effect of diet on the microbiota in dogs [16]. Macronutrient levels seem to affect the microbiota more so than source of the macronutrients (plant vs animal). One study in dogs comparing a low-fiber diet to a diet containing 7.5% beet pulp showed that dogs fed the low fiber diet had lower abundances of Firmicutes and higher abundances of Bacteroidetes and *Fusobacteria* [17]. Another study comparing animal-protein-based diet with plant-based-protein diet in healthy dogs showed no difference in the fecal microbiota with the change in diet [18]. However, this study also showed that the plant-based protein diet fed to dogs with food-responsive enteropathy caused a shift in the microbiota towards being more similar to healthy dogs. Obesity has also been associated with alterations of the microbiota in dogs, with one study of research beagles showing, after 6 months of *ad libitum* feeding, Proteobacteria became the predominant bacterial phylum (76% compared to lean group of dogs with <10%) [19]. Antibiotics perhaps have the most profound effects on the GI microbiota.

Numerous studies of different antibiotics have been shown to alter the microbiota in dogs, with some having long-term impacts [10, 20-23].

Examining the intestinal microbiota composition in dogs with GI disease can help grow our understanding of the pathophysiology of disease. Additionally, quantitating the core species, through use of the DI, could help to monitor microbiota changes in response to treatment or disease progression. However, the microbiota exhibits redundancy in function and can switch metabolic pathways in different circumstances [24], therefore making characterization of the metabolome a potentially more accurate representation of how the microbiota is functioning.

1.3. Metabolomics in health and disease

Metabolomics is the study of the complete set of metabolites (small molecules resulting from cellular metabolism) in a biological sample. These metabolites can give information about physiology, metabolic status, and function of a variety of systems in the body. The application of metabolomics to identify patterns of metabolites in disease states is a classic technique used in human medicine to help identify potential biomarkers and possible therapeutic targets of disease [25]. However, it is only recently that metabolomics has begun to emerge as a powerful tool in veterinary medicine, and yet it has already significantly impacted the field of veterinary diagnostics research.

There is a steadily growing body of research employing untargeted and targeted metabolomics approaches to identify biomarkers of various diseases in animals. Untargeted approaches examine the fullest set possible of metabolites present in a

biological sample, sometimes yielding thousands of identified metabolites. The caveat is that untargeted approaches are, by design, not quantitative and only provide relative differences. Therefore, results should be confirmed with targeted analysis. Targeted analysis is the targeted measurement of specific metabolites, and can be quantitative by the addition of internal standards and calibration to a known amount of pure metabolite. Untargeted approaches tend to be hypothesis generating, and have the ability to relatively quickly guide research towards promising biomarkers of disease.

Some of these biomarkers have been first identified in human medicine and have translated well into veterinary medicine within the last 20 years. One example of such a biomarker is symmetric dimethylarginine (SDMA). SDMA is filtered and excreted by the kidneys and therefore increases in kidney disease when there is decreased glomerular filtration rate. Fleck et al., in 2001, showed that SDMA, measured with targeted high-performance liquid chromatography (HPLC), was increased in serum from humans with chronic renal failure [26]. SDMA was first associated with kidney disease in cats in 2008 by Jepson et. al. who were identifying changes in metabolites associated with bioavailability of nitrate/nitrite [27]. Years later it was found that SDMA is actually a better/earlier indicator of decreased kidney function in cats than the previous standard measure of creatinine [28]. Now, SDMA is a common addition to routine serum biochemistry panels that is offered by commercial reference laboratories and can be measured on in-house biochemistry analyzers.

Other metabolites are of interest particularly because they relate directly to the intestinal microbiome. For example, indoxyl sulfate is a colonic-derived uremic toxin

and is increased in serum of cats with kidney disease [29]. Theoretically, it could be used as a surrogate marker of microbiota health or changes of microbiota function in response to therapy in the future. Other metabolites produced exclusively by the microbiota will likely become important markers of GI health in the future, possibly more important than the microbiota composition.

Metabolomics has been helpful in guiding the discovery of new biomarkers of GI diseases in dogs. Untargeted fecal metabolomics data [12] led to the targeted analysis of lactate, bile acids, sterols, fatty acids, and amino acids in dogs with GI disease [3, 30, 31]. Bile acid changes were confirmed with targeted analysis—fecal secondary bile acids were shown to be decreased in dogs with CE, and it was suggested that this could be due to reduced bacterial conversion from primary to secondary bile acid [3]. Concurrently, in human medicine, the body of research on bile acid diarrhea (secretory diarrhea caused by excess bile acid in the colon) is growing, and the hypothesis was generated that this could potentially be contributing to unresolved diarrhea in dogs as well. Cholestyramine, a bile acid sequestrant, has been used in humans to treat bile acid diarrhea [32], and this drug was recently shown to be effective in two dogs with presumptive bile acid diarrhea [11]. More studies are needed to determine if cholestyramine is an effective treatment for unresolved chronic diarrhea in dogs, but it is promising as an intervention that stemmed from metabolomics studies in dogs and studies in human medicine.

In their discovery phase, metabolite markers are often times not practical to measure in a clinical setting. Initially, they are often measured with HPLC or mass

spectrometers, which are not readily available in the veterinary clinical setting. However, if a metabolite proves exceptionally useful clinically, steps can be taken to develop methods of quantitation compatible with existing biochemistry analyzers (as was done with SDMA) or other rapid analysis methods. Blood lactate, for instance, is sometimes measured in emergency veterinary medicine with a handheld lactate meter that is based on enzymatic amperometric detection [33, 34]. This method of detection has the potential for expansion to measure other metabolites as well, especially those with very different concentrations between health and disease.

1.4. Overview of amino acids

Given the important functions amino acids have in maintaining enterocyte health and interactions with the immune system, they become a promising therapeutic target in gastrointestinal disease states. However, the characterization of amino acid profiles in dogs with GI disease is in its infancy. Abundance of fecal AA were altered in dogs with CE in a preliminary untargeted metabolomics dataset [12]. Additionally, other studies have found altered AA concentrations in serum and plasma of dogs with PLE and IBD [35, 36]. Further characterization of AA profiles in dogs is needed, with additional consideration for interactions with the microbiota.

1.4.1. Quantification of amino acids

Amino acid analysis is used to quantify amino acids in samples originating from a wide variety of sources, such as clinical biological fluids or tissues, purified proteins, or food and feed samples, just to name a few. Separation of the individual amino acids

from one another is required in order to identify them, and some means of detection is required to quantify them. This can be accomplished using many different chromatography separation techniques and different kinds of detection systems.

Chromatography is an important technique that allows for the separation of an amino acid mixture into individual, or groups of, amino acids, and can aid in their identification. Chromatography is comprised of a stationary phase and a mobile phase. The stationary phase is either a solid, or a liquid coating on the surface of a solid, and the mobile phase is either a gas or a liquid. Various strategies and techniques of chromatography have been employed over the years to separate amino acids (AA).

Two relatively older methods of AA separation are paper chromatography and thin-layer chromatography (TLC). The paper chromatography setup is formed by a layer of cellulose or thick filter paper (support) saturated with water, which acts as the stationary liquid phase. The mobile phase is also a liquid that flows through the stationary phase carrying the different amino acids at different rates, which is why it is known as a “liquid-liquid” form of chromatography. Paper chromatography can also be performed in two dimensions, accomplished by rotating the paper 90 degrees and allowing the mobile phase to move the amino acids a second time. TLC is similar to paper chromatography, but instead of a liquid stationary phase, it is a solid. Silica gel or cellulose are typical stationary phases used in amino acid separation with TLC.

Other methods of amino acid separation include ion exchange chromatography (IEC), high-performance liquid chromatography (HPLC) and gas chromatography (GC). IEC methods typically require 30-100 µl sample volume, are optimized with over 65

years of experience, and have the ability to regenerate the columns instead of replacing them. An advantage of IEC over HPLC is that it can measure amino acids in the linear range of 200 pmol-300 nmol, while HPLC has a much smaller range of 0.5-200 pmol (with o-phthalaldehyde derivatization) or 50-2,000 pmol (with phenylisothiocyanate derivatization) [37]. Disadvantages of IEC compared to HPLC include the longer run time per sample and the use of commercial buffers that increase cost of analysis. IEC are generally incorporated into instruments dedicated for amino acid analysis, which can be considered an advantage over HPLC, because problems can arise when columns are frequently changed on non-dedicated HPLC instruments, leading to increased down time. GC methods are rising in popularity, in part because they are frequently paired with mass spectrometry detectors, which have superior sensitivity. Some advantages of GC separation include better resolution than HPLC and shorter analysis times. Disadvantages of GC separation include the need for derivatization of amino acids into volatile derivatives, which can sometimes be problematic (e.g., trimethyl-silylation reagents are susceptible to reacting with water and then having decreased reactivity with amino acids), and constitute an additional expense.

Typically, methods of separation are paired with methods of detection such as ultraviolet-visible (UV-Vis) spectrophotometry, fluorescent spectrophotometry, or mass spectrometry (MS) for the quantitation of amino acids. UV-Vis detection involves measuring the absorption of light at particular wavelengths absorbed by the compounds of interest, usually colored compounds that result from the derivatization of amino acids (e.g., Rhueman’s purple from reaction with ninhydrin). UV-Vis detection has the

advantage of being easily available and accessible (cheap), but the disadvantages of relatively low sensitivity. Fluorescent detection involves the measurement of fluorescence after derivatization, commonly with o-phthalaldehyde (OPA). The advantages of fluorescent detection with OPA include that it is also relatively cheap and provides good sensitivity, but the disadvantages are that the OPA does not react with proline, hydroxyproline, cystine, or homocysteine, rendering them undetectable with this method. MS involves the fragmentation of compounds into ions and their subsequent detection. Advantages of MS include its superior sensitivity and its ability to elucidate unknown compound structures, and its main disadvantage is much higher cost compared to other detection methods.

The sensitivity of each method of chromatography is dependent on derivatization and type of detector, but in general, method sensitivity: paper chromatography < TLC < IEC < HPLC < GC. The working range of paper chromatography for amino acid analysis was around 1-10 µM in 1955, where Hackman and Lazarus used a method to make the chromatogram semi-transparent and then scan the density of the spots [38]. Sensitivity of TLC is better, with detection limits using ninhydrin reagent reported down to 0.001 µg, but in practice, it is difficult to identify spots generated by reaction with ninhydrin, so newer reagents are becoming more popular even though they have much higher detection limits (0.08-5 µg) [39]. IEC with ninhydrin derivatization has been reported to have sensitivity down to 100-500 pmol of amino acid applied to the column, whereas HPLC with fluorescent derivatives can be up to 1,000 times more sensitive [37]. Newer GC methods paired with tandem MS can have detection limits in the femtomoles.

One study comparing amino acid analysis in urine with, 1) IEC and post column ninhydrin derivatization (Biochrom 30 amino acid analyzer), 2) GC-MS, and 3) LC-MS-MS, found that all methods had comparable limits of quantification (LOQ, 2-3 µM, 0.3-30 µM and 0.5-50 µM, respectively) [40]. This study also showed that, while the IEC method had a higher LOQ for some of the amino acids, it also had a much better reproducibility compared to the other two methods (mean ± SD, 7.27 ± 5.22, 21.18 ± 10.94, 18.34 ±14.67, respectively for amino acid analyzer, GC-MS, and LC-MS-MS).

Electrophoresis methods can also be used to separate amino acids from one another based on size and electrical charge. Capillary electrophoresis methods vary widely but can be sensitive to 0.03 mg/L in dried blood spot analysis [41] and 0.33 µM in microchip electrophoresis paired with MS [42]. Capillary electrophoresis paired with time-of-flight MS has been used to measure amino acids in human urine, obtaining limits of detection as low as 20 nM [43].

Both IEC and HPLC methods are utilized in the human clinical setting, oftentimes in hospitals or reference laboratories. However, the use of mass spectrometry paired techniques in these settings is slowly becoming more feasible in cost and will likely become the standard in the future.

1.4.2. Digestion and absorption of amino acids

Dietary proteins are digested through enzymatic hydrolysis into small peptides and free amino acids, which can then be absorbed through the enterocytes of the gastrointestinal tract. Proteases, enzymes which participate in the catabolism of dietary proteins and peptides, are produced in the stomach, pancreas, and small intestine.

Pepsins A, B, and C are endopeptidases that originate in the chief cells of the stomach and are secreted as inactive forms that are then activated by HCl. An endopeptidase cleaves the peptide bond adjacent to the recognized amino acid residue in the interior of the peptide chain. Pepsins recognize aromatic and hydrophobic (most efficient) AA residues. Rennin (also known as chymosin) is also produced in the stomach of some mammals but has weak proteolytic activity and is mainly used by young animals to aid in the clotting of milk proteins to slow passage through the intestinal tract to allow more time for proteases to act on them [44]. Trypsin, chymotrypsin, and elastase are endopeptidases produced by the pancreas and secreted into the small intestine in their inactive forms. Enteropeptidase is released by the mucosal cells in the small intestine and activates trypsinogen to trypsin. Subsequently, trypsin hydrolyses and activates the other pancreatic enzymes (i.e., chymotrypsinogen to chymotrypsin). Elastase breaks down elastin, a protein in connective tissue. All of these pancreatic enzymes have optimal activity in an alkaline environment, or the small intestine after the release of bile and other substances. The stomach enzymes have optimal activity in an acidic environment. Trypsin recognizes arginine and lysine residues, chymotrypsin recognizes aromatic residues and methionine, and elastase recognizes aliphatic amino acid residues. Carboxypeptidase A and B are also produced by the pancreas and are exopeptidases, meaning they cleave the last peptide bond adjacent to the recognized amino acid residue. Carboxypeptidases cleave at the carboxyl terminus and aminopeptidase (produced by mucosa of small intestine) cleaves at the amine terminus. Carboxypeptidase A recognizes aromatic amino acid residues, carboxypeptidase B recognizes arginine and

lysine residues, and aminopeptidase recognizes all amino acids with free amine groups. This coordination of proteases and peptidases working together produces a very characteristic pattern of liberation of free AA in the intestine.

Free AA in the lumen of the small intestine are taken up by enterocytes via several mechanisms: 1) simple diffusion (passive and nonsaturable), 2) Na^+-independent systems (facilitated diffusion; responsible ~40% free AA uptake), 3) Na^+-dependent systems (active transport; responsible ~60% free AA uptake), and 4) γ-glutamyl cycle [45]. The γ-glutamyl cycle and passive transport (simple diffusion) are not common modes of transport of AA by enterocytes. The majority of AA absorption occurs in the small intestine with absorption being most rapid in the duodenum and jejunum and slower in the ileum. Di- and tripeptides in the lumen can be directly transported into the enterocytes through the apical membrane by Na^+-independent (H^+ gradient-driven) peptide transporter 1. There is ample evidence that the rate of absorption into enterocytes is higher for di- and tripeptides than free AA [46]. Once inside the enterocytes, the small peptides are hydrolyzed rapidly by intracellular peptidases to form free AA, which are utilized in multiple pathways. Some evidence exists that basolateral peptide transporters exist but have not been identified.

AA transporters are grouped into solute carrier families, 11 of which have been identified. There are too many AA transporters to list and describe here and they have been described elsewhere [47]. However, it is important to know that they have affinity for different AA, more than one transporter can transport any particular AA, and some transporters may serve as transceptors, which are capable of sensing and signaling AA

availability to regulatory machinery in cells (i.e., the mechanistic target of rapamycin pathway).

1.4.3. Classification of amino acids

An amino acid is a compound that contains both a carboxyl and amino group. There are over 700 amino acids found in nature but only 20 of them are considered proteinogenic [45]. Selenocysteine and pyrrolysine are sometimes referred to as additional proteinogenic amino acids, but they are encoded in a special manner. Non-proteinogenic amino acids are amino acids whose formation is not encoded by the genes of an organism. However, non-proteinogenic can be a misleading term for them, because non-proteinogenic amino acids can sometimes be incorporated into polypeptides or proteins. This is because some of them can be formed during posttranslational modification events on incorporated amino acid residues. Examples of non-proteinogenic amino acids that are formed in post-translational modifications include: hydroxyproline, which is made from the hydroxylation of proline residue by the enzyme peptidyl proline hydroxylase; citrulline, which is made in a central reaction in the urea cycle catalyzed by carbamoyl phosphate synthetase where a carbamoyl phosphate group is donated to ornithine and releases a phosphate group; asymmetric dimethylarginine (ADMA), which is produced from the methylation of arginine residues by S-adenosylmethionine protein N-methyltransferases; 3-methylhistidine, which is formed by the methylation of histidine residues in post-translational modifications of actin and myosin; hydroxyserine and hydroxythreonine from hydroxylation of serine and threonine residues, respectively; hydroxylysine produced by post-translational

modification of lysine during collagen synthesis; and γ-carboxyglutamic acid, a calcium-binding amino acid found in the blood-clotting protein prothrombin. One of the most important post-translational modifications of amino acids is the phosphorylation of serine, threonine, and tyrosine residues to regulate the activity of proteins in the cell [48].

Some non-proteinogenic amino acids are not incorporated into proteins. Examples of these include γ-aminobutyric acid, L-3,4-dihydroxyphenylalanine, ornithine, triiodothyronine, and β-aminoisobutyric acid. β-aminoisobutyric acid is produced by the catabolism of thymine and valine. Other non-proteinogenic amino acids (such as D-amino acids) can be incorporated into non-ribosomal peptides which are synthesized by non-ribosomal peptide synthetases and often have cyclic and/or branched structures.

Still others, such as norleucine, can be misincorporated in place of a genetically encoded amino acid due to infidelity of the protein synthesis process. An example of this is canavanine, which is lethal to rats in high concentrations [49]. Sometimes, misincorporation of a non-proteinogenic amino acid into a protein can cause misfolding of that protein which could potentially lead to a neurodegenerative cascade of misfolding proteins. One example is the misincorporation of β-N-methylamino-L-alanine in the place of serine, which can result in neurodegenerative diseases like amytrophic lateral sclerosis [50, 51]. Another example is the misincorporation of hypoglycin in place of leucine, which is thought to cause birth defects [52]. Homocystine is another example of a non-proteinogenic amino acid. Homocystine is two molecules of homocysteine

biosynthesized from methionine and joined together by a disulfide bond. It differs from the amino acid cysteine in that it has an additional methylene bridge.

Another way to classify amino acids is by nutritionally essential (EAA) or nonessential (NEAA). Traditionally, amino acids were classified based on an animal's dietary need of the amino acid for maintenance and growth [45]. NEAA can be *de novo* synthesized in sufficient quantity and don't need to be provided by the diet, while EAA cannot be synthesized and need to be provided in the diet. There are nine EAAs in dogs: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Interestingly, taurine is also considered an EAA in cats. Additionally, there are some amino acids that can normally be synthesized *de novo* but need to be provided in the diet under certain conditions, such as illness or weaning. These are called conditionally essential AA and, for most mammals, include: arginine, glutamine, glutamic acid, glycine, and proline [45]. Recently however, the term "NEAA" has become a misnomer, because it is recognized that all proteinogenic AA have some dietary requirement, including NEAA [53].

1.4.4. Amino acids and the microbiota

Amino acids can play several roles in the interactions between microbiota and host, sometimes indirectly. Possibly most straightforward, is the use of AA as a substrate by bacteria for metabolism, the products of which can interact with the host. Interestingly, some studies indicate that 10-30% of the dietary intakes of some AA are metabolized by the bacteria in the small intestine [54, 55]. In the human small intestine, some of the most abundant bacterial taxa that ferment amino acids belong to *Clostridium*

clusters, *Bacteroides*, and *Prevotella* [56, 57]. Microbiota can metabolize undigested proteins and AA that reach the large intestine into potentially harmful substances like p-cresol and indoxyl sulfate [29]. They can also metabolize them into beneficial substances like indolepropionate that can increase expression of tight junction proteins and decrease permeability [58]. Furthermore, the microbiota can utilize AAs to synthesize SCFAs and branched-chain fatty acids (BCFAs), which are important modulators of host physiology [59, 60]. For example, SCFA-mediated activation of GPR43 receptor regulates fat accumulation in adipocytes and energy expenditure in other tissues, including liver and muscle, in order to maintain energy homeostasis [61]. Another microbial generated AA metabolite is 5-hydroxy-L-tryptophan, which has been shown to activate the aryl hydrocarbon receptor on host cells regulating the gut-microbiome immune axis [60, 62]. Altered abundance or composition of AA in the gut can affect those bacteria that metabolize AA and the resultant set of metabolites, therefore indirectly affecting gut-microbiome-immune system interactions [62].

Additionally, there is some evidence that the microbiota can alter the physical distribution of amino acids in the lumen of the GI tract, suggesting that the microbiota may be able to affect the bioavailability of AAs [57, 63, 64]. Some bacterial species thrive in the mucosa, meaning their metabolic products will be in closer proximity to transporters located on the apical membrane of the epithelial cells. The microbiota also use AAs to influence the gut environment or other bacterial species. For example, some bacterial species thrive in more acidic environments, and given the chance with abundant amino acids, will utilize them to make acidic products, potentially acidifying the

environment to outcompete other bacteria. Additionally, bacteria can use amino acid residues to make their own proteins, such as bacteriocins, which are antimicrobial peptides that could influence the microbial community composition.

1.4.5. Amino acids and gastrointestinal disease

Studies in humans with gastrointestinal disease have shown alterations in plasma [65, 66] and fecal [67] amino acids, with amino acid concentrations typically decreasing in plasma and increasing in feces. Additionally, studies in dogs and cats with GI disease have shown alterations in serum or plasma amino acid profiles. Tryptophan was decreased in serum of dogs with PLE [36], in plasma of dogs with IBD [35], and in plasma of cats with GI diseases [68]. It is likely that low plasma concentrations of essential amino acids in GI disease could contribute to the inflammatory cycle or clinical signs. Signs of tryptophan deficiency, which include reduced appetite, can occur at intake 25% below daily requirement [69], which could exacerbate weight loss and malnutrition in GI disease. Histidine was also shown to be decreased in plasma of cats with GI disease, was inversely correlated with clinical severity, and suppressed LPS-induced IL-8 expression by feline macrophages [68].

Restoring normal levels of amino acids could support gastrointestinal tract recovery through production of the antioxidant glutathione, polyamines, and tight junction proteins as well as inhibition of pro-inflammatory cytokine production. However, due to the potential for excess amino acids in the GI tract to lead to excessive bacterial utilization and possible production of toxic metabolites, supplementation studies have proceeded with an abundance of caution. While amino acid

supplementation in humans with gastrointestinal disease is still controversial [70], numerous animal models of IBD have shown promising effects of amino acid supplementation [71]. In 25 studies using rodent models of IBD, supplementation with an amino acid had some level of benefit, including increased glutathione and Treg cell expression, and decreased histopathological scores, diarrhea, TNF-α, and NF- kβ [71]. Additionally, in 3 studies using piglet models of IBD, supplementation with sulfur containing amino acids increased tight junction expression and decreased pro-inflammatory cytokines, and supplementation with tryptophan decreased gut permeability and pro-inflammatory cytokines [71]. Whether or not there is any benefit of amino acid supplementation in naturally occurring GI disease remains to be determined.

1.5. Hypothesis and specific objectives

While a future goal may be to supplement amino acids in dogs with GI disease, we first need to extensively characterize amino acid profiles in dogs with and without GI disease as well as to determine physiological explanations for their altered concentrations in disease. The ultimate goal of these studies is to provide additional non-invasive metabolic (and potentially predictive) markers of chronicity/severity of disease, and to elucidate functional deficiencies in amino acid metabolism. Thus, the objective of this study is to assess amino acid concentrations in various biofluids of healthy dogs and dogs with gastrointestinal diseases.

Study Hypotheses:

1. Serum will have different concentrations of amino acids than plasma and whole

blood.

2. Dogs with CE will have decreased serum concentrations of some amino acids when compared to healthy control dogs.
3. Dogs with CE will have increased fecal concentrations of some amino acids when compared to healthy control dogs.
4. Dogs with various gastrointestinal diseases will have altered fecal tryptophan metabolite concentrations when compared to healthy control dogs and will have altered fecal microbiota.

Study Objectives:

1. To analytically validate the assay for measurement of amino acids in dog serum, and to measure amino acids in whole blood, plasma, and serum of healthy dogs.
2. To measure serum amino acids in dogs with chronic enteropathy and healthy control dogs.
3. To measure fecal amino acids in dogs with chronic enteropathy and healthy control dogs.
4. To measure fecal tryptophan metabolites and to characterize the fecal microbiota in healthy dogs and dogs with various gastrointestinal diseases.

2. ANALYTICAL VALIDATION OF AN ASSAY FOR CONCURRENT MEASUREMENT OF AMINO ACIDS IN DOG SERUM AND COMPARISON OF AMINO ACID CONCENTRATIONS BETWEEN WHOLE BLOOD, PLASMA, AND SERUM FROM DOGS

OVERVIEW

Amino acids play an important role in metabolism. To date there has been no comprehensive analytical validation of an assay for the concurrent measurement of a large number of amino acids in dog serum in the literature, which precludes its usefulness in a clinical setting. Amino acids are often measured in plasma or whole blood. However, serum is commonly used for gastrointestinal diagnostic testing in dogs and is therefore convenient to use. This study aimed to analytically validate an assay for the concurrent measurement of amino acids in dog serum and to evaluate differences in amino acid concentrations in whole blood, plasma, and serum in dogs.

Analytical validation of an HPLC assay (Biochrom 30+ Amino Acid Analyzer) for the concurrent measurement of a wide variety of amino acids was performed on fresh or banked serum samples from dogs. Whole blood, plasma, and serum from 36 healthy dogs were analyzed and concentrations between the 3 sample types were compared using Friedman tests followed by Dunn's multiple comparisons tests.

The assay was demonstrated to be precise, reproducible, accurate, linear, and stable for the measurement of the majority of compounds detected in dog serum. Cystine, glutamic acid, and ethanolamine were shown to be unstable at conditions

commonly encountered in clinical settings. Significant differences in concentrations were identified between whole blood, plasma, and serum for 33 of 42 compounds. Concentrations of 23 compounds were higher in serum than in plasma, and 14 compounds had highest concentrations in whole blood. Amino acid profiles in serum and plasma were more similar to each other than to those in whole blood.

In conclusion, while some amino acids are present in similar concentrations in whole blood, plasma, and serum, others are highly dependent on the type of biofluid and measurements warrants strict adherence to sample type-based reference intervals.

2.1. Introduction

Amino acids (AA) are involved in the biosynthesis of functional molecules, the maintenance of tight junction proteins, the nourishment of enterocytes, and the regulation of metabolic pathways, oxidative stress, and inflammation of the gut [71]. Amino acids also have a direct relationship with the microbiota — bacteria in the gut metabolize dietary amino acids and proteins into new metabolites that can then be absorbed by the host. For example, aromatic amino acid metabolites produced by the microbiota, such as indolepropionic acid, positively affect intestinal permeability and systemic immunity [58]. Another example is tryptamine, a bacterial metabolite of dietary tryptophan that has been shown to accelerate gastrointestinal (GI) transit by activating colonic secretion pathways in the host via epithelial G-protein-coupled receptors [72]. Therefore, amino acids have the potential to serve as non-invasive markers of GI health.

One significant hurdle to expanding our knowledge of amino acids in GI diseases in veterinary medicine is that amino acids are not commonly measured in veterinary medicine, and to the authors' knowledge, only one commercial laboratory in the United States offers this service commercially. Plasma amino acids are routinely measured in humans, for example, in newborn infants to detect inborn errors of metabolism. Ion exchange chromatography (IEC) or liquid chromatography coupled to visible light or fluorescence-based detection have been used for more than 50 years for the detection and quantification of amino acids, and are still used today. New methods of detection and quantification involve mass spectrometry or nuclear magnetic resonance-based methods. However, the significantly increased cost to purchase, maintain, and operate these newer types of instruments can be a limiting factor to scalability in veterinary medicine. An extensive validation of IEC-based methods to measure amino acids in plasma or serum from dogs has not yet been published. Even though the majority of human studies utilize plasma as their preferred sample type (AA have been reported to be more stable in plasma than serum [73]), many clinical diagnostic tests for dog and cat gastrointestinal disease currently utilize serum [74, 75]. Therefore, it may be more practical to measure AA in dog serum if possible.

The aims of this study were to analytically validate an assay for the concurrent measurement of a wide range of amino acids in dog serum and to measure and compare amino acid concentrations in whole blood, plasma, and serum from healthy dogs.

2.2. Materials and methods

2.2.1. Sample collection

This study was approved by the TAMU Institutional Animal Care and Use Committee (animal use protocol 2018-0112 CA and 2020-0184 CA). Excess dog serum from samples submitted to the Gastrointestinal Laboratory at Texas A&M University for routine diagnostic testing was used for analytical validation after all personal identifying features had been removed from the samples. For testing of short-term stability, serum was collected prospectively from 4 apparently healthy dogs recruited in October 2021. Additionally, healthy privately-owned pet dogs that had absence of clinical signs based on an owner questionnaire were recruited prospectively in October of 2018. Following recruitment, physical examination was performed, and blood, feces, and urine were collected to rule out clinically relevant abnormalities on complete blood count (CBC), serum biochemistry profile, urinalysis, canine fecal dysbiosis index [15], and gastrointestinal panel (serum cobalamin, folate, trypsin-like immunoreactivity, and pancreatic lipase immunoreactivity). Lithium heparin blood and whole clotted blood (without separator gel, allowed to clot at room temperature for 30 minutes) were centrifuged at 1,800 x g at 4°C for 15 minutes to obtain plasma and serum, respectively. Multiple aliquots of each sample type were made for validation purposes. Within one hour of collection, aliquots of lithium heparin blood, serum, and plasma were stored at 4°C and kept for 24 hours to simulate overnight shipping conditions. Then samples were deproteinized and transferred to −80°C storage until further analysis.

2.2.2. Amino acid analysis

Amino acids and other nitrogenous compounds were measured with a Biochrom 30+ lithium high performance amino acid analyzer (Biochrom Ltd., Cambridge, UK) following manufacturer's guidelines. Biological samples were deproteinized following laboratory protocols (dx.doi.org/10.17504/protocols.io.877hzrn) [PROTOCOL DOI]. Briefly, samples were deproteinized with a 1:1 (v/v) addition of 5% sulfosalicylic acid + 500 µM L-norleucine as an internal standard and subsequently centrifuged at 10,000 x g for 5 minutes at 4°C. After centrifugation the precipitate was discarded. Whole blood required an additional transfer and centrifugation due to the loose nature of the precipitate. Up to 500 µl of supernatant was transferred to a 0.2 µm PVDF centrifugal filter tube and centrifuged at 10,000 x g for 5 minutes at 4°C. The supernatant was then placed on a chilled autosampler and injected into the instrument. Samples were not kept on the autosampler (4°C) for longer than 48 hours prior to being injected on the column. If needed, samples were stored at −80°C prior to being placed on the autosampler. The instrument parameters were set to an injection volume of 30 µl with partial loop fill injection.

2.2.3. Validation

Two milliliters of excess serum were obtained from 8 different dogs and deproteinized according to the previously mentioned protocol. The resulting extract was distributed into 15 aliquots and stored at −80°C. Eight aliquots from the same dog were run consecutively on the same day to test intra-assay variability (precision). The last injection from each dog was used together with the remaining aliquots to test inter-assay

variability (reproducibility) — aliquots from each individual dog were run at least one day apart (and up to two weeks apart) 7 additional times.

Spiking recovery (accuracy/matrix effect) was tested by spiking excess serum from 5 dogs with pure standard (Amino acid standards, physiological, analytical standard, acidics/neutrals, basics, and L-glutamine, 2,500 μM, Millipore Sigma, Burlington, MA) to give a final added concentration of 100 μM, 200 μM, and 300 μM for the low, medium, and high spike, respectively (Table 2.1). Because this would increase the sample volume by more than 5%, the following equation was used to calculate spiking recovery percentage (%R):

$$\%R = \frac{(spiked\ sample\ result - (unspiked\ sample\ result * DF)) * 100\%}{known\ spike\ added\ concentration}$$

$$Where\ DF = \frac{sample\ volume}{total\ volume\ of\ sample\ plus\ spike}$$

Samples were also diluted with equivalent amounts of loading buffer to measure dilutional parallelism (linearity; Table 2.1). Dilutional parallelism was additionally tested with pure standards in standard diluent (lithium loading buffer, Biochrom Inc., UK) due to the wide range of concentrations for the various amino acids in individual biological samples. Standard dilutions were made using amounts listed in Table A-1.

Table 2.1. The components used to make the spiked matrix samples and diluted matrix samples.

	Matrix Spike Level		
	Low	Medium	High
components	220 µl serum	190 µl serum	160 µl serum
	10 µl acidics/neutrals	20 µl acidics/neutrals	30 µl acidics/neutrals
	10 µl basics	20 µl basics	30 µl basics
	10 µl glutamine	20 µl glutamine	30 µl glutamine
	Dilution Factor		
	0.88	0.76	0.64
components	220 µl serum	190 µl serum	160 µl serum
	30 µl lithium loading buffer	60 µl lithium loading buffer	90 µl lithium loading buffer

Acidics/neutrals and basics physiological standards contain all amino acids noted in the assay at a concentration of 2,500 µM with the exception of cystine at 1,250 µM, in 0.1 N HCl. Glutamine was added separately after making solution of 2,500 µM glutamine in lithium loading buffer (Biochrom Inc., UK).

To test stability of amino acids, excess serum from 8 dogs (collected the previous day and shipped on ice to the GI Lab at Texas A&M University) were distributed into 5 aliquots each and stored at −80°C. The first aliquot was deproteinized immediately prior to storage, and the rest of the aliquots were thawed and deproteinized once a week for 4 weeks and returned to −80°C storage. All samples were run as a batch at the end of the 4-week time period. After 61 weeks, the baseline aliquot was used to repeat amino acid analysis to test effects of long-term storage of deproteinized samples at −80°C.

The second part of stability testing examined short-term storage conditions that clinical samples might be exposed to when being collected in a veterinary clinical setting. Fresh serum was collected from 4 dogs to test short-term stability. Blood samples were allowed to clot at room temperature for 30 minutes, then centrifuged at

1,800 x g at 4°C for 15 minutes. Serum was transferred to a clean tube. Aliquots were made and tested under the listed conditions in Table 2.2.

Table 2.2. Short-term stability study design.

serum volume (µl)	ID	Day 0 - collect	Day 1	Day 2	Day 3	Day 7	Day 14	Day 21	Day 28
600	A	dp, run A_e							
	B	store A_e 4°C 72h			run A_e				
	C	store A_e -20°C 1w				run A_e			
200	D	Aliquot, store 4°C 24h	dp, run						
200	E	Aliquot, store 4°C 48h		dp, run					
200	F	Aliquot, store 4°C 72h			dp, run				
200	G	Aliquot, store -20°C 24h	dp, run						
200	H	Aliquot, store -20°C 48h		dp, run					
200	I	Aliquot, store -20°C 1w				dp, run			
200	J	Aliquot, store -20°C 2w					dp, run		
200	K	Aliquot, store -20°C 3w						dp, run	
200	L	Aliquot, store -20°C 4w							dp, run

dp = deproteinize, run = analyze the sample on the amino acid analyzer, subscript e = sample deproteinized on day 0.

Using a previously described objective photometric method [76], hemolysis scores (HS) were assigned to serum and plasma samples from healthy dogs to identify correlations between hemolysis and concentrations of amino acids.

To calculate the lower limit of quantitation (LLOQ), 5 replicates of low concentration standards (2.5 µM, 5 µM, 10 µM, and 15 µM) were analyzed for consistency (coefficient of variation; CV%) and signal to noise ratios (in 4 of the 5 replicates).

2.2.4. Statistical analysis

A Shapiro-Wilk test for normality of the data was performed, and given non-normal distribution, Friedman test with Dunn's *post hoc* multiple comparisons tests were performed to determine differences between amino acid concentrations over time in stability studies, as well as differences of AA concentrations between whole blood, plasma, and serum. Spearman's correlations were performed to analyze correlations between individual amino acid concentrations in serum and plasma, serum biochemistry panel, CBC, clinical data (body condition score, fecal score, age), GI panel, and hemolysis score (HS). These correlations were corrected for false discovery rate using Benjamini-Hochberg correction. All other validation testing was evaluated by calculating coefficients of variation (CV%) and observed to expected ratios (OE%). Data was analyzed using two statistical software programs (JMP Pro v14, SAS Institute Inc., Cary, North Carolina; Graph Pad Prism v 9.0.1, GraphPad Software, San Diego, California).

2.3. Results

2.3.1. Analytical validation for dog serum

Signal to noise ratios above 3 were determined acceptable. Standard concentrations for which this was not achievable in the majority of 4 replicates were deemed below the lower limit of quantitation (LLOQ). Signal to noise ratios are available in Table A-2. The determined LLOQ for the vast majority of compounds was 5 μM, with the exception of urea (30 μM), sarcosine (30 μM), β-aminoisobutyric acid (10 μM), γ-aminobutyric acid (10 μM), hydroxyproline (20 μM), and proline (10 μM). The cutoff LLOQ values have been multiplied by 2 to account for the dilution factor of 2 in serum samples.

2.3.1.1. Intra-assay variability (precision)

A total of 31 compounds were detected with the majority of the eight samples' median concentrations above the detection limit. The range of median CV%'s was 0.2-8.8 with only a few compounds with a CV% above 10% for some of the samples tested: phosphoserine, α-aminoadipic acid, cystathionine, 1-methylhistidine, and 3-methylhistidine (Table A-3).

2.3.1.2. Inter-assay variability (reproducibility)

A total of 32 compounds were detected with the majority of the samples above the detection limit. The range of median CV%'s was 0.7-21.5 with only a few compounds with a CV% above 10% for some of the samples tested: phosphoserine, α-aminoadipic acid, cystathionine, hydroxylysine, 1-methylhistidine, 3-methylhistidine, and carnosine (Table A-4).

2.3.1.3. Spiking recovery (accuracy/matrix effect)

Results of spiking recovery are listed in Table A-5. All recoveries for the following compounds fell within the acceptable range of 80-120%: phosphoethanolamine, aspartic acid, threonine, serine, asparagine, glutamic acid, glycine, citrulline, α-aminobutyric acid, valine, cystine, methionine, cystathionine, isoleucine, leucine, tyrosine, β-alanine, phenylalanine, β-aminoisobutyric acid, γ-aminobutyric acid, ethanolamine, ammonia, hydroxylysine, ornithine, lysine, 1-methylhistidine, histidine, tryptophan, 3-methylhistidine, anserine, carnosine, arginine, hydroxyproline, and proline. A few compounds had one or more recoveries fall outside of the acceptable range of 80-120%: phosphoserine (low spike only), taurine, urea, glutamine (low spike only), sarcosine (low spike only), α-aminoadipic acid (low spike only), alanine (low spike only), and homocysteine (low spike only).

2.3.1.4. Dilutional parallelism (linearity)

The results of sample dilution are listed in Table A-6. A total of 24 compounds were detected in 3 or more of the 5 dogs tested: taurine, urea, threonine, serine, asparagine, glutamic acid, glutamine, glycine, alanine, citrulline, α-aminobutyric acid, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, ammonia, lysine, histidine, tryptophan, carnosine, arginine, and proline. The OE% for the majority of detected compounds fell within the accepted range (80-120%). Recovery was between 80-120% for all 5 dogs tested at all dilutions (down to 0.64 dilution factor) for 12 compounds: urea, threonine, serine, asparagine, glutamine, glycine, alanine, valine, lysine, histidine, arginine, and proline. When expected concentrations were below 26

µM for one or more sample, several compounds fell out of acceptable recovery range: glutamic acid, citrulline, α-aminobutyric acid, tyrosine, phenylalanine, ammonia, tryptophan, and carnosine. Methionine, isoleucine, and leucine had poor recovery at the strongest dilution (0.64 dilution factor) in one or two dogs tested. Taurine had poor recovery at all dilutions tested for one dog, and at 0.64 dilution factor for three dogs.

The results for linearity of standards are listed in Table A-7. Several compounds were linear throughout the full range of standards (2.5-750 µM), including taurine, phosphoethanolamine, aspartic acid, threonine, serine, asparagine, glutamic acid, citrulline, valine, phenylalanine, β-aminoisobutyric acid, and arginine. Several more were linear down to 5 µM (α-aminobutyric acid, methionine, cystathionine, β-alanine, γ-aminobutyric acid, ornithine, and histidine) or 10 µM (phosphoserine, glutamine, alanine, tyrosine, homocystine, hydroxylysine, lysine, 1-methylhistidine, 3-methylhistidine, carnosine, hydroxyproline, and proline). Glycine, cystine, isoleucine, leucine, tryptophan, and anserine were linear down to 15 µM, α-aminoadipic acid and ethanolamine were linear down to 20 µM, ammonia was linear to 50 µM, urea was linear to 125 µM, and sarcosine was linear to 250 µM.

2.3.1.5. Stability

The stability of serum amino acid concentrations for serum stored at −80°C for up to 4 weeks and the stability of deproteinized serum samples stored at −80°C for 61 weeks is presented in Table A-8 and Figure A-9. The CV% for most compounds was acceptable (<10%) with the exception of a few compounds that were present in the samples at very low concentrations. The median CV% for 9 compounds was borderline

acceptable (10-20%): aspartic acid, asparagine, glutamic acid, α-aminobutyric acid, isoleucine, tyrosine, ammonia, histidine, tryptophan. However, only 3 of these (ammonia, histidine, and tryptophan) also showed differences using Friedman's and Dunn's *post hoc* testing after storage for 61 weeks when compared to baseline. It is important to note that these results also include variability due to deproteinization since the samples were deproteinized separately one week apart.

The results of the second short-term stability study are presented in Table A-10, Table A-11, Figure A-12, and Figure A-13. Storage of dog serum at 4°C for up to 72 hours yielded acceptable CV% (<10%), with the exception of those for the measurement of glutamic acid, cystine, ammonia, and hydroxyproline, with ammonia being the only one that also showed a difference with Dunn's test at both 72-hour time points. Storage of dog serum at −20°C for up to 4 weeks showed the same amino acids as above with unacceptable CV%, but also isoleucine, ethanolamine, and carnosine. Ammonia was increased at 4 weeks of storage at −20°C when compared to baseline.

2.3.1.6. Effect of hemolysis

In serum, hemolysis (HS) positively correlated with phosphoserine, aspartic acid, and phosphoethanolamine, and negatively correlated with α-aminobutyric acid and phenylalanine. In plasma, HS positively correlated with aspartic acid and glutamic acid, and negatively correlated with α-aminobutyric acid and phenylalanine.

2.3.1.7. Other correlations

In both serum and plasma, branched chain amino acids (BCAA; isoleucine, leucine and valine) positively correlated with white blood cell count. Additionally, in

both serum and plasma, 3-methylhistidine showed a strong positive correlation with creatinine, and carnosine positively correlated with blood urea nitrogen. Glutamine positively correlated with age in both serum and plasma. Results of correlation analysis are presented in Table A-14 for serum and in Table A-15 for plasma.

2.3.2. Comparison of results in whole blood, plasma, and serum

A total of 51 dogs were screened for inclusion. After biological sample testing, 15 dogs were excluded due to one or more clinically relevant abnormalities, leaving 36 dogs to be analyzed as a healthy group.

At least one difference between whole blood, plasma, and serum concentrations was identified for 33 compounds (Table 2.3). No difference was identified for 6 compounds, and 3 compounds were not detected in any samples. Concentrations of taurine, phosphoethanolamine, aspartic acid, serine, glutamic acid, α-aminoadipic acid, citrulline, cystathionine, tyrosine, ammonia, ornithine, lysine, histidine, and arginine were significantly higher in whole blood than in plasma or serum. Concentrations of asparagine, glutamine, proline, glycine, valine, methionine, leucine, and phenylalanine were significantly higher in serum than in plasma or whole blood. Concentrations for 23 compounds were higher in serum than in plasma. Amino acid profiles of whole blood clustered separately from those of plasma and serum (Figure 2.1).

Table 2.3. Comparison of amino acid concentrations (µM) in whole blood, plasma, and serum of healthy dogs.

	whole blood		plasma		serum		
	interval	median	interval	median	interval	median	p-value
Phser	7-13	9[a]	3-10	3[b]	4-14	9[a]	<0.001
Taur	135-359	208[a]	50-145	85[b]	89-326	175[c]	<0.001
Pea	5-14	9[a]	0-4	2[b]	0-10	4[c]	<0.001
Urea	2656-7943	4867[a]	2961-8632	5109[b]	2937-8585	5149[b]	<0.001
Asp	143-458	295[a]	4-10	6[b]	5-12	9[c]	<0.001
Hypro	2-106	11	2-99	10	0-106	12	0.590
Thr	115-340	178[a]	94-346	173[b]	101-354	182[a]	<0.001
Ser	84-176	120[a]	66-149	105[b]	69-170	112[c]	<0.001
Asn	16-55	35[a]	31-88	61[b]	32-93	64[c]	<0.001
Glu	40-80	58[a]	16-42	25[b]	24-45	34[c]	<0.001
Gln	479-996	658[a]	490-1055	704[a]	494-1079	704[b]	<0.001
AAAA	465-1070	685[a]	1-11	4[b]	1-12	5[b]	<0.001
Pro	81-256	134[a]	74-294	129[a]	80-312	135[b]	<0.001
Gly	139-362	204[a]	118-371	216[a]	128-390	222[b]	<0.001
Ala	241-570	364[a]	192-544	371[b]	207-553	367[a]	<0.001
Citr	27-102	55[a]	20-86	48[b]	20-88	50[b]	<0.001
Aaba	9-48	23[a]	10-53	26[b]	11-53	27[b]	<0.001
Val	114-220	159[a]	101-215	157[a]	106-223	164[b]	<0.001
Cys	1-10	5[a]	2-17	7[b]	3-14	7[b]	<0.001
Met	31-68	46[a]	34-73	46[a]	37-76	49[b]	<0.001
Cysth	4-24	9[a]	2-14	6[b]	2-14	6[b]	<0.001
Ile	39-88	55[a]	35-88	54[b]	38-90	58[a]	<0.001
Leu	69-181	113[a]	68-189	117[a]	75-196	123[b]	<0.001
Tyr	51-90	63[a]	24-73	43[b]	26-76	44[c]	<0.001
B-ala	0-5	0	0-5	0	0-5	0	N/A
Phe	39-76	52[a]	38-81	54[a]	40-86	56[b]	<0.001
Homocys	0-2	0	0-4	0	0-4	0	<0.001
Ethan	0-23	4	0-49	0	0-34	3	0.089
Amm	62-117	83[a]	37-81	51[b]	55-104	67[c]	<0.001
Hylys	10-17	13	9-17	14	10-17	14	0.423
Orn	13-35	21[a]	6-23	12[b]	7-24	12[b]	<0.001

Table 2.3. Continued.

	whole blood		plasma		serum		
	interval	median	interval	median	interval	median	p-value
Lys	157-541	272[a]	57-288	147[b]	64-305	154[c]	<0.001
1-Mhis	4-38	7[a]	4-31	8[b]	5-33	8[b]	<0.001
His	59-96	79[a]	50-84	70[b]	52-88	74[c]	<0.001
Trp	18-52	29[a]	31-110	55[b]	30-114	58[c]	<0.001
3-Mhis	4-19	8[a]	3-22	9[b]	4-22	10[b]	<0.001
Ans	0-10	3	2-19	3	0-16	3	0.144
Car	11-25	16[a]	11-45	25[b]	11-42	26[b]	<0.001
Arg	121-242	182[a]	62-157	109[b]	96-197	147[c]	<0.001

Values listed are in µM. Interval represents the middle 95% of the data. Values not sharing a common letter are significantly different (P<0.05) according to Friedman testing with Dunn's multiple comparisons. Sarcosine, β-aminoisobutyric acid, and γ-aminobutyric acid were not detected in any samples and are therefore not listed in the table.

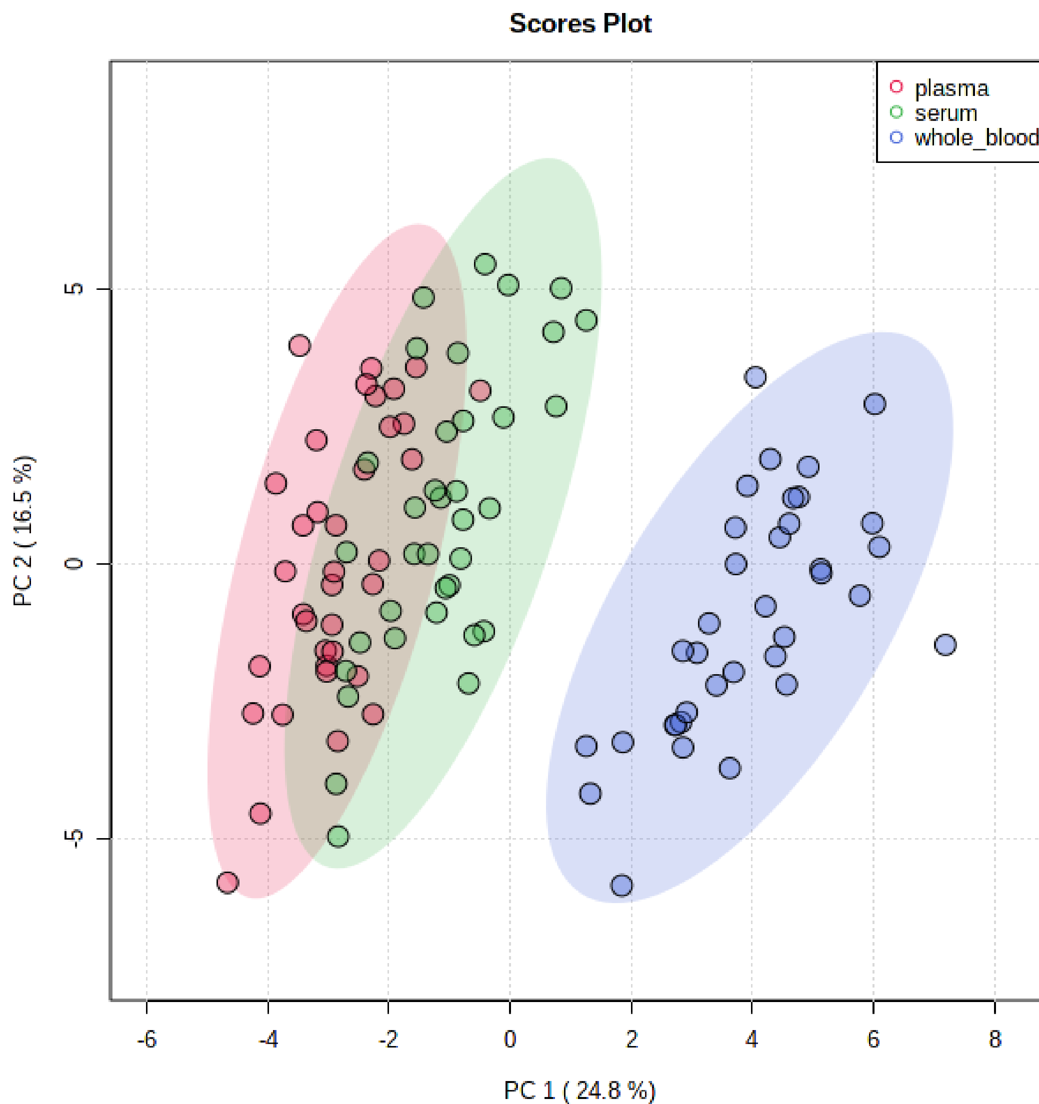

Figure 2.1. Principal component analysis scores 2D plot of amino acid profiles in whole blood, plasma, and serum. Plot was made using Metaboanalyst 5.0 free online software package and autoscaling of the concentration data.

2.4. Discussion

There is growing interest in measuring amino acids in veterinary medicine. However, there are multiple sample types from which circulating free amino acids can be measured, including whole blood, plasma, and serum. Additionally, published

reference intervals in dogs are only available for plasma (most amino acids) and whole blood (taurine only) [77]. This study provides a direct comparison of amino acid concentrations in all three biofluids in healthy dogs.

In summary, this study analytically validated a method to concurrently measure a wide variety of amino acids in dog serum using the Biochrom 30+ Amino Acid Analyzer. This study also showed that amino acid concentrations are generally different between whole blood, plasma, and serum (in 33 out of 39 detected compounds), and that serum contains higher concentrations of many amino acids when compared to plasma.

2.4.1. Analytical validation of dog serum

This study demonstrated precision, reproducibility, accuracy, lack of apparent matrix effect, linearity, and stability for the measurement of amino acids in dog serum using the Biochrom 30+ Amino Acid Analyzer. Out of 42 total compounds that are part of the assay, 31 were consistently detected in the serum from dogs and had acceptable validation parameters (phosphoserine, taurine, urea, aspartic acid, threonine, serine, asparagine, glutamic acid, glutamine, glycine, alanine, citrulline, α-aminobutyric acid, valine, methionine, cystathionine, isoleucine, leucine, tyrosine, phenylalanine, ammonia, hydroxylysine, ornithine, lysine, 1-methylhistidine, histidine, tryptophan, 3-methylhistidine, carnosine, arginine, and proline). Compounds that had a CV% above 10% for some samples for intra- or inter-assay variability tended to have very low concentrations below or near the lower limit of quantitation.

It was important that a thorough stability study be performed on dog serum, because it is known that interspecies differences in enzymatic activity in plasma can

affect the stability of amino acids [78]. Cystine and ethanolamine were highly unstable in dog serum, and therefore, should not be reported for clinical samples using this assay. Previous studies have also reported the instability of cystine, and together with this study suggest that it can only be measured reliably if strict attention is paid to sample collection, storage, and deproteinization conditions with deproteinization occurring within one hour of sample collection [73, 79, 80]. Concentrations of free cystine rapidly decline in serum due to the formation of disulfide bonds to proteins [81]. Consequently, this protein-bound half-cystine is removed from the measurable sample in the deproteinization process. Treatment of the sample with a reducing agent will reverse the binding, however, this will only allow the measurement of total cysteine and not free cystine in the sample. In this study, serum ethanolamine concentrations were highly dependent on time since collection, regardless of storage time or temperature prior to deproteinization, showing a noticeable increase 1 week or more after collection. However, a previous study has shown ethanolamine to be stable in human serum at 4°C and 22°C for at least 24 hours [82]. While unconfirmed, one possibility for the increases seen at longer storage times may be a reaction between ammonia in the serum and ethylene oxide commonly used to sterilize plastic laboratory vessels and tubes, that yields ethanolamine [83]. Additionally, phosphoethanolamine can decompose to ethanolamine and phosphate, leading to increases in ethanolamine concentrations with increasing storage time [84]. However, phosphoethanolamine concentrations were generally low in dog serum (< 10 μM), so it is unlikely that this is a significant contributing factor.

While previous studies have stressed the importance of prompt deproteinization for accuracy [85, 86], the results presented here suggest that deproteinization can be delayed for up to one month if samples are stored at −80°C within one day of collection, without significantly affecting the majority of amino acid concentrations. Long-term storage (i.e., 61 weeks) at −80°C, even with prompt deproteinization, may lead to slight increases in concentrations of ammonia, histidine, and tryptophan. Storage of dog serum at 4°C for up to 72 hours may affect glutamic acid, cystine, and ammonia. While no significant differences of concentrations were noted for glutamic acid or cystine for these storage conditions, this is likely due to a low sample number as glutamic acid concentrations more than doubled in 3 of 4 dogs from baseline to 72 hours of storage at 4°C and cystine concentrations dropped to zero for all 4 dogs by 48 hours of storage at 4°C. The source of the increase in serum glutamic acid concentrations is from the deamidation of glutamine to glutamic acid via enzymes that retain activity in serum until it is deproteinized. This deamidation reaction also releases ammonia, contributing to an increase in ammonia concentrations with increasing storage time. Ammonia was significantly increased after 72 hours of storage at 4°C and this finding is consistent with results from previous studies [87].

While some amino acid concentration changes were seen in serum stored at −20°C for up to 4 weeks prior to deproteinization, these changes were relatively minor compared to the range of concentrations in healthy dogs, with the exception of those for cystine, ethanolamine, and ammonia. Similar to results from previous studies [88], once the serum was deproteinized, no further changes were observed in amino acid

concentrations when stored at 4°C for 72 hours (with the exception of changes in ammonia concentrations) and −20°C for 1 week. Hydroxyproline showed a relatively high coefficient of variation under all storage conditions. However, because assay variability data was not able to be collected for this compound, it is unclear whether this is due to biological variability or assay variability.

Interestingly, serum concentrations of 3-methylhistidine and carnosine were associated with serum concentrations of creatinine and blood urea nitrogen in healthy dogs, both common indices used for assessment of kidney function. 3-methylhistidine is used as an index of muscle turnover in humans, but has a reduced renal clearance in patients with kidney disease [80, 89, 90]. Symmetric dimethylarginine (SDMA) is another methylated amino acid that has recently gained recognition as an early marker for loss of kidney function in veterinary medicine [27]. Carnosine is predominantly present in muscle tissue, is metabolized in the kidneys in humans, and is an indicator of animal protein consumption [91, 92]. This study suggests that 3-methylhistidine and carnosine have potential to be useful in evaluating kidney function, but further studies are needed to confirm this hypothesis.

Although hemolysis was found to correlate with the concentrations for several amino acids in plasma and serum, most were weak correlations. However, aspartic acid showed a moderate positive correlation with increased hemolysis in serum and plasma ($q=0.002$, $\rho=0.410$; $q<0.001$, $\rho=0.490$, respectively). This correlation was expected because aspartic acid is found in much higher concentrations in erythrocytes than in plasma or serum [93, 94]. This suggests that aspartic acid concentrations should be

interpreted with caution in hemolyzed samples. Further studies using spiked concentrations of hemolyzed red blood cells are necessary to determine the effect of hemolysis on amino acid quantification.

2.4.2. Whole blood, plasma, serum

Typically, plasma is more commonly used for amino acid analysis. It is generally believed to be a more stable sample type [73]. To collect serum, blood needs to clot at room temperature, during which time enzymatic reactions can occur (conversion of asparagine to aspartic acid and glutamine to glutamic acid) as well as loss of free cystine due to disulfide binding to proteins [84, 95]. However, one of the benefits to using serum is the ability to use plain red top tubes for collection, mitigating any variation from ratio of lithium heparin to sample. This study showed that the majority of amino acids measured using this method were stable in serum. It is difficult to compare results from different studies that use different sample types, but this study has provided results for whole blood, plasma, and serum in the same samples, which will allow general inferences to be made when comparing the sample types in the future.

Similar to results from previous studies, we found that concentrations of amino acids in serum were generally higher than in plasma [96, 97]. This is due, in part, to the *ex vivo* release of amino acids from erythrocytes, leukocytes, and platelets during the clotting process. Additionally, three amino acids that had large differences in concentration between serum and plasma in dogs (taurine, arginine, and glutamic acid) also had large differences in concentration between serum and plasma in humans [96, 97]. Concentrations of aspartic acid were highest in whole blood due to the majority of

aspartic acid being contained within blood cells [93, 94]. Our results suggest that specific reference intervals for plasma and serum (and whole blood) should not be used interchangeably.

Antibiotic administration can interfere with AA measurement with IEC, causing additional peaks that coelute with AAs of interest, increasing their apparent concentration [98]. Therefore, care was taken to ensure that the healthy dogs utilized for this study were not on any antibiotics or other medications other than monthly flea and heartworm preventatives. Also, food was withheld from all dogs for 8-12 hours prior to blood collection because some amino acids, including BCAA have been shown to increase postprandially [99].

Limitations of this study include the lack of comparison of different types of deproteinization agents. Deproteinization with sulfosalicylic acid may cause the loss of free tryptophan and it is thus preferred to use trichloroacetic acid for the determination of tryptophan [100]. However, in this study, it was of more interest to measure the amino acid profiles as a whole rather than optimizing the method for the measurement of individual amino acids. Additionally, while we did not examine the effect of freeze thaw cycles on AA stability in serum, previous studies have done so, showing that up to 3 freeze thaw cycles on deproteinized serum will have no effect on AA concentrations [101]. However, freeze thaw cycles have been shown to have an effect on serum that has not yet been deproteinized, significantly changing the concentrations of up to 15 different amino acids [82].

2.5. Conclusions

In conclusion, we analytically validated this assay for amino acid measurement in dog serum. For the most accurate measurement of serum amino acids, samples should be allowed to clot at room temperature for 15-30 minutes, centrifuged, and serum immediately frozen. Cystine and ethanolamine concentrations should not be reported under typical clinical collection conditions utilizing this method of sample preparation and analysis. Also, while amino acid profiles of dog serum and plasma are more similar to each other than whole blood, reference intervals for these sample types should not be used interchangeably.

3. ALTERED SERUM AND FECAL AMINO ACID CONCENTRATIONS IN DOGS WITH CHRONIC ENTEROPATHY

OVERVIEW

Amino acids (AA) can affect many metabolic processes and serve as the main energy source for enterocytes, and therefore could serve as an indicator of overall health of the gastrointestinal (GI) tract. Plasma and serum amino acid concentrations were previously reported as altered in dogs and cats with GI disease. However, studies in humans with IBD suggest fecal amino acids may be more useful in discriminating IBD patients from healthy controls. The aims of this study were to measure amino acids in feces and serum of dogs with chronic enteropathy (CE) and healthy control dogs (HC) and to correlate these amino acid profiles with gastrointestinal histopathology and clinical activity.

Serum and feces were collected from HC dogs (n=23 and 29, respectively) and dogs with CE (n=15 and 29, respectively). Additionally, GI biopsies were collected from 13 HC dogs undergoing elective surgical procedures and from 8 dogs with CE. Serum AA concentrations were measured with an amino acid analyzer (Biochrom 30+), and fecal AA concentrations were measured on an Agilent HPLC with pre-column o-phthalaldehyde and fluorenylmethyloxycarbonyl derivatization. Mann-Whitney tests were used to determine differences in AA concentrations between CE and HC dogs. Spearman's correlation analysis was performed between serum and fecal AA

concentrations, and clinical activity index and histopathological scores for stomach, small intestine, and colon.

Serum concentrations of valine were significantly higher in dogs with CE compared to HC, and fecal concentrations of tryptophan were significantly higher in dogs with CE compared to HC dogs. All correlations did not reach the level of significance after correction for multiple comparisons.

In conclusion, both serum and fecal AA profiles are altered in dogs with CE, and further studies are needed to determine the mechanisms behind these alterations.

3.1. Introduction

Amino acids (AA) are important nutrients for enterocytes, but also act as signaling molecules that regulate intestinal permeability, intestinal inflammation, and host-microbiota interactions [71]. Amino acids also play a key role in maintaining intestinal homeostasis by serving as precursors for the production of antioxidants, such as glutathione and nitric oxide. Their importance is exemplified by the negative effects seen in dietary deficiencies of AA. Dietary deficiency of sulfur containing amino acids, methionine, cysteine, and cystine, results in small intestinal atrophy, suppresses mucosal growth, and lowers intestinal glutathione levels in pigs [102]. Dietary deficiency of threonine increases paracellular permeability in pigs [103] and dietary deficiency of tryptophan downregulates antimicrobial peptides and increases susceptibility to DSS-induced inflammation in mice [104]. Therefore, AA may serve as a therapeutic target in

diseases where the intestinal integrity is compromised, such as inflammatory bowel disease (IBD) in humans or chronic enteropathy (CE) in dogs.

Chronic enteropathy in dogs is described as having clinical gastrointestinal signs (i.e., vomiting, diarrhea, weight loss) for more than 3 weeks duration with histological evidence of inflammation in the gastrointestinal (GI) tract and exclusion of enteropathogens. Treatment for these dogs typically includes dietary trials with highly digestible, hydrolyzed or novel protein diets, antimicrobial therapy, steroids, or any combination of the above [7]. Non-invasive biomarkers, such as serum C-reactive protein (CRP), can potentially be used to help monitor response to therapy. It is also of interest to have non-invasive biomarkers that can indicate severity or type of inflammation in cases where obtaining gastrointestinal biopsies is not possible.

Amino acids have begun to be used as biomarkers of GI disease in human medicine. In a study of metabolic profiling of serum and plasma using ^{1}H nuclear magnetic resonance spectroscopy, IBD patients had increased isoleucine, methionine, lysine, glycine, and arginine and decreased valine, tyrosine, serine, and urea compared to healthy controls [105]. A different study showed plasma histidine and tryptophan concentrations were decreased in humans with IBD and had an inverse correlation with disease activity and serum CRP levels [65]. More recently, Kolho et al. discovered that the fecal metabolomic profile was superior to the serum metabolomic profile in discriminating human IBD patients from controls [106]. Furthermore, untargeted metabolomics in feces of dogs with CE showed alterations in a higher number of compounds than in serum [31, 107]. Fecal concentrations of amino acids are generally

increased in human IBD patients [67, 108]. However, these changes in feces may not coincide with changes in disease activity [67]. Plasma citrulline concentration is used in human medicine as a biomarker of functional enterocyte mass independent from intestinal inflammation [109, 110]. Another amino acid, 3-Methylhistidine, has also been used as an indicator of muscle protein catabolism [89, 111] which could be a helpful tool to understand nutritional adequacy in GI disease.

There are a limited number of studies examining the use of amino acids as biomarkers of intestinal disease in dogs [35, 36, 112]. However, to the authors' knowledge, there are no studies measuring fecal amino acid concentrations in a targeted manner in dogs with GI disease. The aims of this study were to measure amino acids in feces and serum of dogs with chronic enteropathy and healthy control dogs and to correlate these amino acid profiles with gastrointestinal histopathology and clinical activity.

3.2. Materials and methods

3.2.1. Sample collection

All samples used in the study were collected retrospectively from banked samples. Dogs were originally enrolled in other studies between February 2019 and March 2021. Serum and gastrointestinal biopsy samples obtained from dogs had prior approval from the relevant Institutional Animal Care and Use Committees (IACUC 2019-0072 CA; CEUA n° 4079150419). All fecal samples obtained were naturally

passed and therefore required no institutional approval. Samples were stored frozen and shipped on dry ice to the Gastrointestinal Lab at Texas A&M University.

3.2.1.1. Healthy dogs

Healthy control (HC, n=41) dogs were privately-owned pet dogs that, based on responses in an owner questionnaire and physical exam performed by a licensed veterinarian, had an absence of clinical signs. Exclusion criteria for HC were gastrointestinal signs, skin disease, or administration of antibiotics or probiotics within the previous four weeks. Gastrointestinal biopsies were collected from a subset of healthy control dogs that were undergoing spay or neuter surgery.

3.2.1.2. Diseased dogs

Dogs with chronic enteropathy (CE, n=34) were defined as having had gastrointestinal signs for at least 3 weeks and were at least one year of age. At the time of enrollment, owners and veterinarians were asked to complete a questionnaire on the medical history and diet of the patient. A physical examination, CBC, serum biochemistry profile, and fecal dysbiosis index were performed on all dogs. Further diagnostic work-up included serum cobalamin and folate (30/34 dogs), serum trypsin-like immunoreactivity and pancreatic lipase immunoreactivity (16/34 dogs), basal-cortisol serum concentration or ACTH-stimulation test (14/34 dogs), fecal flotation (33/34 dogs), *Giardia* spp. antigen ELISA or SNAP test (32/34 dogs), an abdominal ultrasound (32/34 dogs), and gastrointestinal biopsy procedure (8/34 dogs). Exclusion criteria for dogs with CE were administration of antibiotics within the previous one month or presence of endoparasites.

3.2.2. Serum amino acid analysis

Serum samples were deproteinized following published protocol (dx.doi.org/10.17504/protocols.io.877hzrn) [PROTOCOL DOI]. Briefly, samples were deproteinized with 1:1 (v/v) addition of 5% sulfosalicylic acid + 500 µM L-norleucine internal standard and filtered through 0.2 µm PVDF centrifugal filter tube prior to being loaded onto the instrument autosampler for injection and analysis or stored at −80°C for later analysis. Serum amino acids and other nitrogenic compounds were measured with a Biochrom 30+ lithium high performance amino acid analyzer (Biochrom Ltd., Cambridge, UK) following manufacturer's guidelines. Autosampler injection parameters were set to an injection volume of 30 µl with partial loop fill injection.

3.2.3. Fecal amino acid analysis

To prepare the fecal samples for amino acid analysis, 1 g feces was lyophilized overnight. Lyophilized feces were then crushed to a powder and 50 – 60 mg aliquoted into 2 mL plastic snap cap tubes. Next, 400 µl Milli-Q water was added to each fecal sample, vortexed on high speed for one minute, and incubated at 4°C for one hour. Samples were again vortexed briefly and then centrifuged at 15,000 x g for 10 minutes at 4°C. Less than or equal to a volume of 300 µL of supernatant was transferred to a 5,000 Dalton molecular weight cutoff centrifugal filter (Sartorius vivaspin 500µl) and centrifuged at 15,000 x g for 20 minutes at 4°C. In a glass autosampler vial, 20 µL of filtrate was combined with 20 µL internal standard (250 µM in water; norvaline and sarcosine; Sigma-Aldrich, St. Louis, MO, USA) and mixed. Blanks were made with the addition of 20 µL Milli-Q water and 20 µL internal standard added to glass vials, and

standards were made with 20 µL amino acid mixture #1 (250 µM aspartic acid, glycine, alanine, valine, leucine, isoleucine, serine, threonine, tyrosine, proline, arginine, histidine, glutamic acid, cystine, phenylalanine, lysine, and methionine in 0.1 M HCl; Amino Acid Standard, Agilent Technologies, Santa Clara, CA, USA), 4 µL amino acid mixture #2 (250 µM glutamine, asparagine, tryptophan, β-alanine, taurine, citrulline, and ornithine; Sigma-Aldrich, St. Louis, MO, USA), and 20 µL internal standard. Samples, blanks, and standards were stored at −80°C until analysis. Analysis was performed on an Agilent Infinity 1260 HPLC instrument (Agilent Technologies, Inc., Santa Clara, CA, USA) using automated precolumn derivatization with ortho-phthalaldehyde (OPA) and 9-fluorenylmethyl chloroformate (FMOC), an ODS Hypersil™ 200 x 2.1 mm 5 µ particle size column (Thermo Fisher Scientific, Waltham, MA, USA), and fluorescence detection at 450 nm emission wavelength and 340 nm excitation wavelength (except for proline: excitation=266 nm and emission=450 nm) per manufacturer guidelines. Mobile phase A consisted of 20 mM NaAc + 0.018% triethanolamine adjusted to pH 7.2 with 1-2% acetic acid, and mobile phase B consisted of 20% of 100 mM NaAc adjusted to pH 7.2 with 1-2% acetic acid + 40% ACN and 40% MeOH. Acquisition program was set to start with 100% A at 0.45 mL/min, at 17 min 60% B, at 18 min 100% B, with flow rate at 18.5 min set to 0.8 mL/min and at 24 min 0.45 mL/min. Injection volume was set to 5 µL and column temperature set to 40°C.

3.2.4. Statistical analysis

A Shapiro-Wilk test for normality of the data was performed, and given non-normal distribution, Mann-Whitney tests were used to compare serum and fecal amino

acid concentrations between dogs with CE and HC dogs and p-values corrected for multiple comparisons using Benjamini-Hochberg false discovery rate (FDR) correction. Spearman's correlation analysis was performed between serum and fecal amino acid concentrations, and clinical activity index (CCECAI) and World Small Animal Veterinary Association (WSAVA) histopathological scores for gastric, small intestinal, and colonic tissues. These correlations were corrected for false discovery rate using Benjamini-Hochberg FDR correction. The data was analyzed using two statistical software programs (JMP Pro v14, SAS Institute Inc., Cary, North Carolina; Graph Pad Prism v 9.0.1, GraphPad Software, San Diego, California).

3.3. Results

A total of 41 healthy dogs were enrolled. Of these 41 dogs, serum AA concentrations were obtained in 12 dogs, fecal AA concentrations were obtained in 18 dogs, and both serum and fecal AA concentrations were obtained in 11 dogs. GI biopsies were obtained for 13 dogs. CCECAI was available for 24 dogs.

A total of 34 dogs with CE were enrolled. Of these 34 dogs, serum AA concentrations were obtained in 5 dogs, fecal AA concentrations were obtained in 19 dogs, and both serum and fecal AA concentrations were obtained in 10 dogs. GI biopsies were obtained for 8 dogs, and CCECAI was available for 16 dogs. Clinical response to therapy was noted by the enrolling veterinarian for 16 dogs, 7 of which were food-responsive (FRE), 4 of which were antibiotic-responsive (ARE), and 5 of which were

steroid-responsive (SRE). None of the dogs with CE had a serum albumin concentration less than 2.3 g/dL.

3.3.1. Serum and fecal amino acid concentrations

Serum amino acid concentrations for healthy dogs and dogs with CE are shown in Figure 3.1. A total of 30 amino acid or nitrogenous compounds were detected in the samples. Serum amino acid concentrations of valine were significantly higher in dogs with CE when compared to HC dogs (q=0.026). All other compounds in serum were not significantly different between groups.

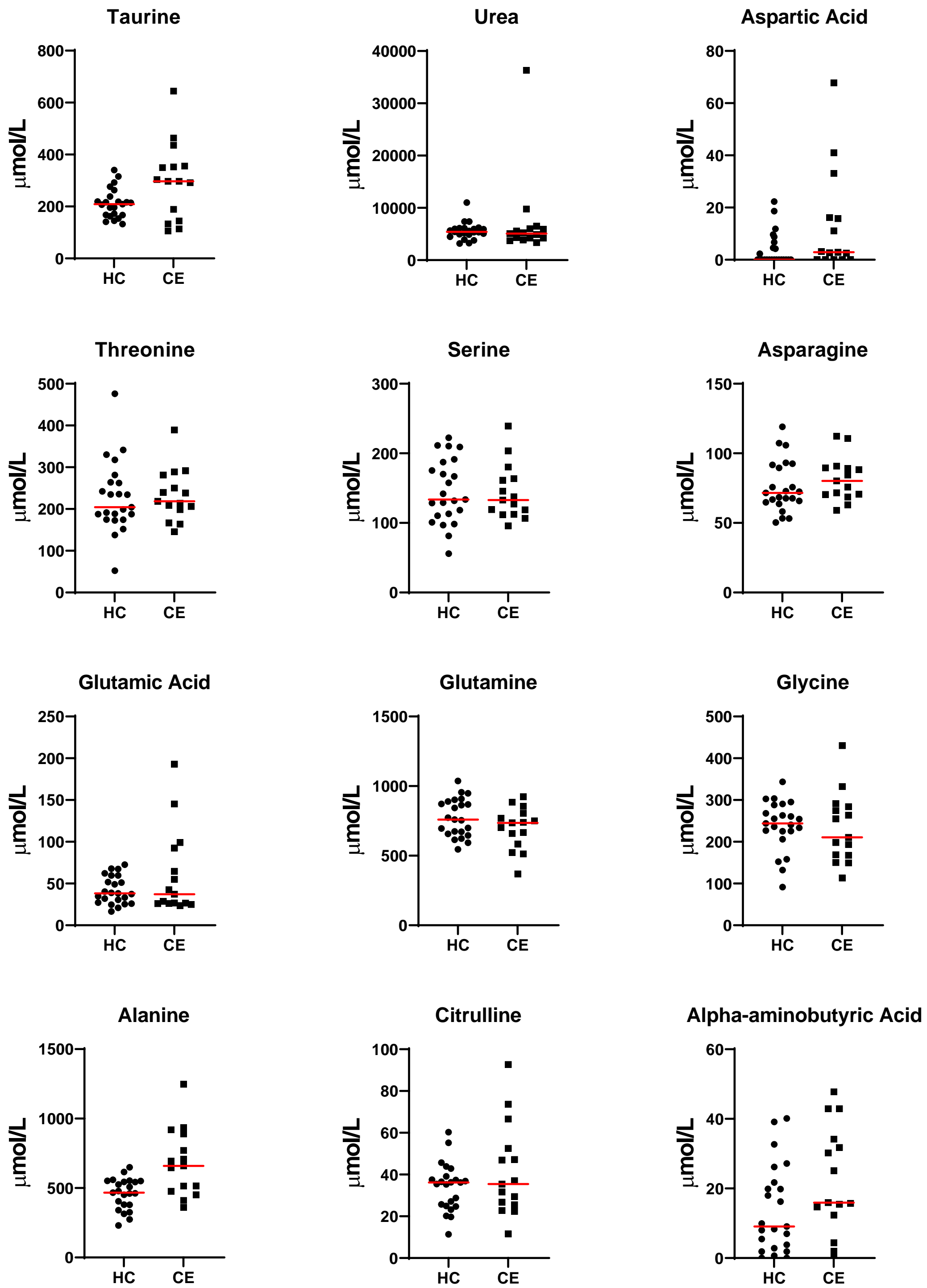

Figure 3.1. Serum amino acid concentrations in healthy dogs (HC) and dogs with chronic enteropathy (CE). Red lines indicate median.

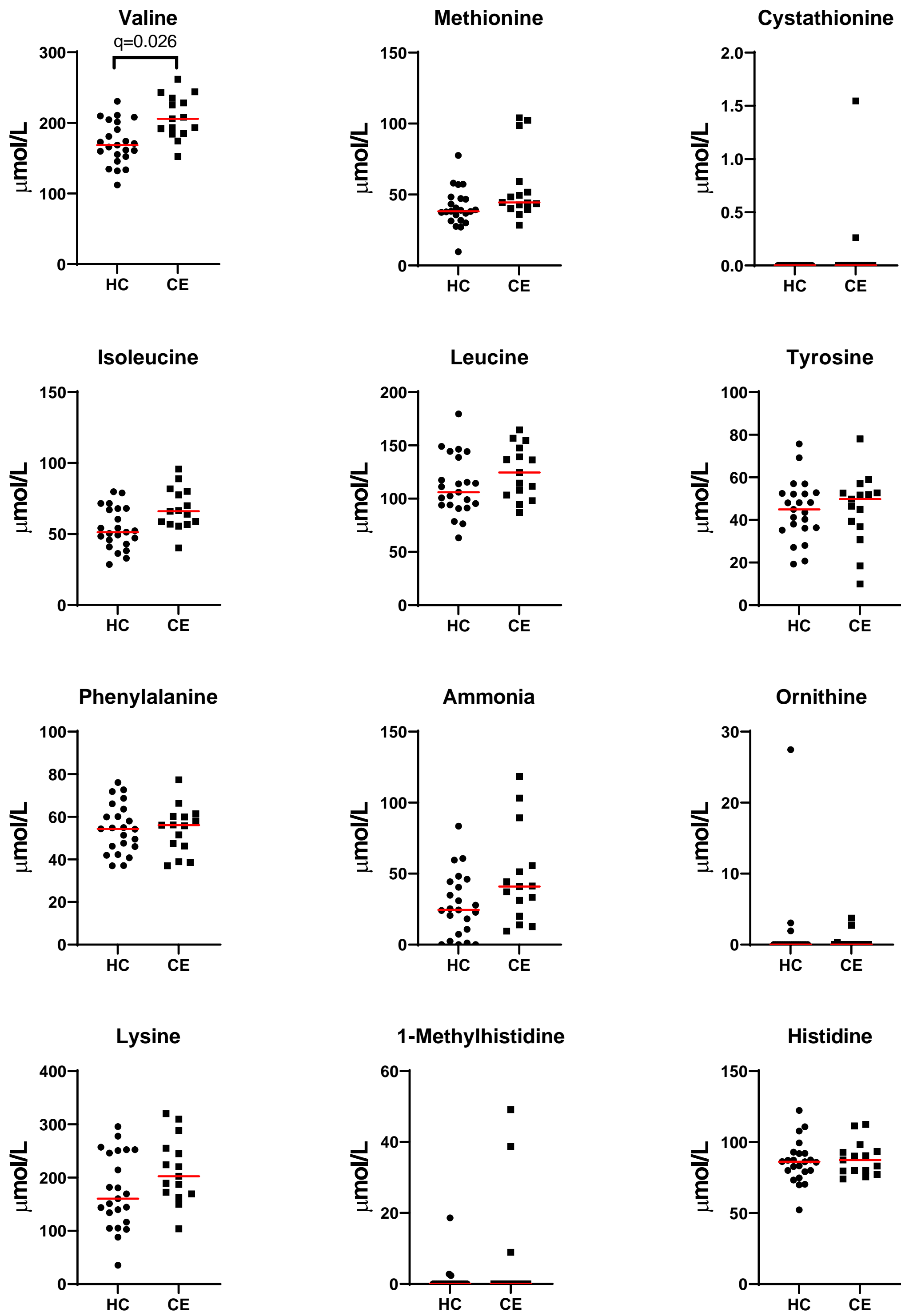

Figure 3.1 Continued

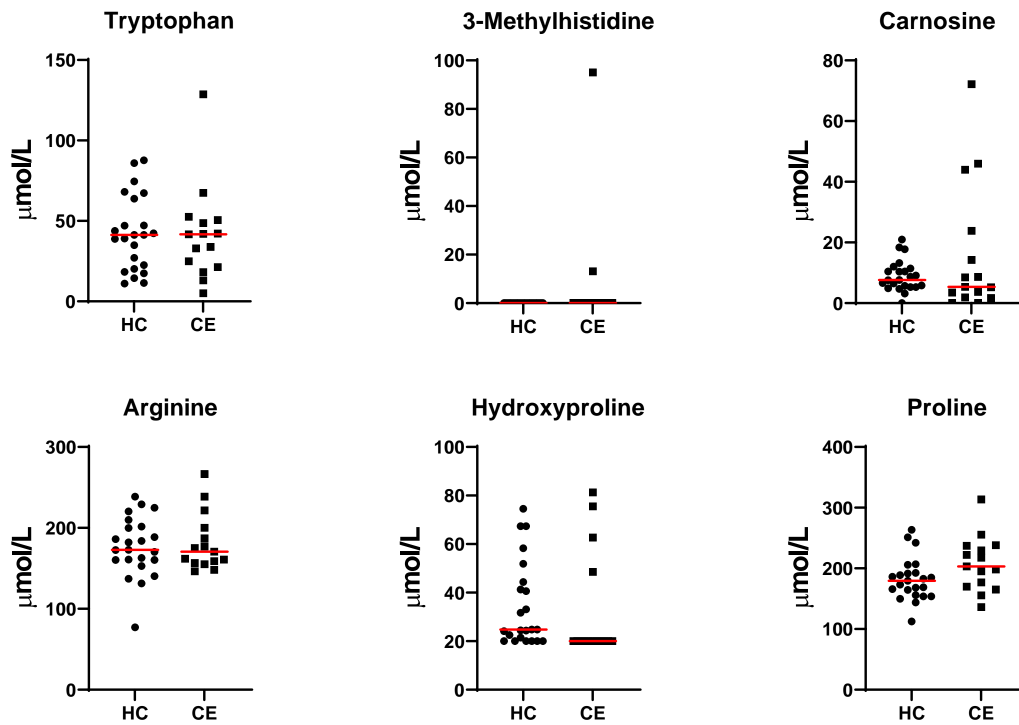

Figure 3.1 Continued

Fecal amino acid concentrations for healthy dogs and dogs with CE are shown in Figure 3.2. A total of 22 amino acids were measured in the samples. Fecal concentrations of tryptophan were significantly higher in dogs with CE when compared to HC dogs. All other amino acids in feces were not significantly different between groups.

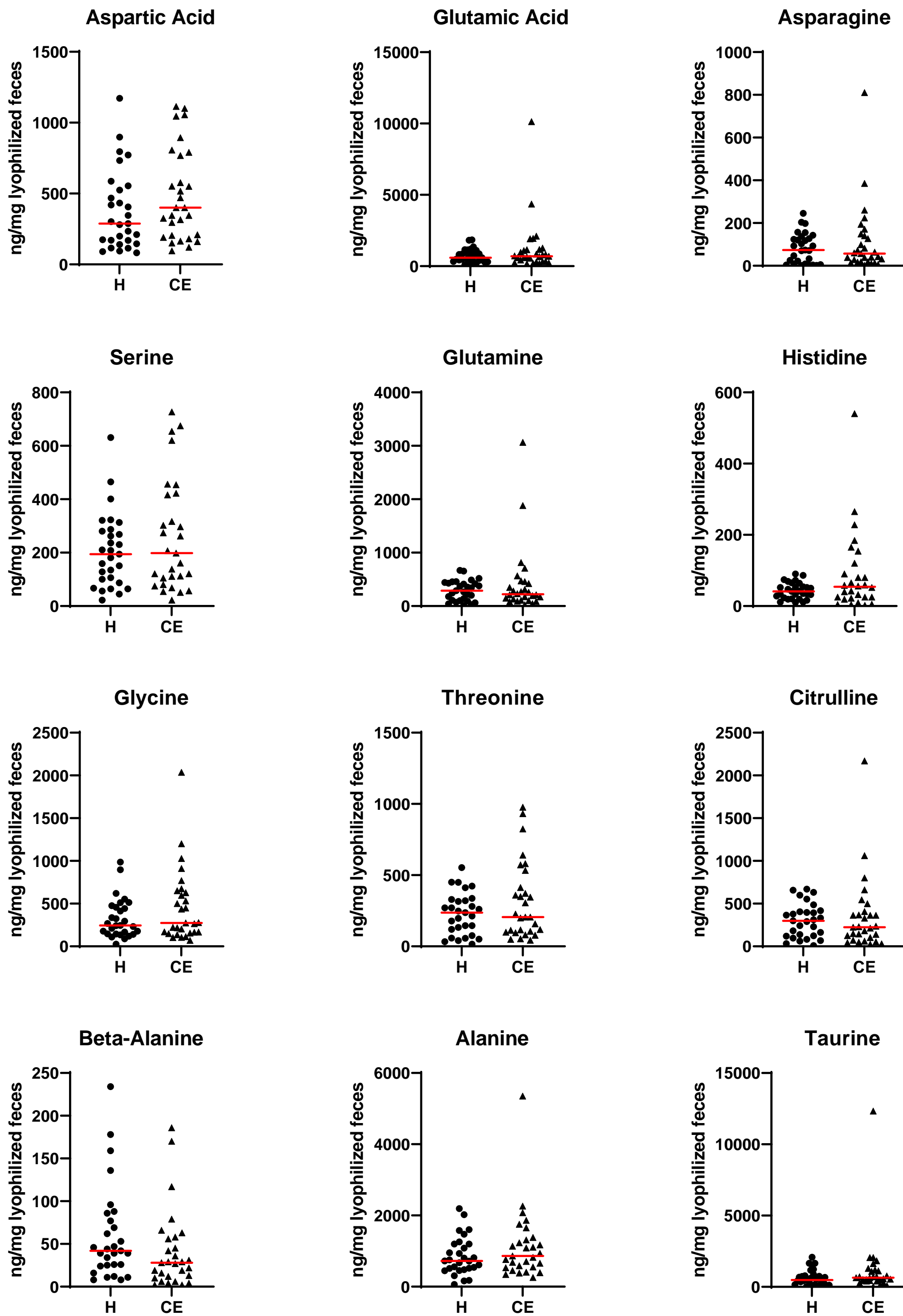

Figure 3.2. Fecal amino acid concentrations in healthy dogs (HC) and dogs with chronic enteropathy (CE). Red lines indicate median.

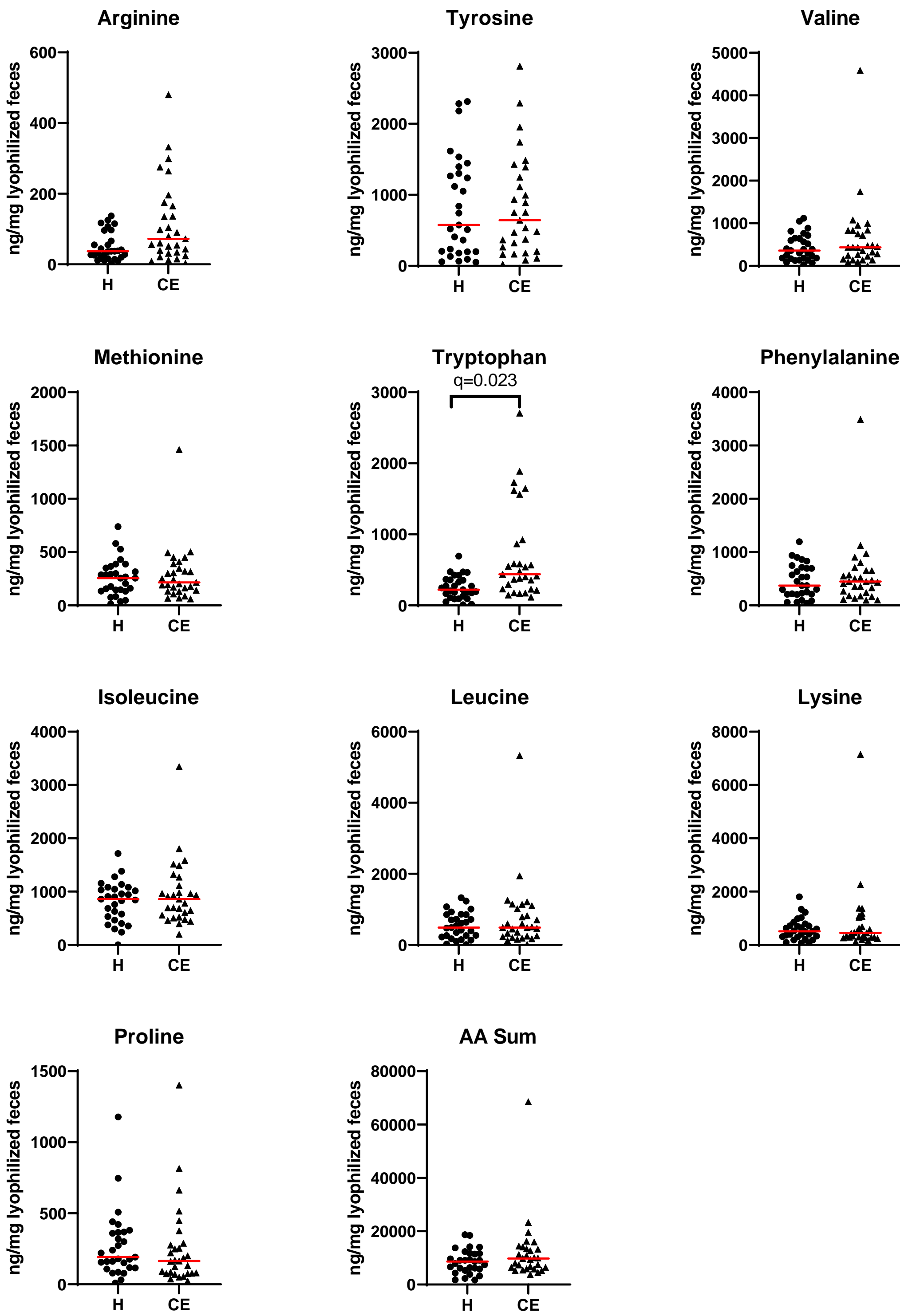

Figure 3.2 Continued

After correction for false discovery rate with Benjamini-Hochberg procedure, none of the correlations between serum and fecal amino acid concentrations and CCECAI and WSAVA scores were statistically significant (Table A-16).

3.4. Discussion

In this study, we aimed to measure amino acid concentrations in serum and feces of dogs with CE and compare them to healthy controls. Serum concentrations of valine and fecal concentrations of tryptophan were significantly higher in dogs with CE compared to HC. This is in contrast to a previous study that identified decreased plasma amino acid (methionine, proline, serine, and tryptophan) concentrations in dogs with IBD [35]. However, this difference may be due to the study only looking at dogs with lymphoplasmacytic inflammation that did not respond to dietary or antibiotic trials. Our study included dogs more representative of the clinical population of dogs with CE, including dogs that responded clinically to dietary or antibiotic trial and possibly including dogs with little to no intestinal inflammation as gastrointestinal biopsy procedure was not performed on all dogs. Another study measured serum amino acid concentrations in dogs with hypoalbuminemia and protein-losing enteropathy (PLE), a severe form of CE, and found serum tryptophan concentrations were decreased in dogs with PLE [36]. All dogs with CE in our study were normoalbuminemic to mildly hypoalbuminemic with albumin concentrations greater than 2.3 g/dL, which may exhibit different amino acid profiles than dogs with moderate to severe hypoalbuminemia,

despite a previous study showing no difference in plasma amino acid concentrations between 4 dogs with hypoalbuminemia and 6 dogs with normoalbuminemia [35].

Decreased serum or plasma concentrations of amino acids, especially histidine and tryptophan, are a common finding in humans with IBD [65, 113, 114]. Plasma concentrations of histidine and tryptophan also inversely correlate with serum CRP levels and disease activity in humans with IBD [65]. Interestingly, plasma concentrations of histidine and tryptophan also inversely correlate with disease activity in cats with chronic GI disease [68]. While no decreases in serum amino acid concentrations were found in our study, this may be due to the inclusion of dogs with food-responsive enteropathy, which tend to have less severe intestinal inflammation. Further studies are needed to determine if type of therapeutic response coincides with different amino acid profiles in dogs with CE.

Similar to the results of our study, serum or plasma concentrations of some amino acids are increased in humans with IBD, such as alanine, methionine, and isoleucine [105, 113]. Valine and isoleucine are branched-chain amino acids, which have been used as biomarkers of cardiometabolic wellness and chronic kidney disease in humans [115, 116]. Additionally, during states of brief starvation, plasma concentrations of branched-chain amino acids increase [99] suggesting their potential use as nutritional markers. Dietary deficiencies in AA could exacerbate damage in the intestines as mentioned previously in dietary deficiency studies. While commercial dog food diets are formulated to meet or exceed nutritional requirements, intestinal inflammation seen in some dogs with CE may necessitate increased dietary nutrients beyond what commercial

food will provide. Alternatively, the dogs may not be able to absorb the nutrients available due to enterocyte damage. Absorption and digestion studies utilizing amino acid isotopes are necessary and the logical next step to understanding the mechanisms behind altered amino acid concentrations in serum and feces of dogs with CE.

One sulfur containing amino acid, methionine, was apparently higher in the serum of dogs with CE, but did not reach the level of significance after FDR correction. Methionine is a precursor to the antioxidant glutathione and inhibits paracellular permeability increase via TNF-α [117, 118]. Although no correlations were significant after correction for multiple comparisons (Table A-16), serum methionine concentrations were positively associated with small intestinal ($\rho=0.698$, $q=0.063$) and stomach ($\rho=0.584$, $q=0.237$) WSAVA histopathological scores, and fecal methionine concentrations were inversely associated with small intestinal score ($\rho=-0.522$, $q=0.595$). More dogs with GI biopsies, both healthy dogs and dogs with CE, would be needed to formulate and validate a model using fecal and serum amino acid concentrations to predict severity of histological inflammation. However, there may be limitations to such a model as new evidence suggests that apparently healthy cats can have mild to moderate levels of intestinal inflammation [119]. Furthermore, only 2 of 13 (15%) healthy dogs for which intestinal biopsies were obtained in this study had no histological abnormalities.

While citrulline is useful as a marker of functional enterocyte mass in humans [109, 110], its usefulness may be limited in dogs. The results of this study showed no correlation between serum citrulline concentrations and histopathological scores, which

is in congruence with previous studies in dogs that showed either no difference in serum citrulline concentrations in dogs with CE compared to healthy controls [120], or increased plasma citrulline concentrations in dogs with parvoviral enteritis [121]. Serum citrulline concentrations may be more useful to quantify degree of extreme intestinal damage, such as necrosis, but these studies have not been done in dogs.

The amino acid tryptophan was increased in feces of dogs with CE in this study. Similarly, humans with IBD were found to have increased amino acid concentrations in feces but did not correlate to disease activity [67, 108]. Tryptophan is an important amino acid for intestinal health as it is required for bacterial indole production and host synthesis of serotonin. Therefore, the increased availability of tryptophan in the feces of dogs with CE may impact the intestinal microbiota. Alternatively, the altered intestinal microbiota found in dogs with CE [4, 122] may impact tryptophan availability in the feces. However, bacterial gene analysis in feces of dogs with CE has shown decreased gene expression of genes responsible for biosynthesis of amino acids and increased expression of genes for glutathione biosynthesis [123], suggesting an excess of amino acids available in the lumen. Further studies on fecal amino acids in dogs with GI disease could elaborate if these decreased concentrations of tryptophan in feces are specific to chronic GI disease or present in acute disease as well.

Limitations of this study included the lack of intestinal biopsies from all animals. Healthy animals were limited because only those that were already undergoing an elective surgical procedure (spay or neuter) were eligible to obtain biopsies. Dogs with CE are often not recommended for biopsy procedure unless they are clinically stable and

have failed dietary and antibiotic trials [7]. However, all biopsy WSAVA scores were given by a single histopathologist, which helps avoid any bias in the scores used. Additionally, the inclusion of dogs with CE not undergoing intestinal biopsy procedure is a better representation of the clinical patient population, as many owners of dogs with CE elect to instead try empirical treatment with steroids if the dog fails to respond to diet or antibiotic therapy. Furthermore, diet was not controlled in this study—dogs consumed a variety of commercial, home-cooked, and veterinary therapeutic diets. There is evidence in humans and dogs that dietary macronutrient composition, source of carbohydrates and proteins, and hydrolyzation of proteins can affect circulating amino acid concentrations [77, 124-129]. However, further studies are required in which effect of diet is directly compared to effect of disease to determine if the effect of disease will overshadow any changes in amino acid profiles related to diet in dogs. Another limitation of our study was that serum for amino acid measurement was not available for all dogs in the study. Although previous studies of human plasma have reported that some amino acids are unstable under extended storage times [78, 85], our validation of serum amino acid measurement in dogs showed that very few amino acids change with extended storage at −80°C (see page 22). Furthermore, samples from our healthy control dogs and dogs with CE were collected in similar time frames and underwent the same storage conditions.

3.5. Conclusions

In conclusion, our study showed dogs with CE have higher serum concentrations of valine and higher fecal concentrations of tryptophan when compared to healthy dogs. Additional studies are needed to determine the mechanisms behind these alterations, such as decreased absorption in the GI tract, increased losses via the intestinal tract, nutritional inadequacies resulting in muscle protein catabolism, or changes in intestinal microbiota utilization of amino acids. In addition, future studies could examine whether predictive modeling with serum or fecal amino acid concentrations can estimate level of histological inflammation in gastrointestinal biopsy samples.

4. TRYPTOPHAN METABOLITE ALTERATIONS IN FECES FROM DOGS WITH GASTROINTESTINAL DISEASES

OVERVIEW

Metabolic products of tryptophan metabolism, of host or bacterial origin, have been shown to regulate gastrointestinal secretion and motility and attenuate intestinal inflammation. The tryptophan metabolic pathway in various gastrointestinal diseases has not been investigated in dogs. Therefore, this study aimed to evaluate the fecal microbiome and tryptophan metabolites in dogs with acute diarrhea (AD; n=10), acute hemorrhagic diarrhea syndrome (AHDS; n=10), and inflammatory bowel disease (IBD; n=8) at initial presentation and during the recovery period.

Samples from diseased dogs were selected retrospectively from dogs enrolled in previous studies. Samples from healthy dogs were collected as controls (HC; n=19). The fecal microbiome was analyzed using 16S rRNA gene sequencing. Tryptophan metabolites were measured with a targeted liquid chromatography tandem mass spectrometry method.

The fecal microbial communities were significantly different between dogs with AD, AHDS, and IBD when compared to healthy dogs, at baseline as well as follow up timepoints within each group. At baseline, dogs with AHDS and IBD had increased concentrations of fecal indole-3-acetamide when compared to HC, whereas dogs with AD had increased concentrations of fecal indole. Indole-3-acetamide concentrations normalized in follow up timepoints of IBD, resembling HC dogs.

This study suggests that AD, AHDS, and IBD disease processes in dogs impacts not only the intestinal microbiota but also important functional metabolites.

4.1. Introduction

Gastrointestinal (GI) disease is a common disease in dogs with estimated prevalence ranging from 10-18% [1]. There are several classifications of GI disease in dogs depending on the severity and duration of symptoms. Acute diarrhea (AD) is defined as having diarrhea for less than two weeks with an absence of enteric pathogens, and acute hemorrhagic diarrhea syndrome (AHDS) has additional presence of blood in the stool. Inflammatory bowel disease (IBD) in dogs is defined as having GI symptoms, such as weight loss, vomiting, and diarrhea, for at least two weeks, failure to respond to dietary trial, along with inflammation present in the GI tract upon histological examination.

The role of the intestinal microbiome in chronic and acute GI diseases in dogs has only recently been explored. Changes in bacterial taxa have been identified in these disease states, such as increased *Escherichia coli* and decreased *Faecalibacterium spp.* in dogs with IBD or AD [2, 10, 15], and decreased *Lactobacillus* and *Streptococcus* in dogs with AHDS [14]. However, how the microbiome changes from disease state back to health is less clear. There are also few studies looking at functional metabolites of the microbiota in GI diseases in dogs. Untargeted metabolomics analysis of feces from dogs with IBD showed decreased abundances of the tryptophan metabolites indoleacetate and

indolepropionate, sparking an interest to measure tryptophan and its metabolites in a targeted manner [12].

Tryptophan has been identified as a metabolite of interest in human and animal models of GI disease. In humans with IBD, tryptophan concentrations in plasma are significantly decreased and inversely correlated with disease activity [65]. In a pig model of dextran sodium sulfate-induced colitis, tryptophan supplementation reduced pro-inflammatory cytokines at the protein and gene expression levels, indicating anti-inflammatory properties [130]. Furthermore, metabolic products of tryptophan produced via host (e.g., serotonin) or bacterial (e.g., indole) metabolism have been shown to regulate GI secretion and motility [131], and attenuate intestinal inflammation [132], respectively. In humans, dietary tryptophan is metabolized in several different pathways: 1) approximately 5% is metabolized by gut microbiota to produce tryptamine, indole, and indole derivatives, 2) approximately 1-2% is metabolized by the body to form serotonin, and 3) approximately 95% is metabolized by the body in the kynurenine pathway [133, 134].

Although the first pathway represents a relatively small proportion of tryptophan metabolism, the resulting metabolites have large impacts on the gastrointestinal host architecture and ecosystem. For example, indole produced via bacterial tryptophan metabolism in the gut regulates growth and virulence of other bacteria in the gut, acting as a signaling molecule and communication tool for the multispecies community [135, 136]. Indole has also been shown to strengthen tight junctions in the epithelium improving the intestinal barrier function, as well as reducing the activation and

expression of proinflammatory chemokine interleukin-8 [132]. Another bacterial tryptophan metabolite, tryptamine, acts as a neurotransmitter that stimulates intestinal motility [137] and can potentially decrease intestinal permeability [138]. Indole and indole derivatives produced via intestinal microbiota can also act as aryl hydrocarbon receptor (AhR) agonists in the GI tract which function to regulate immune response and maintain the mucosal barrier [139].

The second and third pathways of tryptophan metabolism listed above are dependent upon the host metabolism instead of microbial metabolism. Intermediates and end products of these host metabolic pathways for tryptophan have their own physiologic functions. For example, tryptophan is enzymatically converted to serotonin in mucosal enterochromaffin cells and, once released, acts as a local signaling molecule to stimulate motility and secretion [140]. While only 1-2% of the dietary tryptophan is converted into serotonin, approximately 95% of the body's serotonin is produced in the gut and is virtually the sole source of serotonin in the blood [141]. Metabolites in the kynurenine pathway, such as 3-hydroxyanthranilic acid and quinolinic acid, can induce apoptosis of activated T lymphocytes [142]. Therefore, measuring both microbial and host derived products of tryptophan metabolism may help elucidate the downstream functional effects from GI disease.

Our objective of this study was to characterize the fecal microbiota in different GI diseases in dogs (AD, AHDS, and IBD) compared to healthy dogs, as well as over time after initial diagnosis. Additionally, we aimed to characterize tryptophan and its host and bacterial metabolites in the feces of these dogs.

Methods

4.1.1. Study population

Naturally voided fecal samples were collected prospectively from healthy adult dogs (HC; n=19) recruited from students and staff of the College of Veterinary Medicine and Biomedical Sciences at Texas A&M University, College Station, TX in September and October of 2017. These were pet dogs that lived indoors and were fed a variety of diets. Owners consented to the collection of their pet's feces and its use in research, and filled out a questionnaire to give medical history and dietary information about their dog. Dogs were excluded if they had signs of GI disease or antibiotic history in the previous six months. Fecal samples from dogs with AD, AHDS, and IBD were collected retrospectively as banked excess samples having been initially collected for previous studies between October 2013 and January 2018. Institutional approval was not necessary for this study as fecal samples collected prospectively for this study were naturally voided and required no restraint of the animal or were banked excess feces, and other data (such as histopathology) were obtained retrospectively having been collected previously for other studies. The study population is summarized in Table 4.1 and characteristics (i.e., age, body weight, and gender) were compared using Kruskal-Wallis tests and Chi-square test (GraphPad Prism v 8.0.2). All samples were stored at −80°C until further analysis.

Table 4.1. Patient characteristics.

	HC	AD	AHDS	IBD	p-value
Age in years median (range)	3.0 (1.0-12.0)	8.5 (3.0-11.0)	4.0 (0.9-11.0)	5.2 (2.8-9.3)	p = 0.099
Weight in kg median (range)	21.8 (2.3-49.9)	12.5 (5.6-36.0)	12.1 (3.5-41.5)	9.9 (4.1-49.0)	p = 0.933
Gender female/male	10/9	7/3	6/4	4/4	p = 0.793

HC, healthy controls; AD, acute diarrhea; AHDS, acute hemorrhagic diarrhea; IBD, inflammatory bowel disease.

Dogs with acute diarrhea (AD; n=10) had GI signs for less than 3 days at initial presentation and pathogenic causes of disease excluded (e.g., parasites, enteropathogens). Half of the dogs received amoxicillin-clavulanic acid as part of an antibiotic treatment trial [5], and all dogs received symptomatic treatment consisting of maropitant, metamizole, and gastrointestinal diet. Fecal samples were collected at initial presentation, and 6 and 30 days later (AD0, AD6, AD30, respectively). Dogs with AD that were treated with antibiotics were not significantly different from those treated with placebo at any timepoint for any tryptophan metabolites, and therefore were combined for further analysis.

Dogs with acute hemorrhagic diarrhea (AHDS; n=10) met the same criteria for enrollment as the dogs with AD, but their feces visibly contained blood. Half of the dogs received probiotic therapy as part of their treatment regimen [143], and all dogs received supportive therapy as needed, including fluid therapy, antiemetics, and analgesics. Dogs were placed on a gastrointestinal diet until at least day 21. Fecal samples were collected

at initial presentation (0-3 days after symptoms began; AHDS0), and 7, 14, and 90 days after initial presentation (AHDS7, AHDS14, AHDS90, respectively). Dogs with AHDS that were treated with probiotics were not significantly different from those treated with placebo at any timepoint for any tryptophan metabolites, and therefore were combined for further analysis.

Dogs with inflammatory bowel disease (IBD; n=8) had GI signs for more than 3 weeks and pathogenic causes of disease excluded (e.g., parasites, enteropathogens). Dogs were diagnosed after endoscopy and histopathology were found to be consistent with intestinal inflammation. All dogs had low serum cobalamin concentrations (≤ 285 ng/L) and were supplemented orally or parenterally during the study as previously described [144]. Feces were collected prior to biopsy procedure (IBD0), and then 30, 90, and 270 days later (IBD30, IBD90, and IBD270, respectively). None of the dogs received antibiotics in the 6 months prior to sampling or during the entire sampling period, due to the high regulation of antimicrobial usage in their country of origin. Some dogs (n=6) received steroid therapy as part of their treatment regimen after the initial sample was collected.

4.1.2. DNA extraction, sequencing, and analysis

DNA was extracted from a 100 mg aliquot of feces with a commercially available kit following manufacturer instructions (PowerSoil® DNA Isolation Kit, MOBIO Laboratories, Inc., Carlsbad, CA, USA). Extracted DNA was used for Illumina sequencing of the bacterial 16S rRNA genes using primers 515F (5’-GTGYCAGCMGCCGCGGTAA) [145] to 806RB (5’-

GGACTACNVGGGTWTCTAAT) [146] (MR DNA laboratory, Shallowater, TX, USA) as previously described [22, 147].

Sequences of the 16S rRNA genes were processed using Quantitative Insights Into Microbial Ecology 2 (QIIME 2, v 2018.6) [148]. The sequence data was demultiplexed, and an amplicon sequence variant (ASV) table was created using DADA2 [149]. Prior to downstream analysis, sequences assigned as chloroplast, mitochondria, and low abundance ASVs, containing less than 0.01% of the total reads in the dataset were removed. Samples were rarefied to 11,470 sequences per sample, based on the lowest read depth, to normalize sequencing depth across all samples.

Alpha diversity was measured with the Chao1, observed ASVs, and Shannon diversity metrics. Beta diversity was evaluated with the weighted phylogeny-based UniFrac [150] distance metric and visualized using Principal Coordinate Analysis (PCoA) plots. Multivariate analysis was performed on the ASV tables using ANOSIM (Analysis of Similarity) test within PRIMER 7 software (PRIMER-E Ltd., Luton, UK) to analyze differences in microbial communities between the different groups of dogs and across timepoints.

To compare differences in individual taxa, any taxonomic group not present in at least half of the samples in at least one group was considered a rare taxa, and removed from the dataset for this analysis. All remaining statistical analysis was performed in GraphPad Prism v 8.0.2 software (San Diego, CA, USA). For comparisons between groups at baseline, Kruskal-Wallis tests were used with Benjamini & Hochberg False Discovery Rate correction for multiple comparisons [151]. Significance was set at q <

0.05. *Post hoc* Dunn's multiple comparison tests were performed to determine which groups were different from healthy dogs at baseline. To compare changes in bacterial taxa within diseased groups of dogs over time, Friedman tests were performed with Benjamini & Hochberg False Discovery Rate, and significance set to $q < 0.05$. *Post hoc* Dunn's multiple comparison tests were used to compare follow up sampling to baseline.

4.1.3. Metabolite extraction and analysis

Metabolites were extracted from a 50-60 mg aliquot of feces using a methanol:chloroform:water based extraction method. Briefly, 800 µL ice cold methanol:chloroform (1:1, v:v) was added to samples in a bead based lysis tube (Bertin, Rockville, MD). Samples were extracted on a Precyllys 24 (Bertin) tissue homogenizer for 30 seconds at an intensity setting of 6,000, then centrifuged at 15,000 rcf for 5 minutes at 4°C. The supernatant was collected and samples were homogenized a second time with 800 µL ice cold methanol:chloroform combining supernatant with supernatant from the first extraction. Then 600 µL ice cold water spiked with indole-3-acetic acid 2-2-d2 internal standard (Cambridge Isotope Laboratories, Tewksbury, MA, USA) was added to the combined extract, vortexed, and centrifuged at 4,000 rcf for 8 minutes at 4°C to separate the phases. The upper aqueous layer was passed through a 0.2 µm nylon filter (Merck Millipore, Burlington, MA). Then 500 µL of the filtered aqueous phase was passed through a 3 kDa molecular weight cutoff filter (Amicon Ultra, Thermo Scientific, Waltham, MA). The flow through was collected and solvent removed by vacuum centrifugation. Samples were resuspended in 50 µL methanol:water (1:1,v:v) for analysis. External standards L-tryptophan (indole-d5), 15N-anthranilic acid, and 2-C13

indole (Cambridge Isotope Laboratories, Tewksbury, MA, USA) were used to verify retention times and calculate standard curves.

Metabolite analysis was performed at the TAMU Integrated Metabolomics Analysis Core. Targeted liquid chromatography tandem mass spectrometry analysis was performed on a TSQ Altis mass spectrometer (Thermo Scientific) coupled to a binary pump HPLC (Vanquish, Thermo Scientific). Samples were maintained at 4°C before injection, and the injection volume was 10 µL. Chromatographic separation was achieved on a Synergi Fusion 4 µm, 150 mm x 2 mm reverse phase column (Phenomenex, Torrance, CA) maintained at 30°C using a solvent gradient method. Solvent A was water (0.1% formic acid) and solvent B was methanol (0.1% formic acid). The gradient method used was 0-5 min (10% B to 40% B), 5-7 min (40% B to 95% B), 7-9 min (95% B), 9-9.1 min (95% B to 10% B), 9.1-13 min (10% B). The flow rate was set at 0.4 mL/min. Instrument parameters for acquisition and target transitions are listed in Table A-17. Sample acquisition and data analysis were performed in TraceFinder software (Thermo Scientific).

Kruskal-Wallis with Dunn's multiple comparisons tests were performed to determine which groups were different from healthy dogs at baseline and follow up timepoints. To compare changes in tryptophan metabolites within diseased groups of dogs over time, mixed-effects analysis with Holm-Sidak's multiple comparison tests were performed when missing data points were present (AHDS, IBD). When there were no missing data points, Friedman test with Dunn's multiple comparisons tests were performed to compare changes over time within groups (AD). Spearman's correlation

tests were used to identify associations between ASVs and tryptophan metabolites at baseline in all groups. Benjamini & Hochberg False Discovery Rate was used to correct for multiple comparisons with significance set at q < 0.05. All statistical analysis was performed in GraphPad Prism v 8.0.2 software (San Diego, CA, USA).

4.2. Results

4.2.1. Bacterial sequencing

4.2.1.1. Baseline comparison

Alpha diversity, described by Chao 1, observed ASVs (Figure 4.1), and Shannon Index, was significantly affected by groups according to Kruskal Wallis testing (q<0.05). However, when *post hoc* analysis was applied, the only difference identified was for Shannon Index between healthy controls and dogs with AHDS (p=0.006).

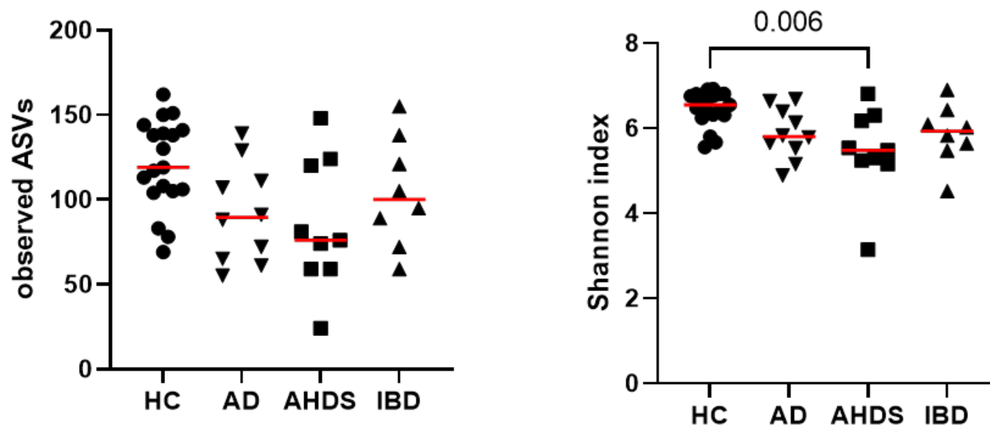

Figure 4.1. Observed ASVs and Shannon Index at baseline from the dogs with acute diarrhea (AD), acute hemorrhagic diarrhea (AHDS), inflammatory bowel disease (IBD), and the healthy controls (HC). In *post hoc* analysis, Shannon Index was significantly decreased in dogs with AHDS compared to HC.

Presence of gastrointestinal disease significantly impacted the fecal microbial communities, as indicated by the healthy dogs clustering separately from disease groups on the PCoA plots (Figure 4.2). ANOSIM test revealed significant differences in microbial communities of dogs with AD, AHDS, and IBD when compared to healthy control dogs ($P_{unweighted} \leq 0.002$, $P_{weighted} = 0.001$; Table 4.2).

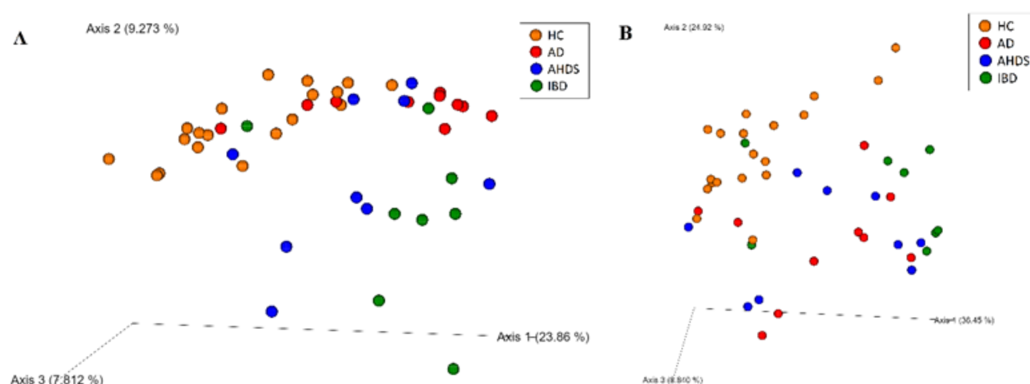

Figure 4.2. PCoA plots of fecal microbial communities at baseline from the dogs with AD, AHDS, IBD, and the healthy controls. A) 3D plot of unweighted UniFrac distances. B) 3D plot of weighted UniFrac distances.

Table 4.2. ANOSIM results for unweighted and weighted distance matrices between healthy control dogs (HC) and dogs with acute diarrhea (AD), acute hemorrhagic diarrhea (AHDS), and inflammatory bowel disease (IBD) at baseline

Groups compared	Unweighted ANOSIM		Weighted ANOSIM	
	R statistic	p-value	R statistic	p-value
HC, AD	0.225	0.002	0.525	0.001
HC, AHDS	0.244	0.002	0.543	0.001
HC, IBD	0.533	0.001	0.577	0.001

Based on univariate statistics (Table 4.3), all diseased groups had decreased abundance of phylum Bacteroidetes, driven by decreases in genera *Bacteroides* and *Prevotella,* compared to healthy dogs. Other short chain fatty acid (SCFA)-producing

genera were also decreased in intestinal disease, including *Faecalibacterium* (IBD), *Megamonas* (AD and AHDS), and an unclassified Lachnospiraceae genus (AD, AHDS, and IBD). Dogs with IBD had increased *Enterococcus*, dogs with AD had increased *Lactobacillus*, and both dogs with AHDS and IBD had increased *Streptococcus* compared to healthy dogs. Several families of bacteria within the Proteobacteria phylum were also altered in disease. Abundance of Helicobacteraceae were decreased in dogs with AD, abundance of Succinivibrionaceae were decreased in dogs with AD and IBD, abundance of Enterobacteriaceae were increased in dogs with AD, and abundance of Pasteurellaceae were increased in dogs with AHDS.

Table 4.3. Relative percentages of the most abundant bacterial groups for baseline of dogs with acute diarrhea (AD), acute hemorrhagic diarrhea (AHDS), and inflammatory bowel disease (IBD), as well as healthy controls (HC). Medians not sharing a common superscript differ (P<0.05).

Taxa	HC		AD		AHDS		IBD		HC vs AD vs AHDS vs IBD	
	Median	Range	Median	Range	Median	Range	Median	Range	P value*	Q value**
Actinobacteria	$1.38^{a,b}$	0-9.12	0.06^{a}	0-3.85	$0.63^{a,b}$	0-5.71	1.6^{b}	0.3-9.69	0.035	0.088
Actinobacteria (class)	0^{b}	0-4.56	0^{b}	0-0	$0.11^{a,b}$	0-0.52	0.56^{a}	0-1.34	0.001	0.005
Actinomycetales	0^{a}	0-0	$0^{a,c}$	0-0	$0.11^{b,c}$	0-0.52	0.43^{b}	0-1.34	<0.001	0.001
Corynebacteriaceae	0^{b}	0-0	0^{b}	0-0	$0^{a,b}$	0-0.16	0.06^{a}	0-0.31	0.001	0.004
Corynebacterium	0^{b}	0-0	0^{b}	0-0	$0^{a,b}$	0-0.16	0.06^{a}	0-0.31	0.001	0.005
Unclassified‡	0^{b}	0-0	0^{b}	0-0	$0^{a,b}$	0-0.16	0.06^{a}	0-0.31	0.001	0.005
Coriobacteriia	1.35	0-4.56	0.06	0-3.85	0.18	0-5.28	0.72	0-8.65	0.145	0.181
Coriobacteriales	1.35	0-4.56	0.06	0-3.85	0.18	0-5.28	0.72	0-8.65	0.145	0.171
Coriobacteriaceae	1.35	0-4.56	0.06	0-3.85	0.18	0-5.28	0.72	0-8.65	0.145	0.160
Collinsella	1.26	0-4.56	0.03	0-3.85	0.18	0-5.15	0.72	0-8.65	0.121	0.171
stercoris	1.08	0-3.84	0.03	0-3.85	0.17	0-5.15	0.72	0-8.65	0.143	0.199
Slackia	0	0-0.24	0	0-0.45	0	0-0.33	0	0-0.84	0.739	0.739
Unclassified	0	0-0.24	0	0-0.45	0	0-0.33	0	0-0.84	0.739	0.739
Bacteroidetes	28.41^{a}	10.31-44.71	0.41^{b}	0-24.81	4.39^{b}	0.27-26.39	0.44^{b}	0-35.14	<0.001	0.001
Bacteroidia	28.41^{a}	10.31-44.71	$0.41^{a,b}$	0-24.81	$4.39^{a,b}$	0.27-26.39	$0.28^{a,b}$	0-35.14	<0.001	0.001
Bacteroidales	28.41^{a}	10.31-44.71	0.41^{b}	0-24.81	4.39^{b}	0.27-26.39	0.28^{b}	0-35.14	<0.001	0.001
Bacteroidaceae	20.82^{a}	0.85-44.59	0.41^{b}	0-13.47	4.03^{b}	0.27-11.6	0.27^{b}	0-32.99	<0.001	0.001
Bacteroides	20.82^{a}	0.85-44.59	0.41^{b}	0-13.47	4.03^{b}	0.27-11.6	0.27^{b}	0-32.99	<0.001	0.001
Other‡‡	5.2^{a}	0-30.38	0.13^{a}	0-9.3	0.74^{a}	0-2.9	0^{a}	0-15.88	0.046	0.078
Unclassified	8.06^{a}	0-40.45	0.22^{b}	0-6.47	$2.73^{a,b}$	0-10.85	0.04^{b}	0-9.95	<0.001	0.002
plebeius	2.85^{a}	0-11.85	0^{b}	0-0.19	0^{b}	0-0.4	$0.03^{a,b}$	0-7.33	<0.001	0.001

Table 4.3. Continued

Taxa	HC		AD		AHDS		IBD		HC vs AD vs AHDS vs IBD	
	Median	Range	Median	Range	Median	Range	Median	Range	P value*	Q value**
Prevotellaceae	3.75[a]	0-10.74	0[b]	0-2.24	0[a,b]	0-0.17	0[b]	0-0.86	0.001	0.004
Prevotella	3.75[a]	0-10.74	0[b]	0-2.24	0[a,b]	0-0.17	0[b]	0-0.86	0.001	0.005
copri	3.75[a]	0-10.74	0[b]	0-2.24	0[b]	0-0.17	0[b]	0-0.86	0.001	0.003
[Paraprevotellaceae][‡‡‡]	2.01	0-8.51	0	0-11.34	0.26	0-7.56	0	0-1.29	0.080	0.098
[Prevotella]	2.01	0-8.51	0	0-11.34	0.26	0-7.56	0	0-1.29	0.062	0.095
Unclassified	2.01	0-8.51	0	0-11.34	0.26	0-7.56	0	0-1.29	0.062	0.096
Firmicutes	36.29	15.8-85.83	58.93	13.75-84.75	63.81	12.74-98.81	74.58	27.31-92.09	0.082	0.137
Bacilli	0.78	0-5.19	0.76	0.17-56.5	1.43	0.23-43.97	10.58	0.56-74.62	0.073	0.145
Lactobacillales	0.21[a]	0-2.66	0.71[a,b]	0.17-56.28	0.76[a,b]	0.23-43.14	9.9[b]	0.56-73.81	0.001	0.002
Enterococcaceae	0[b]	0-0.99	0.27[a,b]	0-3.91	0[b]	0-2.21	1.08[a]	0.21-71.85	<0.001	0.001
Other	0[b]	0-0	0[b]	0-0	0[b]	0-0	0.2[a]	0-3.34	<0.001	0.001
Other	0[b]	0-0	0[b]	0-0	0[b]	0-0	0.2[a]	0-3.34	<0.001	0.001
Enterococcus	0[a]	0-0.99	0.27[a,b]	0-3.91	0[a,b]	0-2.21	1.08[b]	0-68.51	0.001	0.005
Unclassified	0[a]	0-0.99	0.27[a,b]	0-3.91	0[a,b]	0-2.21	0.58[b]	0-68.51	0.006	0.016
Lactobacillaceae	0[b]	0-2.29	0.27[a]	0-55.8	0[b]	0-0	0.15[a,b]	0-0.96	0.001	0.003
Lactobacillus	0[b]	0-2.29	0.27[a]	0-55.8	0[b]	0-0	0.06[a,b]	0-0.96	0.001	0.005
Unclassified	0[b]	0-2.29	0.27[a]	0-55.8	0[b]	0-0	0.06[a,b]	0-0.96	0.001	0.004
Streptococcaceae	0.14[a]	0-1.4	0.07[a,b]	0-15.9	0.76[b]	0.23-42.56	0.75[b]	0.29-33.23	0.002	0.006
Streptococcus	0.14[a]	0-1.4	0.07[a,b]	0-15.9	0.44[b]	0.23-42.56	0.75[b]	0.29-33.23	0.002	0.007
Unclassified	0.14[a]	0-1.4	0.07[a,b]	0-15.9	0.44[b]	0.23-42.56	0.75[b]	0.29-33.23	0.002	0.009
Turicibacterales	0.17	0-2.62	0	0-4	0	0-1.08	0.1	0-0.71	0.210	0.227
Turicibacteraceae	0.17	0-2.62	0	0-4	0	0-1.08	0.1	0-0.71	0.210	0.220
Turicibacter	0.17	0-2.62	0	0-4	0	0-1.08	0.1	0-0.71	0.210	0.255

Table 4.3. Continued

Taxa	HC		AD		AHDS		IBD		HC vs AD vs AHDS vs IBD	
	Median	Range	Median	Range	Median	Range	Median	Range	P value*	Q value**
Unclassified	0.17	0-2.62	0	0-4	0	0-1.08	0.1	0-0.71	0.210	0.269
Clostridia	31.14	14.24-57.79	36.47	11.85-73.86	32.21	11.97-97.23	33.98	17.4-82.58	0.564	0.627
Clostridiales	31.14	14.24-57.79	36.47	11.85-73.86	32.21	11.97-97.23	33.98	17.4-82.58	0.564	0.564
Unclassified	0.32[a]	0-1.39	0[b]	0-0.59	0[a,b]	0-1.81	0[b]	0-0.55	0.007	0.014
Unclassified	0.32[a]	0-1.39	0[b]	0-0.59	0[a,b]	0-1.81	0[b]	0-0.55	0.007	0.016
Unclassified	0.32[a]	0-1.39	0[b]	0-0.59	0[a,b]	0-1.81	0[b]	0-0.55	0.007	0.017
Clostridiaceae	7.95[a]	1.87-16.9	12.96[a,b]	3.87-47.07	18.06[b]	2.86-92.37	12.28[a,b]	5.81-56.96	0.025	0.035
Other	5.75	0.99-14.21	6.26	0-21.46	2.71	0-37.3	7.28	0.97-43.74	0.530	0.546
Other	5.75	0.99-14.21	6.26	0-21.46	2.71	0-37.3	7.28	0.97-43.74	0.530	0.559
Unclassified	1.6	0-5.49	2.72	0-8.26	2.31	0-7.27	2.86	0.87-11.69	0.227	0.266
Unclassified	1.6	0-5.49	2.72	0-8.26	2.31	0-7.27	2.86	0.87-11.69	0.227	0.276
Clostridium	0.45[a]	0-4.13	3.26[b]	0-28.39	10.36[b]	0.11-87.95	3.47[a,b]	0.22-11.26	<0.001	0.003
Other	0.36[a]	0-3.87	2.17[b]	0-21.49	8.61[b]	0-87.95	2.01[a,b]	0-9.49	0.001	0.003
Unclassified	0[a]	0-0.26	0[a,b]	0-3.86	0[a,b]	0-2.99	0.48[b]	0-1.98	0.012	0.026
Lachnospiraceae	12.82	2.34-25.68	9.37	2.48-38.7	3.83	0-45.28	18.88	2.9-27.81	0.257	0.257
Other	2.43	0.21-6.63	3.05	0.89-14.24	2.23	0-11.75	4.14	0.31-9.31	0.248	0.281
Other	2.43	0.21-6.63	3.05	0.89-14.24	2.23	0-11.75	4.14	0.31-9.31	0.248	0.293
Unclassified	2.17[a]	0-3.9	0[b]	0-2.09	0[b]	0-1.64	0[b]	0-4.27	<0.001	0.003
Unclassified	2.17[a]	0-3.9	0[b]	0-2.09	0[b]	0-1.64	0[b]	0-4.27	<0.001	0.002
Blautia	4.18	1.14-12.4	3.78	0-16.84	0.57	0-26.66	5.63	0.33-16.9	0.346	0.367
Unclassified	1.12	0-6.34	0	0-4.58	0	0-3.02	0	0-4.72	0.171	0.230
producta	3.37	1.14-9.56	3.44	0-12.32	0	0-26.66	4.31	0.33-14.2	0.214	0.269
Dorea	0	0-2.21	0.13	0-2.28	0.3	0-1.33	0.54	0-2.42	0.273	0.299

Table 4.3. Continued

Taxa	HC		AD		AHDS		IBD		HC vs AD vs AHDS vs IBD	
	Median	Range	Median	Range	Median	Range	Median	Range	P value*	Q value**
Unclassified	0	0-2.21	0.13	0-2.28	0.3	0-1.33	0.54	0-2.42	0.273	0.313
Epulopiscium	0[b]	0-0.84	0.08[a]	0-1.59	0[b]	0-0	0[a,b]	0-1.47	0.003	0.008
Unclassified	0[b]	0-0.84	0.08[a]	0-1.59	0[b]	0-0	0[a,b]	0-1.47	0.003	0.009
[Ruminococcus]	2.76	0.43-4.76	1.95	0-12.41	0.96	0-8.87	3.26	0.31-11.33	0.191	0.243
gnavus	2.3	0-4.76	1.54	0-12.41	0.96	0-8.87	2.07	0.31-11.33	0.287	0.319
Peptostreptococcaceae	0[a]	0-4.75	3.12[b]	0-10.77	0.92[a,b]	0-5.94	1.06[a,b]	0-4.26	0.010	0.017
Unclassified	0[a]	0-4.75	3.12[b]	0-10.77	0.92[a,b]	0-5.94	1.06[a,b]	0-4.26	0.008	0.016
Unclassified	0[a]	0-4.75	3.12[b]	0-10.77	0.92[a,b]	0-5.94	1.06[a,b]	0-4.26	0.008	0.017
Ruminococcaceae	3.29[a]	0.1-10.88	0.2[b]	0-4.41	0.4[a,b]	0-4.52	0[b]	0-5.11	0.002	0.007
Unclassified	0.61[a]	0-3.45	0[a]	0-2.27	0.26[a]	0-1.63	0[a]	0-1.64	0.029	0.056
Unclassified	0.61[a]	0-3.45	0[a]	0-2.27	0.26[a]	0-1.63	0[a]	0-1.64	0.029	0.057
Faecalibacterium	2.96[a]	0-9.38	0[a,b]	0-3.68	0.03[a,b]	0-2.89	0[b]	0-3.47	0.024	0.048
prausnitzii	2.96[a]	0-9.38	0[a,b]	0-3.68	0.03[a,b]	0-2.89	0[b]	0-3.47	0.024	0.049
Veillonellaceae	4.34[a]	0.06-9.69	0[b]	0-2.48	0.15[b]	0-0.32	0.08[a,b]	0-8.67	<0.001	0.001
Megamonas	3.2[a]	0.06-7.48	0[b]	0-2.48	0.1[b]	0-0.27	0.06[a,b]	0-8.67	<0.001	0.001
Unclassified	3.2[a]	0.06-7.48	0[b]	0-2.48	0.1[b]	0-0.27	0.02[b]	0-8.67	<0.001	0.001
Phascolarctobacterium	0.18[a]	0-1.53	0[b]	0-0.45	0[a,b]	0-0.15	0[b]	0-0	0.005	0.012
Unclassified	0.18[a]	0-1.53	0[b]	0-0.45	0[a,b]	0-0.15	0[b]	0-0	0.005	0.014
Erysipelotrichi	5[a]	0.28-40.85	1.51[a,b]	0.2-8.76	0.17[b]	0-5.83	3.02[a,b]	0.07-20.22	0.018	0.045
Erysipelotrichales	5[a]	0.28-40.85	1.51[a,b]	0.2-8.76	0.17[b]	0-5.83	3.02[a,b]	0.07-20.22	0.018	0.033
Erysipelotrichaceae	5[a]	0.28-40.85	1.51[a,b]	0.2-8.76	0.17[b]	0-5.83	3.02[a,b]	0.07-20.22	0.018	0.028
Unclassified	0.48	0-3.36	0.12	0-2.69	0	0-2.07	0.06	0-0.99	0.054	0.087
Unclassified	0.48	0-3.36	0.12	0-2.69	0	0-2.07	0.06	0-0.99	0.054	0.087

Table 4.3. Continued

Taxa	HC		AD		AHDS		IBD		HC vs AD vs AHDS vs IBD	
	Median	Range	Median	Range	Median	Range	Median	Range	P value*	Q value**
Catenibacterium	0.61	0-34.6	0	0-6.07	0	0-0.18	0.25	0-20.22	0.082	0.121
Unclassified	0.61	0-34.6	0	0-6.07	0	0-0.18	0.25	0-20.22	0.082	0.122
[Eubacterium]	0.75	0-6.24	0.18	0-3.03	0	0-5.45	0.43	0-1.74	0.193	0.243
biforme	0.07	0-6.24	0	0-0.89	0	0-0.44	0.09	0-1.72	0.477	0.516
dolichum	0.04	0-1.05	0	0-3.03	0	0-5.33	0.01	0-0.91	0.683	0.701
Fusobacteria	23.65	1.01-44.81	25.41	0.69-70.44	24.18	0.24-69.22	3.14	0-57.25	0.126	0.157
Fusobacteriia	23.65	1.01-44.81	25.41	0.69-70.44	24.18	0.24-69.22	3.14	0-57.25	0.126	0.181
Fusobacteriales	23.65	1.01-44.81	25.41	0.69-70.44	24.18	0.24-69.22	3.14	0-57.25	0.126	0.168
Fusobacteriaceae	23.65	1.01-44.81	25.41	0.69-70.44	24.18	0.24-69.22	3.14	0-57.25	0.126	0.146
Fusbacterium	23.65	0.96-44.81	18.68	0.69-70.44	24.18	0.24-69.06	3.14	0-57.25	0.143	0.194
Unclassified	23.65	0.96-44.81	18.68	0.69-70.44	24.18	0.24-69.06	3.14	0-57.25	0.143	0.199
Proteobacteria	5.95	0.29-15.45	5.17	0.5-46.13	5.72	0.17-34.73	4.74	0-36.96	0.948	0.948
Betaproteobacteria	3.16	0-7.52	0.14	0-8.05	0.46	0-6.58	0.27	0-4.38	0.130	0.181
Burkholderiales	3.16	0-7.52	0.14	0-8.05	0.46	0-6.58	0.27	0-4.38	0.130	0.168
Alcaligenaceae	3.16[a]	0-7.52	0.14[a,b]	0-8.05	0.46[a,b]	0-6.58	0.09[b]	0-4.38	0.044	0.057
Sutterella	3.16[a]	0-7.52	0.14[a,b]	0-8.05	0.46[a,b]	0-6.58	0[b]	0-4.38	0.035	0.062
Unclassified	3.16[a]	0-7.52	0.14[a,b]	0-8.05	0.46[a,b]	0-6.58	0[b]	0-4.38	0.035	0.065
Epsilonproteobacteria	0.86[a]	0-4.27	0[b]	0-0.56	0[a,b]	0-4.06	0[a,b]	0-1.27	0.002	0.005
Campylobacterales	0.86[a]	0-4.27	0[b]	0-0.56	0[a,b]	0-4.06	0[a,b]	0-1.27	0.002	0.005
Helicobacteraceae	0.78[a]	0-3.57	0[b]	0-0	0[a,b]	0-4.06	0[a,b]	0-1.27	0.005	0.012
Helicobacter	0.78[a]	0-3.57	0[b]	0-0	0[a,b]	0-4.06	0[a,b]	0-1.27	0.005	0.012
Unclassified	0.78[a]	0-3.57	0[b]	0-0	0[a,b]	0-2.9	0[a,b]	0-0.65	0.004	0.013
Gammaproteobacteria	1.34	0-8.19	1.85	0.24-46.05	0.68	0-34.47	0.71	0-36.62	0.694	0.694

Table 4.3. Continued

Taxa	HC		AD		AHDS		IBD		HC vs AD vs AHDS vs IBD	
	Median	Range	Median	Range	Median	Range	Median	Range	P value*	Q value**
Aeromonadales	0.19[a]	0-5.66	0[b]	0-0	0[a,b]	0-4.52	0[b]	0-0	0.006	0.015
Succinivibrionaceae	0.19[a]	0-5.66	0[b]	0-0	0[a,b]	0-4.52	0[b]	0-0	0.006	0.013
Enterobacteriales	0.1[a]	0-8.19	1.85[b]	0.24-46.05	0.17[a,b]	0-34.43	0.71[a,b]	0-36.62	0.023	0.037
Enterobacteriaceae	0.1[a]	0-8.19	1.85[b]	0.24-46.05	0.17[a,b]	0-34.43	0.71[a,b]	0-36.62	0.023	0.033
Unclassified	0.1[a]	0-8.19	1.85[b]	0.24-45.45	0.17[a,b]	0-34.15	0.67[a,b]	0-36.62	0.038	0.064
Unclassified	0.1[a]	0-8.19	1.85[b]	0.24-45.45	0.17[a,b]	0-34.15	0.67[a,b]	0-36.62	0.038	0.067
Pasteurellales	0[b]	0-0.01	0[b]	0-0	0[a]	0-9.63	0[a,b]	0-0.33	0.012	0.026
Pasteurellaceae	0[b]	0-0.01	0[b]	0-0	0[a]	0-9.63	0[a,b]	0-0.33	0.012	0.021
Other	0[b]	0-0	0[b]	0-0	0[a]	0-8.09	0[a,b]	0-0.33	0.003	0.009
Other	0[b]	0-0	0[b]	0-0	0[a]	0-8.09	0[a,b]	0-0.33	0.003	0.010

*P values obtained with Kruskal Wallis testing.

**Q values are the adjusted P values after Benjamini and Hochberg False discovery rate correction for multiple comparisons.

‡Unclassified=matches (97%) similarity to a reference sequence undefined at the given taxonomic level.

‡‡Other=ambiguous assignment because QIIME cannot distinguish between taxa within that taxonomic level.

‡‡‡Square brackets=proposed taxonomic grouping according to the Greengenes database used in the QIIME pipeline.

Highlighted cells indicate significant P or Q values.

4.2.1.2. AD over time

Alpha diversity, described by Chao 1, observed ASVs (Figure 4.3), and Shannon Index, was significantly different between all AD timepoints and HC ($q<0.05$). *Post hoc* analysis revealed that all three measures of alpha diversity were significantly lower in AD0 and AD30 when compared to HC. When comparing follow up samples with the AD baseline, no significant changes were observed over time.

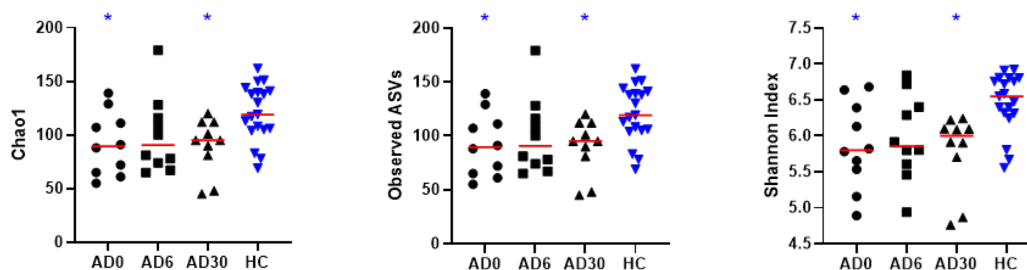

Figure 4.3. Chao1, Observed ASVs, and Shannon Index in acute diarrhea (AD) over time compared to healthy controls (HC) and compared between timepoints. Groups significantly different ($p<0.05$, Kruskal-Wallis testing with Dunn's *post hoc* tests) from HC are indicated with blue asterisks. There were no differences between baseline acute diarrhea (AD0) and follow up time points at day 6 (AD6) and day 30 (AD30; Friedman testing with Dunn's *post hoc* tests).

ANOSIM test of PCoA plots (Figure 4.4) revealed significant differences between HC and AD0 ($P_{unweighted, weighted}=0.001$, $R_{unweighted}=0.492$, $R_{weighted}=0.525$), AD6 ($P_{unweighted}=0.001$, $P_{weighted}=0.002$, $R_{unweighted}=0.401$, $R_{weighted}=0.324$), and AD30 ($P_{unweighted, weighted}=0.001$, $R_{unweighted}=0.378$, $R_{weighted}=0.481$), but not differences between AD0 and subsequent time points. Based on univariate statistics (Table 4.4), follow up timepoints at day 6 and day 30 were not significantly different from baseline, for dogs with AD. However, differences in bacterial taxa between healthy controls and follow up

timepoints persisted to day 6 (e.g., *Enterococcus*) and day 30 (e.g., *Bacteroides*, *Lactobacillus*, *Megamonas*). Several families of bacteria within the Firmicutes phylum were altered in the dogs with AD compared to HC. Abundance of Clostridiaceae were increased at day 6 and day 30, abundance of Peptostreptococcaceae were increased at day 0 and day 6, and abundance of Ruminococcaceae and Veillonellaceae were decreased at all time points in dogs with AD compared to HC. Within the phylum Proteobacteria, abundance of the genus *Sutterella* was decreased at day 6 and day 30 in dogs with AD compared to HC. Furthermore, abundance of the family Enterobacteriaceae was significantly increased at all time points in dogs with AD compared to HC dogs.

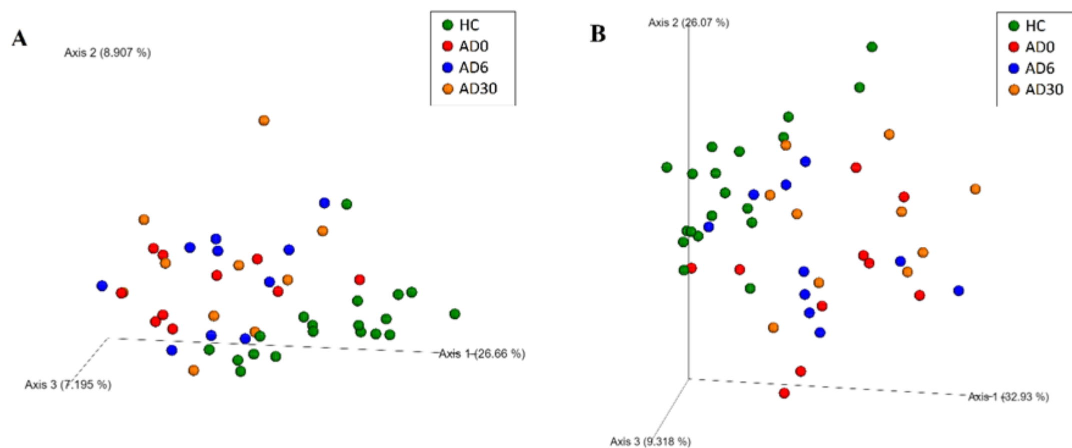

Figure 4.4. PCoA plots of fecal microbial communities from dogs with acute diarrhea (AD) over time and healthy controls (HC). A) 3D plot of unweighted UniFrac distances. B) 3D plot of weighted UniFrac distances.

Table 4.4. Relative percentages of the most abundant bacterial groups for healthy control dogs (HC) and dogs with acute diarrhea (AD) over time. Medians with asterisks were significantly different from healthy controls (Kruskal Wallis; q<0.05). Follow up timepoints were not significantly different from baseline within the acute diarrhea group (Friedman; q>0.05).

Taxa	HC		AD0		AD6		AD30		AD0 vs AD6, AD30		HC vs AD0, AD6, AD30	
	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Actinobacteria	1.38	0-9.12	0.06	0-3.85	0.64	0-6.19	0.85	0-6	0.252	0.999	0.168	0.257
Coriobacteriia	1.35	0-4.56	0.06	0-3.85	0.64	0-6.19	0.85	0-3.27	0.221	0.999	0.173	0.257
Coriobacteriales	1.35	0-4.56	0.06	0-3.85	0.64	0-6.19	0.85	0-3.27	0.221	0.999	0.173	0.257
Coriobacteriaceae	1.35	0-4.56	0.06	0-3.85	0.64	0-6.19	0.85	0-3.27	0.221	0.999	0.173	0.257
Collinsella	1.26	0-4.56	0.03	0-3.85	0.48	0-6.19	0.85	0-3.27	0.221	0.999	0.126	0.209
stercoris	1.08	0-3.84	0.03	0-3.85	0.48	0-6.19	0.85	0-3.27	0.221	0.999	0.146	0.239
Bacteroidetes	28.41	10.31-44.71	0.41*	0-24.81	3.5*	0-24.86	4.59*	0-39.65	0.897	0.999	<0.001	0.005
Bacteroidia	28.41	10.31-44.71	0.41*	0-24.81	3.5*	0-24.86	4.59*	0-38.58	0.897	0.999	<0.001	0.005
Bacteroidales	28.41	10.31-44.71	0.41*	0-24.81	3.5*	0-24.86	4.59*	0-38.58	0.897	0.999	<0.001	0.005
Bacteroidaceae	20.82	0.85-44.59	0.41*	0-13.47	3.5*	0-24.86	4.47*	0-38.58	0.806	0.999	<0.001	0.005
Bacteroides	20.82	0.85-44.59	0.41*	0-13.47	3.5*	0-24.86	4.47*	0-38.58	0.806	0.999	<0.001	0.005
Other	5.2	0-30.38	0.13	0-9.3	0.45	0-7.35	0.46	0-9.9	0.587	0.999	0.151	0.244
Unclassified	8.06	0-40.45	0.22*	0-6.47	1.41	0-16.43	1.95*	0-34.93	0.740	0.999	0.002	0.007
plebeius	2.85	0-11.85	0*	0-0.19	0*	0-5.77	0*	0-0	0.778	0.999	<0.001	0.005
Prevotellaceae	3.75	0-10.74	0*	0-2.24	0	0-7.39	0	0-6.7	0.653	0.999	0.007	0.015
Prevotella	3.75	0-10.74	0*	0-2.24	0	0-7.39	0	0-6.7	0.653	0.999	0.007	0.015
copri	3.75	0-10.74	0*	0-2.24	0	0-7.39	0	0-6.7	0.653	0.999	0.007	0.015
[Paraprevotellaceae]	2.01	0-8.51	0	0-11.34	0	0-2.81	0*	0-1.23	0.241	0.999	0.018	0.036
[Prevotella]	2.01	0-8.51	0	0-11.34	0*	0-1.05	0*	0-1.23	0.407	0.999	0.007	0.015
Unclassified	2.01	0-8.51	0	0-11.34	0*	0-1.05	0*	0-1.23	0.407	0.999	0.007	0.015

Table 4.4. Continued.

Taxa	HC		AD0		AD6		AD30		AD0 vs AD6, AD30		HC vs AD0, AD6, AD30	
	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Firmicutes	36.29	15.8-85.83	58.93	13.75-84.75	50.2	31.96-92.07	63.38*	15.66-97.97	0.830	0.999	0.042	0.075
Bacilli	0.78	0-5.19	0.76	0.17-56.5	1.97	0.59-28.26	2.1	0.51-70.43	0.710	0.999	0.162	0.254
Lactobacillales	0.21	0-2.66	0.71*	0.17-56.28	1.09*	0.59-28.1	1.38*	0.17-69.7	0.830	0.999	<0.001	0.005
Enterococcaceae	0	0-0.99	0.27*	0-3.91	0.45*	0-20.57	0.09	0-1.55	0.330	0.999	0.001	0.005
Enterococcus	0	0-0.99	0.27*	0-3.91	0.45*	0-18.43	0.09	0-1.55	0.330	0.999	0.005	0.013
Unclassified	0	0-0.99	0.27*	0-3.91	0.45*	0-18.43	0.09	0-0.73	0.330	0.999	0.005	0.013
Lactobacillaceae	0	0-2.29	0.27*	0-55.8	0.15	0-3.75	0.31*	0-51.65	0.741	0.999	0.001	0.005
Lactobacillus	0	0-2.29	0.27*	0-55.8	0.15	0-3.75	0.31*	0-51.65	0.741	0.999	0.001	0.005
Unclassified	0	0-2.29	0.27*	0-55.8	0.15	0-3.75	0.31*	0-51.65	0.741	0.999	0.001	0.005
Streptococcaceae	0.14	0-1.4	0.07	0-15.9	0.34	0-3.78	0.47	0-63.64	0.282	0.999	0.332	0.416
Streptococcus	0.14	0-1.4	0.07	0-15.9	0.23	0-2.03	0.29	0-63.64	0.212	0.999	0.615	0.697
Unclassified	0.14	0-1.4	0.07	0-15.9	0.23	0-2.03	0.29	0-63.64	0.212	0.999	0.615	0.697
Turicibacterales	0.17	0-2.62	0	0-4	0.15	0-3.03	0.19	0-1.88	0.845	0.999	0.633	0.697
Turicibacteraceae	0.17	0-2.62	0	0-4	0.15	0-3.03	0.19	0-1.88	0.845	0.999	0.633	0.697
Turicibacter	0.17	0-2.62	0	0-4	0.15	0-3.03	0.19	0-1.88	0.845	0.999	0.633	0.697
Unclassified	0.17	0-2.62	0	0-4	0.15	0-3.03	0.19	0-1.88	0.845	0.999	0.633	0.697
Clostridia	31.14	14.24-57.79	36.47	11.85-73.86	45.36	4.89-86.98	53.88	14.8-88.34	0.710	0.999	0.071	0.123
Clostridiales	31.14	14.24-57.79	36.47	11.85-73.86	45.36	4.89-86.98	53.88	14.8-88.34	0.710	0.999	0.071	0.123
Unclassified	0.32	0-1.39	0*	0-0.59	0*	0-0.48	0	0-0.59	0.309	0.999	0.003	0.010
Unclassified	0.32	0-1.39	0*	0-0.59	0*	0-0.48	0	0-0.59	0.309	0.999	0.003	0.010
Unclassified	0.32	0-1.39	0*	0-0.59	0*	0-0.48	0	0-0.59	0.309	0.999	0.003	0.010
Clostridiaceae	7.95	1.87-16.9	12.96	3.87-47.07	16.71*	3.98-46.72	21.51*	7.46-74.58	0.368	0.999	0.004	0.011
Other	5.75	0.99-14.21	6.26	0-21.46	11.1	1.23-24.46	8.88	2.42-34.66	0.974	0.999	0.186	0.269

Table 4.4. Continued.

Taxa	HC		AD0		AD6		AD30		AD0 vs AD6, AD30		HC vs AD0, AD6, AD30	
	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Other	5.75	0.99-14.21	6.26	0-21.46	11.1	1.23-24.46	8.88	2.42-34.66	0.974	0.999	0.186	0.269
Unclassified	1.6	0-5.49	2.72	0-8.26	2.75*	0.99-9.18	2.99*	0.71-10.64	0.974	0.999	0.026	0.051
Unclassified	1.6	0-5.49	2.72	0-8.26	2.75*	0.99-9.18	2.99*	0.71-10.64	0.974	0.999	0.026	0.051
Clostridium	0.45	0-4.13	3.26*	0-28.39	0.48	0-40.84	1.02	0-66.82	0.316	0.999	0.028	0.054
Other	0.36	0-3.87	2.17*	0-21.49	0.48	0-33.77	0.86	0-54.39	0.436	0.999	0.017	0.035
Unclassified	0	0-0.26	0	0-3.86	0.1	0-7.07	0	0-9.92	0.847	0.999	0.035	0.066
Lachnospiraceae	12.82	2.34-25.68	9.37	2.48-38.7	18.4	0.83-38.62	16.1	0.5-37.36	0.710	0.999	0.767	0.818
Other	2.43	0.21-6.63	3.05	0.89-14.24	7.6	0-14.12	7.75	0-10.94	0.601	0.999	0.275	0.349
Other	2.43	0.21-6.63	3.05	0.89-14.24	7.6	0-14.12	7.75	0-10.94	0.601	0.999	0.275	0.349
Unclassified	2.17	0-3.9	0*	0-2.09	0.28*	0-1.89	0.58*	0-1.83	0.406	0.999	<0.001	0.005
Unclassified	2.17	0-3.9	0*	0-2.09	0.28*	0-1.89	0.58*	0-1.83	0.406	0.999	<0.001	0.005
Blautia	4.18	1.14-12.4	3.78	0-16.84	6.31	0-13.51	4.71	0-15.49	0.974	0.999	0.921	0.921
Unclassified	1.12	0-6.34	0	0-4.58	1.11	0-4.35	0.31	0-2.41	0.117	0.999	0.156	0.248
producta	3.37	1.14-9.56	3.44	0-12.32	5.85	0-12.13	3.5	0-15.49	0.897	0.999	0.784	0.828
Dorea	0	0-2.21	0.13	0-2.28	0	0-2.63	0	0-2.78	0.477	0.999	0.876	0.884
Unclassified	0	0-2.21	0.13	0-2.28	0	0-2.63	0	0-2.78	0.477	0.999	0.876	0.884
Epulopiscium	0	0-0.84	0.08*	0-1.59	0[a]	0-0.26	0[a]	0-0.84	0.025	0.999	0.010	0.021
Unclassified	0	0-0.84	0.08*	0-1.59	0[a]	0-0.26	0[a]	0-0.84	0.025	0.999	0.010	0.021
[Ruminococcus]	2.76	0.43-4.76	1.95	0-12.41	2.96	0.46-9.33	1.87	0-12.41	0.135	0.999	0.809	0.846
Other	0	0-2.07	0	0-0.93	0.09	0-0.78	0	0-2.31	0.140	0.999	0.534	0.629
gnavus	2.3	0-4.76	1.54	0-12.41	2.65	0-8.54	1.22	0-12.14	0.187	0.999	0.820	0.849
Peptostreptococcaceae	0	0-4.75	3.12*	0-10.77	1.68*	0-9.45	0.86	0-12.7	0.874	0.999	0.005	0.013

Table 4.4. Continued.

Taxa	HC		AD0		AD6		AD30		AD0 vs AD6, AD30		HC vs AD0, AD6, AD30	
	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Unclassified	0	0-4.75	3.12*	0-10.77	1.68	0-9.45	0.8	0-12.7	0.874	0.999	0.005	0.013
Unclassified	0	0-4.75	3.12*	0-10.77	1.68	0-9.45	0.8	0-12.7	0.874	0.999	0.005	0.013
Ruminococcaceae	3.29	0.1-10.88	0.2*	0-4.41	0.24*	0-3.45	0.1*	0-2.9	0.488	0.999	0.002	0.007
Unclassified	0.61	0-3.45	0*	0-2.27	0.17	0-1.13	0.1*	0-1.04	0.862	0.999	0.007	0.015
Unclassified	0.61	0-3.45	0*	0-2.27	0.17	0-1.13	0.1*	0-1.04	0.862	0.999	0.007	0.015
Faecalibacterium	2.96	0-9.38	0	0-3.68	0.02	0-3.1	0*	0-2.66	0.597	0.999	0.041	0.075
prausnitzii	2.96	0-9.38	0	0-3.68	0.02	0-3.1	0*	0-2.66	0.597	0.999	0.041	0.075
Veillonellaceae	4.34	0.06-9.69	0*	0-2.48	0.07*	0-7.2	0.03*	0-17.3	0.542	0.999	<0.001	0.005
Megamonas	3.2	0.06-7.48	0*	0-2.48	0.03*	0-6.93	0.03*	0-17.3	0.851	0.999	<0.001	0.005
Unclassified	3.2	0.06-7.48	0*	0-2.48	0.03*	0-6.93	0.03*	0-17.3	0.851	0.999	<0.001	0.005
Phascolarctobacterium	0.18	0-1.53	0*	0-0.45	0	0-0.27	0*	0-0	0.667	0.999	0.002	0.007
Unclassified	0.18	0-1.53	0*	0-0.45	0	0-0.27	0*	0-0	0.667	0.999	0.002	0.007
Erysipelotrichi	5	0.28-40.85	1.51	0.2-8.76	3	0.1-11.87	1.32	0.32-19.06	0.436	0.999	0.202	0.269
Erysipelotrichales	5	0.28-40.85	1.51	0.2-8.76	3	0.1-11.87	1.32	0.32-19.06	0.436	0.999	0.202	0.269
Erysipelotrichaceae	5	0.28-40.85	1.51	0.2-8.76	3	0.1-11.87	1.32	0.32-19.06	0.436	0.999	0.202	0.269
Unclassified	0.48	0-3.36	0.12	0-2.69	0	0-3.44	0.41	0-1.79	0.260	0.999	0.339	0.416
Unclassified	0.48	0-3.36	0.12	0-2.69	0	0-3.44	0.41	0-1.79	0.260	0.999	0.339	0.416
Allobaculum	0	0-4.39	0	0-1.39	0.06	0-3.03	0.22	0-3.8	0.284	0.999	0.705	0.759
Unclassified	0	0-4.39	0	0-1.39	0.06	0-3.03	0.22	0-3.8	0.284	0.999	0.705	0.759
Catenibacterium	0.61	0-34.6	0	0-6.07	0	0-3.32	0	0-13.65	0.930	0.999	0.075	0.126
Unclassified	0.61	0-34.6	0	0-6.07	0	0-3.32	0	0-13.65	0.930	0.999	0.075	0.126
[Eubacterium]	0.75	0-6.24	0.18	0-3.03	1.13	0-8.04	0.37	0-1.39	0.314	0.999	0.389	0.468
biforme	0.07	0-6.24	0	0-0.89	0	0-7.85	0.03	0-1.27	0.934	0.999	0.635	0.697

Table 4.4. Continued.

Taxa	HC		AD0		AD6		AD30		AD0 vs AD6, AD30		HC vs AD0, AD6, AD30	
	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
dolichum	0.04	0-1.05	0	0-3.03	0.14	0-2.42	0.2	0-1.03	0.404	0.999	0.440	0.523
Fusobacteria	23.65	1.01-44.81	25.41	0.69-70.44	27.77	0.22-39.94	6.72	0-43.96	0.092	0.999	0.197	0.269
Fusobacteriia	23.65	1.01-44.81	25.41	0.69-70.44	27.77	0.22-39.94	6.72	0-43.96	0.092	0.999	0.197	0.269
Fusobacteriales	23.65	1.01-44.81	25.41	0.69-70.44	27.77	0.22-39.94	6.72	0-43.96	0.092	0.999	0.197	0.269
Fusobacteriaceae	23.65	1.01-44.81	25.41	0.69-70.44	27.77	0.22-39.94	6.72	0-43.96	0.092	0.999	0.197	0.269
Fusobacterium	23.65	0.96-44.81	18.68	0.69-70.44	27.77	0.22-39.94	6.72	0-43.96	0.222	0.999	0.254	0.330
Unclassified	23.65	0.96-44.81	18.68	0.69-70.44	27.77	0.22-39.94	6.72	0-43.96	0.222	0.999	0.254	0.330
Proteobacteria	5.95	0.29-15.45	5.17	0.5-46.13	5.65	0.15-61.77	3.73	0.67-33.8	0.601	0.999	0.826	0.849
Betaproteobacteria	3.16	0-7.52	0.14	0-8.05	0.02*	0-3.23	0.14*	0-1.34	0.309	0.999	0.002	0.007
Burkholderiales	3.16	0-7.52	0.14	0-8.05	0.02*	0-3.23	0.14*	0-1.34	0.309	0.999	0.002	0.007
Alcaligenaceae	3.16	0-7.52	0.14	0-8.05	0.02*	0-3.23	0.14*	0-1.34	0.309	0.999	0.002	0.007
Sutterella	3.16	0-7.52	0.14	0-8.05	0.02*	0-3.23	0.1*	0-1.34	0.495	0.999	0.002	0.007
Unclassified	3.16	0-7.52	0.14	0-8.05	0.02*	0-3.23	0.1*	0-1.34	0.495	0.999	0.002	0.007
Epsilonproteobacteria	0.86	0-4.27	0*	0-0.56	0*	0-0.24	0*	0-2.23	>0.999	0.999	<0.001	0.005
Campylobacterales	0.86	0-4.27	0*	0-0.56	0*	0-0.24	0*	0-2.23	>0.999	0.999	<0.001	0.005
Helicobacteraceae	0.78	0-3.57	0*	0-0	0*	0-0.06	0*	0-2.23	>0.999	0.999	<0.001	0.005
Helicobacter	0.78	0-3.57	0*	0-0	0*	0-0.06	0*	0-2.23	>0.999	0.999	<0.001	0.005
Unclassified	0.78	0-3.57	0*	0-0	0*	0-0.06	0*	0-2.23	>0.999	0.999	<0.001	0.005
Gammaproteobacteria	1.34	0-8.19	1.85	0.24-46.05	5.62	0-61.6	2.96	0-33.8	0.974	0.999	0.362	0.440
Aeromonadales	0.19	0-5.66	0*	0-0	0*	0-0	0*	0-0	>0.999	0.999	<0.001	0.005
Succinivibrionaceae	0.19	0-5.66	0*	0-0	0*	0-0	0*	0-0	>0.999	0.999	<0.001	0.005
Enterobacteriales	0.1	0-8.19	1.85*	0.24-46.05	5.62*	0-61.46	2.58*	0-33.8	0.830	0.999	0.004	0.011
Enterobacteriaceae	0.1	0-8.19	1.85*	0.24-46.05	5.62*	0-61.46	2.58*	0-33.8	0.830	0.999	0.004	0.011

Table 4.4. Continued.

Taxa	HC		AD0		AD6		AD30		AD0 vs AD6, AD30		HC vs AD0, AD6, AD30	
	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Unclassified	0.1	0-8.19	1.85*	0.24-45.45	5.62*	0-60.73	1.99*	0-29.81	0.830	0.999	0.004	0.011
Unclassified	0.1	0-8.19	1.85*	0.24-45.45	5.62*	0-60.73	1.99*	0-29.81	0.830	0.999	0.004	0.011

First column p- values were obtained with Friedman testing, while second column were obtained with Kruskal Wallis testing.

Q values are the adjusted P values after Benjamini and Hochberg False Discovery Rate correction for multiple comparisons.

‡Unclassified=matches (97%) similarity to a reference sequence undefined at the given taxonomic level.

‡‡Other=ambiguous assignment because QIIME cannot distinguish between taxa within that taxonomic level.

‡‡‡Square brackets=proposed taxonomic grouping according to the Greengenes database used in the QIIME pipeline.

Highlighted cells indicate significant P or Q values.

4.2.1.3. AHDS over time

Alpha diversity, described by Chao 1 and observed ASVs (Figure 4.5), was not significantly different between AHDS at baseline and follow up time points as well as between HC and all AHDS time points (q>0.05). However, there was a difference between all groups including healthy controls in Shannon Index (q<0.01), and *post hoc* analysis revealed healthy controls had higher Shannon Index than AHDS0 and AHDS90 (q<0.05).

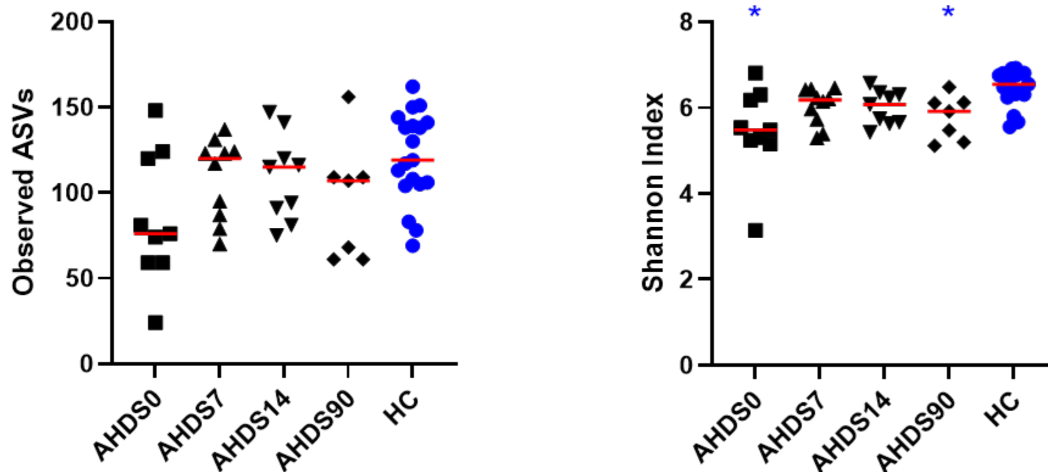

Figure 4.5. Observed ASVs and Shannon Index in acute hemorrhagic diarrhea (AHDS) over time compared to healthy controls (HC) and compared between baseline and follow up time points. Groups significantly different (Kruskal-Wallis testing with Dunn's *post hoc* tests) from HC are indicated with blue asterisks. There were no differences between baseline acute hemorrhagic diarrhea (AHDS0) and follow up time points at day 7 (AHDS7), day 14 (AHDS14), and day 90 (AHDS90; Mixed-effects analysis with Holm-Sidak's *post hoc* tests).

ANOSIM of PCoA plots (Figure 4.6) revealed significant differences between HC and AHDS0 ($P_{unweighted,weighted}$=0.001, $R_{unweighted}$=0.398, $R_{weighted}$ =0.543), AHDS7

($P_{unweighted,weighted}$ =0.001, $R_{unweighted}$=0.504, $R_{weighted}$ =0.594), AHDS14 ($P_{unweighted}$=0.005, $P_{weighted}$=0.001, $R_{unweighted}$=0.253, $R_{weighted}$ =0.609), and AHDS90 ($P_{unweighted,weighted}$=0.001, $R_{unweighted}$=0.438, $R_{weighted}$ =0.625). Significant differences in microbial communities were also found between baseline of AHDS and AHDS7 ($P_{unweighted}$=0.011, $P_{weighted}$=0.032, $R_{unweighted}$=0.208, $R_{weighted}$ =0.167) and AHDS14 ($P_{unweighted}$=0.016, $P_{weighted}$=0.002, $R_{unweighted}$=0.205, $R_{weighted}$ =0.316). Based on univariate statistics (Table 4.5), follow up timepoints at day 7, 14, and 90 were not significantly different from baseline, for dogs with AHDS. However, differences in bacterial taxa between healthy controls and follow up timepoints persisted to day 7 (e.g., *Prevotella*, *Lactobacillus*, *Streptococcus*), day 14 (e.g., *Blautia*), and day 90 (e.g., *Bacteroides*, Clostridia, *Megamonas*, *Fusobacterium*). Furthermore, changes within the class Clostridia were driven by increased abundance of Clostridiaceae (AHDS7, 14, and 90) and Lachnospiraceae (AHDS14), and decreased abundance of Ruminococcaceae (AHDS0 and 7) and Veillonellaceae (AHDS0, 7, 14, and 90) when compared to HC dogs.

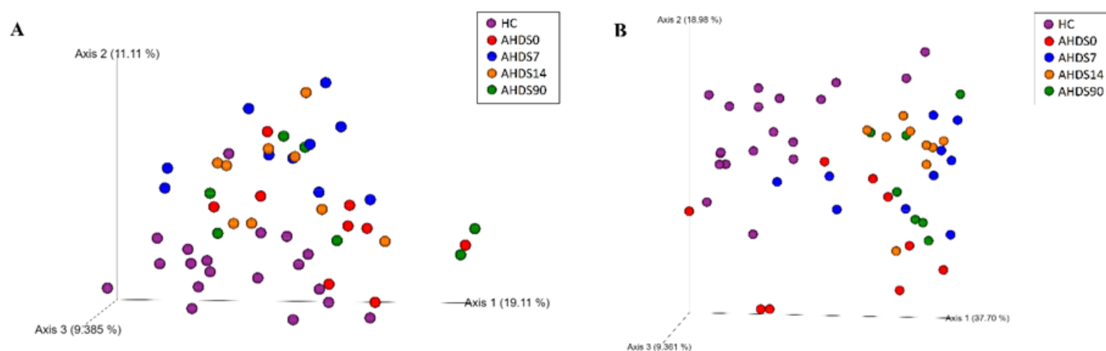

Figure 4.6. PCoA plots of fecal microbial communities from the dogs with acute hemorrhagic diarrhea (AHDS) over time and the healthy controls (HC). A) 3D plot of unweighted UniFrac distances. B) 3D plot of weighted UniFrac distances.

Table 4.5. Relative percentages of the most abundant bacterial groups for healthy control dogs (HC) and dogs with acute hemorrhagic diarrhea (AHDS) over time. Medians with asterisks were significantly different from healthy controls (Kruskal Wallis; q<0.05). Follow up timepoints were not significantly different from baseline within the AHDS group (Mixed Effect Model; q>0.05).

Taxa	HC Day 0		AHDS0		AHDS7		AHDS14		AHDS90		AHDS0 vs AHDS7, AHDS14, AHDS90		HC vs AHDS0, AHDS7, AHDS14, AHDS90	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Actinobacteria	1.38	0-9.12	0.63	0-5.71	5.07*	0.1-10.01	2.76	0.24-9.64	2.79	0-10.02	0.192	0.465	0.030	0.054
Actinobacteria (class)	0	0-4.56	0.11	0-0.52	0.31	0-3.2	0	0-2.29	0.22	0-1.29	0.275	0.473	0.122	0.169
Actinomycetales	0	0-0	0.11*	0-0.52	0	0-0.43	0	0-0.28	0.17*	0-1.29	0.249	0.465	0.006	0.014
Bifidobacteriales	0	0-4.56	0	0-0	0.03	0-3.2	0	0-2.01	0	0-0.05	0.146	0.456	0.135	0.176
Bifidobacteriaceae	0	0-4.56	0	0-0	0.03	0-3.2	0	0-2.01	0	0-0.05	0.146	0.456	0.135	0.176
Bifidobacterium	0	0-4.56	0	0-0	0.03	0-3.2	0	0-2.01	0	0-0.05	0.146	0.456	0.135	0.176
Unclassified‡	0	0-4.56	0	0-0	0.03	0-3.2	0	0-2.01	0	0-0.05	0.146	0.456	0.097	0.150
Coriobacteriia	1.35	0-4.56	0.18	0-5.28	3.96	0.1-7.53	2.48	0-8.12	2.79	0-9.66	0.227	0.465	0.090	0.142
Coriobacteriales	1.35	0-4.56	0.18	0-5.28	3.96	0.1-7.53	2.48	0-8.12	2.79	0-9.66	0.227	0.465	0.090	0.142
Coriobacteriaceae	1.35	0-4.56	0.18	0-5.28	3.96	0.1-7.53	2.48	0-8.12	2.79	0-9.66	0.227	0.465	0.090	0.142
Collinsella	1.26	0-4.56	0.18	0-5.15	3.1	0.1-7.06	2.34	0-8.12	2.48	0-9.28	0.261	0.471	0.110	0.157
Unclassified	0	0-0.93	0	0-0.37	0.31*	0-1.53	0	0-0.88	0.08	0-0.46	0.239	0.465	0.030	0.054
stercoris	1.08	0-3.84	0.17	0-5.15	2.84	0.1-6.64	1.96	0-8.12	2.26	0-8.81	0.287	0.473	0.101	0.153
Slackia	0	0-0.24	0	0-0.33	0.25*	0-1.38	0.23*	0-0.55	0.23	0-0.78	0.118	0.456	0.008	0.017
Unclassified	0	0-0.24	0	0-0.33	0.25*	0-1.38	0.23*	0-0.55	0.23	0-0.78	0.118	0.456	0.008	0.017
Bacteroidetes	28.41	10.31-44.71	4.39*	0.27-26.39	1.29*	0-15.26	5.44*	0-13.36	1.63*	0-9.72	0.544	0.596	<0.001	0.003

Table 4.5. Continued.

Taxa	HC Day 0		AHDS0		AHDS7		AHDS14		AHDS90		AHDS0 vs AHDS7, AHDS14, AHDS90		HC vs AHDS0, AHDS7, AHDS14, AHDS90	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Bacteroidia	28.41	10.31-44.71	4.39*	0.27-26.39	1.29*	0-15.26	5.44*	0-13.36	1.63*	0-9.72	0.544	0.596	<0.001	0.003
Bacteroidales	28.41	10.31-44.71	4.39*	0.27-26.39	1.29*	0-15.26	5.44*	0-13.36	1.63*	0-9.72	0.544	0.596	<0.001	0.003
Bacteroidaceae	20.82	0.85-44.59	4.03*	0.27-11.6	0.88*	0-9.61	3.61*	0-12.13	0.62*	0-7.18	0.500	0.590	<0.001	0.003
Bacteroides	20.82	0.85-44.59	4.03*	0.27-11.6	0.88*	0-9.61	3.61*	0-12.13	0.62*	0-7.18	0.500	0.590	<0.001	0.003
Other[‡‡]	5.2	0-30.38	0.74	0-2.9	0.5	0-2.35	0.22	0-3.37	0.39	0-4.2	0.963	0.963	0.082	0.139
Unclassified	8.06	0-40.45	2.73	0-10.85	0.38*	0-7.74	1.16*	0-8.76	0.6*	0-2.97	0.456	0.553	<0.001	0.003
plebius	2.85	0-11.85	0*	0-0.4	0*	0-0.03	0*	0-2.04	0*	0-0	0.157	0.459	<0.001	0.003
Prevotellaceae	3.75	0-10.74	0*	0-0.17	0*	0-0.12	0.05	0-3.92	0	0-1.79	0.112	0.456	0.011	0.023
Prevotella	3.75	0-10.74	0*	0-0.17	0*	0-0.12	0.05	0-3.92	0	0-1.79	0.112	0.456	0.011	0.023
copri	3.75	0-10.74	0*	0-0.17	0*	0-0.12	0.05	0-3.92	0	0-1.79	0.110	0.456	0.007	0.016
[Paraprevotellaceae][‡‡‡]	2.01	0-8.51	0.26	0-7.56	0.27	0-2.75	0.24	0-2.23	0.1	0-2.55	0.478	0.575	0.581	0.615
[Prevotella]	2.01	0-8.51	0.26	0-7.56	0.06	0-2.75	0.11	0-2.23	0.1	0-1.54	0.386	0.489	0.398	0.433
Unclassified	2.01	0-8.51	0.26	0-7.56	0.06	0-2.75	0.11	0-2.23	0.1	0-1.54	0.386	0.489	0.398	0.433
Firmicutes	36.29	15.8-85.83	63.81[a]	12.74-98.81	82.89[a]*	47.53-96.93	82.83[a]*	70.66-91.93	79.99[a]*	72.04-98.38	0.035	0.456	<0.001	0.003
Bacilli	0.78	0-5.19	1.43	0.23-43.97	6.04	0.24-47.67	1.94	0.15-21.22	1.68	0.17-4.46	0.125	0.456	0.170	0.208
Bacillales	0	0-0.02	0	0-0.19	0	0-1.48	0	0-0.03	0	0-0.6	0.279	0.473	0.137	0.177

Table 4.5. Continued.

Taxa	HC Day 0		AHDS0		AHDS7		AHDS14		AHDS90		AHDS0 vs AHDS7, AHDS14, AHDS90		HC vs AHDS0, AHDS7, AHDS14, AHDS90	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Lactobacillales	0.21	0-2.66	0.76*	0.23-43.14	5.27*	0.24-43.37	1*	0.15-21.22	0.39	0-2.41	0.088	0.456	0.001	0.003
Lactobacillaceae	0	0-2.29	0	0-0	0.27*	0-21.21	0	0-3.53	0	0-0	0.181	0.465	<0.001	0.003
Lactobacillus	0	0-2.29	0	0-0	0.27*	0-21.21	0	0-3.18	0	0-0	0.222	0.465	<0.001	0.003
Unclassified	0	0-2.29	0	0-0	0.27*	0-15.55	0	0-3.18	0	0-0	0.200	0.465	<0.001	0.003
Streptococcaceae	0.14	0-1.4	0.76*	0.23-42.56	1.53*	0-37.04	0.34	0-21.22	0.28	0-1.95	0.223	0.465	0.003	0.009
Streptococcus	0.14	0-1.4	0.44*	0.23-42.56	1.29*	0-36.47	0.34	0-21.22	0.28	0-1.95	0.241	0.465	0.006	0.014
Unclassified	0.14	0-1.4	0.44*	0.23-42.56	1.29*	0-36.3	0.34	0-21.22	0.28	0-1.95	0.242	0.465	0.006	0.014
Turicibacterales	0.17	0-2.62	0	0-1.08	0	0-4.31	0	0-1.62	0.08	0-4.2	0.350	0.473	0.205	0.242
Turicibacteraceae	0.17	0-2.62	0	0-1.08	0	0-4.31	0	0-1.62	0.08	0-4.2	0.350	0.473	0.205	0.242
Turicibacter	0.17	0-2.62	0	0-1.08	0	0-4.31	0	0-1.62	0.08	0-4.2	0.350	0.473	0.205	0.242
Unclassified	0.17	0-2.62	0	0-1.08	0	0-4.31	0	0-1.62	0.08	0-4.2	0.350	0.473	0.205	0.242
Clostridia	31.14	14.24-57.79	32.21[a]	11.97-97.23	60.34[a]*	22.63-87.21	72.69[a]*	54.24-82.5	72.54[a]*	54.46-87.99	0.028	0.456	<0.001	0.003
Clostridiales	31.14	14.24-57.79	32.21[a]	11.97-97.23	60.34[a]*	22.63-87.21	72.69[a]*	54.24-82.5	72.54[a]*	54.46-87.99	0.028	0.456	<0.001	0.003
Unclasified	0.32	0-1.39	0	0-1.81	0.25	0-0.65	0.17	0-2.21	0	0-0.92	0.532	0.596	0.383	0.424
Unclassified	0.32	0-1.39	0	0-1.81	0.25	0-0.65	0.17	0-2.21	0	0-0.92	0.532	0.596	0.383	0.424
Unclassified	0.32	0-1.39	0	0-1.81	0.25	0-0.65	0.17	0-2.21	0	0-0.92	0.532	0.596	0.383	0.424
Clostridiaceae	7.95	1.87-16.9	18.06	2.86-92.37	29.38*	4.86-57.67	38.4*	3.78-52.22	41.2*	2.63-55.44	0.718	0.724	<0.001	0.003

Table 4.5. Continued.

Taxa	HC Day 0		AHDS0		AHDS7		AHDS14		AHDS90		AHDS0 vs AHDS7, AHDS14, AHDS90		HC vs AHDS0, AHDS7, AHDS14, AHDS90	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Other	5.75	0.99-14.21	2.71[a]	0-37.3	19.19[a,b]*	4.37-44.83	30.37[b]*	0-38.06	23.98[a,b]	2.14-36.51	0.031	0.456	<0.001	0.003
Other	5.75	0.99-14.21	2.71[a]	0-37.3	19.19[a,b]*	4.37-44.83	30.37[b]*	0-38.06	23.98[a,b]	2.14-36.51	0.031	0.456	<0.001	0.003
Unclassified	1.6	0-5.49	2.31	0-7.27	5.79*	0-14.75	7.2*	1-10.48	5.48*	0.32-17.1	0.093	0.456	<0.001	0.003
Unclassified	1.6	0-5.49	2.31	0-7.27	5.79*	0-14.75	7.2*	1-10.48	5.48*	0.32-17.1	0.093	0.456	<0.001	0.003
Clostridium	0.45	0-4.13	10.36*	0.11-87.95	2.79	0-6.12	0.86	0-3.76	0.54	0-36.03	0.158	0.459	0.005	0.013
Other	0.36	0-3.87	8.61*	0-87.95	1.59	0-6.12	0.86	0-2.92	0.54	0-36.03	0.155	0.459	0.004	0.011
Lachnospiraceae	12.82	2.34-25.68	3.83	0-45.28	20.69	3.16-39.94	31.27*	17.18-56.19	21.57	1.08-82.44	0.129	0.456	0.001	0.003
Other	2.43	0.21-6.63	2.23	0-11.75	8.63*	1.47-13.05	14.57*	5.2-19.22	9.75	0-59.89	0.112	0.456	<0.001	0.003
Other	2.43	0.21-6.63	2.23	0-11.75	8.63*	1.47-13.05	14.57*	5.2-19.22	9.75	0-59.89	0.112	0.456	<0.001	0.003
Unclassified	2.17	0-3.9	0*	0-1.64	0.87	0-2.51	0.84	0-1.91	0.11*	0-3.92	0.647	0.665	0.002	0.006
Unclassified	2.17	0-3.9	0*	0-1.64	0.87	0-2.51	0.84	0-1.91	0.11*	0-3.92	0.647	0.665	0.002	0.006
Blautia	4.18	1.14-12.4	0.57	0-26.66	8.13	0.55-17.21	11.65*	1.46-32.28	7.84	0-18.93	0.247	0.465	0.011	0.023
Unclassified	1.12	0-6.34	0	0-3.02	1.96	0-5.25	3.06	0-5.41	0.85	0-5.06	0.086	0.456	0.129	0.173
producta	3.37	1.14-9.56	0	0-26.66	6.37	0.55-13.5	7.73	1.02-32.28	5.93	0-14.05	0.364	0.478	0.018	0.036
Dorea	0	0-2.21	0.3	0-1.33	0.19	0-0.93	0.63	0-2.29	0.58	0-2.19	0.333	0.473	0.805	0.832
Unclassified	0	0-2.21	0.3	0-1.33	0.19	0-0.93	0.63	0-2.29	0.58	0-2.19	0.333	0.473	0.805	0.832

Table 4.5. Continued.

Taxa	HC Day 0		AHDS0		AHDS7		AHDS14		AHDS90		AHDS0 vs AHDS7, AHDS14, AHDS90		HC vs AHDS0, AHDS7, AHDS14, AHDS90	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
[Ruminococcus]	2.76	0.43-4.76	0.96	0-8.87	2.93	0-7.33	4.45	0.31-7.05	0.76	0-3.96	0.264	0.471	0.097	0.150
Other	0	0-2.07	0	0-0.78	0.42	0-1.63	0.46	0-1.87	0	0-0.96	0.104	0.456	0.056	0.099
Unclassified	0	0-1.05	0	0-0.4	0.25	0-3.03	0	0-1.6	0.28	0-1.29	0.253	0.465	0.169	0.208
gnavus	2.3	0-4.76	0.96	0-8.87	1.65	0-5.06	2.66	0-7.05	0.29	0-3.68	0.328	0.473	0.129	0.173
Peptostreptococcaceae	0	0-4.75	0.92	0-5.94	0.43	0-17.58	0.79	0-7.38	1.73	0-31.57	0.367	0.478	0.125	0.172
Unclassified	0	0-4.75	0.92	0-5.94	0.43	0-17.58	0.79	0-7.38	1.73	0-31.57	0.356	0.473	0.086	0.141
Unclassified	0	0-4.75	0.92	0-5.94	0.43	0-17.58	0.79	0-7.38	1.73	0-31.57	0.356	0.473	0.086	0.141
Ruminococcaceae	3.29	0.1-10.88	0.4*	0-4.52	0.27*	0.04-2.37	0.4	0.08-3.78	0.58	0-1.73	0.566	0.605	0.015	0.030
Unclassified	0.61	0-3.45	0.26	0-1.63	0.18	0-1.43	0.36	0-1.12	0.11	0-1.53	0.654	0.665	0.109	0.157
Unclassified	0.61	0-3.45	0.26	0-1.63	0.18	0-1.43	0.36	0-1.12	0.11	0-1.53	0.654	0.665	0.109	0.157
Faecalibacterium	2.96	0-9.38	0.03	0-2.89	0.04	0-1.42	0	0-2.92	0.28	0-0.95	0.391	0.489	0.103	0.153
prausnitzii	2.96	0-9.38	0.03	0-2.89	0.04	0-1.42	0	0-2.92	0.28	0-0.95	0.391	0.489	0.103	0.153
Veillonellaceae	4.34	0.06-9.69	0.15*	0-0.32	0.05*	0-0.69	0.18*	0.06-1.39	0.15*	0-1.22	0.247	0.465	<0.001	0.003
Megamonas	3.2	0.06-7.48	0.1*	0-0.27	0.01*	0-0.22	0.1*	0-1.05	0.15*	0-1.15	0.224	0.465	<0.001	0.003
Unclassified	3.2	0.06-7.48	0.1*	0-0.27	0.01*	0-0.22	0.1*	0-1.05	0.15*	0-1.15	0.224	0.465	<0.001	0.003
Phascolarctobacterium	0.18	0-1.53	0	0-0.15	0	0-0.61	0	0-0.29	0	0-0.75	0.558	0.601	0.170	0.208
Unclassified	0.18	0-1.53	0	0-0.15	0	0-0.61	0	0-0.29	0	0-0.75	0.558	0.601	0.170	0.208

Table 4.5. Continued.

Taxa	HC Day 0		AHDS0		AHDS7		AHDS14		AHDS90		AHDS0 vs AHDS7, AHDS14, AHDS90		HC vs AHDS0, AHDS7, AHDS14, AHDS90	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Erysipelotrichi	5	0.28-40.85	0.17*	0-5.83	2.5	0.57-17.47	6.11	0.71-15	5.93	0-26.44	0.213	0.465	0.029	0.053
Erysipelotrichales	5	0.28-40.85	0.17*	0-5.83	2.5	0.57-17.47	6.11	0.71-15	5.93	0-26.44	0.213	0.465	0.029	0.053
Erysipelotrichaceae	5	0.28-40.85	0.17*	0-5.83	2.5	0.57-17.47	6.11	0.71-15	5.93	0-26.44	0.213	0.465	0.029	0.053
Unclassified	0.48	0-3.36	0	0-2.07	0.53	0-6.39	1.06	0-7.66	0	0-3.66	0.145	0.456	0.075	0.128
Unclassified	0.48	0-3.36	0	0-2.07	0.53	0-6.39	1.06	0-7.66	0	0-3.66	0.145	0.456	0.075	0.128
Allobaculum	0	0-4.39	0	0-1.43	0.48	0-2.55	1.22	0-3.3	0.62	0-1.16	0.072	0.456	0.291	0.337
Unclassified	0	0-4.39	0	0-1.43	0.48	0-2.55	1.22	0-3.3	0.62	0-1.16	0.072	0.456	0.291	0.337
Catenibacterium	0.61	0-34.6	0	0-0.18	0.22	0-5.63	0.23	0-6.81	0.24	0-19.82	0.327	0.473	0.112	0.157
Unclassified	0.61	0-34.6	0	0-0.18	0.22	0-5.63	0.23	0-6.81	0.24	0-19.82	0.327	0.473	0.112	0.157
Coprobacillus	0	0-0.15	0	0-0.5	0	0-1.6	0	0-1.44	0	0-1.49	0.601	0.631	0.310	0.352
Unclassified	0	0-0.15	0	0-0.5	0	0-1.6	0	0-1.44	0	0-1.49	0.601	0.631	0.310	0.352
[Eubacterium]	0.75	0-6.24	0	0-5.45	0.88	0.13-11.1	1.4	0-3.64	0.74	0-5.61	0.512	0.593	0.167	0.208
biforme	0.07	0-6.24	0	0-0.44	0.5	0-10.94	0.07	0-3.64	0.1	0-5.54	0.302	0.473	0.497	0.531
dolichum	0.04	0-1.05	0	0-5.33	0.07	0-1.05	0.17	0-1.4	0	0-0.65	0.512	0.593	0.590	0.620
Fusobacteria	23.65	1.01-44.81	24.18	0.24-69.22	4.81	0.31-37.21	3.48*	0-17.95	6.63*	0-13.55	0.070	0.456	0.005	0.013
Fusobacteriia	23.65	1.01-44.81	24.18	0.24-69.22	4.81	0.31-37.21	3.48*	0-17.95	6.63*	0-13.55	0.070	0.456	0.005	0.013
Fusobacteriales	23.65	1.01-44.81	24.18	0.24-69.22	4.81	0.31-37.21	3.48*	0-17.95	6.63*	0-13.55	0.070	0.456	0.005	0.013

Table 4.5. Continued.

Taxa	HC Day 0		AHDS0		AHDS7		AHDS14		AHDS90		AHDS0 vs AHDS7, AHDS14, AHDS90		HC vs AHDS0, AHDS7, AHDS14, AHDS90	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Fusobacteriaceae	23.65	1.01-44.81	24.18	0.24-69.22	4.81	0.31-37.21	3.48*	0-17.95	6.63*	0-13.55	0.070	0.456	0.005	0.013
Fusobacterium	23.65	0.96-44.81	24.18	0.24-69.06	4.81*	0.31-37.21	3.48*	0-17.95	6.63*	0-13.55	0.055	0.456	0.002	0.006
Unclassified	23.65	0.96-44.81	24.18	0.24-69.06	4.81*	0.31-37.21	3.48*	0-17.95	6.63*	0-13.55	0.055	0.456	0.002	0.006
Proteobacteria	5.95	0.29-15.45	5.72	0.17-34.73	0.63*	0-5.63	0.42*	0.09-26.12	1.9	0.05-22.96	0.104	0.456	0.002	0.006
Alphaproteobacteria	0	0-0	0	0-0	0	0-0.08	0	0-0	0*	0-0.14	0.127	0.456	0.003	0.009
Betaproteobacteria	3.16	0-7.52	0.46	0-6.58	0.12*	0-1.33	0.2*	0-0.82	0*	0-1.19	0.077	0.456	<0.001	0.003
Burkholderiales	3.16	0-7.52	0.46	0-6.58	0.12*	0-1.33	0.2*	0-0.82	0*	0-1.19	0.077	0.456	<0.001	0.003
Alcaligenaceae	3.16	0-7.52	0.46	0-6.58	0.12*	0-1.33	0.2*	0-0.82	0*	0-1.19	0.077	0.456	<0.001	0.003
Sutterella	3.16	0-7.52	0.46	0-6.58	0.12*	0-1.33	0.2*	0-0.82	0*	0-1.19	0.077	0.456	<0.001	0.003
Unclassified	3.16	0-7.52	0.46	0-6.58	0.12*	0-1.33	0.2*	0-0.82	0*	0-1.19	0.077	0.456	<0.001	0.003
Epsilonproteobacteria	0.86	0-4.27	0*	0-4.06	0*	0-0.27	0*	0-0	0*	0-0.08	0.348	0.473	<0.001	0.003
Campylobacterales	0.86	0-4.27	0*	0-4.06	0*	0-0.27	0*	0-0	0*	0-0.08	0.348	0.473	<0.001	0.003
Helicobacteraceae	0.78	0-3.57	0	0-4.06	0*	0-0.27	0*	0-0	0	0-0.08	0.348	0.473	<0.001	0.003
Helicobacter	0.78	0-3.57	0	0-4.06	0*	0-0.27	0*	0-0	0	0-0.08	0.348	0.473	<0.001	0.003
Unclassified	0.78	0-3.57	0	0-2.9	0*	0-0.27	0*	0-0	0	0-0.08	0.348	0.473	0.003	0.009
Gammaproteobacteria	1.34	0-8.19	0.68	0-34.47	0.06	0-5.16	0.16	0-25.92	0.62	0-22.82	0.221	0.465	0.402	0.433
Aeromonadales	0.19	0-5.66	0	0-4.52	0*	0-0	0	0-0.92	0	0-0.31	0.401	0.491	0.024	0.046

Table 4.5. Continued.

Taxa	HC Day 0		AHDS0		AHDS7		AHDS14		AHDS90		AHDS0 vs AHDS7, AHDS14, AHDS90		HC vs AHDS0, AHDS7, AHDS14, AHDS90	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Succinivibrionaceae	0.19	0-5.66	0	0-4.52	0*	0-0	0	0-0.92	0	0-0.31	0.401	0.491	0.024	0.046
Enterobacteriales	0.1	0-8.19	0.17	0-34.43	0.06	0-5.16	0.1	0-25.92	0.31	0-22.77	0.303	0.473	0.838	0.838
Enterobacteriaceae	0.1	0-8.19	0.17	0-34.43	0.06	0-5.16	0.1	0-25.92	0.31	0-22.77	0.303	0.473	0.838	0.838
Unclassified	0.1	0-8.19	0.17	0-34.15	0.06	0-5.16	0.1	0-23.79	0.31	0-22.77	0.297	0.473	0.838	0.838
Unclassified	0.1	0-8.19	0.17	0-34.15	0.06	0-5.16	0.1	0-23.79	0.31	0-22.77	0.297	0.473	0.838	0.838
Pasteurellales	0	0-0.01	0*	0-9.63	0	0-0	0	0-0	0	0-0.05	0.201	0.465	0.008	0.017
Pasteurellaceae	0	0-0.01	0*	0-9.63	0	0-0	0	0-0	0	0-0.05	0.201	0.465	0.008	0.017
Other	0	0-0	0*	0-8.09	0	0-0	0	0-0	0	0-0	0.231	0.465	<0.001	0.003
Other	0	0-0	0*	0-8.09	0	0-0	0	0-0	0	0-0	0.231	0.465	<0.001	0.003

First column p- values were obtained with mixed effects testing, while second column were obtained with Kruskal Wallis testing.

Q values are the adjusted P values after Benjamini and Hochberg False Discovery Rate correction for multiple comparisons.

‡Unclassified=matches (97%) similarity to a reference sequence undefined at the given taxonomic level.

‡‡Other=ambiguous assignment because QIIME cannot distinguish between taxa within that taxonomic level.

‡‡‡Square brackets=proposed taxonomic grouping according to the Greengenes database used in the QIIME pipeline.

Highlighted cells indicate significant P or Q values.

4.2.1.4. IBD over time

Alpha diversity, described by Chao 1, observed ASVs (Figure 4.7), and Shannon Index was not significantly different between IBD at baseline and follow up time points as well as between HC and all IBD time points (q>0.05).

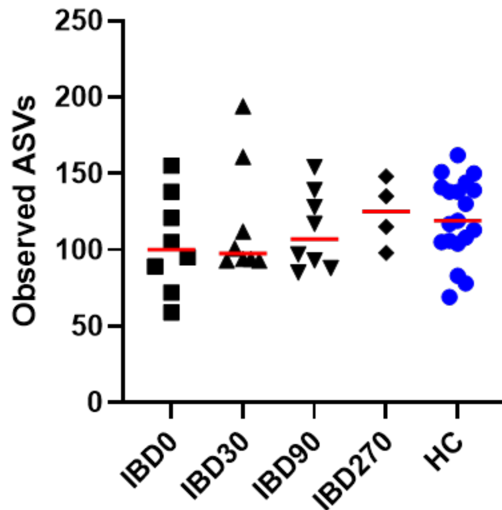

Figure 4.7.Observed ASVs in inflammatory bowel disease (IBD) over time compared to healthy controls (HC) and compared between baseline and follow up time points. No differences were observed (Kruskal-Wallis testing with Dunn's *post hoc* tests) between HC and any time point of IBD, including day 0 (IBD0), day 30 (IBD30), day 90 (IBD90), and day 270 (IBD270). Additionally, no differences were observed between IBD0 and any follow up time point (Mixed-effects analysis with Holm-Sidak's *post hoc* tests).

ANOSIM of PCoA plots (Figure 4.8) revealed significant differences between HC and IBD0 ($P_{unweighted,weighted}$=0.001, $R_{unweighted}$=0.608, $R_{weighted}$ =0.577), IBD30 ($P_{unweighted,weighted}$=0.001, $R_{unweighted}$=0.528, $R_{weighted}$ =0.488), IBD90 ($P_{unweighted,weighted}$=0.001, $R_{unweighted}$=0.407, $R_{weighted}$ =0.593), and IBD270 ($P_{unweighted}$=0.005, $P_{weighted}$=0.006, $R_{unweighted}$=0.463, $R_{weighted}$ =0.528), but no differences

between IBD0 and subsequent timepoints. Based on univariate statistics (Table 4.6), follow up timepoints at day 30, 90, and 270 were not significantly different from baseline, for dogs with IBD. However, differences in bacterial taxa between healthy controls and follow up timepoints persisted to day 30 (e.g., *Prevotella*, *Megamonas*), day 90 (e.g., *Bacteroides*, *Streptococcus*), and day 270 (e.g., Clostridia, *Sutterella*, Firmicutes).

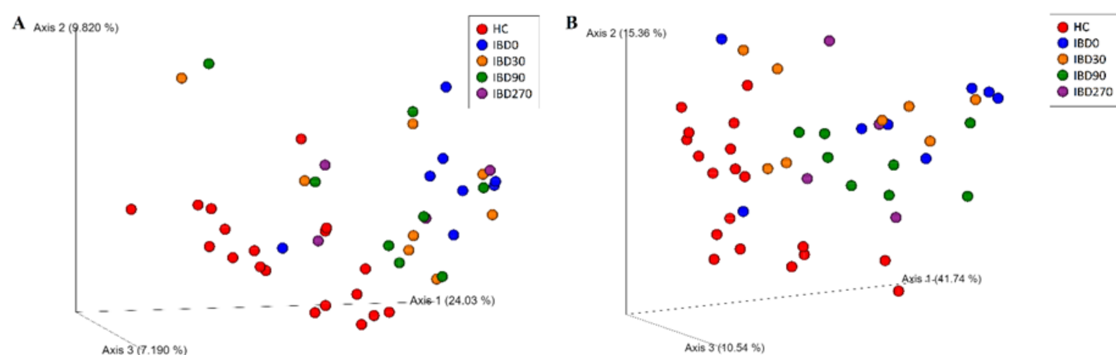

Figure 4.8. PCoA plots of fecal microbial communities from the dogs with inflammatory bowel disease (IBD) over time and the healthy controls (HC). A) 3D plot of unweighted UniFrac distances. B) 3D plot of weighted UniFrac distances.

Table 4.6. Relative percentages of the most abundant bacterial groups for healthy control dogs (HC) and dogs with inflammatory bowel disease (IBD) over time. Medians with asterisks were significantly different from healthy controls (Kruskal Wallis; q<0.05). Follow up timepoints were not significantly different from baseline within the IBD group (Mixed Effect Model; q>0.05).

Taxa	HC Day 0		IBD0		IBD30		IBD90		IBD270		IBD0 vs IBD30, IBD90, IBD270		HC vs IBD0, IBD30, IBD90, IBD270	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Actinobacteria	1.38	0-9.12	1.6	0.3-9.69	4.31	0-11.35	6.96*	0.1-15.35	6.3	2.24-6.86	0.223	0.558	0.033	0.060
Actinobacteria (class)	0	0-4.56	0.56*	0-1.34	0.14*	0-4.05	0.36*	0-2.43	0.14	0-0.51	0.337	0.492	0.012	0.037
Actinomycetales	0	0-0	0.43*	0-1.34	0.13*	0-1.53	0.04	0-0.73	0.14	0-0.51	0.566	0.613	<0.001	0.007
Corynebacteriaceae	0	0-0	0.06*	0-0.31	0	0-0.15	0	0-0	0	0-0.23	0.072	0.571	0.009	0.029
Corynebacterium	0	0-0	0.06*	0-0.31	0	0-0.15	0	0-0	0	0-0.23	0.072	0.548	0.009	0.029
Unclassified‡	0	0-0	0.06*	0-0.31	0	0-0.15	0	0-0	0	0-0.23	0.072	0.577	0.009	0.029
Bifidobacteriales	0	0-4.56	0	0-0.9	0	0-3.78	0.26	0-2.43	0	0-0	0.26	0.495	0.200	0.239
Bifidobacteriaceae	0	0-4.56	0	0-0.9	0	0-3.78	0.26	0-2.43	0	0-0	0.26	0.571	0.200	0.239
Bifidobacterium	0	0-4.56	0	0-0.9	0	0-3.78	0.26	0-2.43	0	0-0	0.26	0.548	0.200	0.239
Unclassified	0	0-4.56	0	0-0.01	0	0-1.31	0.26	0-2.43	0	0-0	0.111	0.577	0.117	0.167
Coriobacteriia	1.35	0-4.56	0.72	0-8.65	4.23	0-7.3	5.44*	0.1-13.54	6.29	1.97-6.36	0.149	0.492	0.027	0.055
Coriobacteriales	1.35	0-4.56	0.72	0-8.65	4.23	0-7.3	5.44*	0.1-13.54	6.29	1.97-6.36	0.149	0.495	0.027	0.055
Coriobacteriaceae	1.35	0-4.56	0.72	0-8.65	4.23	0-7.3	5.44*	0.1-13.54	6.29	1.97-6.36	0.149	0.571	0.027	0.055
Collinsella	1.26	0-4.56	0.72	0-8.65	3.04	0-6.93	5.08*	0.1-13.54	5.60	1.97-5.79	0.132	0.548	0.032	0.060
stercoris	1.08	0-3.84	0.72	0-8.65	2.01	0-6.28	4.93	0.1-13.54	5.50	1.97-5.79	0.104	0.577	0.054	0.087
Slackia	0	0-0.24	0	0-0.84	0.11	0-0.39	0	0-0.42	0.46	0-0.74	0.318	0.548	0.199	0.239
Unclassified	0	0-0.24	0	0-0.84	0.11	0-0.39	0	0-0.42	0.46	0-0.74	0.318	0.577	0.199	0.239

Table 4.6. Continued.

Taxa	HC Day 0		IBD0		IBD30		IBD90		IBD270		IBD0 vs IBD30, IBD90, IBD270		HC vs IBD0, IBD30, IBD90, IBD270	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Bacteroidetes	28.41	10.31-44.71	0.44*	0-35.14	2.48*	0.2-19.64	5.71*	1.85-11.26	3.57*	0.22-21	0.855	0.855	<0.001	0.007
Bacteroidia	28.41	10.31-44.71	0.28*	0-35.14	2.48*	0.2-19.64	5.71*	1.85-11.26	3.57	0.22-21	0.851	0.851	<0.001	0.007
Bacteroidales	28.41	10.31-44.71	0.28*	0-35.14	2.48*	0.2-19.64	5.71*	1.85-11.26	3.57	0.22-21	0.851	0.851	<0.001	0.007
Bacteroidaceae	20.82	0.85-44.59	0.27*	0-32.99	2.39*	0.18-18.19	4.92*	0.21-11.13	1.02	0.22-20.63	0.915	0.915	<0.001	0.007
Bacteroides	20.82	0.85-44.59	0.27*	0-32.99	2.39*	0.18-18.19	4.92*	0.21-11.13	1.02	0.22-20.63	0.915	0.915	<0.001	0.007
Other[‡‡]	5.20	0-30.38	0	0-15.88	0.05	0-3	0.10	0-3.31	0.24	0-3.84	0.548	0.639	0.051	0.083
Unclassified	8.06	0-40.45	0.04*	0-9.95	0.24*	0-15.19	1.82	0-11.13	0.55	0.22-15.93	0.577	0.653	0.003	0.018
plebeius	2.85	0-11.85	0.03	0-7.33	0.04	0-5	0.20	0-3.97	0.24	0-0.86	0.464	0.609	0.055	0.087
Porphyromonadaceae	0	0-0.78	0	0-0	0.01	0-1.41	0	0-0.95	0	0-0	0.224	0.571	0.112	0.165
Prevotellaceae	3.75	0-10.74	0*	0-0.86	0*	0-3.47	0.07	0-2.72	0.19	0-5.09	0.23	0.571	0.014	0.040
Prevotella	3.75	0-10.74	0*	0-0.86	0*	0-3.47	0.07	0-2.72	0.19	0-5.09	0.23	0.548	0.014	0.040
copri	3.75	0-10.74	0*	0-0.86	0*	0-3.47	0.07	0-2.72	0.19	0-5.09	0.23	0.577	0.014	0.040
[Prevotella][‡‡‡]	2.01	0-8.51	0	0-1.29	0	0-0.49	0	0-1.8	0*	0-0	0.543	0.634	0.017	0.043
Unclassified	2.01	0-8.51	0	0-1.29	0	0-0.49	0	0-1.8	0*	0-0	0.543	0.639	0.017	0.043
[Paraprevotellaceae]	2.01	0-8.51	0	0-1.29	0	0-0.49	0	0-1.8	0*	0-0	0.543	0.635	0.017	0.043
Firmicutes	36.29	15.8-85.83	74.58*	27.31-92.09	74.9*	46.28-92.09	76.6*	72.62-87.62	74.46*	63.9-88.87	0.517	0.646	<0.001	0.007

Table 4.6. Continued.

Taxa	HC Day 0		IBD0		IBD30		IBD90		IBD270		IBD0 vs IBD30, IBD90, IBD270		HC vs IBD0, IBD30, IBD90, IBD270	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Bacilli	0.78	0-5.19	10.58*	0.56-74.62	2.82	0.49-38.95	6.48	0.24-64.69	4.35	0.47-24.39	0.443	0.492	0.020	0.049
Lactobacillales	0.21	0-2.66	9.90*	0.56-73.81	0.99*	0.12-38.61	5.76*	0.24-64.69	1.80	0.41-23.21	0.419	0.495	<0.001	0.007
Enterococcaceae	0	0-0.99	1.08*	0.21-71.85	0.17	0-0.66	0.17	0-2.35	0	0-0	0.207	0.571	<0.001	0.007
Other	0	0-0	0.20*	0-3.34	0	0-0	0	0-0	0	0-0	0.17	0.548	<0.001	0.007
Other	0	0-0	0.2*	0-3.34	0	0-0	0	0-0	0	0-0	0.17	0.577	<0.001	0.007
Enterococcus	0	0-0.99	1.08*	0-68.51	0.17	0-0.66	0.17	0-2.35	0	0-0	0.208	0.548	<0.001	0.007
Unclassified	0	0-0.99	0.58*	0-68.51	0.17	0-0.66	0.17	0-2.35	0	0-0	0.215	0.577	0.005	0.022
Lactobacillaceae	0	0-2.29	0.15	0-0.96	0	0-30.45	0	0-9.31	0.17	0-1.8	0.501	0.633	0.045	0.076
Lactobacillus	0	0-2.29	0.06	0-0.96	0	0-30.45	0	0-9.31	0.17	0-1.8	0.499	0.619	0.090	0.134
Unclassified	0	0-2.29	0.06	0-0.96	0	0-28.95	0	0-9.31	0.17	0-1.8	0.506	0.625	0.09	0.134
Streptococcaceae	0.14	0-1.4	0.75*	0.29-33.23	0.48	0-37.86	0.61*	0.24-64.49	0.9	0.27-23.02	0.676	0.705	0.007	0.028
Streptococcus	0.14	0-1.4	0.75*	0.29-33.23	0.37	0-37.86	0.61*	0.24-64.49	0.90	0.27-23.02	0.675	0.736	0.008	0.028
Unclassified	0.14	0-1.4	0.75*	0.29-33.23	0.37	0-37.86	0.61*	0.24-64.49	0.90	0.27-23.02	0.675	0.727	0.008	0.028
Turicibacterales	0.17	0-2.62	0.10	0-0.71	0.71	0-2.38	0	0-0.17	0.62	0-5.1	0.124	0.495	0.191	0.239
Turicibacteraceae	0.17	0-2.62	0.1	0-0.71	0.71	0-2.38	0	0-0.17	0.62	0-5.1	0.124	0.571	0.191	0.239
Turicibacter	0.17	0-2.62	0.10	0-0.71	0.71	0-2.38	0	0-0.17	0.62	0-5.1	0.124	0.548	0.191	0.239
Unclassified	0.17	0-2.62	0.10	0-0.71	0.71	0-2.38	0	0-0.17	0.62	0-5.1	0.124	0.577	0.191	0.239
Clostridia	31.14	14.24-57.79	33.98	17.4-82.58	49.56*	40.96-79.98	59.32*	20.57-70.1	56.39*	40.92-85.56	0.361	0.492	0.001	0.007
Clostridiales	31.14	14.24-57.79	33.98	17.4-82.58	49.56*	40.96-79.98	59.26*	20.57-70.1	56.39*	40.92-85.56	0.362	0.495	0.001	0.007
Other	0	0-0.87	0	0-0.94	0	0-1.03	0	0-0	0.06	0-1.27	0.368	0.571	0.169	0.220

Table 4.6. Continued.

Taxa	HC Day 0		IBD0		IBD30		IBD90		IBD270		IBD0 vs IBD30, IBD90, IBD270		HC vs IBD0, IBD30, IBD90, IBD270	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Other	0	0-0.87	0	0-0.94	0	0-1.03	0	0-0	0.06	0-1.27	0.368	0.548	0.169	0.220
Other	0	0-0.87	0	0-0.94	0	0-1.03	0	0-0	0.06	0-1.27	0.368	0.577	0.169	0.220
Unclassified	0.32	0-1.39	0	0-0.55	0.4	0-2.67	0.12	0-1.65	0.16	0-0.73	0.231	0.571	0.162	0.219
Unclassified	0.32	0-1.39	0	0-0.55	0.40	0-2.67	0.12	0-1.65	0.16	0-0.73	0.231	0.548	0.162	0.219
Unclassified	0.32	0-1.39	0	0-0.55	0.40	0-2.67	0.12	0-1.65	0.16	0-0.73	0.231	0.577	0.162	0.219
Clostridiaceae	7.95	1.87-16.9	12.28	5.81-56.96	15.61	6.64-56.33	24.09*	2.94-45.79	25.72*	11.83-64.05	0.624	0.681	0.005	0.022
Other	5.75	0.99-14.21	7.28	0.97-43.74	12.39	0.56-37.97	18.92*	0.8-33.46	17.96	8.33-48.78	0.362	0.548	0.033	0.060
Other	5.75	0.99-14.21	7.28	0.97-43.74	12.39	0.56-37.97	18.92*	0.8-33.46	17.96	8.33-48.78	0.362	0.577	0.033	0.060
Unclassified	1.60	0-5.49	2.86	0.87-11.69	3.78*	2.32-9.62	4.63	0.95-6.79	6.62*	2.18-11.88	0.426	0.548	0.004	0.021
Unclassified	1.60	0-5.49	2.86	0.87-11.69	3.78*	2.32-9.62	4.63	0.95-6.79	6.62*	2.18-11.88	0.426	0.577	0.013	0.039
Clostridium	0.45	0-4.13	3.47*	0.22-11.26	1.14	0.37-8.74	0.77	0-5.54	1.48	0.51-3.39	0.297	0.548	0.035	0.063
Other	0.36	0-3.87	2.01	0-9.49	1.14*	0.37-5	0.29	0-3.3	0.99	0.51-1.19	0.273	0.577	0.029	0.058
Unclassified	0	0-0.26	0.48*	0-1.98	0	0-1.05	0	0-1.03	0	0-0.84	0.147	0.577	0.012	0.037
Lachnospiraceae	12.82	2.34-25.68	18.88	2.9-27.81	23.73*	13.51-66.62	24.75*	12-32.97	25.7*	19.86-29.76	0.219	0.571	0.003	0.018
Other	2.43	0.21-6.63	4.14	0.31-9.31	4.91	1.44-11.47	7.01*	1.26-14.11	7.14*	5.39-13.98	0.211	0.548	0.008	0.028
Other	2.43	0.21-6.63	4.14	0.31-9.31	4.91	1.44-11.47	7.01*	1.26-14.11	7.14*	5.39-13.98	0.211	0.577	0.008	0.028
Unclassified	2.17	0-3.9	0*	0-4.27	0.34	0-3.73	1.64	0-4.55	0.69	0-2.46	0.073	0.548	0.038	0.066
Unclassified	2.17	0-3.9	0*	0-4.27	0.34	0-3.73	1.64	0-4.55	0.69	0-2.46	0.073	0.577	0.038	0.066

Table 4.6. Continued.

Taxa	HC Day 0		IBD0		IBD30		IBD90		IBD270		IBD0 vs IBD30, IBD90, IBD270		HC vs IBD0, IBD30, IBD90, IBD270	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Blautia	4.18	1.14-12.4	5.63	0.33-16.9	10.42	1.12-24.2	8.64	4.46-22.5	9.92	8.15-10.94	0.331	0.548	0.024	0.051
Unclassified	1.12	0-6.34	0	0-4.72	0.61	0-5.71	2.69	0-5.84	2.68	0-4.24	0.376	0.577	0.510	0.535
producta	3.37	1.14-9.56	4.31	0.33-14.2	7.55*	0.58-24.2	6.89	2.55-20.57	6.55	5.29-10.94	0.359	0.577	0.020	0.049
Dorea	0	0-2.21	0.54	0-2.42	0.82	0-2.61	0.58	0-2.84	0.52	0-1.36	0.818	0.841	0.117	0.167
Unclassified	0	0-2.21	0.54	0-2.42	0.82	0-2.61	0.58	0-2.84	0.52	0-1.36	0.818	0.838	0.117	0.167
[Ruminococcus]	2.76	0.43-4.76	3.26	0.31-11.33	4.59	1.97-50.85	3.73	1.05-8.95	4.62*	4.23-8.66	0.389	0.548	0.040	0.068
Unclassified	0	0-1.05	0	0-2.5	0	0-2.1	0.20	0-1.63	0.51	0-0.85	0.911	0.911	0.794	0.800
gnavus	2.30	0-4.76	2.07	0.31-11.33	3.35	1.75-50.85	3.23	1.05-8.95	4.11	3.58-7.81	0.373	0.577	0.063	0.099
Peptostreptococcaceae	0	0-4.75	1.06	0-4.26	0.53	0-3.03	0	0-3.67	0.17	0-0.82	0.377	0.571	0.320	0.359
Unclassified	0	0-4.75	1.06	0-4.26	0.53	0-3.03	0	0-3.67	0.17	0-0.82	0.364	0.548	0.258	0.292
Unclassified	0	0-4.75	1.06	0-4.26	0.53	0-3.03	0	0-3.67	0.17	0-0.82	0.364	0.577	0.258	0.292
Ruminococcaceae	3.29	0.1-10.88	0*	0-5.11	0.03*	0-4.45	0.18	0-6.35	0.08	0-1.39	0.556	0.635	0.002	0.013
Unclassified	0.61	0-3.45	0*	0-1.64	0*	0-2.55	0.09	0-4.05	0*	0-0.17	0.591	0.665	0.004	0.021
Unclassified	0.61	0-3.45	0*	0-1.64	0*	0-2.55	0.09	0-4.05	0*	0-0.17	0.591	0.653	0.004	0.021
Faecalibacterium	2.96	0-9.38	0*	0-3.47	0	0-1.09	0	0-2.1	0	0-1.39	0.705	0.746	0.017	0.043
prausnitzii	2.96	0-9.38	0*	0-3.47	0	0-1.09	0	0-2.1	0	0-1.39	0.705	0.74	0.017	0.043
Veillonellaceae	4.34	0.06-9.69	0.08*	0-8.67	0.07*	0-3.85	0.68	0-8.83	0.1	0.1-5.1	0.337	0.571	0.007	0.028
Megamonas	3.20	0.06-7.48	0.06*	0-8.67	0.07*	0-3.85	0.30	0-8.83	0.10	0-4.96	0.374	0.548	0.008	0.028

Table 4.6. Continued.

Taxa	HC Day 0		IBD0		IBD30		IBD90		IBD270		IBD0 vs IBD30, IBD90, IBD270		HC vs IBD0, IBD30, IBD90, IBD270	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Unclassified	3.20	0.06-7.48	0.02*	0-8.67	0.07*	0-3.85	0.24	0-8.83	0.10	0-4.96	0.380	0.577	0.007	0.028
Phascolarctobacterium	0.18	0-1.53	0*	0-0	0*	0-0.06	0*	0-0.12	0	0-0.11	0.341	0.548	0.005	0.022
Unclassified	0.18	0-1.53	0*	0-0	0*	0-0.06	0*	0-0.12	0	0-0.11	0.341	0.577	0.005	0.022
Erysipelotrichi	5.00	0.28-40.85	3.02	0.07-20.22	3.04	0.99-11.44	7.6	1.06-27.31	4.58	0.92-18.8	0.219	0.492	0.432	0.457
Erysipelotrichales	5.00	0.28-40.85	3.02	0.07-20.22	3.04	0.99-11.44	7.60	1.06-27.31	4.58	0.92-18.8	0.219	0.495	0.432	0.457
Erysipelotrichaceae	5	0.28-40.85	3.02	0.07-20.22	3.04	0.99-11.44	7.6	1.06-27.31	4.58	0.92-18.8	0.219	0.571	0.432	0.457
Unclassified	0.48	0-3.36	0.06	0-0.99	1.22	0-6.98	0.59	0-4.06	0.76	0.09-3.23	0.292	0.548	0.160	0.219
Unclassified	0.48	0-3.36	0.06	0-0.99	1.22	0-6.98	0.59	0-4.06	0.76	0.09-3.23	0.292	0.577	0.160	0.219
Allobaculum	0	0-4.39	0	0-3.48	0	0-1.99	0.77	0-4.09	2.58	0-3.43	0.222	0.548	0.374	0.405
Unclassified	0	0-4.39	0	0-3.48	0	0-1.99	0.77	0-4.09	2.58	0-3.43	0.222	0.577	0.374	0.405
Catenibacterium	0.61	0-34.6	0.25	0-20.22	0.08	0-0.99	1.77	0-22.43	0.54	0-7.86	0.292	0.548	0.694	0.710
Unclassified	0.61	0-34.6	0.25	0-20.22	0.08	0-0.99	1.77	0-22.43	0.54	0-7.86	0.292	0.577	0.694	0.710
[Eubacterium]	0.75	0-6.24	0.43	0-1.74	0.79	0-4.46	0.91	0.35-15.64	1.08	0.09-4.27	0.356	0.548	0.536	0.557
biforme	0.07	0-6.24	0.09	0-1.72	0	0-1.19	0.09	0-15.21	0.34	0-4.27	0.310	0.577	0.750	0.762
dolichum	0.04	0-1.05	0.01	0-0.91	0.25	0-4.46	0.39	0-1.06	0.37	0-0.8	0.304	0.577	0.230	0.269
Fusobacteria	23.65	1.01-44.81	3.14*	0-57.25	9.03	0.11-44.36	6.86*	0.51-16.82	5.57	1.78-29.73	0.412	0.646	0.023	0.051
Fusobacteriia	23.65	1.01-44.81	3.14*	0-57.25	9.03	0.11-44.36	6.86*	0.51-16.82	5.57	1.78-29.73	0.412	0.492	0.023	0.051
Fusobacteriales	23.65	1.01-44.81	3.14*	0-57.25	9.03	0.11-44.36	6.86*	0.51-16.82	5.57	1.78-29.73	0.412	0.495	0.023	0.051

Table 4.6. Continued.

Taxa	HC Day 0		IBD0		IBD30		IBD90		IBD270		IBD0 vs IBD30, IBD90, IBD270		HC vs IBD0, IBD30, IBD90, IBD270	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Fusobacteriaceae	23.65	1.01-44.81	3.14*	0-57.25	9.03	0.11-44.36	6.86*	0.51-16.82	5.57	1.78-29.73	0.412	0.571	0.023	0.051
Fusobacterium	23.65	0.96-44.81	3.14*	0-57.25	9.03	0.11-44.36	6.86*	0.51-16.82	5.57	1.78-29.73	0.412	0.548	0.023	0.051
Unclassified	23.65	0.96-44.81	3.14*	0-57.25	9.03	0.11-44.36	6.86*	0.51-16.82	5.57	1.78-29.73	0.412	0.577	0.023	0.051
Proteobacteria	5.95	0.29-15.45	4.74	0-36.96	3.44	0.11-10.27	2.22	0-3.75	1.19	0.74-2.48	0.211	0.558	0.038	0.066
Betaproteobacteria	3.16	0-7.52	0.27	0-4.38	0.31*	0-0.8	0*	0-0.32	0*	0-0.17	0.188	0.492	<0.001	0.007
Burkholderiales	3.16	0-7.52	0.27	0-4.38	0.31*	0-0.8	0*	0-0.32	0*	0-0.17	0.188	0.495	<0.001	0.007
Alcaligenaceae	3.16	0-7.52	0.09*	0-4.38	0.31*	0-0.8	0*	0-0.32	0*	0-0.17	0.379	0.571	<0.001	0.007
Sutterella	3.16	0-7.52	0*	0-4.38	0.31*	0-0.8	0*	0-0.32	0*	0-0.17	0.399	0.548	<0.001	0.007
Unclassified	3.16	0-7.52	0*	0-4.38	0.31*	0-0.8	0*	0-0.32	0*	0-0.17	0.399	0.577	<0.001	0.007
Epsilonproteobacteria	0.86	0-4.27	0	0-1.27	0	0-1.3	0.20	0-2.44	0.03	0-0.55	0.326	0.492	0.048	0.079
Campylobacterales	0.86	0-4.27	0	0-1.27	0	0-1.3	0.20	0-2.44	0.03	0-0.55	0.326	0.495	0.048	0.079
Helicobacteraceae	0.78	0-3.57	0	0-1.27	0	0-1.13	0.01	0-2.44	0.03	0-0.55	0.428	0.571	0.205	0.242
Helicobacter	0.78	0-3.57	0	0-1.27	0	0-1.13	0.01	0-2.44	0.03	0-0.55	0.546	0.634	0.164	0.220
Unclassified	0.78	0-3.57	0	0-0.65	0*	0-0.41	0	0-1.73	0	0-0.06	0.499	0.625	0.024	0.051
Gammaproteobacteria	1.34	0-8.19	0.71	0-36.62	1.97	0-10.27	0.93	0-3.09	1.12	0.08-2.48	0.317	0.492	0.923	0.923
Aeromonadales	0.19	0-5.66	0*	0-0	0	0-0.07	0	0-2.2	0.04	0-0.45	0.363	0.495	0.033	0.060
Succinivibrionaceae	0.19	0-5.66	0*	0-0	0	0-0.07	0	0-2.2	0.04	0-0.45	0.363	0.571	0.033	0.060
Succinivibrio	0	0-3.06	0	0-0	0	0-0.07	0	0-0.31	0.04	0-0.45	0.198	0.548	0.082	0.125
Unclassified	0	0-3.06	0	0-0	0	0-0.07	0	0-0.31	0.04	0-0.45	0.198	0.577	0.082	0.125
Enterobacteriales	0.10	0-8.19	0.71	0-36.62	1.97	0-10.2	0.70	0-3.09	1.12	0-1.65	0.381	0.495	0.241	0.277

Table 4.6. Continued.

Taxa	HC Day 0		IBD0		IBD30		IBD90		IBD270		IBD0 vs IBD30, IBD90, IBD270		HC vs IBD0, IBD30, IBD90, IBD270	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	P value	Q value	P value	Q value
Enterobacteriaceae	0.1	0-8.19	0.71	0-36.62	1.97	0-10.2	0.7	0-3.09	1.12	0-1.65	0.381	0.571	0.241	0.277
Unclassified	0.10	0-8.19	0.67	0-36.62	1.97	0-10.2	0.70	0-3.09	1.12	0-1.65	0.394	0.548	0.334	0.368
Unclassified	0.10	0-8.19	0.67	0-36.62	1.97	0-10.2	0.70	0-3.09	1.12	0-1.65	0.394	0.577	0.334	0.368

First column p- values were obtained with mixed effects testing, while second column were obtained with Kruskal Wallis testing.

Q values are the adjusted P values after Benjamini and Hochberg False Discovery Rate correction for multiple comparisons.

‡Unclassified=matches (97%) similarity to a reference sequence undefined at the given taxonomic level.

‡‡Other=ambiguous assignment because QIIME cannot distinguish between taxa within that taxonomic level.

‡‡‡Square brackets=proposed taxonomic grouping according to the Greengenes database used in the QIIME pipeline.

Highlighted cells indicate significant P or Q values.

4.2.2. Tryptophan metabolites

4.2.2.1. Baseline comparison

At baseline, several tryptophan metabolites were altered in GI disease groups when compared to healthy control dogs (Figure 4.9). In dogs with AD, fecal concentrations of indole were increased (q=0.034) and concentrations of indole-3-acetaldehyde were decreased (q=0.024) when compared to healthy dogs. In dogs with AHDS, fecal concentrations of anthranilic acid, indole, indole-3-acetamide, and tryptophan were all significantly increased when compared to healthy dogs (q=0.025, q=0.002, q=0.003, and q<0.001, respectively). In addition, dogs with AHDS had decreased fecal concentrations of indole-3-acetic acid and tryptamine (q=0.032 and q=0.027, respectively) when compared to healthy dogs. In dogs with IBD, fecal concentrations of indole-3-acetamide were significantly increased (q=0.002) when compared to healthy dogs at baseline.

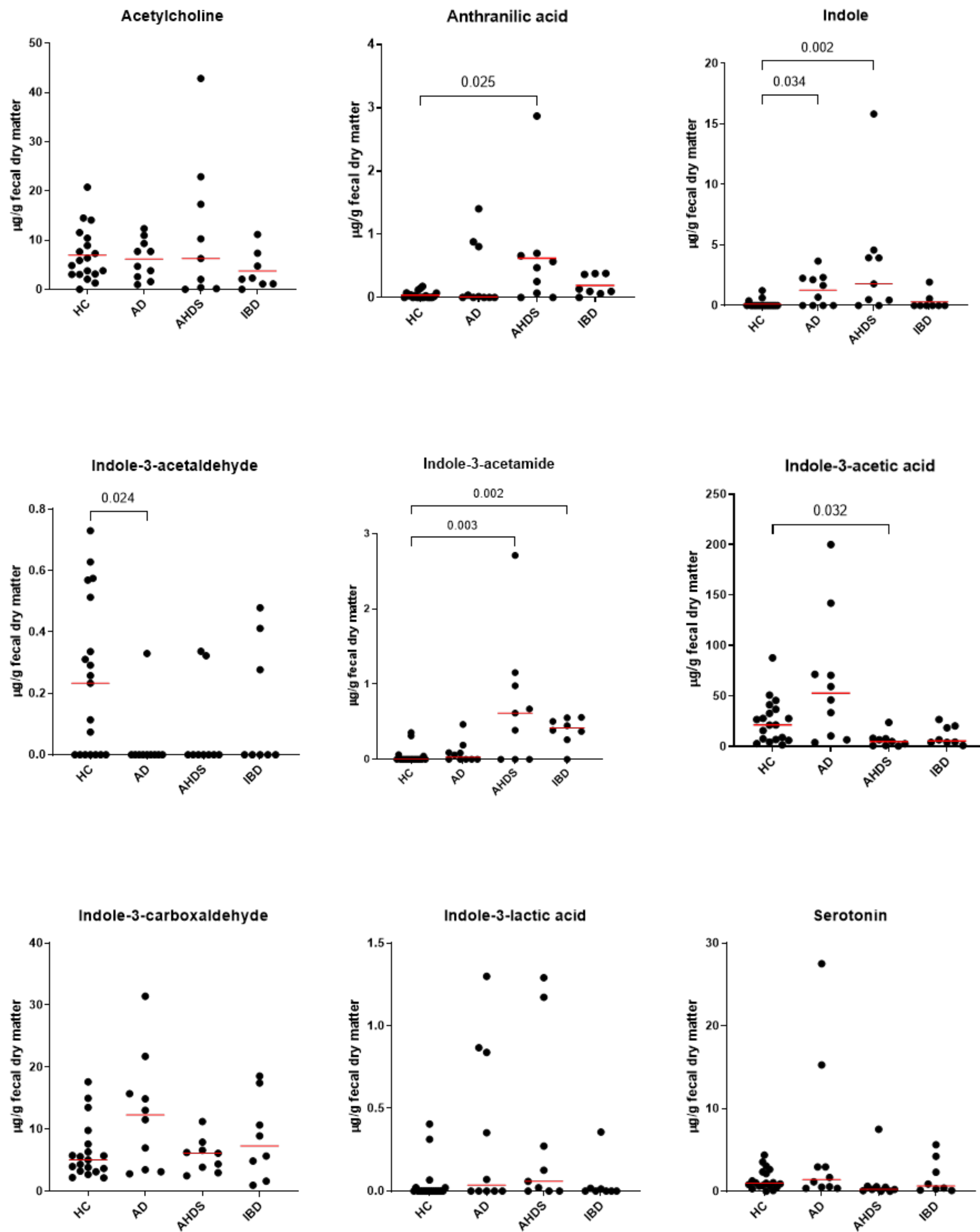

Figure 4.9. Fecal concentrations of tryptophan metabolites at baseline from the dogs with acute diarrhea (AD), acute hemorrhagic diarrhea (AHDS), inflammatory bowel disease (IBD), and the healthy control dogs (HC).

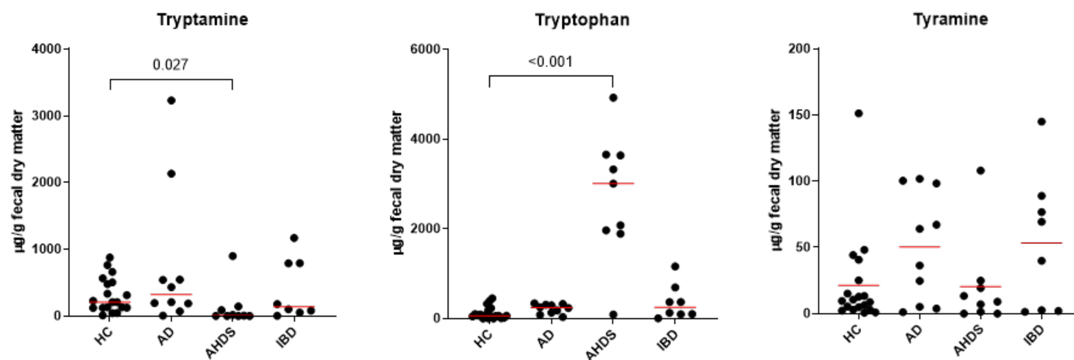

Figure 4.9. Continued.

4.2.2.2. AD over time

Fecal concentrations of tryptophan metabolites in dogs with acute diarrhea over time are plotted in Figure 4.10. When compared to healthy control dogs, fecal concentrations of indole were increased at baseline (AD0; q=0.014) and fecal concentrations of anthranilic acid were decreased at day 6 (AD6; q=0.036). Fecal concentrations of indole-3-acetaldehyde were persistently decreased in dogs with acute diarrhea at day 6 of follow up when compared to healthy control dogs (AD0, q=0.011; AD6, q=0.006). Fecal concentrations of tryptophan were significantly increased in dogs with AD at day 30 (AD30) compared to healthy control dogs (q<0.001) as well as compared to dogs with AD at baseline (q=0.015). All other tryptophan metabolites did not significantly change in follow up samples compared to baseline.

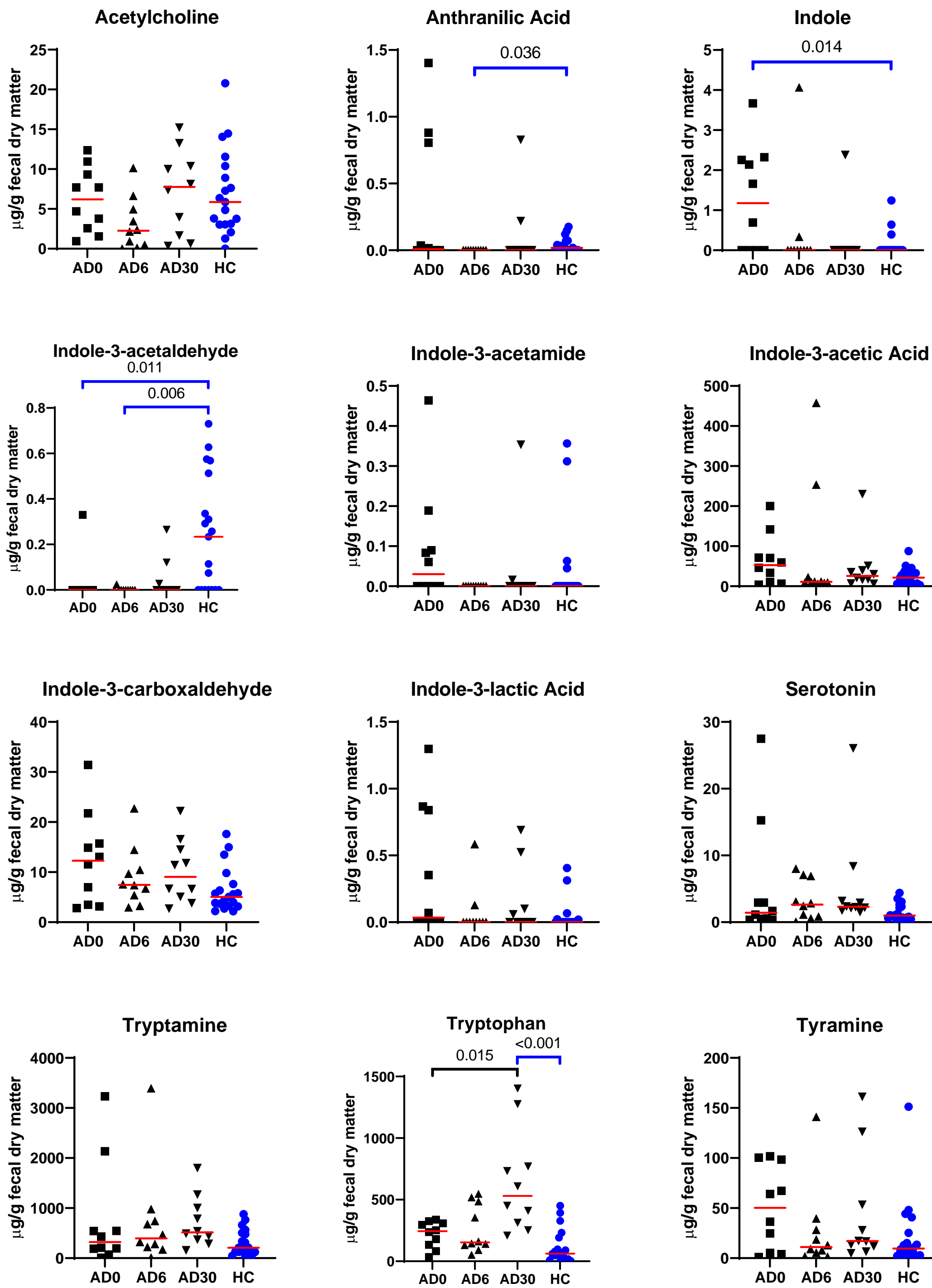

Figure 4.10. Fecal concentrations of tryptophan metabolites in dogs with acute diarrhea (AD) over time. Groups significantly different (Kruskal-Wallis testing with

Dunn's *post hoc* tests) from HC are indicated with blue bars. Differences between AD follow up timepoints and AD baseline (Friedman testing with Dunn's *post hoc* tests) are indicated with black bars. Red lines indicate medians.

4.2.2.3. AHDS over time

Fecal concentrations of tryptophan metabolites in dogs with acute hemorrhagic diarrhea over time are plotted in Figure 4.11. When compared to healthy control dogs, fecal concentrations of indole-3-acetamide and tryptophan were increased at baseline (AHDS0; q=0.009 and q<0.001, respectively), fecal concentrations of indole-3-acetic acid were decreased at baseline (q=0.014), and fecal concentrations of indole-3-acetaldehyde were decreased from day 7 to day 90 of follow up (AHDS7, AHDS14, and AHDS90; q=0.001, q=0.002, and q=0.034, respectively). Fecal concentrations of indole were persistently increased in dogs with acute hemorrhagic diarrhea to day 14 of follow up when compared to healthy control dogs (AHDS0, q=0.004; AHDS7, q=0.006; AHDS14, q=0.021). Additionally, when compared to baseline, fecal concentrations of tryptamine were significantly increased at day 14 (AHDS14, q=0.021) and fecal concentrations of tryptophan were significantly decreased in all three follow up timepoints (AHDS7, q<0.001; AHDS14, q<0.001; AHDS90, q=0.005).

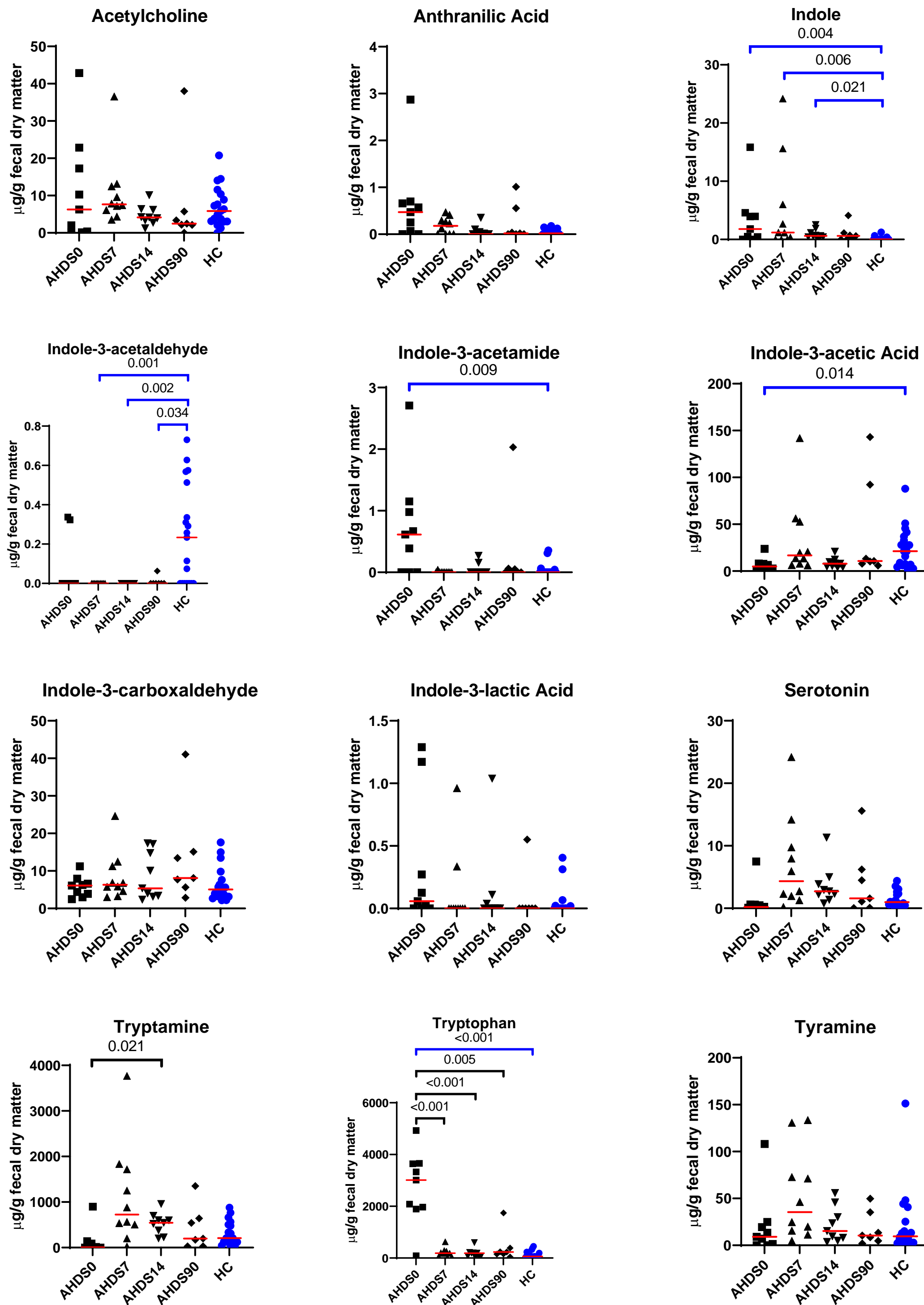

Figure 4.11. Fecal concentrations of tryptophan metabolites in dogs with acute hemorrhagic diarrhea (AHDS) over time. Groups significantly different (Kruskal-

Wallis testing with Dunn's *post hoc* tests) from HC are indicated with blue bars. Differences between AHDS follow up timepoints and AHDS baseline (mixed-effects analysis with Holm-Sidak's multiple comparison tests) are indicated with black bars. Red lines indicate medians.

4.2.2.4. IBD over time

Fecal concentrations of tryptophan metabolites in dogs with inflammatory bowel disease over time are plotted in Figure 4.12. When compared to healthy control dogs, fecal concentrations of indole-3-acetamide were increased at baseline in dogs with IBD (IBD0, $q<0.001$), and tryptamine concentrations were increased at day 30 and day 270 (IBD30, $q=0.014$; IBD270, $q=0.004$). In dogs with IBD, fecal concentrations of indole-3-acetamide decreased significantly at day 30, day 90, and day 270 follow up samples when compared to baseline ($q=0.002$, $q=0.002$, and $q=0.005$, respectively). All other tryptophan metabolites did not significantly change in follow up samples compared to baseline in dogs with IBD.

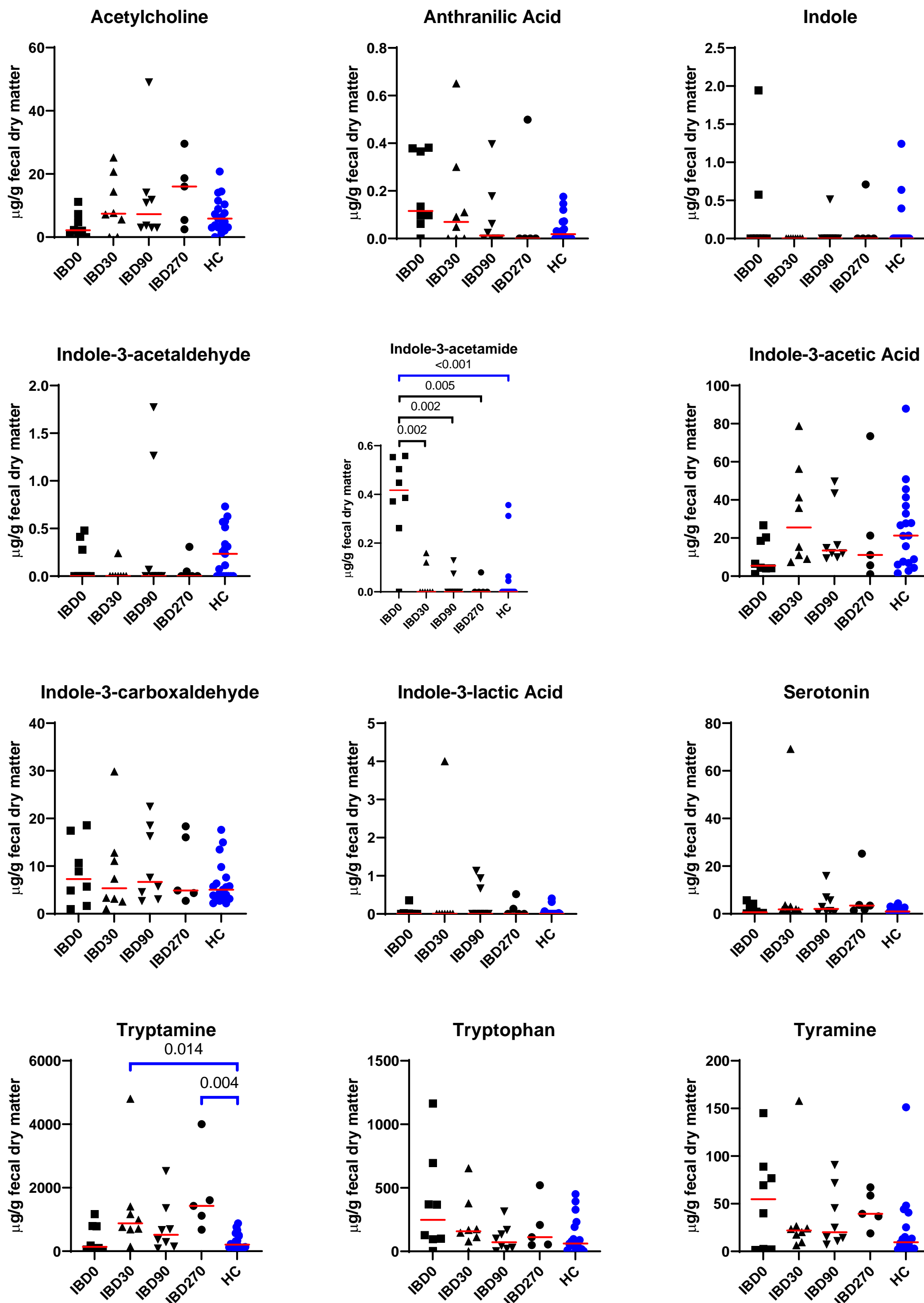

Figure 4.12. Fecal concentrations of tryptophan metabolites in dogs with inflammatory bowel disease (IBD) over time. Groups significantly different (Kruskal-

Wallis testing with Dunn's *post hoc* tests) from HC are indicated with blue bars. Differences between IBD follow up timepoints and IBD baseline (mixed-effects analysis with Holm-Sidak's multiple comparison tests) are indicated with black bars. Red lines indicate medians.

4.2.2.5. Correlation of metabolites with bacterial groups

Several bacterial groups strongly correlated with tryptophan metabolites at baseline sampling (Table 4.7). Fecal concentrations of indole-3-acetamide positively correlated with abundance of *Corynebacterium* (ρ=0.465, q=0.035) and negatively correlated with abundance of *Bacteroides* (ρ=-0.542, q=0.035) and *Megamonas* spp. (ρ=-0.492, q=0.035). Fecal concentrations of indole-3-acetaldehyde positively correlated with abundance of *Colinsella* (ρ=0.448, q=0.046) and *Turicibacter* (ρ=0.471, q=0.035). Fecal concentrations of indole-3-acetamide negatively correlated with abundance of *Bacteroides* (ρ=-0.542, q=0.035) and *Megamonas* spp. (ρ=-0.492, q=0.035). Fecal concentrations of indole-3-carboxaldehyde negatively correlated with abundance of *Bacteroides* (ρ=-0.449, q=0.046) and *Megamonas* spp. (ρ=-0.452, q=0.046). Fecal concentrations of tryptophan negatively correlated with abundance of *Bacteroides* (ρ=-0.538, q=0.035) and *Megamonas* spp. (ρ=-0.437, q=0.046), and positively correlated with abundance of *Clostridium* (ρ=0.529, q=0.035). Fecal concentrations of tyramine positively correlated with abundance of *Enterococcus* (ρ=0.525, q=0.035) and *Blautia* (ρ=0.445, q=0.046). Fecal concentrations of serotonin and tryptamine also positively correlated with abundance of *Blautia* (ρ=0.472, q=0.035; ρ=0.456, q=0.035; respectively).

Table 4.7. Spearman's correlations between tryptophan metabolites and bacterial taxa. Only significant correlations are listed.

Metabolite	Bacterial Taxa	R value	Q-value
anthranilic acid	*Prevotella copri*	-0.447	0.046
indole	*Bacteroides plebeius*	-0.440	0.046
indole	Other Pasteurellaceae genus	0.498	0.035
indole	Other Pasteurellaceae sp.	0.498	0.035
indole-3-acetaldehyde	Coriobacteriia	0.440	0.046
indole-3-acetaldehyde	Coriobacteriales	0.440	0.046
indole-3-acetaldehyde	Coriobacteriaceae	0.440	0.046
indole-3-acetaldehyde	*Collinsella*	0.448	0.046
indole-3-acetaldehyde	*Collinsella stercoris*	0.441	0.046
indole-3-acetaldehyde	Turicibacterales	0.471	0.035
indole-3-acetaldehyde	Turicibacteraceae	0.471	0.035
indole-3-acetaldehyde	*Turicibacter*	0.471	0.035
indole-3-acetaldehyde	Unclassified *Turicibacter* sp.	0.471	0.035
indole-3-acetaldehyde	Unclassified *Blautia* sp.	0.553	0.035
indole-3-acetaldehyde	Erysipelotrichi	0.500	0.035
indole-3-acetaldehyde	Erysipelotrichales	0.500	0.035
indole-3-acetaldehyde	Erysipelotrichaceae	0.500	0.035
indole-3-acetamide	Actinomycetales	0.620	0.035
indole-3-acetamide	Corynebacteriaceae	0.465	0.035
indole-3-acetamide	*Corynebacterium*	0.465	0.035
indole-3-acetamide	Unclassified *Corynebacterium* sp.	0.465	0.035
indole-3-acetamide	Bacteroidetes	-0.508	0.035
indole-3-acetamide	Bacteroidia	-0.544	0.035
indole-3-acetamide	Bacteroidales	-0.544	0.035
indole-3-acetamide	Bacteroidaceae	-0.542	0.035
indole-3-acetamide	*Bacteroides*	-0.542	0.035
indole-3-acetamide	Unclassified Lachnospiraceae genus	-0.443	0.046
indole-3-acetamide	Unclassified Lachnospiraceae sp.	-0.443	0.046
indole-3-acetamide	Ruminococcaceae	-0.515	0.035
indole-3-acetamide	*Megamonas*	-0.471	0.035
indole-3-acetamide	Unclassified *Megamonas* sp.	-0.492	0.035
indole-3-acetamide	Unclassified Erysipelotrichaceae genus	-0.493	0.035
indole-3-acetamide	Unclassified Erysipelotrichaceae sp.	-0.493	0.035
indole-3-carboxaldehyde	Bacteroidetes	-0.474	0.035
indole-3-carboxaldehyde	Bacteroidia	-0.440	0.046
indole-3-carboxaldehyde	Bacteroidales	-0.440	0.046
indole-3-carboxaldehyde	Bacteroidaceae	-0.449	0.046

Table 4.7. Continued.

Metabolite	Bacterial Taxa	R value	Q-value
indole-3-carboxaldehyde	*Bacteroides*	-0.449	0.046
indole-3-carboxaldehyde	Lactobacillaceae	0.447	0.046
indole-3-carboxaldehyde	*Megamonas*	-0.445	0.046
indole-3-carboxaldehyde	Unclassified *Megamonas* sp.	-0.452	0.046
serotonin	*Blautia*	0.472	0.035
serotonin	*Blautia producta*	0.490	0.035
tryptamine	*Blautia*	0.456	0.035
tryptamine	*Blautia producta*	0.470	0.035
tryptophan	Actinomycetales	0.481	0.035
tryptophan	Bacteroidetes	-0.519	0.035
tryptophan	Bacteroidia	-0.510	0.035
tryptophan	Bacteroidales	-0.510	0.035
tryptophan	Bacteroidaceae	-0.538	0.035
tryptophan	*Bacteroides*	-0.538	0.035
tryptophan	Unclassified *Bacteroides* sp.	-0.444	0.046
tryptophan	*Bacteroides plebeius*	-0.474	0.035
tryptophan	Clostridiaceae	0.497	0.035
tryptophan	*Clostridium*	0.529	0.035
tryptophan	Other *Clostridium* sp.	0.492	0.035
tryptophan	*Megamonas*	-0.447	0.046
tryptophan	Unclassified *Megamonas* sp.	-0.437	0.046
tyramine	Lactobacillales	0.447	0.046
tyramine	Enterococcaceae	0.550	0.035
tyramine	*Enterococcus*	0.525	0.035
tyramine	Unclassified Enterococcus sp.	0.587	0.035
tyramine	*Blautia*	0.445	0.046
tyramine	*Blautia producta*	0.453	0.046

4.3. Discussion

Studies examining changes in metabolic pathways, such as tryptophan metabolism, in dogs with GI disease are lacking. We identified changes in the microbiota as well as in tryptophan metabolites in the feces of dogs with GI diseases, both compared to healthy control dogs and over time within each disease group. Dogs

with GI disease at initial presentation (baseline) had distinctly different microbial communities and different concentrations of tryptophan metabolites when compared to healthy control dogs.

At initial presentation (baseline), dogs with AHDS had a lower microbial diversity (Shannon index) than healthy dogs, and all diseased groups (AD, AHDS, IBD) had a significantly different microbial community composition (ANOSIM) and clustered separately (PCoA) from healthy dogs. These differences were driven by several key bacterial groups. All diseased groups had significantly decreased abundance of *Bacteroides* when compared to healthy dogs. Other bacterial groups were increased in diseased dogs, such as *Enterococcus* in dogs with IBD, *Lactobacillus* in dogs with AD, and *Streptococcus* in dogs with AHDS or IBD. Previous studies have identified similar changes in bacterial taxa in these disease states in dogs [2, 4, 10, 14, 15]. Decreased proportions of Bacteroidetes are commonly observed in dogs with AD [4, 10] and dogs with IBD [14]. However, decreases in Bacteroidetes in dogs with AHDS are uncommon [4] and one study even showed a decreased in *Streptococcus* in contrast to our study showing an increase [14]. These discrepancies could be due to low sample number in the current study and should be interpreted with caution.

In this study, in addition to changes in the microbiota at baseline, fecal concentrations of metabolites from the tryptophan metabolism pathways were also altered in disease states at baseline. Dogs with AD had an increased fecal concentration of indole and decreased concentration of indole-3-acetaldehyde. Dogs with AHDS had an increased fecal concentration of anthranilic acid, indole, indole-3acetamide, and

tryptophan and decreased concentration of indole-3-acetic acid and tryptamine. Dogs with IBD had an increased fecal concentration of indole-3-acetamide. Changes in these metabolites in the feces was expected based on previous untargeted metabolomics analysis in dogs with IBD, which showed fecal indoleacetate and indolepropionate to be decreased in diseased dogs [152]. Additionally, tryptophan concentrations are significantly decreased in the serum or plasma and inversely correlated with disease activity in both humans and cats with IBD [65, 68, 153]. The increased concentrations of tryptophan in feces of dogs with AHDS may be due to several reasons: 1) decreased digestion and absorption of dietary tryptophan (potentially due to increased transit time), 2) decreased bacterial or host utility of dietary tryptophan in metabolic pathways, or 3) increased elimination of tryptophan via intestinal secretions or sloughing of epithelial cells. Furthermore, dogs with AD had increasing fecal concentrations of tryptophan with follow up sampling and were significantly increased compared to healthy dogs at day 30. More mechanistic studies are needed to determine why fecal concentrations of tryptophan are increased in AHDS and AD. However, tryptophan supplementation holds promise as a therapeutic treatment for GI disease. In a dextran-induced colitis model in pigs, tryptophan supplementation at 80% recommended daily dietary intake decreased intestinal permeability, reduced symptoms, and reduced expression of pro-inflammatory cytokines [130]. Supplementation of tryptophan or other amino acids, while shown to have positive effects in animal models of GI disease, have not been pursued in large human clinical trials [154].

Another metabolite altered in dogs with GI disease was tryptamine, which can be produced via host or bacterial enzymes and acts as a neurotransmitter and stimulates intestinal motility [138]. Tryptamine also decreased monolayer permeability in an *in vitro* study using T84 cells [155]. Tryptamine was decreased in dogs with AHDS at baseline compared to healthy dogs and returned to normal levels by day 90 of follow up. Similarly, fecal concentrations of tryptophan decreased to levels resembling healthy dogs by day 7 of follow up in dogs with AHDS, indicating a more rapid return to normal levels. This is in contrast to the slow partial recovery of the intestinal microbiota in dogs with AHDS. At day 90 of follow up, microbial communities still clustered separately from healthy dogs, and abundances of several bacterial groups were still significantly different compared to the healthy control group (i.e., *Bacteroides*, *Clostridia*, *Megamonas*, and *Fusobacterium*). This supports the idea of functional redundancy in the intestinal microbiota [24]. Further supporting this idea, all tryptophan metabolites that were different from healthy dogs at baseline in any disease state were no longer different from healthy dogs at the final follow up time point, and all disease groups had at least one major bacterial group that remained different from healthy controls at the final follow up time point (e.g., *Bacteroides*, *Lactobacillus*, and *Megamonas* in AD30; *Clostridia*, *Suterella* in IBD270). Longer follow up periods are necessary to determine the true recovery of metabolites and microbiota in dogs with AD, AHDS, and IBD.

Another metabolite increased in feces of dogs with AHDS was anthranilic acid, a metabolite in the tryptophan-kynurenine pathway produced by the host. Anthranilic acid is increased in serum of human patients with active Crohn’s disease [153] and has been

demonstrated to inhibit intestinal barrier dysfunction and NF-κβ activation [156]. Increased activity of the tryptophan-kynurenine pathway is associated with increased intestinal inflammation and disease activity in humans with IBD [153, 157]. Unfortunately, kynurenic acid and kynurenine were not measured in the current study, but should be included in future analysis of dogs with GI disease to better understand the role of the tryptophan-kynurenine metabolism pathway in disease.

Indole and several indole derivatives were altered in feces of dogs with GI disease. Indole was increased at baseline in dogs with AD and AHDS, indole-3-acetamide was increased at baseline in dogs with AHDS and IBD, indole-3-acetaldehyde was decreased at baseline in dogs with AD, and indole-3-acetic acid was decreased at baseline in dogs with AHDS. Indole and indole derivatives produced by microbial metabolism of tryptophan in the intestinal lumen serve an important role in maintaining intestinal homeostasis. Indole increased expression of genes involved in strengthening the mucosal barrier and mucin production and expression of anti-inflammatory interleukin-10, and decreased expression of proinflammatory interleukin-8 [132]. Many indole derivatives are AhR agonists which function as signaling molecules to regulate immune response and maintain the mucosal barrier [139]. Bacteria also produce indole as a way of communication to other bacteria through intercellular signaling pathways which can help maintain a balanced multispecies community [135]. Indole and indole derivatives may be useful as a monitoring tool in dogs with GI disease to evaluate changes in the microbiota functional capacity since they can only be produced via microbial pathways.

There are limitations to this study, including the small number of animals enrolled in each disease group and missing data points in follow up sampling. However, the results are very descriptive and can be used to justify larger sampling sizes in future studies. The administration of antibiotics in half of the dogs with AD and probiotics in half of the dogs with AHDS presents as a confounding factor that should be taken into consideration in the interpretation of the bacterial sequencing results. However, when comparing tryptophan metabolites between groups that received these treatments versus those that did not, there were no differences at any timepoint, suggesting that the functional metabolites of the microbiota were unaffected. However, follow-up studies are needed to confirm these initial results in a larger population without confounding factors. Furthermore, administration of steroids in dogs with IBD is common, so our study cohort (6 out of 8 dogs received steroids) represent the patient population. Additionally, with the dogs being owned pets, and the retrospective nature of the study, it was impossible to control for diet of the dogs. While diet has been shown to affect the intestinal microbiota [16], previous studies have also shown that the effect size of disease is larger than that of commercial dietary differences [107, 158]. Other variables such as gender, weight, and age were shown to not be different between the groups analyzed (Table 4.1).

4.4. Conclusions

This study showed dogs with acute diarrhea, acute hemorrhagic diarrhea, and inflammatory bowel disease all had significantly different microbial communities from

healthy dogs. Several tryptophan metabolites were altered in the feces of these dogs, and fecal concentrations of these metabolites recovered more quickly than did the fecal microbiota. This suggests that AD, AHDS, and IBD disease processes in dogs impacts not only the intestinal microbiota but also functional metabolites.

5. CONCLUSIONS

The work presented in this dissertation described amino acid profiles and tryptophan metabolite concentrations in feces of dogs with chronic and acute gastrointestinal diseases. In general, serum concentrations of valine and fecal concentrations of tryptophan were higher in dogs with chronic enteropathy than in healthy control dogs, neither of which corresponded to clinical activity or histopathological scores. Additionally, dogs with acute and chronic gastrointestinal disease exhibited alterations in fecal tryptophan metabolites that correlated with changes in abundance of some bacterial taxa in the feces. Some of these alterations persisted in follow up timepoints.

None of the changes seen were vastly different between disease and healthy groups, and cutoff values, as used in diagnostic assays, would not be applicable to this data. Examples of biomarkers that would be useful could be predictive in nature (predictive of relapse in chronic diarrhea patients that are in remission, predictive of patient outcome, predictive of acute diarrhea becoming a chronic disease) or could be useful monitoring tools (to monitor response to treatment) or even could be used to identify a subset of the disease population that would benefit from additional/alternative therapies. One such example of the later kind of biomarker is bile acids. Recent work measuring fecal bile acids in dogs with chronic enteropathy has identified a subset of patients that seem to have bile acid malabsorption [3]. Furthermore, a case report of two dogs with presumptive bile acid diarrhea showed that they clinically responded to

cholestyramine (a bile acid sequestrant) administration [11]. From our data, it certainly appears that some dogs had serum or fecal amino acid concentrations outside of the range of concentrations from healthy dogs. However, more work needs to be done in order to tell if this represents a subpopulation of dogs that would benefit from any amino acid-related interventions.

While this work was very descriptive, much still needs to be done to investigate the mechanisms behind these alterations. The question of "cause or effect" always comes into play in descriptive studies. However, we simply cannot answer any questions of causality with the results from these studies. Doing so would help pinpoint therapeutic targets of disease and is necessary to move forward with this work. For instance, if decreased digestion or absorption is found to be the cause of increased fecal tryptophan concentrations, one could target therapeutics that would aid in digestion or absorption first rather than supplementing tryptophan to a GI tract that will have trouble absorbing it.

Surprisingly, 5-27% of dogs with CE will have poor long-term response to conventional treatments (i.e., diet, antibiotics, steroids) [1]. Therefore, it is important to investigate novel modes of therapy. Dogs that respond well to dietary change could be considered to have a food allergy, where the immune system is reacting inappropriately to dietary proteins. However, hydrolyzed protein diets inadvertently aid in digestion, because the proteins are already broken down to some extent. The main methods used to identify maldigestion include analysis of feces for undigested and unabsorbed dietary components (e.g., steatorrhea, fecal nitrogen), and measurement of pancreatic enzymes

in the feces (e.g., fecal elastin). However, measurements in feces will generally be inaccurate due to the bacterial metabolic activity in the colon. Novel methods using stable isotopes have recently been used to measure protein digestion and amino acid absorption in healthy and diseased states in humans, rodents, and pigs [159, 160]. Isotopes are chemical elements that contain the same number of protons and different number of neutrons. Stable isotopes do not radioactively decay, meaning they do not emit any particle or radiation, and are stable. Some stable isotopes, such as ^{12}C and ^{14}N are abundant in nature, while others, such as ^{13}C and ^{15}N, have low natural abundance. Those with low natural abundance are often used as tracers to label amino acids (e.g., L-[ring-$^{13}C_6$]phenylalanine) in studies of amino acid metabolism. They are called tracers because they can be used to trace the corresponding atom in the unlabeled molecule (e.g., L-[ring-$^{12}C_6$]phenylalanine), or the tracee. This is due to the principle that the tracer and tracee molecules will behave the same way in the body. The relative amount of tracer to tracee is known as the tracer to tracee ratio or isotope enrichment. Enterally administered ^{15}N-labelled spirulina protein, and L-[ring-$^{2}H_5$]phenylalanine [159, 160] or L-[1-^{13}C]phenylalanine [161] with a protein meal has been used previously to quantify protein digestibility in humans and pigs, respectively. The plasma enrichments of ^{15}N-Phe (Phe from spirulina/Phe from food) and of $^{2}H_5$-Phe or ^{13}C-Phe (Phe from free labelled AA/Phe from food) are calculated, and a ratio of the two will give protein digestibility. Because the calculation is comprised of ratios, the method is independent of metabolism of phenylalanine by the enterocytes or elsewhere in the body. For accurate measurement of absorption, an inert amino acid, or one that will not be utilized

by the host cells, should be used. Kaur et al. did this by using two differently labelled isotopes of allo-isoleucine; one administered enterally and the other intravenously [161]. Then, fraction absorbed can be calculated by dividing plasma concentrations of enteral by parenteral allo-isoleucine isomers.

These stable isotope methods of measuring protein and amino acid digestion and absorption have not previously been performed in dogs and could provide valuable insight into these critical gastrointestinal functions in dogs, in health and disease. Studies are currently underway in our lab to measure the absorption and digestion of amino acids and proteins in dogs with chronic enteropathy. This will aid in pinpointing causality for alterations of AA profiles seen in serum and feces.

In regards to supplementation of amino acids, there are several factors that raise concerns and prevent supplementation studies from being the immediate next step. First, free AA are not absorbed as readily as peptides, and a study in growing rats showed that feeding an isonitrogenous diet of free AA versus in-tact protein led to 62% less weight gain and increased urinary nitrogen excretion [162]. This suggests that for the most efficient use of orally supplemented amino acids, they would need to be incorporated into proteins. In addition, if dogs with GI disease have less digestion and/or absorption capacity, oral administration of any AA or protein supplementation would be required in excess for an appropriate amount of the desired AA to reach circulation. However, excess amino acids or proteins in the GI tract would be available for bacterial utilization, potentially increasing the risk of toxic metabolite production, such as p-cresol sulfate or indoxyl sulfate. Amino acid supplementation could also exacerbate comorbidities such

as kidney disease. In one study of dogs with proteinuric chronic kidney disease, AA supplementation led to an increase in body weight and serum albumin concentration but may have prevented a decrease in proteinuria and urea concentration [163]. Finally, and perhaps the most concerning factor, is that amino acid supplementation in humans with gastrointestinal disease remains a controversial topic and has shown no clear clinical benefit [70]. Although studies of amino acid supplementation in animal models of GI disease appear to be promising [71], any supplementation studies in dogs with GI disease should proceed with an abundance of caution.

Clinical implications of altered tryptophan metabolites in feces of dogs with gastrointestinal disease is not immediately clear. However, these findings have the potential to be incorporated into existing therapies. For example, probiotics are becoming a more common adjunctive therapy for dogs with GI disease. Future studies could investigate whether the addition of specific bacterial strains that support tryptophan metabolism to beneficial products would aid in clinical response to treatment. Furthermore, increased tryptophan metabolism through the kynurenine pathway is associated with endoscopic inflammation in humans with ulcerative colitis [157]. It would be interesting to see if the same holds true in dogs with CE.

In summary, altered serum and fecal amino acid profiles and tryptophan metabolites were identified in dogs with gastrointestinal diseases in addition to altered fecal microbiota. Future studies should aim to uncover the mechanisms behind these alterations to help identify potential therapeutic targets. Studies are currently underway

investigating the absorption and digestion of amino acids and proteins in dogs with chronic gastrointestinal disease.

6. REFERENCES

1. Dandrieux JRS, Mansfield CS. Chronic enteropathy in canines: Prevalence, impact and management strategies. Vet Med (Auckl). 2019;10:203-14. doi: 10.2147/VMRR.S162774.

2. Guard BC, Barr JW, Reddivari L, Klemashevich C, Jayaraman A, Steiner JM, et al. Characterization of microbial dysbiosis and metabolomic changes in dogs with acute diarrhea. PLoS One. 2015;10(5):e0127259. doi: 10.1371/journal.pone.0127259.

3. Guard BC, Honneffer JB, Jergens AE, Jonika MM, Toresson L, Lawrence YA, et al. Longitudinal assessment of microbial dysbiosis, fecal unconjugated bile acid concentrations, and disease activity in dogs with steroid-responsive chronic inflammatory enteropathy. J Vet Intern Med. 2019;33(3):1295-305. doi: 10.1111/jvim.15493.

4. Suchodolski JS, Markel ME, Garcia-Mazcorro JF, Unterer S, Heilmann RM, Dowd SE, et al. The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease. PLoS One. 2012;7(12):e51907. doi: 10.1371/journal.pone.0051907.

5. Werner M, Suchodolski JS, Straubinger RK, Wolf G, Steiner JM, Lidbury JA, et al. Effect of amoxicillin-clavulanic acid on clinical scores, intestinal microbiome, and amoxicillin-resistant escherichia coli in dogs with uncomplicated acute diarrhea. J Vet Intern Med. 2020;34(3):1166-76. doi: 10.1111/jvim.15775.

6. Dandrieux JRS. Inflammatory bowel disease versus chronic enteropathy in dogs: Are they one and the same? J Small Anim Pract. 2016;57(11):589-99. doi: 10.1111/jsap.12588.

7. Simpson KW, Jergens AE. Pitfalls and progress in the diagnosis and management of canine inflammatory bowel disease. Vet Clin North Am Small Anim Pract. 2011;41(2):381-98. doi: 10.1016/j.cvsm.2011.02.003.

8. Rossi G, Pengo G, Caldin M, Piccionello AP, Steiner JM, Cohen ND, et al. Comparison of microbiological, histological, and immunomodulatory parameters in response to treatment with either combination therapy with prednisone and metronidazole or probiotic vsl#3 strains in dogs with idiopathic inflammatory bowel disease. PLoS One. 2014;9(4):e94699. doi: 10.1371/journal.pone.0094699.

9. Rossi G, Cerquetella M, Gavazza A, Galosi L, Berardi S, Mangiaterra S, et al. Rapid resolution of large bowel diarrhea after the administration of a combination of a high-fiber diet and a probiotic mixture in 30 dogs. Vet Sci. 2020;7(1):21. doi: 10.3390/vetsci7010021.

10. Chaitman J, Ziese A-L, Pilla R, Minamoto Y, Blake AB, Guard BC, et al. Fecal microbial and metabolic profiles in dogs with acute diarrhea receiving either fecal microbiota transplantation or oral metronidazole. Front Vet Sci. 2020;7(192). doi: 10.3389/fvets.2020.00192.

11. Toresson L, Steiner JM, Suchodolski JS. Cholestyramine treatment in two dogs with presumptive bile acid diarrhoea: A case report. Canine Med Genet. 2021;8(1):1. doi: 10.1186/s40575-021-00099-x.

12. Honneffer J, Guard B, Steiner JM, Suchodolski JS. Untargeted metabolomics reveals disruption within bile acid, cholesterol, and tryptophan metabolic pathways in dogs with idiopathic inflammatory bowel disease. Gastroenterol. 2015;148(4):S715-S.

13. Handl S, Dowd SE, Garcia-Mazcorro JF, Steiner JM, Suchodolski JS. Massive parallel 16s rrna gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. FEMS Microbiol Ecol. 2011;76(2):301-10. doi: 10.1111/j.1574-6941.2011.01058.x.

14. Markel M, Berghoff N, Unterer S, Oliveira-Barros LM, Grellet A, Allenspach K, et al. Characterization of fecal dysbiosis in dogs with chronic enteropathies and acute hemorrhagic diarrhea. J Vet Intern Med. 2012;26(3):765-6. doi: 10.1111/j.1939-1676.2012.00937.x.

15. AlShawaqfeh MK, Wajid B, Minamoto Y, Markel M, Lidbury JA, Steiner JM, et al. A dysbiosis index to assess microbial changes in fecal samples of dogs with chronic inflammatory enteropathy. FEMS Microbiol Ecol. 2017;93(11):fix136. doi: 10.1093/femsec/fix136.

16. Pilla R, Suchodolski JS. The role of the canine gut microbiome and metabolome in health and gastrointestinal disease. Front Vet Sci. 2020;6:498-. doi: 10.3389/fvets.2019.00498.

17. Swanson KS, Dowd SE, Suchodolski JS, Middelbos IS, Vester BM, Barry KA, et al. Phylogenetic and gene-centric metagenomics of the canine intestinal microbiome reveals similarities with humans and mice. ISME J. 2011;5(4):639-49. doi: 10.1038/ismej.2010.162.

18. Bresciani F, Minamoto Y, Suchodolski JS, Galiazzo G, Vecchiato CG, Pinna C, et al. Effect of an extruded animal protein-free diet on fecal microbiota of dogs with food-responsive enteropathy. J Vet Intern Med. 2018;32(6):1903-10. doi: 10.1111/jvim.15227.

19. Park HJ, Lee SE, Kim HB, Isaacson RE, Seo KW, Song KH. Association of obesity with serum leptin, adiponectin, and serotonin and gut microflora in beagle dogs. J Vet Intern Med. 2015;29(1):43-50. doi: 10.1111/jvim.12455.

20. Suchodolski JS, Dowd SE, Westermarck E, Steiner JM, Wolcott RD, Spillmann T, et al. The effect of the macrolide antibiotic tylosin on microbial diversity in the canine small intestine as demonstrated by massive parallel 16s rrna gene sequencing. BMC Microbiol. 2009;9:210. doi: 10.1186/1471-2180-9-210.

21. Igarashi H, Maeda S, Ohno K, Horigome A, Odamaki T, Tsujimoto H. Effect of oral administration of metronidazole or prednisolone on fecal microbiota in dogs. PLoS One. 2014;9(9):e107909. doi: 10.1371/journal.pone.0107909.

22. Pilla R, Gaschen FP, Barr JW, Olson E, Honneffer J, Guard BC, et al. Effects of metronidazole on the fecal microbiome and metabolome in healthy dogs. J Vet Intern Med. 2020;34(5):1853-66. doi: 10.1111/jvim.15871.

23. Manchester AC, Webb CB, Blake AB, Sarwar F, Lidbury JA, Steiner JM, et al. Long-term impact of tylosin on fecal microbiota and fecal bile acids of healthy dogs. J Vet Intern Med. 2019;33(6):2605-17. doi: 10.1111/jvim.15635.

24. Tian L, Wang X-W, Wu A-K, Fan Y, Friedman J, Dahlin A, et al. Deciphering functional redundancy in the human microbiome. Nat Commun. 2020;11(1):6217. doi: 10.1038/s41467-020-19940-1.

25. Kennedy AD, Wittmann BM, Evans AM, Miller LA, Toal DR, Lonergan S, et al. Metabolomics in the clinic: A review of the shared and unique features of untargeted metabolomics for clinical research and clinical testing. J Mass Spectrom. 2018;53(11):1143-54. doi: 10.1002/jms.4292.

26. Fleck C, Janz A, Schweitzer F, Karge E, Schwertfeger M, Stein G. Serum concentrations of asymmetric (adma) and symmetric (sdma) dimethylarginine in renal failure patients. Kidney Int Suppl. 2001;78:S14-8. doi: 10.1046/j.1523-1755.2001.59780014.x.

27. Jepson RE, Syme HM, Vallance C, Elliott J. Plasma asymmetric dimethylarginine, symmetric dimethylarginine, l-arginine, and nitrite/nitrate concentrations in cats with chronic kidney disease and hypertension. J Vet Intern Med. 2008;22(2):317-24. doi: 10.1111/j.1939-1676.2008.0075.x.

28. Hall JA, Yerramilli M, Obare E, Yerramilli M, Jewell DE. Comparison of serum concentrations of symmetric dimethylarginine and creatinine as kidney function biomarkers in cats with chronic kidney disease. J Vet Intern Med. 2014;28(6):1676-83. doi: 10.1111/jvim.12445.

29. Summers SC, Quimby JM, Isaiah A, Suchodolski JS, Lunghofer PJ, Gustafson DL. The fecal microbiome and serum concentrations of indoxyl sulfate and p-cresol

sulfate in cats with chronic kidney disease. J Vet Intern Med. 2019;33(2):662-9. doi: 10.1111/jvim.15389.

30. Blake AB, Guard BC, Honneffer JB, Lidbury JA, Steiner JM, Suchodolski JS. Altered microbiota, fecal lactate, and fecal bile acids in dogs with gastrointestinal disease. PloS One. 2019;14(10). doi: 10.1371/journal.pone.0224454.

31. Honneffer JB. Microbiota and metabolomic changes across various canine gastrointestinal diseases [Doctoral dissertation]: Texas A & M University; 2017.

32. Camilleri M. Advances in understanding of bile acid diarrhea. Expert Rev Gastroenterol Hepatol. 2014;8(1):49-61. doi: 10.1586/17474124.2014.851599.

33. Sharkey LC, Wellman ML. Use of lactate in small animal clinical practice. Clin Lab Med. 2015;35(3):567-77. doi: 10.1016/j.cll.2015.05.006.

34. Pang DS, Boysen S. Lactate in veterinary critical care: Pathophysiology and management. J Am Anim Hosp Assoc. 2007;43:270-9. doi: 10.5326/0430270.

35. Tamura Y, Ohta H, Kagawa Y, Osuga T, Morishita K, Sasaki N, et al. Plasma amino acid profiles in dogs with inflammatory bowel disease. J Vet Intern Med. 2019;33(4):1602-7. doi: 10.1111/jvim.15525.

36. Kathrani A, Allenspach K, Fascetti AJ, Larsen JA, Hall EJ. Alterations in serum amino acid concentrations in dogs with protein-losing enteropathy. J Vet Intern Med. 2018;32(3):1026-32. doi: 10.1111/jvim.15116.

37. Walker V, Mills G. Quantitative methods for amino acid analysis in biological fluids. Ann Clin Biochem. 1995;32:28-57.

38. Hackman R, Lazarus M. Quantitative analysis of amino acids using paper chromatography. Aust J Biol Sci. 1956;9:281-92.

39. Ukil S, Samanta T, Laskar, S. Identification of amino acids on thin-layer chromatography plates with a new reagent. Orient J Chem. 2013;29(2).

40. Kaspar H, Dettmer K, Chan Q, Daniels S, Nimkar S, Daviglus ML, et al. Urinary amino acid analysis: A comparison of itraq®–lc–ms/ms, gc–ms, and amino acid analyzer. J Chromatogr B. 2009;877(20):1838-46. doi: 10.1016/j.jchromb.2009.05.019.

41. Jeong J-S, Kim S-K, Park S-R. Amino acid analysis of dried blood spots for diagnosis of phenylketonuria using capillary electrophoresis-mass spectrometry equipped with a sheathless electrospray ionization interface. Anal Bioanal Chem. 2013;405(25):8063-72. doi: 10.1007/s00216-013-6999-6.

42. Li X, Xiao D, Sanders T, Tchounwou PB, Liu Y-M. Fast quantification of amino acids by microchip electrophoresis–mass spectrometry. Anal Bioanal Chem. 2013;405(25):8131-6. doi: 10.1007/s00216-013-7260-z.

43. Ramautar R, Mayboroda OA, Derks RJ, van Nieuwkoop C, van Dissel JT, Somsen GW, et al. Capillary electrophoresis-time of flight-mass spectrometry using noncovalently bilayer-coated capillaries for the analysis of amino acids in human urine. Electrophoresis. 2008;29(12):2714-22. doi: 10.1002/elps.200700929.

44. Malpress FH. Rennin and the gastric secretion of normal infants. Nature. 1967;215(5103):855-7. doi: 10.1038/215855a0.

45. Wu G. Principles of animal nutrition: Taylor & Francis Group, LLC; 2018.

46. Webb KE, Jr. Amino acid and peptide absorption from the gastrointestinal tract. Fed Proc. 1986;45(8):2268-71.

47. Wu G. Amino acids: Biochemistry and nutrition. Boca Raton, FL: Taylor & Francis Group, LLC; 2013.

48. Ardito F, Giuliani M, Perrone D, Troiano G, Lo Muzio L. The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (review). Int J Mol Med. 2017;40(2):271-80. doi: 10.3892/ijmm.2017.3036.

49. Thomas DA, Rosenthal GA. Toxicity and pharmacokinetics of the nonprotein amino acid l-canavanine in the rat. Toxicol Appl Pharmacol. 1987;91(3):395-405. doi: 10.1016/0041-008X(87)90061-5.

50. Onselen RV, Downing S, Kemp G, Downing T. Investigating β-n-methylamino-l-alanine misincorporation in human cell cultures: A comparative study with known amino acid analogues. Toxins. 2017;9(12):400. doi: 10.3390/toxins9120400.

51. Dunlop RA, Cox PA, Banack SA, Rodgers KJ. The non-protein amino acid bmaa is misincorporated into human proteins in place of l-serine causing protein misfolding and aggregation. PloS One. 2013;8(9):e75376-e. doi: 10.1371/journal.pone.0075376.

52. Hicks S, Tanaka K, Boches F, Sulis C, Warshaw JB. Hypoglycin effects on leucine incorporation into protein: Mechanism for teratogenesis? Pediatr Res. 1974;8(4):440-. doi: 10.1203/00006450-197404000-00600.

53. Hou Y, Wu G. Nutritionally nonessential amino acids: A misnomer in nutritional sciences. Adv Nutr. 2017;8(1):137-9. doi: 10.3945/an.116.012971.

54. Davila A-M, Blachier F, Gotteland M, Andriamihaja M, Benetti P-H, Sanz Y, et al. Re-print of "intestinal luminal nitrogen metabolism: Role of the gut microbiota and consequences for the host". Pharmacol Res. 2013;69(1):114-26. doi: 10.1016/j.phrs.2013.01.003.

55. Dai ZL, Zhang J, Wu G, Zhu WY. Utilization of amino acids by bacteria from the pig small intestine. Amino Acids. 2010;39(5):1201-15. doi: 10.1007/s00726-010-0556-9.

56. Oliphant K, Allen-Vercoe E. Macronutrient metabolism by the human gut microbiome: Major fermentation by-products and their impact on host health. Microbiome. 2019;7(1):91. doi: 10.1186/s40168-019-0704-8.

57. Neis EPJG, Dejong CHC, Rensen SS. The role of microbial amino acid metabolism in host metabolism. Nutrients. 2015;7(4):2930-46. doi: 10.3390/nu7042930.

58. Dodd D, Spitzer MH, Van Treuren W, Merrill BD, Hryckowian AJ, Higginbottom SK, et al. A gut bacterial pathway metabolizes aromatic amino acids into nine circulating metabolites. Nature. 2017;551:648. doi: 10.1038/nature24661.

59. Portune KJ, Beaumont M, Davila A-M, Tomé D, Blachier F, Sanz Y. Gut microbiota role in dietary protein metabolism and health-related outcomes: The two sides of the coin. Trends Food Sci Technol. 2016;57:213-32. doi: 10.1016/j.tifs.2016.08.011.

60. Sridharan GV, Choi K, Klemashevich C, Wu C, Prabakaran D, Pan LB, et al. Prediction and quantification of bioactive microbiota metabolites in the mouse gut. Nat Commun. 2014;5:5492. doi: 10.1038/ncomms6492.

61. Kimura I, Ozawa K, Inoue D, Imamura T, Kimura K, Maeda T, et al. The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor gpr43. Nat Commun. 2013;4(1):1829. doi: 10.1038/ncomms2852.

62. Ma N, Ma X. Dietary amino acids and the gut-microbiome-immune axis: Physiological metabolism and therapeutic prospects. Compr Rev Food Sci Food Saf. 2019;18(1):221-42. doi: 10.1111/1541-4337.12401.

63. Macfarlane GT, Allison C, Gibson SA, Cummings JH. Contribution of the microflora to proteolysis in the human large intestine. J Appl Bacteriol. 1988;64(1):37-46. doi: 10.1111/j.1365-2672.1988.tb02427.x.

64. Whitt DD, Demoss RD. Effect of microflora on the free amino acid distribution in various regions of the mouse gastrointestinal tract. Appl Microbiol. 1975;30(4):609-15. doi: 10.1128/am.30.4.609-615.1975.

65. Hisamatsu T, Okamoto S, Hashimoto M, Muramatsu T, Andou A, Uo M, et al. Novel, objective, multivariate biomarkers composed of plasma amino acid profiles for the diagnosis and assessment of inflammatory bowel disease. PLoS One. 2012;7(1):e31131. doi: 10.1371/journal.pone.0031131.

66. Torinsson Naluai A, Saadat Vafa L, Gudjonsdottir AH, Arnell H, Browaldh L, Nilsson S, et al. Altered peripheral amino acid profile indicate a systemic impact of active celiac disease and a possible role of amino acids in disease pathogenesis. PLoS One. 2018;13(3):e0193764. doi: 10.1371/journal.pone.0193764.

67. Bosch S, Struys EA, van Gaal N, Bakkali A, Jansen EW, Diederen K, et al. Fecal amino acid analysis can discriminate de novo treatment-naive pediatric inflammatory

bowel disease from controls. J Pediatr Gastroenterol Nutr. 2018;66(5):773-8. doi: 10.1097/mpg.0000000000001812.

68. Sakai K, Maeda S, Yonezawa T, Matsuki N. Decreased plasma amino acid concentrations in cats with chronic gastrointestinal diseases and their possible contribution in the inflammatory response. Vet Immunol Immunopathol. 2018;195:1-6. doi: 10.1016/j.vetimm.2017.11.001.

69. Moehn S, Pencharz PB, Ball RO. Lessons learned regarding symptoms of tryptophan deficiency and excess from animal requirement studies. J Nutr. 2012;142(12):2231S-5S. doi: 10.3945/jn.112.159061.

70. Severo JS, da Silva Barros VJ, Alves da Silva AC, Luz Parente JM, Lima MM, Moreira Lima AÂ, et al. Effects of glutamine supplementation on inflammatory bowel disease: A systematic review of clinical trials. Clin Nutr ESPEN. 2021. doi: 10.1016/j.clnesp.2020.12.023.

71. Liu Y, Wang X, Hu C-AA. Therapeutic potential of amino acids in inflammatory bowel disease. Nutrients. 2017;9(9):920. doi: 10.3390/nu9090920.

72. Bhattarai Y, Williams BB, Battaglioli EJ, Whitaker WR, Till L, Grover M, et al. Gut microbiota-produced tryptamine activates an epithelial g-protein-coupled receptor to increase colonic secretion. Cell Host Microbe. 2018;23(6):775-85.e5. doi: 10.1016/j.chom.2018.05.004.

73. Dewolfe MS, Baskurt S, Cochrane WA. Automatic amino acid analysis of blood serum and plasma. Clin Biochem. 1967;1:75-81. doi: 10.1016/S0009-9120(67)80009-2.

74. Dossin O. Laboratory tests for diagnosis of gastrointestinal and pancreatic diseases. Top Companion Anim Med. 2011;26(2):86-97. doi: 10.1053/j.tcam.2011.02.005.

75. Allenspach K. Diagnosis of small intestinal disorders in dogs and cats. Clin Lab Med. 2015;35(3):521-34. doi: 10.1016/j.cll.2015.05.003.

76. Appierto V, Callari M, Cavadini E, Morelli D, Daidone MG, Tiberio P. A lipemia-independent nanodrop((r))-based score to identify hemolysis in plasma and serum samples. Bioanalysis. 2014;6(9):1215-26. doi: 10.4155/bio.13.344.

77. Delaney SJ, H. KP, Rogers QR, Fascetti AJ. Plasma and whole blood taurine in normal dogs of varying size fed commercially prepared food. J Anim Physiol Anim Nutr. 2003;87(2003):236-44. doi: 10.1046/j.1439-0396.2003.00433.x.

78. Van Eijk HM, Dejong CH, Deutz NE, Soeters PB. Influence of storage conditions on normal plasma amino-acid concentrations. Clin Nutr. 1994;13(6):374-80. doi: 10.1016/0261-5614(94)90028-0.

79. Takehana S, Yoshida H, Ozawa S, Yamazaki J, Shimbo K, Nakayama A, et al. The effects of pre-analysis sample handling on human plasma amino acid concentrations. Clin Chim Acta. 2016;455:68-74. doi: 10.1016/j.cca.2016.01.026.

80. Hansen B, DiBartola SP, Chew DJ, Brownie C, Berrie HK. Amino acid profiles in dogs with chronic renal failure fed two diets. Am J Vet Res. 1992;53(3):335-41.

81. Downes AM. The fate of intravenous doses of free and plasma protein-bound [^{35}s]cystine in the sheep. Aust J Biol Sci. 1961;14:427-39.

82. An Z, Shi C, Li P, Liu L. Stability of amino acids and related amines in human serum under different preprocessing and pre-storage conditions based on itraq®-lc-ms/ms. Biol Open. 2021;10(2). doi: 10.1242/bio.055020.

83. Yu BL, Han J, Hammond M, Wang X, Zhang Q, Clausen A, et al. Kinetic modeling of the release of ethylene oxide from sterilized plastic containers and its interaction with monoclonal antibodies. PDA J Pharm Sci Technol. 2017;71(1):11-9. doi: 10.5731/pdajpst.2016.005819.

84. Holtrop S, Abeling NGG, van Gennip AH. Recommendations to improve the quality of diagnostic quantitative analysis of amino acids in plasma and urine using cation-exchange liquid chromatography with post column ninhydrin reaction and detection. ERNDIM. 2002.

85. Schaefer A, Piquard F, Haberey P. Plasma amino-acids analysis: Effects of delayed samples preparation and of storage. Clin Chim Acta. 1987;164(2):163-9. doi: 10.1016/0009-8981(87)90067-2.

86. Li J, Piao C, Jin H, Wongpanit K, Manabe N. Delayed deproteinization causes methodological errors in amino acid levels in plasma stored at room temperature or -20'c. Asian-Aust J Anim Sci. 2009;22(12):1703-8. doi: 10.5713/AJAS.2009.90156.

87. Hester JR, Korzun WJ, Mabry LU. Blood ammonia stability revisited. Clin Lab Sci. 2015;28(3):173. doi: 10.29074/ascls.28.3.173.

88. Olek K, Uhlhaas S, Wardenbach P, Yamaguchi M. Influence of storing conditions on the amino acid concentration in human serum (author's transl). J Clin Chem Clin Biochem. 1979;17(9):599-604.

89. Kochlik B, Gerbracht C, Grune T, Weber D. The influence of dietary habits and meat consumption on plasma 3-methylhistidine—a potential marker for muscle protein turnover. Mol Nutr Food Res. 2018;62(9):1701062. doi: 10.1002/mnfr.201701062.

90. Young VR, Munro HN. Ntau-methylhistidine (3-methylhistidine) and muscle protein turnover: An overview. Fed Proc. 1978;37(9):2291-300.

91. Peters V, Klessens CQF, Baelde HJ, Singler B, Veraar KAM, Zutinic A, et al. Intrinsic carnosine metabolism in the human kidney. Amino Acids. 2015;47(12):2541-50. doi: 10.1007/s00726-015-2045-7.

92. Mitry P, Wawro N, Rohrmann S, Giesbertz P, Daniel H, Linseisen J. Plasma concentrations of anserine, carnosine and pi-methylhistidine as biomarkers of habitual meat consumption. Eur J Clin Nutr. 2019;73(5):692-702. doi: 10.1038/s41430-018-0248-1.

93. Seip M, Lindemann R, Gjesdahl P, Gjessing LR. Amino acid concentrations in plasma and erythrocytes in aregeneratory and haemolytic anaemias. Scand J Haematol. 1975;15(3):178-86. doi: 10.1111/j.1600-0609.1975.tb01072.x.

94. Fukuda K, Nishi Y, Usui T. Free amino acid concentrations in plasma, erythrocytes, granulocytes, and lymphocytes in umbilical cord blood, children, and adults. J Pediatr Gastroenterol Nutr. 1984;3(3):432-9. doi: 10.1097/00005176-198406000-00022.

95. Perry TL, Hansen S. Technical pitfalls leading to errors in the quantitation of plasma amino acids. Clin Chim Acta. 1969;25(1):53-8. doi: 10.1016/0009-8981(69)90226-5.

96. Davids M, Peters JH, de Jong S, Teerlink T. Measurement of nitric oxide-related amino acids in serum and plasma: Effects of blood clotting and type of anticoagulant. Clin Chim Acta. 2013;421:164-7. doi: 10.1016/j.cca.2013.03.009.

97. Sotelo-Orozco J, Chen S-Y, Hertz-Picciotto I, Slupsky CM. A comparison of serum and plasma blood collection tubes for the integration of epidemiological and metabolomics data. Front Mol Biosci. 2021;8. doi: 10.3389/fmolb.2021.682134.

98. Williams AP. General problems associated with the analysis of amino acids by automated ion-exchange chromatography. J Chromatogr A. 1986;373:175-90. doi: 10.1016/S0021-9673(00)80212-5.

99. Holecek M. Branched-chain amino acids in health and disease: Metabolism, alterations in blood plasma, and as supplements. Nutr Metab (Lond). 2018;15:33. doi: 10.1186/s12986-018-0271-1.

100. Parvy P, Bardet J, Gasquet M, Rabier D, Kamoun P. Stability of free amino acids in sulfosalicylic filtrates. Clin Chem. 1995;41(3):465-6.

101. Kornhuber ME, Balabanova S, Heiligensetzer GV, Kornhuber C, Zettlmeissl H, Kornhuber AW. Stability of human blood serum aminoacids after storage at different ph and temperature conditions. Clin Chim Acta. 1991;197(3):189-200. doi: 10.1016/0009-8981(91)90139-4.

102. Bauchart-Thevret C, Stoll B, Burrin DG. Intestinal metabolism of sulfur amino acids. Nutr Res Rev. 2009;22(2):175-87. doi: 10.1017/s0954422409990138.

103. Hamard A, Mazurais D, Boudry G, Le Huërou-Luron I, Sève B, Le Floc'h N. A moderate threonine deficiency affects gene expression profile, paracellular permeability

and glucose absorption capacity in the ileum of piglets. J Nutr Biochem. 2010;21(10):914-21. doi: 10.1016/j.jnutbio.2009.07.004.

104. Hashimoto T, Perlot T, Rehman A, Trichereau J, Ishiguro H, Paolino M, et al. Ace2 links amino acid malnutrition to microbial ecology and intestinal inflammation. Nature. 2012;487(7408):477-81. doi: 10.1038/nature11228.

105. Schicho R, Shaykhutdinov R, Ngo J, Nazyrova A, Schneider C, Panaccione R, et al. Quantitative metabolomic profiling of serum, plasma, and urine by (1)h nmr spectroscopy discriminates between patients with inflammatory bowel disease and healthy individuals. J Proteome Res. 2012;11(6):3344-57. doi: 10.1021/pr300139q.

106. Kolho KL, Pessia A, Jaakkola T, de Vos WM, Velagapudi V. Faecal and serum metabolomics in paediatric inflammatory bowel disease. J Crohns Colitis. 2017;11(3):321-34. doi: 10.1093/ecco-jcc/jjw158.

107. Minamoto Y, Otoni CC, Steelman SM, Buyukleblebici O, Steiner JM, Jergens AE, et al. Alteration of the fecal microbiota and serum metabolite profiles in dogs with idiopathic inflammatory bowel disease. Gut Microbes. 2015;6(1):33-47. doi: 10.1080/19490976.2014.997612.

108. Marchesi JR, Holmes E, Khan F, Kochhar S, Scanlan P, Shanahan F, et al. Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. J Proteome Res. 2007;6(2):546-51. doi: 10.1021/pr060470d.

109. Crenn P, Messing B, Cynober L. Citrulline as a biomarker of intestinal failure due to enterocyte mass reduction. Clin Nutr. 2008;27(3):328-39. doi: 10.1016/j.clnu.2008.02.005.

110. Papadia C, Sherwood RA, Kalantzis C, Wallis K, Volta U, Fiorini E, et al. Plasma citrulline concentration: A reliable marker of small bowel absorptive capacity independent of intestinal inflammation. Am J Gastroenterol. 2007;102(7). doi: 10.1111/j.1572-0241.2007.01239.x.

111. Young VR, Haverberg LN, Bilmazes C, Munro HN. Potential use of 3-methylhistidine excretion as an index of progressive reduction in muscle protein catabolism during starvation. Metabolism. 1973;22(11):1429-36. doi: 10.1016/0026-0495(73)90257-6.

112. Xu J, Verbrugghe A, Lourenco M, Janssens GP, Liu DJ, Van de Wiele T, et al. Does canine inflammatory bowel disease influence gut microbial profile and host metabolism? BMC Vet Res. 2016;12(1):114. doi: 10.1186/s12917-016-0736-2.

113. Ooi M, Nishiumi S, Yoshie T, Shiomi Y, Kohashi M, Fukunaga K, et al. Gc/ms-based profiling of amino acids and tca cycle-related molecules in ulcerative colitis. Inflamm Res. 2011;60(9):831-40. doi: 10.1007/s00011-011-0340-7.

114. Kohashi M, Nishiumi S, Ooi M, Yoshie T, Matsubara A, Suzuki M, et al. A novel gas chromatography mass spectrometry-based serum diagnostic and assessment approach to ulcerative colitis. J Crohns Colitis. 2014;8(9):1010-21. doi: 10.1016/j.crohns.2014.01.024.

115. Batch BC, Shah SH, Newgard CB, Turer CB, Haynes C, Bain JR, et al. Branched chain amino acids are novel biomarkers for discrimination of metabolic wellness. Metabolism. 2013;62(7):961-9. doi: 10.1016/j.metabol.2013.01.007.

116. Kumar M, Bitla A, Raju KVN, Manohar S, Kumar V, Narasimha SR. Branched chain amino acid profile in early chronic kidney disease. Saudi J Kidney Dis Transpl. 2012;23(6):1202-7. doi: 10.4103/1319-2442.103560.

117. He F, Wu C, Li P, Li N, Zhang D, Zhu Q, et al. Functions and signaling pathways of amino acids in intestinal inflammation. Biomed Res Int. 2018;2018. doi: 10.1155/2018/9171905.

118. Martín-Venegas R, Brufau MT, Guerrero-Zamora AM, Mercier Y, Geraert PA, Ferrer R. The methionine precursor dl-2-hydroxy-(4-methylthio)butanoic acid protects intestinal epithelial barrier function. Food Chem. 2013;141(3):1702-9. doi: 10.1016/j.foodchem.2013.04.081.

119. Marsilio S, Ackermann MR, Lidbury JA, Suchodolski JS, Steiner JM. Results of histopathology, immunohistochemistry, and molecular clonality testing of small intestinal biopsy specimens from clinically healthy client-owned cats. J Vet Intern Med. 2019;33(2):551-8. doi: 10.1111/jvim.15455.

120. Gerou-Ferriani M, Allen R, Noble PM, German AJ, Caldin M, Batchelor DJ. Determining optimal therapy of dogs with chronic enteropathy by measurement of serum citrulline. J Vet Intern Med. 2018;32(3):993-8. doi: 10.1111/jvim.15124.

121. Dossin O, Rupassara SI, Weng HY, Williams DA, Garlick PJ, Schoeman JP. Effect of parvoviral enteritis on plasma citrulline concentration in dogs. J Vet Intern Med. 2011;25(2):215-21. doi: 10.1111/j.1939-1676.2010.0671.x.

122. Minamoto Y, Minamoto T, Isaiah A, Sattasathuchana P, Buono A, Rangachari VR, et al. Fecal short-chain fatty acid concentrations and dysbiosis in dogs with chronic enteropathy. J Vet Intern Med. 2019. doi: 10.1111/jvim.15520.

123. Bermudez-Sanchez S, Pilla R, Gramenzi A, Marsilio F, Steiner JM, Lidbury JA, et al., editors. Fecal microbial metabolism is altered in dogs with chronic enteropathy. ECVIM-CA 29th Annual congress; 2019.

124. Schmidt JA, Rinaldi S, Scalbert A, Ferrari P, Achaintre D, Gunter MJ, et al. Plasma concentrations and intakes of amino acids in male meat-eaters, fish-eaters, vegetarians and vegans: A cross-sectional analysis in the epic-oxford cohort. Eur J Clin Nutr. 2016;70(3):306-12. doi: 10.1038/ejcn.2015.144.

125. Donadelli RA, Pezzali JG, Oba PM, Swanson KS, Coon C, Varney J, et al. A commercial grain-free diet does not decrease plasma amino acids and taurine status but increases bile acid excretion when fed to labrador retrievers. Translational animal science. 2020;4(3):txaa141-txaa. doi: 10.1093/tas/txaa141.

126. Pezzali JG, Acuff HL, Henry W, Alexander C, Swanson KS, Aldrich CG. Effects of different carbohydrate sources on taurine status in healthy beagle dogs. Journal of animal science. 2020;98(2):skaa010. doi: 10.1093/jas/skaa010.

127. Fernstrom JD, Wurtman RJ, Hammarstrom-Wiklund B, Rand WM, Munro HN, Davidson CS. Diurnal variations in plasma concentrations of tryptophan, tyrosine, and other neutral amino acids: Effect of dietary protein intake. Am J Clin Nutr. 1979;32(9):1912-22. doi: 10.1093/ajcn/32.9.1912.

128. Hernell O, Lönnerdal B. Nutritional evaluation of protein hydrolysate formulas in healthy term infants: Plasma amino acids, hematology, and trace elements. Am J Clin Nutr. 2003;78(2):296-301. doi: 10.1093/ajcn/78.2.296.

129. Morifuji M, Ishizaka M, Baba S, Fukuda K, Matsumoto H, Koga J, et al. Comparison of different sources and degrees of hydrolysis of dietary protein: Effect on plasma amino acids, dipeptides, and insulin responses in human subjects. J Agric Food Chem. 2010;58(15):8788-97. doi: 10.1021/jf101912n.

130. Kim CJ, Kovacs-Nolan JA, Yang CB, Archbold T, Fan MZ, Mine Y. L-tryptophan exhibits therapeutic function in a porcine model of dextran sodium sulfate (dss)-induced colitis. J Nutr Biochem. 2010;21(6):468-75. doi: 10.1016/j.jnutbio.2009.01.019.

131. Costedio MM, Hyman N, Mawe GM. Serotonin and its role in colonic function and in gastrointestinal disorders. Dis Colon Rectum. 2007;50(3):376-88. doi: 10.1007/s10350-006-0763-3.

132. Bansal T, Alaniz RC, Wood TK, Jayaraman A. The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation. Proc Natl Acad Sci U S A. 2010;107(1):228-33. doi: 10.1073/pnas.0906112107.

133. Taleb S. Tryptophan dietary impacts gut barrier and metabolic diseases. Front Immunol. 2019;10(2113). doi: 10.3389/fimmu.2019.02113.

134. Gao J, Xu K, Liu H, Liu G, Bai M, Peng C, et al. Impact of the gut microbiota on intestinal immunity mediated by tryptophan metabolism. Front Cell Infect Microbiol. 2018;8:13. doi: 10.3389/fcimb.2018.00013.

135. Lee JH, Lee J. Indole as an intercellular signal in microbial communities. FEMS Microbiol Rev. 2010;34(4):426-44. doi: 10.1111/j.1574-6976.2009.00204.x.

136. Lee J-H, Wood TK, Lee J. Roles of indole as an interspecies and interkingdom signaling molecule. Trends Microbiol. 2015;23(11):707-18. doi: 10.1016/j.tim.2015.08.001.

137. Takaki M, Mawe GM, Barasch JM, Gershon MD, Gershon MD. Physiological responses of guinea-pig myenteric neurons secondary to the release of endogenous serotonin by tryptamine. Neuroscience. 1985;16(1):223-40. doi: 10.1016/0306-4522(85)90059-4.

138. Galligan JJ. Beneficial actions of microbiota-derived tryptophan metabolites. Neurogastroenterol Motil. 2018;30(2):e13283. doi: 10.1111/nmo.13283.

139. Hubbard TD, Murray IA, Perdew GH. Indole and tryptophan metabolism: Endogenous and dietary routes to ah receptor activation. Drug Metab Dispos. 2015;43(10):1522-35. doi: 10.1124/dmd.115.064246.

140. Spiller R. Recent advances in understanding the role of serotonin in gastrointestinal motility in functional bowel disorders: Alterations in 5-ht signalling and metabolism in human disease. Neurogastroenterol Motil. 2007;19(s2):25-31. doi: 10.1111/j.1365-2982.2007.00965.x.

141. Gershon MD, Tack J. The serotonin signaling system: From basic understanding to drug development for functional gi disorders. Gastroenterol. 2007;132(1):397-414. doi: 10.1053/j.gastro.2006.11.002.

142. Fallarino F, Grohmann U, Vacca C, Bianchi R, Orabona C, Spreca A, et al. T cell apoptosis by tryptophan catabolism. Cell Death Differ. 2002;9(10):1069-77. doi: 10.1038/sj.cdd.4401073.

143. Ziese AL, Suchodolski JS, Hartmann K, Busch K, Anderson A, Sarwar F, et al. Effect of probiotic treatment on the clinical course, intestinal microbiome, and toxigenic clostridium perfringens in dogs with acute hemorrhagic diarrhea. PLoS One. 2018;13(9):e0204691. doi: 10.1371/journal.pone.0204691.

144. Toresson L, Steiner JM, Razdan P, Spodsberg E, Olmedal G, Suchodolski JS, et al. Comparison of efficacy of oral and parenteral cobalamin supplementation in normalising low cobalamin concentrations in dogs: A randomised controlled study. Vet J. 2018;232:27-32. doi: 10.1016/j.tvjl.2017.12.010.

145. Parada AE, Needham DM, Fuhrman JA. Every base matters: Assessing small subunit rrna primers for marine microbiomes with mock communities, time series and global field samples. Environ Microbiol. 2016;18(5):1403-14. doi: 10.1111/1462-2920.13023.

146. Apprill A, McNally S, Parsons R, Weber L. Minor revision to v4 region ssu rrna 806r gene primer greatly increases detection of sar11 bacterioplankton. Aquat Microb Ecol. 2015;75(2):129-37. doi: 10.3354/ame01753.

147. Isaiah A, Parambeth JC, Steiner JM, Lidbury JA, Suchodolski JS. The fecal microbiome of dogs with exocrine pancreatic insufficiency. Anaerobe. 2017;45:50-8. doi: 10.1016/j.anaerobe.2017.02.010.

148. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using qiime 2. Nat Biotechnol. 2019;37(8):852-7. doi: 10.1038/s41587-019-0209-9.

149. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. Dada2: High-resolution sample inference from illumina amplicon data. Nat Methods. 2016;13(7):581-3. doi: 10.1038/nmeth.3869.

150. Lozupone C, Knight R. Unifrac: A new phylogenetic method for comparing microbial communities. Appl Environ Microbiol. 2005;71(12):8228-35. doi: 10.1128/AEM.71.12.8228-8235.2005.

151. Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc Series B Stat Methodol. 1995:289-300. doi: 10.1111/j.2517-6161.1995.tb02031.x.

152. Honneffer JB, Steiner JM, Lidbury JA, Suchodolski JS. Variation of the microbiota and metabolome along the canine gastrointestinal tract. Metabolomics. 2017;13(3):26. doi: 10.1007/s11306-017-1165-3.

153. Nikolaus S, Schulte B, Al-Massad N, Thieme F, Schulte DM, Bethge J, et al. Increased tryptophan metabolism is associated with activity of inflammatory bowel diseases. Gastroenterol. 2017;153(6):1504-16.e2. doi: 10.1053/j.gastro.2017.08.028.

154. Bao XY, Feng ZM, Yao JM, Li TJ, Yin YL. Roles of dietary amino acids and their metabolites in pathogenesis of inflammatory bowel disease. Mediators Inflamm. 2017;2017. doi: 10.1155/2017/6869259.

155. Venkatesh M, Mukherjee S, Wang H, Li H, Sun K, Benechet AP, et al. Symbiotic bacterial metabolites regulate gastrointestinal barrier function via the xenobiotic sensor pxr and toll-like receptor 4. Immunity. 2014;41(2):296-310. doi: 10.1016/j.immuni.2014.06.014.

156. Zhao Z, Lu L, Li W. N-(3′,4′-dimethoxycinnamonyl) anthranilic acid alleviates severe acute pancreatitis by inhibiting intestinal barrier dysfunction and nf-κb activation. Drug Dev Res. 2021;82(3):458-64. doi: 10.1002/ddr.21769.

157. Sofia MA, Ciorba MA, Meckel K, Lim CK, Guillemin GJ, Weber CR, et al. Tryptophan metabolism through the kynurenine pathway is associated with endoscopic inflammation in ulcerative colitis. Inflamm Bowel Dis. 2018;24(7):1471-80. doi: 10.1093/ibd/izy103.

158. Suchodolski JS, Dowd SE, Wilke V, Steiner JM, Jergens AE. 16s rrna gene pyrosequencing reveals bacterial dysbiosis in the duodenum of dogs with idiopathic inflammatory bowel disease. PLoS One. 2012;7(6):e39333. doi: 10.1371/journal.pone.0039333.

159. Engelen MP, Com G, Anderson PJ, Deutz NE. New stable isotope method to measure protein digestibility and response to pancreatic enzyme intake in cystic fibrosis. Clin Nutr. 2014;33(6):1024-32. doi: 10.1016/j.clnu.2013.11.004.

160. van der Meij BS, Deutz NEP, Rodriguez RE, Engelen MPKJ. Early signs of impaired gut function affect daily functioning in patients with advanced cancer undergoing chemotherapy. JPEN J Parenter Enteral Nutr. 2021;45(4):752-60. doi: 10.1002/jpen.1941.

161. Kaur A, Ten Have GAM, Hritzo B, Deutz NEP, Olsen C, Moroni M. Morphological and functional impairment in the gut in a partial body irradiation minipig model of gi-ars. Int J Radiat Biol. 2018:1-52. doi: 10.1080/09553002.2018.1552377.

162. Daenzer M, Petzke KJ, Bequette BJ, Metges CC. Whole-body nitrogen and splanchnic amino acid metabolism differ in rats fed mixed diets containing casein or its corresponding amino acid mixture. J Nutr. 2001;131(7):1965-72. doi: 10.1093/jn/131.7.1965.

163. Zatelli A, D'Ippolito P, Roura X, Zini E. Short-term effects of dietary supplementation with amino acids in dogs with proteinuric chronic kidney disease. Can Vet J. 2017;58(12):1287-93.

APPENDIX A

SUPPLEMENTAL TABLES AND FIGURES

Table A-1. Standard dilutions table. Preparation of standards ranging in concentration from 2.5 to 750 µM.

			Std A (acids/ neutrals)	Std B (bases)	Glutamine	L-norleucine	loading buffer	final volume	dilution factor
	initial conc µmol/L	stock solutions:	2500	2500	2500	2500			
						***			
STD#	Final Conc (µM)		Amount needed in µL						
1	750		150	150	150	20	30	500	3.33
2	600		120	120	120	20	120	500	4.16
3	500		100	100	100	20	180	500	5
4	250		50	50	50	20	330	500	10
5	125		25	25	25	20	405	500	20
6	50		10	10	10	20	450	500	50
7	25		5	5	5	20	465	500	100
	25* no IS		12.5	12.5	12.5	0	1212.5	1250	
8	20		400 µl (of 25* no IS)			20	80	500	125
9	15		300 µl (of 25* no IS)			20	180	500	166.67
10	10		200 µl (of 25* no IS)			20	280	500	250
11	5		100 µl (of 25* no IS)			20	380	500	500
12	2.5		50 µl (of 25* no IS)			20	430	500	1000
*** Note: the concentration of L-norleucine will always be 100 µM in all Final concentrations									

Table A-2. Signal to noise ratios for 4 replicates of low concentration standards. Signal to noise ratios above 3 were determined acceptable.

	Average Signal to Noise			
Compound/Concentration (μM)	**2.5**	**5**	**10**	**15**
phosphoserine	26.6	53.3	100.3	145.8
taurine	17.1	31.9	61.1	88.0
phosphoethanolamine	10.2	19.9	35.8	51.7
urea	0.0	0.0	2.4	3.0
aspartic acid	12.1	22.9	41.8	60.4
threonine	7.4	13.7	25.2	36.7
serine	7.8	13.8	25.4	36.1
asparagine	3.4	5.2	10.1	13.7
glutamic acid	3.9	5.8	13.8	18.7
glutamine	3.9	6.1	13.7	18.6
sarcosine	0.0	0.0	1.5	2.8
α-aminoadipic acid	4.4	8.1	16.5	23.0
glycine	5.4	10.3	19.9	29.3
alanine	4.9	8.7	16.5	24.3
citrulline	6.7	10.9	18.0	26.3
α-aminobutyric acid	4.3	10.8	19.7	28.3
valine	5.5	9.7	18.6	25.4
cystine	7.7	13.6	26.3	38.2
methionine	8.3	15.2	28.3	41.2
cystathionine	7.8	14.2	27.1	38.9
isoleucine	14.3	24.5	44.2	62.9
leucine	13.7	23.8	44.5	63.5
tyrosine	9.0	16.4	30.7	44.5
β-alanine	3.1	4.4	7.4	10.8
phenylalanine	7.2	13.4	25.5	37.4
β-aminoisobutyric acid	2.8	3.9	5.8	8.0
homocystine	11.0	23.6	44.8	65.0
γ-aminobutyrci acid	2.0	4.9	10.3	16.9
ethanolamine	21.1	20.1	21.3	23.7
ammonia	14.8	18.8	25.5	33.5
hydroxylysine	13.7	20.8	31.7	43.5
ornithine	13.0	28.2	53.8	78.8
lysine	12.0	23.4	45.0	65.5
1-methylhistidine	9.6	18.9	35.9	51.5
histidine	9.5	18.6	37.3	54.7
tryptophan	3.3	6.7	14.1	0.0*

Table A-2. Continued.

Compound/Concentration (μM)	Average Signal to Noise 2.5	5	10	15
3-methylhistidine	7.2	14.4	30.4	45.6
anserine	3.7	5.5	9.9	16.0
carnosine	3.6	6.1	10.9	15.3
arginine	6.6	12.3	22.5	33.1
hydroxyproline	1.5	2.9	3.7	5.5
proline	1.7	4.0	5.3	7.6

*SN could not be calculated due to close proximity of surrounding peaks

Table A-3. Intra-assay variability (precision).

Compound	median [range] µM[a]	median [range] CV%[b]
phosphoserine	5 [4-12]	5.7 [1.7-22.0]
taurine	206 [107-510]	1.0 [0.2-1.5]
urea	6961 [2081-14595]	0.3 [0.2-2.4]
aspartic acid	10 [6-23]	2.0 [0.7-2.9]
threonine	163 [85-401]	0.2 [0.2-0.8]
serine	120 [100-153]	0.3 [0.2-0.8]
asparagine	60 [23-76]	3.1 [1.8-5.7]
glutamic acid	62 [23-194]	1.7 [0.5-2.5]
glutamine	579 [384-775]	0.4 [0.2-0.6]
α-aminoadipic acid	9 [6-13]	4.7 [2.3-24.1]
glycine	214 [151-253]	0.2 [0.1-0.3]
alanine	468 [202-622]	0.2 [0.1-0.3]
citrulline	57 [20-103]	0.6 [0.3-3.4]
α-aminobutyric acid	26 [11-43]	1.3 [0.5-2.6]
valine	190 [86-228]	0.4 [0.2-0.8]
methionine	50 [33-62]	1.4 [1.0-4.3]
cystathionine	9 [0-15]	8.8 [4.2-17.6]
isoleucine	63 [42-108]	0.7 [0.4-1.3]
leucine	127 [72-173]	0.3 [0.1-0.5]
tyrosine	43 [21-59]	1.3 [0.8-4.7]
phenylalanine	66 [44-105]	1.0 [0.7-1.5]
ammonia	68 [49-303]	2.7 [0.6-5.2]
ornithine	18 [10-46]	1.5 [0.7-3.6]
lysine	186 [92-259]	0.3 [0.2-1.0]
1-methylhistidine	14 [4-105]	5.7 [0.5-32.1]
histidine	83 [61-100]	1.1 [0.7-1.6]
tryptophan	55 [29-101]	2.8 [1.0-4.4]
3-methylhistidine	12 [8-44]	6.1 [1.6-17.8]
carnosine	27 [14-117]	4.1 [2.7-7.1]
arginine	150 [107-213]	0.5 [0.2-1.0]
proline	136 [75-154]	1.3 [0.8-2.1]

Repeatability and reproducibility of the assay. [a]Concentrations of samples used (the median and range of the median of eight replicates from eight different animals). [b]Coefficient of variation, calculated from eight replicates from each of eight animals. Compounds that were not detected in half or more of samples were excluded from analysis (phosphoethanolamine, sarcosine, cystine, β-alanine, β-aminoisobutyric acid, homocystine, γ-aminobutyric acid, ethanolamine, hydroxylysine, anserine, and hydroxyproline).

Table A-4. Inter-assay variability (reproducibility).

Compound	median [range] µM[a]	median [range] CV%[b]
phosphoserine	5 [4-13]	9.5 [3.3-32.3]
taurine	209 [110-505]	1.1 [0.8-1.7]
urea	7135 [2131-14482]	4.9 [3.9-6.0]
aspartic acid	10 [6-22]	4.2 [1.2-8.6]
threonine	164 [81-394]	1.3 [0.6-1.7]
serine	119 [100-153]	1.7 [0.8-2.1]
asparagine	63 [24-78]	4.2 [2.6-8.7]
glutamic acid	63 [24-195]	2.5 [1.3-3.9]
glutamine	628 [421-849]	3.3 [3.1-3.9]
α-aminoadipic acid	8 [3-13]	21.5 [12.6-37.3]
glycine	214 [151-252]	1.0 [0.5-1.4]
alanine	469 [200-618]	0.7 [0.5-1.5]
citrulline	57 [19-104]	2.1 [1.6-3.9]
α-aminobutyric acid	26 [10-43]	3.7 [1.7-6.7]
valine	192 [86-230]	0.9 [0.6-2.2]
methionine	51 [33-70]	3.2 [2.3-4.9]
cystathionine	9 [2-15]	9.7 [4.1-23.6]
isoleucine	64 [42-109]	1.0 [0.6-2.5]
leucine	127 [72-175]	0.8 [0.4-1.6]
tyrosine	44 [21-59]	1.9 [1.3-4.7]
phenylalanine	66 [45-105]	1.7 [1.2-2.3]
ammonia	81 [62-309]	3.9 [2.0-5.3]
hydroxylysine	12 [10-20]	10.1 [3.2-16.4]
ornithine	18 [10-46]	3.0 [1.2-5.4]
lysine	187 [92-260]	0.7 [0.4-2.1]
1-methylhistidine	14 [8-106]	7.0 [0.7-14.2]
histidine	83 [62-102]	1.7 [0.8-2.3]
tryptophan	56 [29-103]	3.4 [2.2-5.0]
3-methylhistidine	12 [9-44]	8.4 [4.3-12.1]
carnosine	29 [20-119]	7.4 [5.0-10.5]
arginine	150 [107-214]	2.1 [1.8-2.4]
proline	137 [74-152]	2.8 [1.7-5.4]

Repeatability and reproducibility of the assay. [a]Concentrations of samples used (the median and range of the median of eight replicates from eight different animals). [b]Coefficient of variation, calculated from eight replicates from each of eight animals. Compounds that were not detected in half or more of samples were excluded from analysis (phosphoethanolamine, sarcosine, cystine, β-alanine, β-aminoisobutyric acid, homocystine, γ-aminobutyric acid, ethanolamine, anserine, and hydroxyproline).

Table A-5. Spiking recovery. Concentrations (µM) and percent recovery (%R) are listed for each of 5 dogs for low (100 µM), medium (200 µM, and high (300 µM) spike conditions.

phosphoserine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	0	59	187	327	59	94	109
dog 2	0	49	189	342	49	95	114
dog 3	0	60	203	348	60	101	116
dog 4	0	52	201	358	52	100	119
dog 5	0	52	198	342	52	99	114
			mean %R		54	98	114

taurine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	192	302	413	511	133	133	129
dog 2	81	195	294	409	124	117	119
dog 3	143	237	338	436	112	115	115
dog 4	139	244	346	461	122	120	124
dog 5	138	251	348	448	129	121	120
			mean %R		124	121	122

phosphoethanolamine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	0	81	219	343	81	110	114
dog 2	0	88	193	316	88	96	105
dog 3	0	89	197	307	89	99	102
dog 4	0	82	191	315	82	95	105
dog 5	0	90	200	309	90	100	103
			mean %R		86	100	106

Table A-5. Continued.

urea

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	11908	10526	9354	8123	46	152	167
dog 2	3327	3086	2823	2519	158	147	130
dog 3	5545	5095	4616	3993	215	201	148
dog 4	6457	5851	5369	4664	169	231	177
dog 5	10268	9650	8442	7315	614	319	248
			mean %R		241	210	174

aspartic acid

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	2	104	210	318	102	104	106
dog 2	0	93	200	315	93	100	105
dog 3	6	113	223	332	107	109	109
dog 4	0	100	213	329	100	106	110
dog 5	0	99	205	317	99	102	106
			mean %R		100	104	107

threonine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	364	428	496	550	108	110	106
dog 2	197	283	363	453	110	107	109
dog 3	161	258	347	433	116	112	110
dog 4	398	461	532	596	111	115	114
dog 5	267	351	421	503	116	109	111
			mean %R		112	111	110

Table A-5. Continued.

serine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	111	207	296	388	110	106	106
dog 2	104	197	290	391	106	105	108
dog 3	110	209	303	397	112	110	109
dog 4	95	194	295	396	111	112	112
dog 5	90	190	282	382	111	107	108
			mean %R		110	108	109

asparagine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	168	227	315	393	79	94	95
dog 2	48	142	243	349	100	103	106
dog 3	48	144	245	349	102	105	106
dog 4	38	138	247	354	104	109	110
dog 5	63	165	253	352	110	102	104
			mean %R		99	103	104

glutamic acid

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	33	124	228	326	96	102	102
dog 2	13	108	206	316	97	98	103
dog 3	68	157	256	350	98	102	102
dog 4	49	142	246	345	99	104	104
dog 5	29	129	220	325	103	99	102
			mean %R		98	101	103

Table A-5. Continued.

glutamine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	396	459	506	573	111	102	106
dog 2	642	686	713	754	121	112	115
dog 3	667	714	732	767	127	113	113
dog 4	687	721	757	798	117	117	119
dog 5	600	667	679	722	139	111	112
			mean %R		123	111	113

sarcosine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	0	85	171	312	85	85	104
dog 2	0	52	151	248	52	75	83
dog 3	0	95	186	272	95	93	91
dog 4	0	84	183	293	84	91	98
dog 5	0	81	160	275	81	80	92
			mean %R		79	85	93

α-aminoadipic acid

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	0	85	190	302	85	95	101
dog 2	0	71	173	286	71	87	95
dog 3	0	83	191	304	83	95	101
dog 4	0	73	181	298	73	91	99
dog 5	0	81	184	295	81	92	98
			mean %R		79	92	99

Table A-5. Continued.

glycine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	200	277	357	446	101	103	106
dog 2	199	286	367	459	111	108	111
dog 3	358	430	496	559	115	112	110
dog 4	212	296	384	473	109	111	112
dog 5	161	257	342	430	116	110	109
			mean %R		110	109	110

alanine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	755	798	819	818	133	123	112
dog 2	355	422	488	560	109	109	111
dog 3	402	471	530	585	118	112	109
dog 4	242	324	408	491	110	112	112
dog 5	577	641	668	708	133	114	113
			mean %R		121	114	111

citrulline

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	11	111	216	322	101	104	105
dog 2	44	146	243	351	107	105	108
dog 3	66	164	260	365	107	105	108
dog 4	35	139	243	354	108	109	110
dog 5	64	166	262	363	110	107	107
			mean %R		107	106	108

Table A-5. Continued.

α-aminobutyric acid

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	23	124	225	331	103	104	106
dog 2	41	143	239	349	107	104	108
dog 3	5	107	213	319	102	104	105
dog 4	21	126	231	340	108	108	109
dog 5	34	139	236	343	109	105	107
			mean %R		106	105	107

valine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	336	416	487	544	121	116	110
dog 2	193	277	357	447	107	105	108
dog 3	172	255	340	426	104	105	105
dog 4	122	213	308	404	105	108	109
dog 5	330	414	469	536	124	109	108
			mean %R		112	109	108

cystine

sample	no spike	low spike (50 µM)	medium spike (100 µM)	high spike (150 µM)	low %R	medium %R	high %R
dog 1	11	61	114	165	102	105	105
dog 2	0	43	98	155	87	98	104
dog 3	0	42	97	149	83	97	99
dog 4	0	44	96	157	88	96	105
dog 5	0	45	96	154	91	96	103
			mean %R		90	98	103

Table A-5. Continued.

methionine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	67	163	262	359	104	105	105
dog 2	56	156	253	360	106	105	108
dog 3	27	128	232	337	104	106	107
dog 4	46	147	252	359	107	108	110
dog 5	59	164	256	357	111	105	106
			mean %R		107	106	107

cystathionine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	0	89	198	308	89	99	103
dog 2	0	90	197	313	90	99	104
dog 3	0	92	199	311	92	100	104
dog 4	0	98	207	322	98	103	107
dog 5	0	101	205	315	101	103	105
			mean %R		94	101	105

isoleucine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	158	256	343	425	117	111	108
dog 2	62	158	253	357	103	103	106
dog 3	50	146	246	346	102	104	105
dog 4	27	128	231	340	105	105	108
dog 5	92	192	280	378	111	105	106
			mean %R		107	106	106

Table A-5. Continued.

leucine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	198	284	366	446	110	108	107
dog 2	116	211	303	402	109	107	109
dog 3	87	184	278	377	108	106	107
dog 4	58	159	262	371	108	109	111
dog 5	198	293	369	454	119	109	109
			mean %R		111	108	109

tyrosine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	28	126	224	328	101	101	103
dog 2	34	133	233	341	103	104	106
dog 3	35	132	231	334	101	102	104
dog 4	14	120	222	338	108	106	110
dog 5	34	137	233	340	108	104	106
			mean %R		104	103	106

β-alanine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	0	97	219	345	97	110	115
dog 2	0	104	207	318	104	103	106
dog 3	0	97	213	305	97	106	102
dog 4	0	105	203	312	105	102	104
dog 5	0	102	203	321	102	102	107
			mean %R		101	105	107

Table A-5. Continued.

phenylalanine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	49	141	240	339	98	101	102
dog 2	53	150	247	352	103	103	106
dog 3	45	144	242	342	105	104	104
dog 4	37	138	238	346	106	105	108
dog 5	54	154	246	349	106	102	105
			mean %R		104	103	105

β-aminoisobutyric acid

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	0	66	179	292	66	89	97
dog 2	0	102	202	314	102	101	105
dog 3	0	98	194	304	98	97	101
dog 4	0	93	189	308	93	95	103
dog 5	0	102	199	315	102	99	105
			mean %R		92	96	102

homocystine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	0	76	195	310	76	98	103
dog 2	0	62	184	316	62	92	105
dog 3	0	74	187	311	74	93	104
dog 4	0	61	185	313	61	92	104
dog 5	0	71	187	314	71	94	105
			mean %R		69	94	104

Table A-5. Continued.

γ-aminoisobutyric acid

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	0	92	201	314	92	101	105
dog 2	0	91	197	315	91	99	105
dog 3	0	102	200	308	102	100	103
dog 4	0	84	196	309	84	98	103
dog 5	0	99	201	312	99	101	104
			mean %R		94	100	104

ethanolamine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	0	115	230	348	115	115	116
dog 2	0	114	218	328	114	109	109
dog 3	0	130	222	326	130	111	109
dog 4	0	112	219	330	112	110	110
dog 5	0	127	221	325	127	110	108
			mean %R		120	111	110

ammonia

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	17	136	220	340	121	104	110
dog 2	18	117	224	331	101	105	107
dog 3	48	155	251	349	113	107	106
dog 4	45	136	249	351	96	107	107
dog 5	33	144	236	344	116	106	108
			mean %R		109	106	107

Table A-5. Continued.

hydroxylysine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	0	89	199	311	89	100	104
dog 2	0	76	190	310	76	95	103
dog 3	0	92	196	308	92	98	103
dog 4	0	75	189	309	75	95	103
dog 5	0	86	194	306	86	97	102
			mean %R		84	97	103

ornithine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	22	125	234	340	106	109	109
dog 2	0	81	199	326	81	99	109
dog 3	0	110	213	330	110	106	110
dog 4	0	86	204	328	86	102	109
dog 5	0	103	215	331	103	107	110
			mean %R		97	105	109

lysine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	315	410	487	542	134	124	114
dog 2	225	301	389	481	103	109	112
dog 3	158	262	340	432	123	110	110
dog 4	86	183	288	392	107	111	112
dog 5	403	483	535	594	128	114	112
			mean %R		119	114	112

Table A-5. Continued.

1-methylhistidine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	147	244	340	422	114	114	109
dog 2	0	91	200	318	91	100	106
dog 3	0	105	204	316	105	102	105
dog 4	0	96	207	323	96	103	108
dog 5	13	119	222	327	108	106	106
			mean %R		103	105	107

histidine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	70	162	258	352	100	102	102
dog 2	66	156	258	370	98	104	109
dog 3	71	177	265	366	114	106	107
dog 4	78	170	267	376	101	104	109
dog 5	77	179	272	373	111	107	108
			mean %R		105	105	107

tryptophan

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	27	118	222	316	94	101	100
dog 2	43	123	213	309	85	90	94
dog 3	45	130	214	309	91	90	93
dog 4	20	104	203	301	87	94	96
dog 5	74	159	237	327	94	90	93
			mean %R		90	93	95

Table A-5. Continued.

3-methylhistidine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	19	127	236	347	110	111	111
dog 2	0	90	196	317	90	98	106
dog 3	0	104	206	317	104	103	106
dog 4	0	96	207	322	96	104	107
dog 5	0	103	209	315	103	104	105
			mean %R		100	104	107

anserine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	0	99	206	316	99	103	105
dog 2	0	93	194	307	93	97	102
dog 3	0	100	189	300	100	94	100
dog 4	0	89	198	306	89	99	102
dog 5	0	99	198	303	99	99	101
			mean %R		96	99	102

carnosine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	27	171	279	373	147	129	119
dog 2	19	116	205	330	100	95	106
dog 3	26	147	231	334	124	106	106
dog 4	17	118	234	346	103	111	112
dog 5	30	139	244	330	112	110	104
			mean %R		117	110	109

Table A-5. Continued.

arginine

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	230	330	421	480	128	123	111
dog 2	154	232	322	419	97	103	107
dog 3	215	302	373	457	113	105	106
dog 4	167	248	340	434	101	107	109
dog 5	172	266	342	429	115	106	106
			mean %R		111	109	108

hydroxyproline

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	17	124	219	306	109	103	98
dog 2	0	89	206	330	89	103	110
dog 3	102	216	306	404	125	114	113
dog 4	0	91	223	346	91	111	115
dog 5	0	122	179	339	122	90	113
			mean %R		107	104	110

proline

sample	no spike	low spike (100 µM)	medium spike (200 µM)	high spike (300 µM)	low %R	medium %R	high %R
dog 1	258	278	357	436	50	80	90
dog 2	107	200	299	404	105	109	112
dog 3	145	247	335	428	119	112	112
dog 4	93	195	289	388	113	109	109
dog 5	170	259	335	437	109	103	109
			mean %R		99	103	107

Table A-6. Sample matrix dilution (linearity). Percent recovery is listed for each of 5 dogs for three dilutions. The following amino acids were not detected at any dilution in any sample and therefore graphs were excluded: phosphoserine, phosphoethanolamine, α-aminoadipic acid, cystathionine, β-alanine, β-aminoisobutyric acid, homocystine, γ-aminobutyric acid, ethanolamine, hydroxylysine, and anserine. DF, dilution factor; Obs, observed; Exp, expected; % R, percent recovery.

taurine

	DF	Obs	Exp	% R
dog 1	1	192		
	0.88	222	169	132
	0.76	190	146	130
	0.64	149	123	121
dog 2	1	81		
	0.88	68	71	96
	0.76	52	61	85
	0.64	35	52	67
dog 3	1	143		
	0.88	119	126	95
	0.76	91	108	84
	0.64	73	91	80
dog 4	1	139		
	0.88	118	122	96
	0.76	91	105	86
	0.64	62	89	70
dog 5	1	138		
	0.88	127	122	104
	0.76	100	105	95
	0.64	70	88	79

urea

	DF	Obs	Exp	% R
dog 1	1	11908		
	0.88	10796	10479	103
	0.76	9702	9050	107
	0.64	8123	7621	107
dog 2	1	3327		
	0.88	3002	2928	103
	0.76	2658	2529	105
	0.64	2260	2129	106
dog 3	1	5545		
	0.88	5097	4880	104
	0.76	4379	4214	104
	0.64	3716	3549	105
dog 4	1	6457		
	0.88	6015	5682	106
	0.76	5152	4907	105
	0.64	4356	4132	105
dog 5	1	10268		
	0.88	9664	9036	107
	0.76	8184	7803	105
	0.64	6777	6571	103

aspartic acid

	DF	Obs	Exp	% R
dog 1	1	2		
	0.88	0	2	3
	0.76	0	2	0
	0.64	0	1	0
dog 2	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 3	1	6		
	0.88	4	6	76
	0.76	0	5	3
	0.64	0	4	0
dog 4	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 5	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-

Table A-6. Continued.

threonine

	DF	Obs	Exp	% R
dog 1	1	364		
	0.88	329	320	103
	0.76	286	276	104
	0.64	236	233	102
dog 2	1	197		
	0.88	177	173	102
	0.76	155	150	103
	0.64	129	126	102
dog 3	1	161		
	0.88	153	142	108
	0.76	126	123	103
	0.64	100	103	97
dog 4	1	398		
	0.88	366	350	105
	0.76	311	302	103
	0.64	258	255	102
dog 5	1	267		
	0.88	244	235	104
	0.76	211	203	104
	0.64	168	171	98

serine

	DF	Obs	Exp	% R
dog 1	1	111		
	0.88	100	98	102
	0.76	84	84	100
	0.64	68	71	96
dog 2	1	104		
	0.88	93	91	102
	0.76	82	79	104
	0.64	66	66	100
dog 3	1	110		
	0.88	105	97	108
	0.76	85	84	102
	0.64	68	70	97
dog 4	1	95		
	0.88	87	84	104
	0.76	72	72	100
	0.64	56	61	93
dog 5	1	90		
	0.88	81	79	102
	0.76	69	68	100
	0.64	54	58	94

asparagine

	DF	Obs	Exp	% R
dog 1	1	168		
	0.88	135	148	91
	0.76	120	128	94
	0.64	101	108	94
dog 2	1	48		
	0.88	42	43	98
	0.76	40	37	109
	0.64	29	31	93
dog 3	1	48		
	0.88	39	42	92
	0.76	37	36	101
	0.64	28	30	91
dog 4	1	38		
	0.88	37	34	110
	0.76	29	29	101
	0.64	23	24	95
dog 5	1	63		
	0.88	48	55	87
	0.76	47	48	99
	0.64	38	40	94

Table A-6. Continued.

glutamic acid

	DF	Obs	Exp	% R
dog 1	1	33		
	0.88	26	29	92
	0.76	22	25	89
	0.64	18	21	85
dog 2	1	13		
	0.88	13	12	112
	0.76	8	10	82
	0.64	2	8	18
dog 3	1	68		
	0.88	58	60	97
	0.76	51	51	100
	0.64	41	43	94
dog 4	1	49		
	0.88	46	43	106
	0.76	36	38	95
	0.64	30	32	94
dog 5	1	29		
	0.88	23	25	90
	0.76	21	22	97
	0.64	14	19	76

glutamine

	DF	Obs	Exp	% R
dog 1	1	396		
	0.88	353	348	101
	0.76	306	301	102
	0.64	252	253	99
dog 2	1	642		
	0.88	587	565	104
	0.76	514	488	105
	0.64	431	411	105
dog 3	1	667		
	0.88	618	587	105
	0.76	533	507	105
	0.64	441	427	103
dog 4	1	687		
	0.88	638	605	106
	0.76	542	522	104
	0.64	454	440	103
dog 5	1	600		
	0.88	550	528	104
	0.76	474	456	104
	0.64	383	384	100

glycine

	DF	Obs	Exp	% R
dog 1	1	200		
	0.88	175	176	99
	0.76	154	152	101
	0.64	125	128	97
dog 2	1	199		
	0.88	181	175	103
	0.76	157	152	104
	0.64	131	128	103
dog 3	1	358		
	0.88	331	315	105
	0.76	282	272	104
	0.64	234	229	102
dog 4	1	212		
	0.88	194	187	104
	0.76	162	161	100
	0.64	133	136	98
dog 5	1	161		
	0.88	147	142	104
	0.76	123	122	100
	0.64	96	103	93

Table A-6. Continued.

alanine

	DF	Obs	Exp	% R
dog 1	1	755		
	0.88	709	664	107
	0.76	625	574	109
	0.64	520	483	108
dog 2	1	355		
	0.88	326	312	105
	0.76	286	270	106
	0.64	241	227	106
dog 3	1	402		
	0.88	375	354	106
	0.76	321	306	105
	0.64	267	257	104
dog 4	1	242		
	0.88	227	213	106
	0.76	191	184	104
	0.64	157	155	101
dog 5	1	577		
	0.88	535	508	105
	0.76	463	439	106
	0.64	378	370	102

citrulline

	DF	Obs	Exp	% R
dog 1	1	11		
	0.88	7	10	75
	0.76	5	8	58
	0.64	2	7	28
dog 2	1	44		
	0.88	40	39	102
	0.76	34	34	101
	0.64	25	28	89
dog 3	1	66		
	0.88	61	58	105
	0.76	52	50	105
	0.64	40	42	96
dog 4	1	35		
	0.88	32	31	106
	0.76	24	26	92
	0.64	18	22	79
dog 5	1	64		
	0.88	57	56	102
	0.76	48	48	99
	0.64	38	41	93

α-aminobutyric acid

	DF	Obs	Exp	% R
dog 1	1	23		
	0.88	19	20	91
	0.76	14	18	81
	0.64	10	15	65
dog 2	1	41		
	0.88	36	36	102
	0.76	31	31	102
	0.64	20	26	77
dog 3	1	5		
	0.88	4	4	97
	0.76	5	4	122
	0.64	0	3	0
dog 4	1	21		
	0.88	20	19	106
	0.76	14	16	85
	0.64	10	14	76
dog 5	1	34		
	0.88	30	30	101
	0.76	25	26	96
	0.64	19	22	85

Table A-6. Continued.

valine

	DF	Obs	Exp	% R
dog 1	1	336		
	0.88	319	296	108
	0.76	278	255	109
	0.64	229	215	107
dog 2	1	193		
	0.88	178	170	104
	0.76	154	147	105
	0.64	131	124	106
dog 3	1	172		
	0.88	159	151	105
	0.76	133	131	102
	0.64	111	110	101
dog 4	1	122		
	0.88	113	108	105
	0.76	95	93	102
	0.64	79	78	101
dog 5	1	330		
	0.88	307	290	106
	0.76	265	251	106
	0.64	215	211	102

cystine

	DF	Obs	Exp	% R
dog 1	1	11		
	0.88	10	10	96
	0.76	7	9	84
	0.64	4	7	53
dog 2	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 3	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 4	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 5	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-

methionine

	DF	Obs	Exp	% R
dog 1	1	67		
	0.88	59	59	101
	0.76	49	51	96
	0.64	38	43	89
dog 2	1	56		
	0.88	50	50	100
	0.76	42	43	98
	0.64	32	36	88
dog 3	1	27		
	0.88	24	24	100
	0.76	17	21	83
	0.64	9	17	55
dog 4	1	46		
	0.88	41	40	101
	0.76	30	35	85
	0.64	20	29	67
dog 5	1	59		
	0.88	53	52	101
	0.76	43	45	96
	0.64	33	38	86

Table A-6. Continued.

isoleucine

	DF	Obs	Exp	% R
dog 1	1	158		
	0.88	151	139	108
	0.76	131	120	109
	0.64	106	101	104
dog 2	1	62		
	0.88	54	55	99
	0.76	45	47	96
	0.64	32	40	82
dog 3	1	50		
	0.88	41	44	93
	0.76	34	38	89
	0.64	25	32	77
dog 4	1	27		
	0.88	25	23	106
	0.76	18	20	88
	0.64	14	17	81
dog 5	1	92		
	0.88	84	81	103
	0.76	70	70	99
	0.64	52	59	87

leucine

	DF	Obs	Exp	% R
dog 1	1	198		
	0.88	181	174	104
	0.76	156	150	104
	0.64	126	126	99
dog 2	1	116		
	0.88	106	102	104
	0.76	87	88	98
	0.64	67	74	91
dog 3	1	87		
	0.88	76	77	99
	0.76	59	66	89
	0.64	44	56	78
dog 4	1	58		
	0.88	53	51	104
	0.76	40	44	90
	0.64	24	37	64
dog 5	1	198		
	0.88	180	174	103
	0.76	151	151	101
	0.64	117	127	93

tyrosine

	DF	Obs	Exp	% R
dog 1	1	28		
	0.88	22	24	89
	0.76	17	21	80
	0.64	12	18	69
dog 2	1	34		
	0.88	33	30	109
	0.76	25	26	97
	0.64	18	22	85
dog 3	1	35		
	0.88	29	31	96
	0.76	20	26	75
	0.64	15	22	66
dog 4	1	14		
	0.88	12	12	99
	0.76	9	10	83
	0.64	2	9	20
dog 5	1	34		
	0.88	32	30	107
	0.76	24	26	92
	0.64	18	22	81

Table A-6. Continued.

phenylalanine

	DF	Obs	Exp	% R
dog 1	1	49		
	0.88	41	44	95
	0.76	35	38	94
	0.64	28	32	88
dog 2	1	53		
	0.88	51	47	109
	0.76	42	41	103
	0.64	34	34	99
dog 3	1	45		
	0.88	43	40	109
	0.76	35	34	102
	0.64	26	29	91
dog 4	1	37		
	0.88	32	32	99
	0.76	24	28	86
	0.64	19	24	79
dog 5	1	54		
	0.88	49	48	102
	0.76	42	41	102
	0.64	31	35	89

ammonia

	DF	Obs	Exp	% R
dog 1	1	17		
	0.88	23	15	154
	0.76	23	13	179
	0.64	7	11	62
dog 2	1	18		
	0.88	19	16	118
	0.76	19	14	139
	0.64	13	12	109
dog 3	1	48		
	0.88	44	42	104
	0.76	40	36	109
	0.64	32	31	103
dog 4	1	45		
	0.88	37	40	94
	0.76	31	34	91
	0.64	24	29	84
dog 5	1	33		
	0.88	32	29	111
	0.76	28	25	112
	0.64	14	21	68

ornithine

	DF	Obs	Exp	% R
dog 1	1	22		
	0.88	17	19	89
	0.76	12	17	71
	0.64	6	14	45
dog 2	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 3	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 4	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 5	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-

Table A-6. Continued.

lysine

	DF	Obs	Exp	% R
dog 1	1	315		
	0.88	309	277	112
	0.76	268	239	112
	0.64	217	201	108
dog 2	1	225		
	0.88	205	198	103
	0.76	178	171	104
	0.64	146	144	102
dog 3	1	158		
	0.88	146	139	105
	0.76	123	120	102
	0.64	97	101	96
dog 4	1	86		
	0.88	77	75	102
	0.76	63	65	97
	0.64	46	55	85
dog 5	1	403		
	0.88	369	355	104
	0.76	319	307	104
	0.64	257	258	99

1-methylhistidine

	DF	Obs	Exp	% R
dog 1	1	147		
	0.88	140	130	108
	0.76	119	112	106
	0.64	96	94	102
dog 2	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 3	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 4	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 5	1	13		
	0.88	11	11	95
	0.76	7	10	75
	0.64	2	8	25

histidine

	DF	Obs	Exp	% R
dog 1	1	70		
	0.88	65	62	105
	0.76	55	53	104
	0.64	45	45	100
dog 2	1	66		
	0.88	61	58	106
	0.76	49	50	98
	0.64	39	42	92
dog 3	1	71		
	0.88	66	62	106
	0.76	54	54	99
	0.64	43	45	94
dog 4	1	78		
	0.88	71	69	103
	0.76	58	59	97
	0.64	49	50	98
dog 5	1	77		
	0.88	70	68	102
	0.76	60	59	103
	0.64	45	49	92

Table A-6. Continued.

tryptophan

	DF	Obs	Exp	% R
dog 1	1	27		
	0.88	29	24	121
	0.76	20	20	100
	0.64	14	17	83
dog 2	1	43		
	0.88	45	38	117
	0.76	34	33	103
	0.64	29	28	103
dog 3	1	45		
	0.88	44	40	110
	0.76	36	34	105
	0.64	30	29	106
dog 4	1	20		
	0.88	14	17	84
	0.76	9	15	63
	0.64	2	13	19
dog 5	1	74		
	0.88	67	65	103
	0.76	57	56	102
	0.64	53	47	112

3-methylhistidine

	DF	Obs	Exp	% R
dog 1	1	19		
	0.88	15	17	85
	0.76	11	15	71
	0.64	4	12	33
dog 2	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 3	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 4	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 5	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-

carnosine

	DF	Obs	Exp	% R
dog 1	1	27		
	0.88	42	24	173
	0.76	33	21	157
	0.64	22	17	124
dog 2	1	19		
	0.88	13	17	79
	0.76	16	14	110
	0.64	6	12	49
dog 3	1	26		
	0.88	17	23	73
	0.76	15	20	75
	0.64	13	17	79
dog 4	1	17		
	0.88	12	15	83
	0.76	10	13	77
	0.64	7	11	66
dog 5	1	30		
	0.88	27	26	102
	0.76	21	23	92
	0.64	18	19	96

Table A-6. Continued.

arginine

	DF	Obs	Exp	% R
dog 1	1	230		
	0.88	235	202	116
	0.76	197	175	113
	0.64	161	147	109
dog 2	1	154		
	0.88	138	135	102
	0.76	120	117	103
	0.64	98	98	100
dog 3	1	215		
	0.88	201	190	106
	0.76	167	164	102
	0.64	137	138	100
dog 4	1	167		
	0.88	154	147	104
	0.76	130	127	102
	0.64	107	107	100
dog 5	1	172		
	0.88	157	151	104
	0.76	134	131	102
	0.64	108	110	98

hydroxyproline

	DF	Obs	Exp	% R
dog 1	1	17		
	0.88	14	15	91
	0.76	0	13	0
	0.64	0	11	0
dog 2	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 3	1	102		
	0.88	72	90	80
	0.76	66	78	85
	0.64	49	66	74
dog 4	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-
dog 5	1	0		
	0.88	0	0	-
	0.76	0	0	-
	0.64	0	0	-

proline

	DF	Obs	Exp	% R
dog 1	1	258		
	0.88	187	227	82
	0.76	186	196	95
	0.64	153	165	92
dog 2	1	107		
	0.88	102	94	109
	0.76	81	82	100
	0.64	82	69	119
dog 3	1	145		
	0.88	143	128	112
	0.76	111	110	101
	0.64	96	93	103
dog 4	1	93		
	0.88	93	82	114
	0.76	69	70	98
	0.64	53	59	90
dog 5	1	170		
	0.88	156	149	104
	0.76	129	129	100
	0.64	98	109	90

Table A-7. Linearity of standards. Cells with a tan color under expected concentrations columns indicate typical range of expected concentrations in healthy dog serum. Exp, expected; Obs, observed; OE%, observed to expected ratio.

phosphoserine		
Exp	Obs	OE%
750	738	98
600	606	101
500	516	103
250	261	104
125	127	102
50	51	102
25	24	94
20	19	96
15	14	94
10	9	89
5	4	75
2.5	0.9	37

taurine		
Exp	Obs	OE%
750	744	99.2
600	601	100.2
500	509	101.8
250	257	102.8
125	126	100.5
50	50	100.6
25	24	94.8
20	19	96.5
15	15	98.1
10	10	97.4
5	5	93.2
2.5	2.2	87.2

phosphoethanolamine		
Exp	Obs	OE%
750	746	99
600	600	100
500	506	101
250	255	102
125	124	99
50	50	100
25	24	94
20	20	99
15	15	99
10	10	100
5	5	103
2.5	2.3	93

urea		
Exp	Obs	OE%
750	748	100
600	593	99
500	505	101
250	254	102
125	116	93
50	37	74
25	27	107
20	27	133
15	17	113
10	14	138
5	0	0
2.5	0	0

aspartic acid		
Exp	Obs	OE%
750	746	99
600	598	100
500	506	101
250	254	102
125	125	100
50	50	100
25	24	96
20	19	97
15	15	102
10	10	102
5	5	104
2.5	2.7	107

threonine		
Exp	Obs	OE%
750	746	99
600	599	100
500	506	101
250	255	102
125	124	99
50	51	102
25	24	94
20	19	97
15	15	98
10	10	97
5	5	94
2.5	2.4	97

Table A-7. Continued.

serine		
Exp	Obs	OE%
750	747	100
600	599	100
500	505	101
250	254	102
125	124	99
50	51	101
25	24	95
20	19	97
15	15	100
10	10	99
5	5	98
2.5	2.2	90

asparagine		
Exp	Obs	OE%
750	742	99
600	600	100
500	511	102
250	259	104
125	129	103
50	49	98
25	23	93
20	19	96
15	14	94
10	10	102
5	5	94
2.5	2.9	117

glutamic acid		
Exp	Obs	OE%
750	749	100
600	597	100
500	502	100
250	255	102
125	124	99
50	51	102
25	24	97
20	20	100
15	15	99
10	10	104
5	4	84
2.5	2.1	83

glutamine		
Exp	Obs	OE%
750	744	99
600	596	99
500	508	102
250	253	101
125	127	102
50	51	101
25	24	95
20	20	99
15	14	95
10	10	96
5	3	62
2.5	0.8	34

sarcosine		
Exp	Obs	OE%
750	734	98
600	603	101
500	517	103
250	286	114
125	150	120
50	42	85
25	16	64
20	16	78
15	15	100
10	8	83
5	0	0
2.5	0.0	0

α-aminoadipic acid		
Exp	Obs	OE%
750	745	99
600	598	100
500	507	101
250	258	103
125	129	103
50	51	103
25	27	108
20	21	104
15	8	55
10	3	35
5	1	12
2.5	0.0	1

Table A-7. Continued.

glycine		
Exp	Obs	OE%
750	745	99
600	598	100
500	506	101
250	255	102
125	127	102
50	52	103
25	26	103
20	21	104
15	13	88
10	8	78
5	3	64
2.5	1.1	42

alanine		
Exp	Obs	OE%
750	745	99
600	600	100
500	507	101
250	255	102
125	126	101
50	51	103
25	25	99
20	20	102
15	13	84
10	8	80
5	4	72
2.5	1.1	44

citrulline		
Exp	Obs	OE%
750	747	100
600	596	99
500	504	101
250	256	102
125	125	100
50	51	101
25	24	97
20	20	99
15	16	106
10	10	104
5	5	108
2.5	2.0	80

α-aminobutyric acid		
Exp	Obs	OE%
750	746	99
600	599	100
500	506	101
250	254	102
125	125	100
50	51	102
25	24	97
20	20	100
15	14	96
10	9	93
5	4	85
2.5	0.4	17

valine		
Exp	Obs	OE%
750	747	100
600	597	100
500	505	101
250	255	102
125	125	100
50	50	100
25	24	95
20	19	97
15	15	99
10	10	103
5	5	91
2.5	2.1	86

cystine		
Exp	Obs	OE%
375	373	100
300	298	99
250	252	101
125	128	103
62.5	62	100
25	26	105
12.5	15	116
10	11	108
7.5	6	81
5	4	76
2.5	1	34
1.25	0.2	14

Table A-7. Continued.

methionine		
Exp	Obs	OE%
750	747	100
600	597	99
500	505	101
250	254	102
125	124	99
50	50	100
25	24	96
20	20	98
15	15	101
10	10	104
5	6	114
2.5	3.3	130

cystathionine		
Exp	Obs	OE%
750	746	100
600	598	100
500	506	101
250	254	102
125	124	99
50	51	101
25	24	97
20	20	99
15	15	99
10	10	97
5	5	90
2.5	1.9	76

isoleucine		
Exp	Obs	OE%
750	747	100
600	599	100
500	505	101
250	255	102
125	125	100
50	51	101
25	25	98
20	20	100
15	12	80
10	7	70
5	2	34
2.5	0.5	20

leucine		
Exp	Obs	OE%
750	746	99
600	599	100
500	506	101
250	254	102
125	125	100
50	51	102
25	24	97
20	20	99
15	12	80
10	7	72
5	2	36
2.5	0.3	10

tyrosine		
Exp	Obs	OE%
750	746	99
600	599	100
500	506	101
250	253	101
125	125	100
50	51	102
25	24	95
20	20	101
15	14	93
10	9	90
5	4	78
2.5	1.2	50

β-alanine		
Exp	Obs	OE%
750	746	99
600	597	99
500	507	101
250	255	102
125	123	98
50	53	105
25	25	98
20	19	94
15	15	99
10	10	95
5	5	108
2.5	3.6	143

Table A-7. Continued.

phenylalanine		
Exp	Obs	OE%
750	747	100
600	597	100
500	505	101
250	252	101
125	124	99
50	51	102
25	24	97
20	20	100
15	15	98
10	9	90
5	4	80
2.5	2.1	85

β-aminoisobutyric acid		
Exp	Obs	OE%
750	747	100
600	594	99
500	504	101
250	251	100
125	123	98
50	53	105
25	24	96
20	22	109
15	17	111
10	10	96
5	4	85
2.5	2.4	94

homocystine		
Exp	Obs	OE%
750	741	99
600	603	100
500	512	102
250	262	105
125	130	104
50	50	100
25	25	100
20	19	96
15	14	91
10	8	81
5	3	54
2.5	0.0	1

γ-aminobutyric acid		
Exp	Obs	OE%
750	745	99
600	602	100
500	508	102
250	256	102
125	126	100
50	48	96
25	25	99
20	19	95
15	13	85
10	8	84
5	5	103
2.5	3.6	142

ethanolamine		
Exp	Obs	OE%
750	745	99
600	602	100
500	508	102
250	256	102
125	126	101
50	48	97
25	26	104
20	21	105
15	38	251
10	32	322
5	26	516
2.5	26.4	1054

ammonia		
Exp	Obs	OE%
750	743	99
600	604	101
500	510	102
250	257	103
125	126	101
50	46	93
25	19	77
20	18	90
15	9	60
10	5	47
5	1	29
2.5	2.1	83

Table A-7. Continued.

hydroxylysine		
Exp	Obs	OE%
750	742	99
600	604	101
500	512	102
250	258	103
125	127	101
50	48	96
25	23	92
20	18	88
15	13	87
10	8	83
5	4	70
2.5	1.2	47

ornithine		
Exp	Obs	OE%
750	744	99
600	602	100
500	508	102
250	257	103
125	127	101
50	50	99
25	25	102
20	20	99
15	15	98
10	9	94
5	4	80
2.5	1.1	42

lysine		
Exp	Obs	OE%
750	744	99
600	602	100
500	508	102
250	257	103
125	126	101
50	50	99
25	25	102
20	20	98
15	14	96
10	9	92
5	4	79
2.5	1.3	52

1-methylhistidine		
Exp	Obs	OE%
750	743.7	99.2
600	603.2	100.5
500	509	101.8
250	256.4	102.6
125	126.1	100.9
50	49.7	99.4
25	25.2	100.8
20	19.7	98.5
15	14.5	96.4
10	9.2	92.2
5	3.9	78.0
2.5	1.1	44.8

histidine		
Exp	Obs	OE%
750	745	99
600	595	99
500	508	102
250	253	101
125	123	99
50	49	98
25	25	101
20	20	102
15	15	98
10	10	97
5	4	84
2.5	1.8	73

tryptophan		
Exp	Obs	OE%
750	743	99
600	610	102
500	509	102
250	264	106
125	131	105
50	50	100
25	25	100
20	20	98
15	14	94
10	7	75
5	2	34
2.5	0.0	0

Table A-7. Continued.

3-methylhistidine		
Exp	Obs	OE%
750	743	99
600	602	100
500	510	102
250	259	103
125	127	101
50	50	101
25	26	105
20	20	98
15	15	97
10	8	82
5	3	52
2.5	0.2	7

anserine		
Exp	Obs	OE%
750	744	99
600	601	100
500	508	102
250	256	102
125	127	102
50	51	102
25	26	104
20	20	102
15	16	105
10	7	67
5	2	44
2.5	0.7	29

carnosine		
Exp	Obs	OE%
750	744	99
600	598	100
500	508	102
250	260	104
125	127	102
50	52	104
25	21	84
20	17	85
15	14	96
10	9	91
5	3	69
2.5	0.6	26

arginine		
Exp	Obs	OE%
750	743	99
600	603	100
500	510	102
250	258	103
125	126	101
50	49	98
25	26	102
20	19	94
15	15	98
10	9	95
5	5	98
2.5	2.3	94

hydroxyproline		
Exp	Obs	OE%
750	762	102
600	585	97
500	483	97
250	252	101
125	116	93
50	48	95
25	29	117
20	17	85
15	17	114
10	12	119
5	10	203
2.5	4.1	163

proline		
Exp	Obs	OE%
750	759	101
600	589	98
500	487	97
250	250	100
125	120	96
50	48	95
25	26	105
20	23	115
15	15	97
10	8	80
5	6	128
2.5	2.8	111

Table A-8. Stability of amino acids in dog serum stored at −80°C and their coefficients of variation (CV%).

Compound	median [range] µM[a]	median [range] CV%[b]
taurine	130 [60-262]	6.5 [3.1-24.8]
urea	3495 [1840-10693]	3.5 [1.9-4.2]
aspartic acid	0 [0-27]	11.8 [11.8-11.8]
threonine	170 [37-294]	3.0 [2.3-8.2]
serine	98 [29-145]	3.0 [2.4-11.4]
asparagine	59 [20-92]	10.8 [8.8-22.6]
glutamic acid	30 [13-62]	13.8 [4.0-35.7]
glutamine	667 [230-959]	3.2 [2.0-4.5]
glycine	165 [63-237]	3.0 [2.0-8.5]
alanine	414 [198-897]	2.6 [1.6-4.4]
citrulline	37 [5-57]	8.4 [4.3-49.2]
α-aminobutyric acid	23 [2-90]	11.7 [5.0-79.9]
valine	183 [124-445]	3.1 [1.8-4.0]
cystine	0 [0-4]	39.6 [39.6-39.6]
methionine	54 [7-63]	5.9 [3.9-51.1]
isoleucine	57 [26-194]	14.1 [5.1-38.7]
leucine	124 [67-332]	4.2 [3.5-10.7]
tyrosine	26 [5-51]	13.2 [5.5-58.5]
phenylalanine	48 [34-105]	5.8 [3.6-6.5]
ethanolamine	0 [0-0]	223.6 [223.6-223.6]
ammonia	38 [27-84]	12.6 [6.0-20.3]
ornithine	0 [0-26]	42.6 [32.9-52.2]
lysine	145 [41-295]	3.6 [2.8-9.0]
1-methylhistidine	0 [0-5]	68.1 [56.3-79.9]
histidine	48 [28-64]	10.7 [8.6-13.3]
tryptophan	35 [18-130]	18.3 [5.6-36.3]
3-methylhistidine	0 [0-0.3]	141.1 [141.1-141.1]
carnosine	8 [5-20]	34.4 [21.2-64.5]
arginine	153 [45-210]	5.5 [4.6-8.2]
hydroxyproline	0 [0-5]	223.6 [93.0-223.6]
proline	124 [33-192]	6.3 [3.4-13.3]

Stability at −80°C. Amino acids excluded from the table (not detected in all samples): phosphoserine, phosphoethanolamine, sarcosine, α-aminoadipic acid, cystathionine, β-alanine, β-aminoisobutyric acid, homocystine, γ-aminobutyric acid, hydroxylysine, anserine. [a]Concentrations of samples used (the median and range of the median of concentrations from eight dogs across six different timepoints of storage at −80°C). [b]Coefficient of variation, calculated from the same six timepoints: baseline, storage at −80°C for 1, 2 3, and 4 weeks prior to deproteinization, and storage at −80°C for 61 weeks following deproteinization.

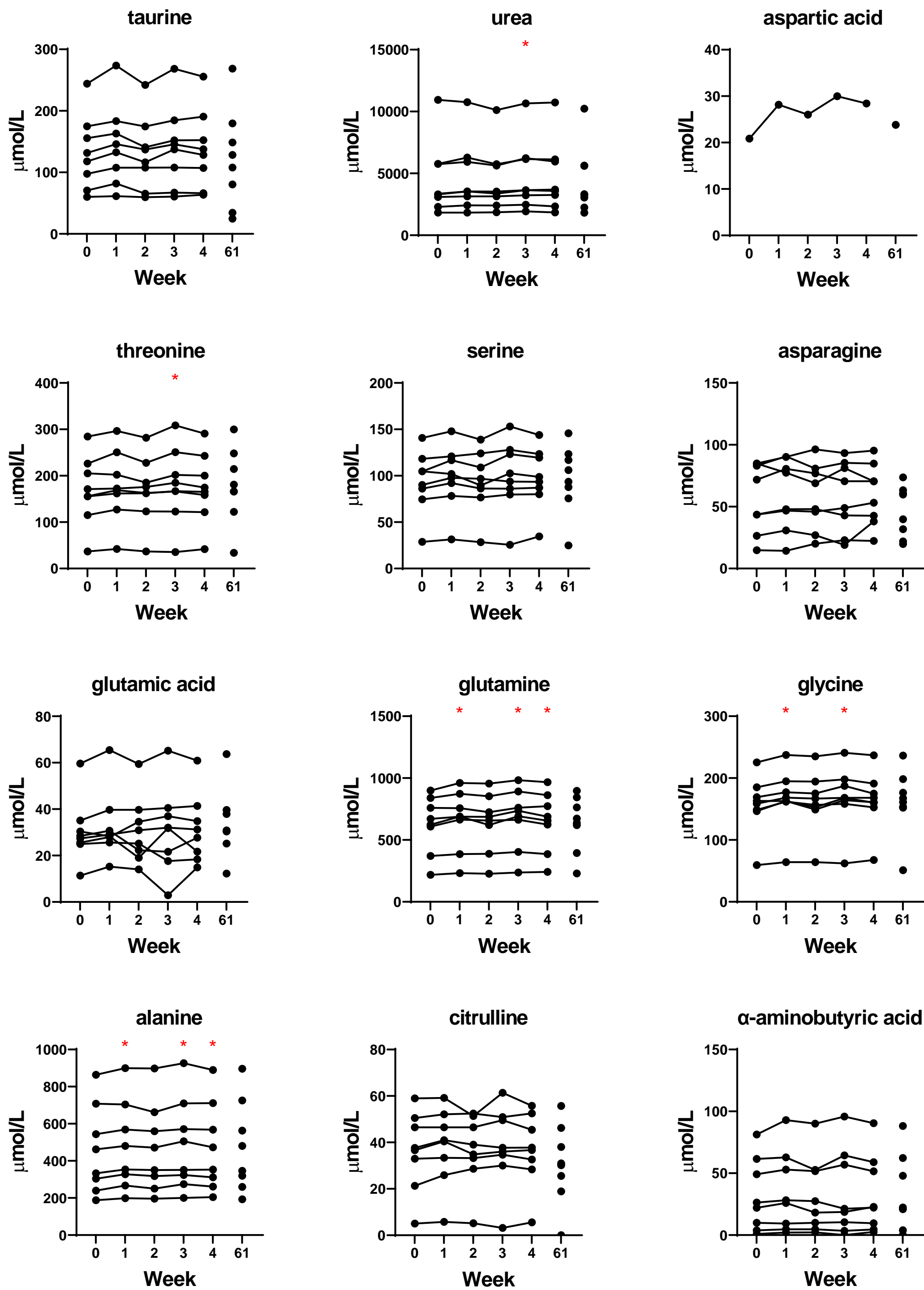

Figure A-9. Stability of amino acids in dog serum stored at −80°C. Red asterisks indicate significance difference ($p<0.05$) compared to week 0.

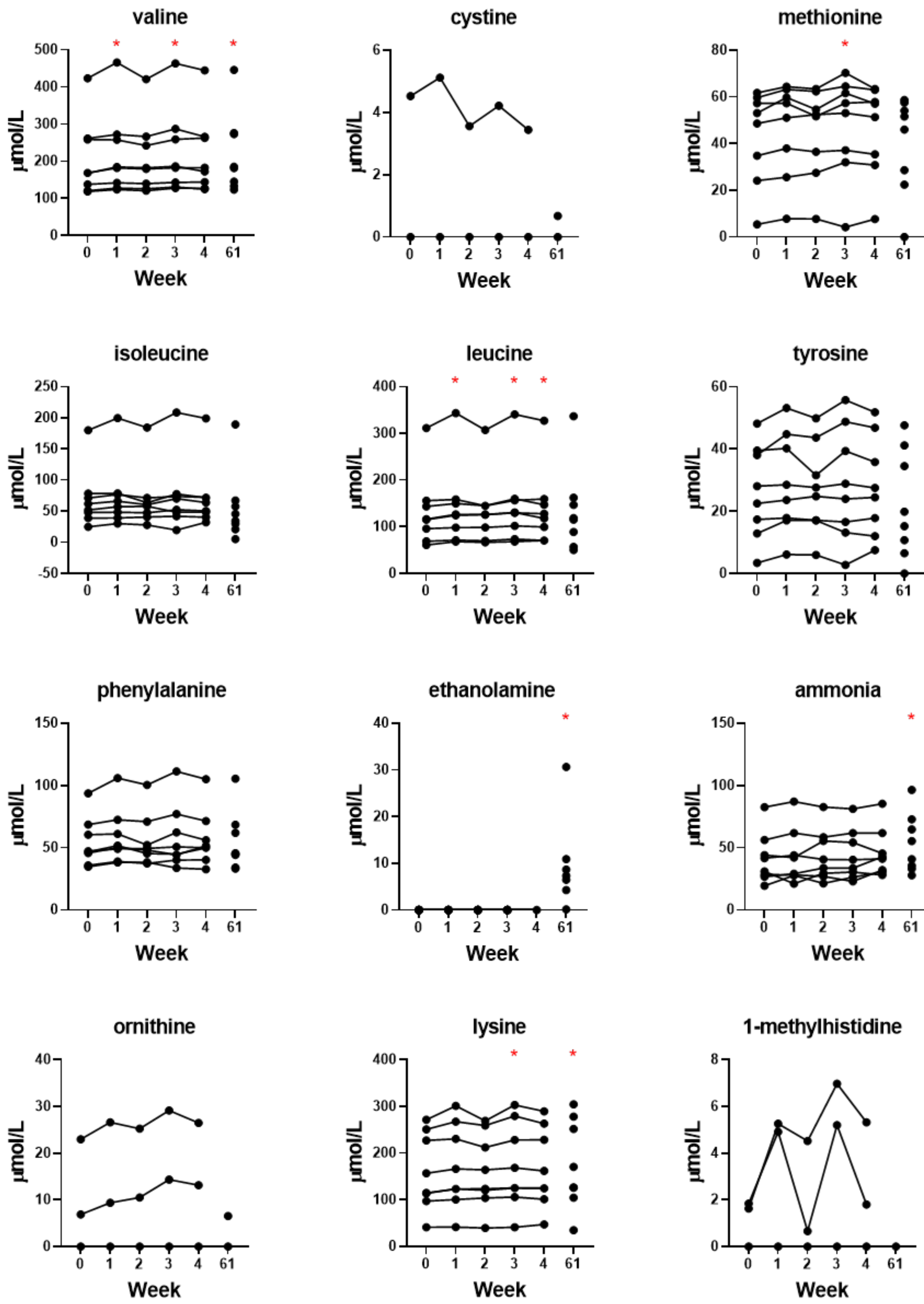

Figure A-9. Continued.

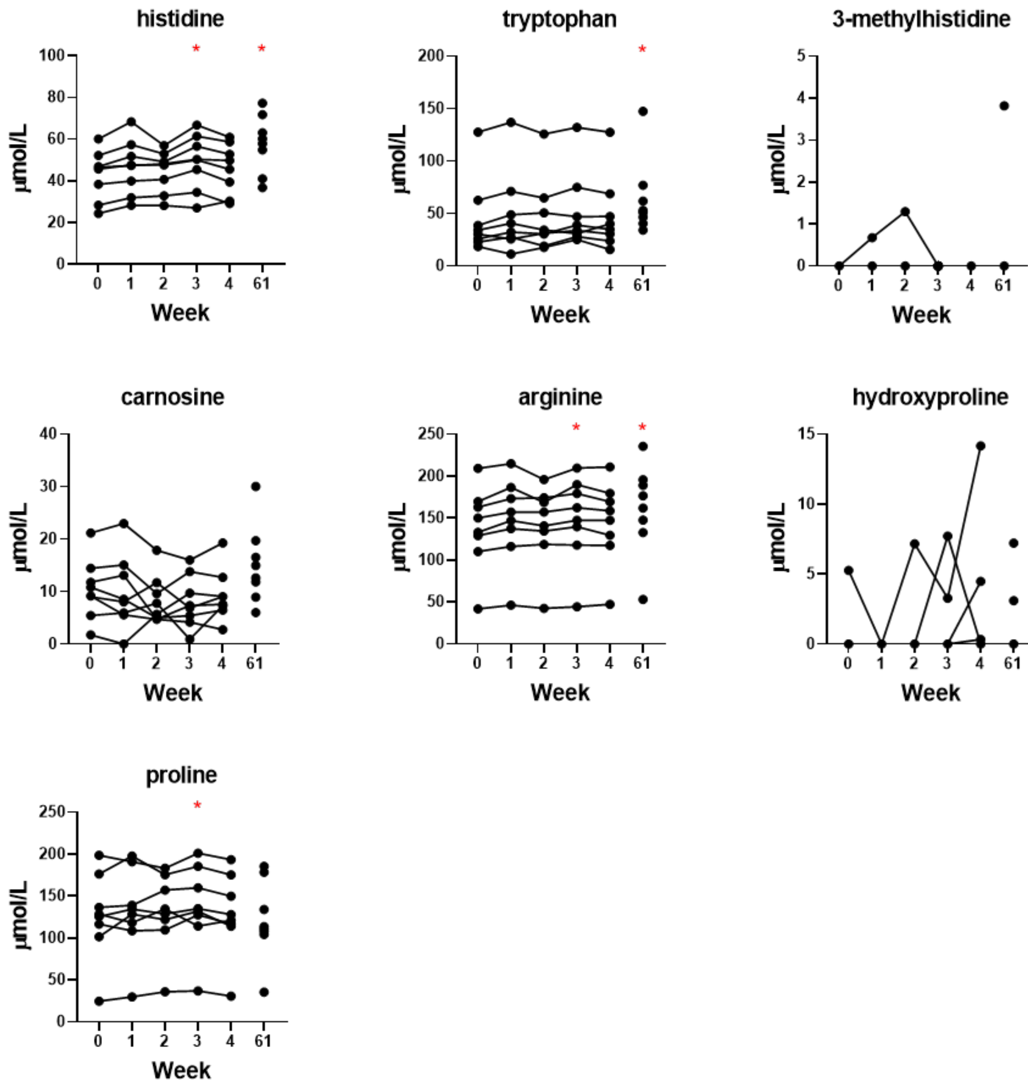

Figure A-9. Continued.

Table A-10. Stability of amino acids in dog serum stored at 4°C and their coefficients of variation (CV%).

Compound	Median [range] µM[a]	Median [range] CV%[b]
taurine	173 [107-204]	3.7 [1.9-7.1]
urea	6479 [5323-7450]	2.6 [0.8-5.7]
threonine	271 [157-369]	2.3 [1.7-3.6]
serine	124 [80-176]	3.4 [1.9-4.9]
asparagine	69 [38-95]	4.9 [4.2-8.1]
glutamic acid	13 [8-22]	41.7 [25.2-54.1]
glutamine	813 [788-932]	2.8 [1.2-4.2]
glycine	311 [284-420]	3.8 [2.0-4.9]
alanine	453 [418-550]	1.8 [0.8-3.5]
citrulline	55 [40-70]	2.6 [1.4-3.8]
α-aminobutyric acid	1 [0-19]	5.7 [0.0-45.9]
valine	165 [131-231]	3.1 [1.4-3.9]
cystine	0.4 [0-5]	117.8 [103.2-123.2]
methionine	58 [44-68]	4.0 [2.3-4.7]
isoleucine	23 [1-45]	9.7 [4.9-92.9]
leucine	123 [93-191]	4.0 [1.6-6.3]
tyrosine	38 [33-43]	3.8 [3.2-6.3]
phenylalanine	44 [39-51]	4.8 [2.7-7.9]
ammonia	24 [9-27]	54.8 [34.8-67.9]
lysine	116 [56-217]	4.2 [3.4-6.5]
histidine	58 [52-74]	4.8 [1.9-7.0]
tryptophan	76 [51-90]	4.5 [1.3-4.9]
carnosine	26 [14-29]	6.7 [3.1-8.3]
arginine	145 [115-154]	5.0 [2.9-9.1]
hydroxyproline	24 [17-68]	38.8 [25.9-110.1]
proline	294 [251-372]	2.9 [1.5-6.2]

Stability of the assay at 4°C. Compounds excluded from the table because they were not detected in any sample: phosphoserine, phosphoethanolamine, aspartic acid, sarcosine, α-aminoadipic acid, cystathionine, β-alanine, β-aminoisobutyric acid, homocystine, γ-aminobutyric acid, ethanolamine, hydroxylysine, ornithine, 1-methyhistidine, 3-methylhistidine, and anserine. [a]Concentrations of samples used (the median and range of the median of concentrations from four dogs across five different timepoints of storage at 4°C). [b]Coefficient of variation, calculated from the same five timepoints: baseline, storage at 4°C for 24, 48, and 72 hours prior to deproteinization, and storage at 4°C for 72 hours following deproteinization.

Table A-11. Stability of amino acids in dog serum stored at −20°C and their coefficients of variation (CV%).

Compound	Median [range] μM[a]	Median [range] CV%[b]
taurine	174 [110-207]	5.1 [4.9-5.4]
urea	6594 [5379-7613]	2.9 [1.9-4.2]
threonine	276 [161-378]	2.5 [1.4-3.8]
serine	125 [80-181]	3.2 [1.5-4.6]
asparagine	72 [39-93]	4.7 [4.1-8.9]
glutamic acid	9[4-12]	37.2 [18.2-45.8]
glutamine	819 [799-935]	2.3 [2.1-3.6]
glycine	308 [280-426]	2.8 [2.2-3.8]
alanine	452 [414-550]	3.1 [2.5-3.8]
citrulline	54 [39-69]	4.5 [4.3-5.3]
α-aminobutyric acid	1 [0-19]	4.9 [0.0-55.9]
valine	167 [129-237]	2.4 [1.1-3.1]
cystine	0.6 [0-4]	126.1 [112.9-150.3]
methionine	57 [44-70]	3.7 [2.5-5.3]
isoleucine	22 [0.6-42]	18.7 [8.9-126.0]
leucine	126 [94-198]	3.9 [1.7-7.0]
tyrosine	40 [35-46]	8.0 [5.3-8.4]
phenylalanine	45 [38-51]	4.8 [2.9-7.4]
ethanolamine	0 [0-0]	173.2 [173.2-264.6]
ammonia	12 [6-17]	61.5 [44.6-89.7]
lysine	118 [53-217]	3.1 [2.3-6.2]
histidine	57 [53-74]	4.0 [2.4-5.4]
tryptophan	77 [51-92]	4.5 [4.1-5.9]
carnosine	26 [13-29]	23.9 [21.9-48.2]
arginine	147 [106-154]	3.0 [1.6-4.0]
hydroxyproline	36 [26-56]	33.3 [24.9-45.1]
proline	276 [238-362]	6.8 [6.6-7.2]

Stability of the assay at −20°C. Compounds excluded from the table because they were not detected in any sample: phosphoserine, phosphoethanolamine, aspartic acid, sarcosine, α-aminoadipic acid, cystathionine, β-alanine, β-aminoisobutyric acid, homocystine, γ-aminobutyric acid, hydroxylysine, ornithine, 1-methyhistidine, 3-methylhistidine, and anserine. [a]Concentrations of samples used (the median and range of the median of concentrations from four dogs across eight different timepoints of storage at −20°C). [b]Coefficient of variation, calculated from the same eight timepoints: baseline, storage at −20°C for 24 and 48 hours, and 1, 2, 3, and 4 weeks prior to deproteinization, and storage at −20°C for 1 week following deproteinization.

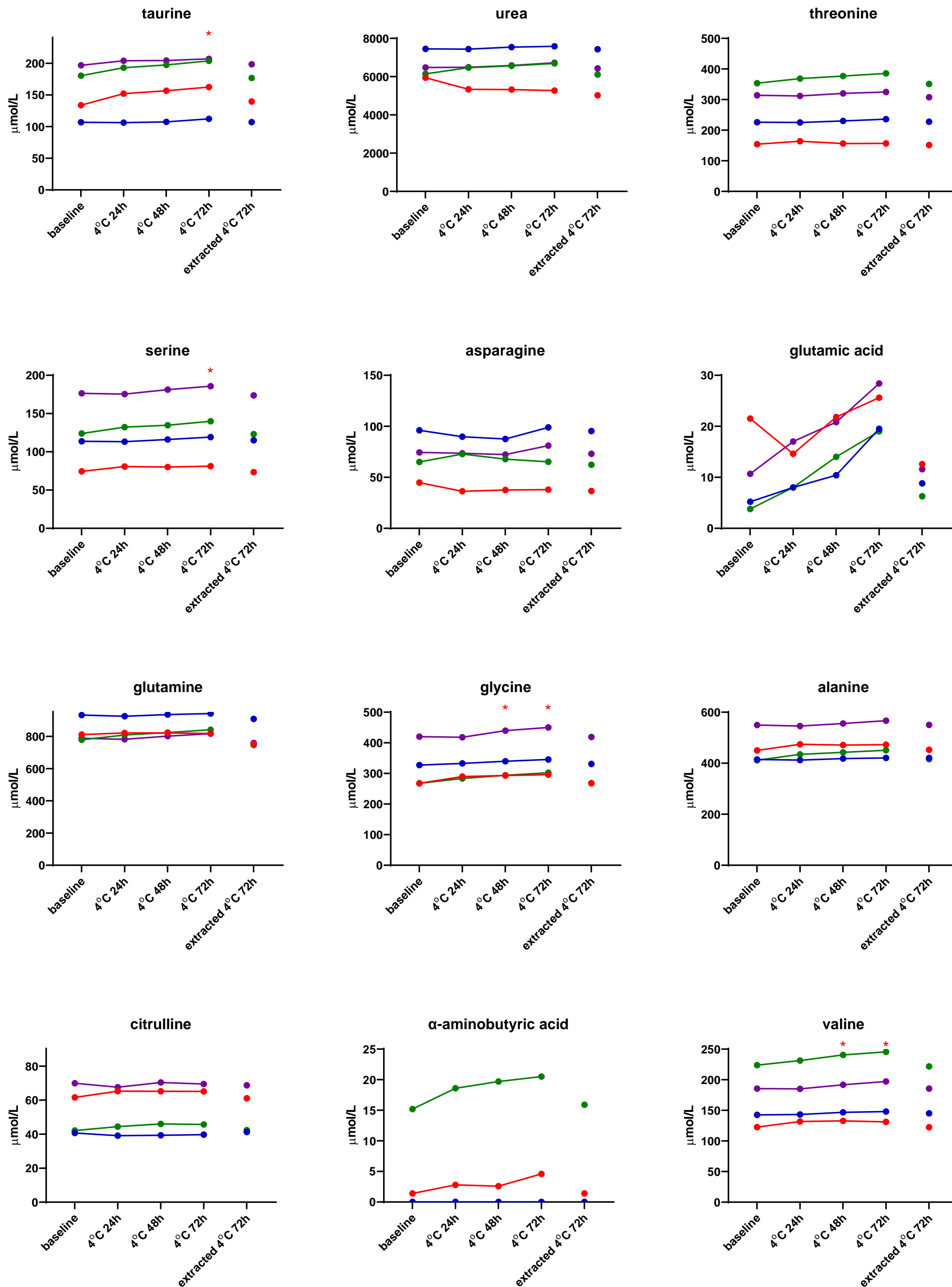

Figure A-12. Stability of amino acids in dog serum stored at 4°C. Red asterisks indicate significance ($p<0.05$) compared to baseline.

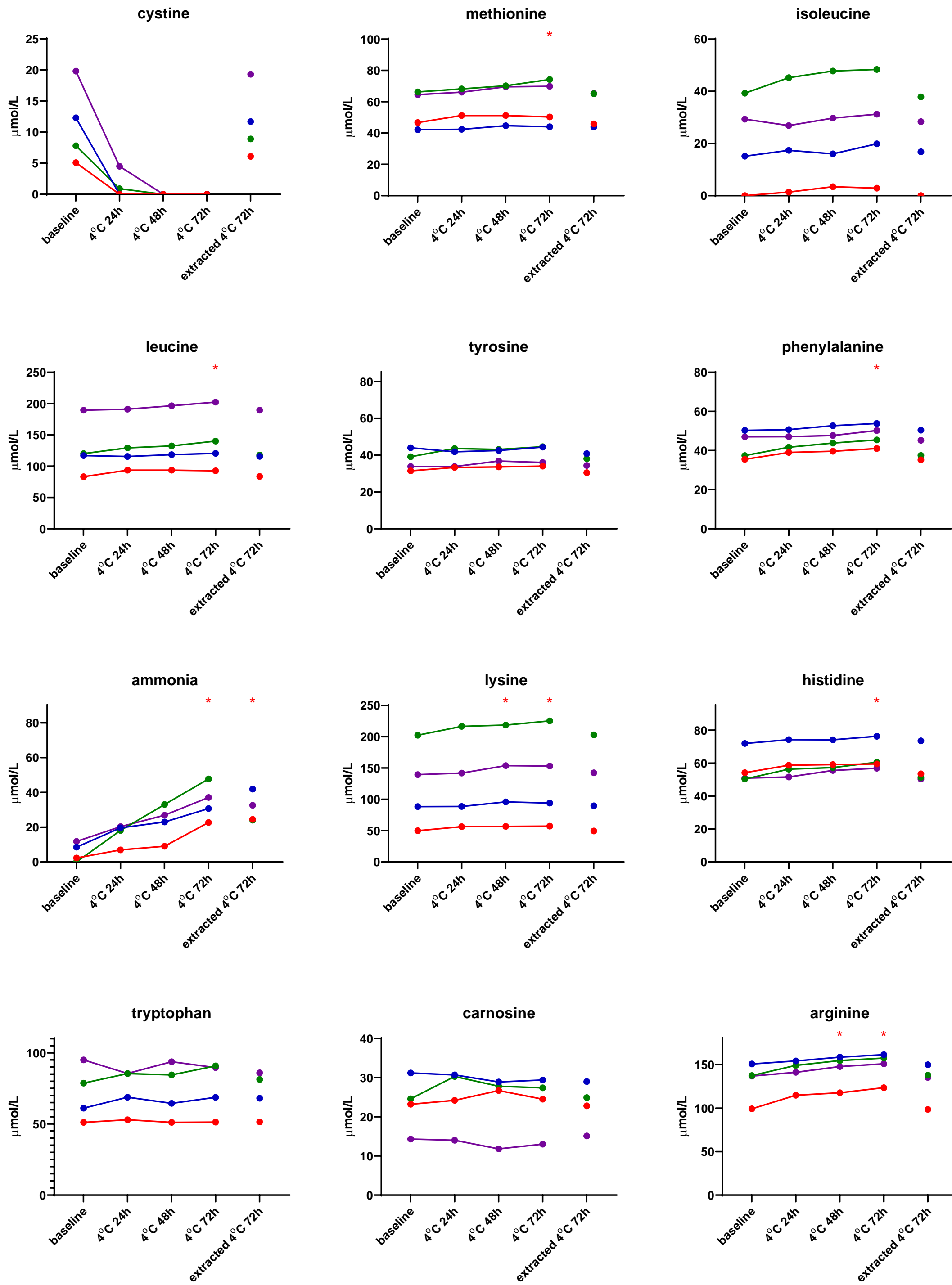

Figure A-12. Continued.

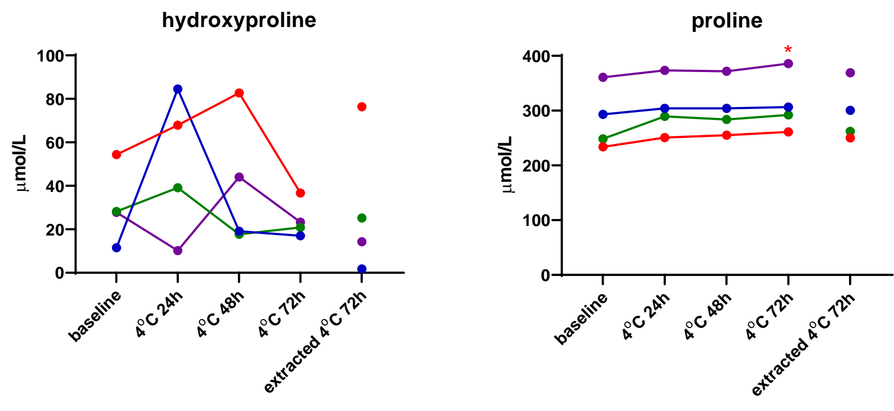

Figure A-12. Continued.

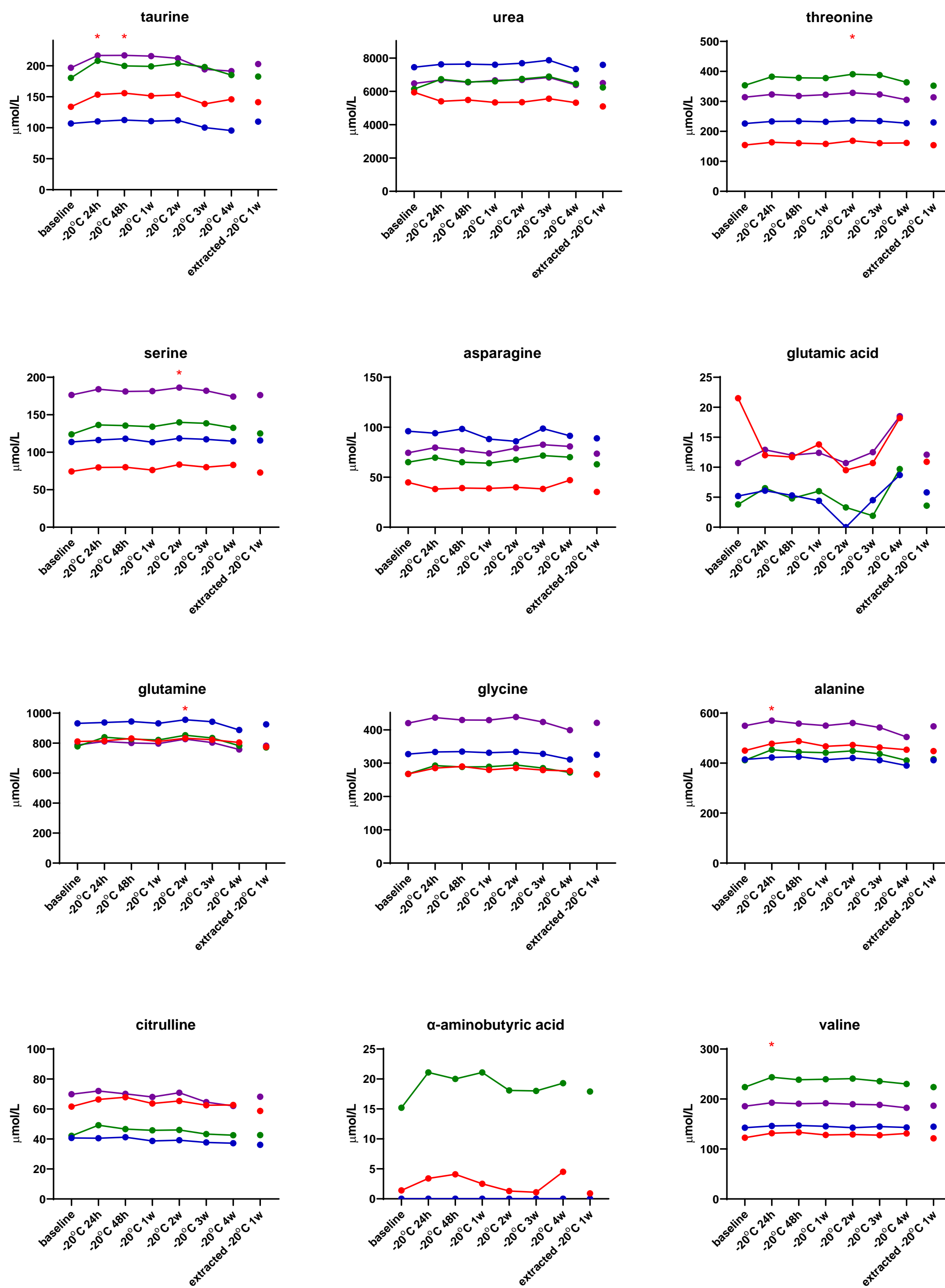

Figure A-13. Stability of amino acids in dog serum stored at −20°C. Red asterisks indicate significance ($p<0.05$) compared to baseline.

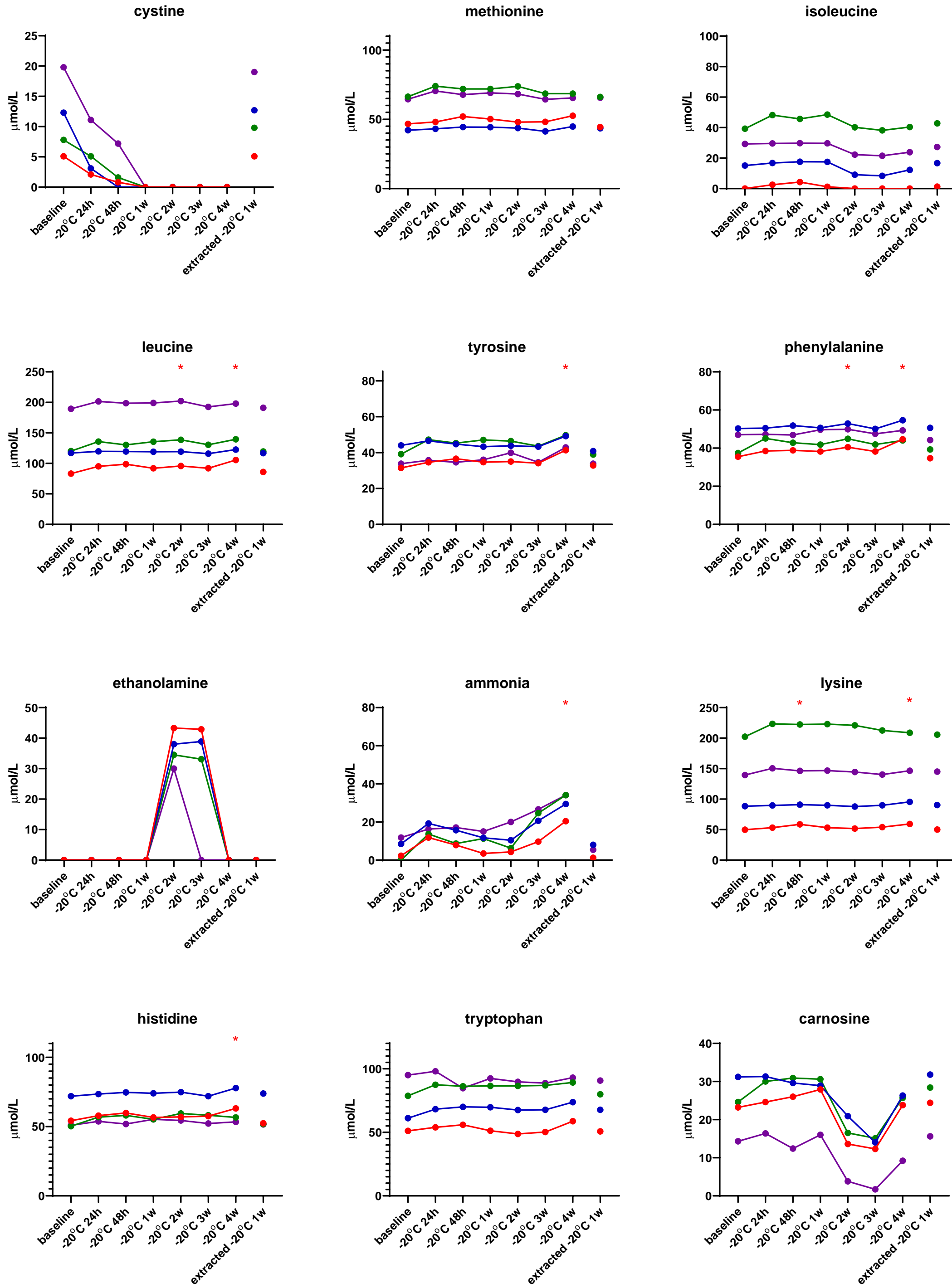

Figure A-13. Continued.

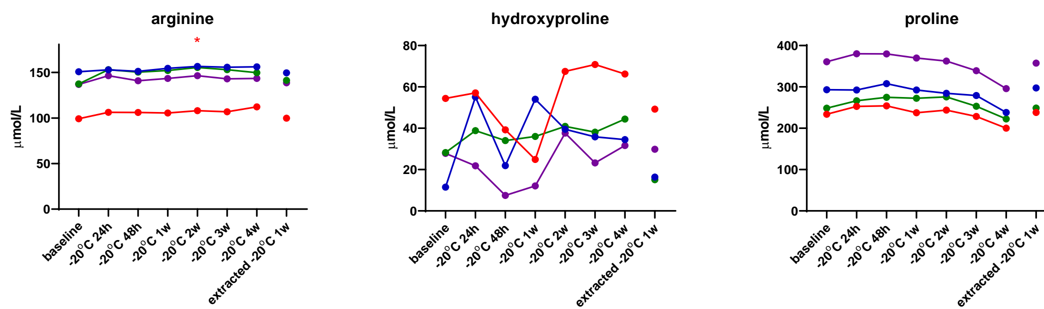

Figure A-13. Continued.

Table A-14. Spearman's correlation of serum amino acids. This table shows spearman's rank and q-values for each correlation of individual amino acid concentrations in serum to serum biochemistry panel (blue), complete blood count (orange), clinical data (body condition score, BCS; fecal score; age of the dog; green), GI panel (purple), and hemolysis score (red). Only significant (q<0.05) comparisons are shown.

Variable	by Variable	Spearman ρ	p-value	direction	q-value
Urea	BUN	0.9743	<.0001	++++++++++	2.46E-05
Gly	PHOS	0.5622	<.0001	++++++	-1.3E-05
3-Mhis	CREA	0.6795	<.0001	+++++++	-5.1E-05
Hypro	PHOS	0.5545	<.0001	++++++	-8.9E-05
Glu	age	0.5163	0.0001	+++++	-0.00013
Citr	CALA	0.5157	0.0001	+++++	-0.00016
Ile	Total WBC	0.5132	0.0001	+++++	-0.0002
Leu	CHOL	0.5113	0.0001	+++++	-0.00024
3-Mhis	CALA	0.5084	0.0001	+++++	-0.00018
Citr	ALP	0.5011	0.0002	+++++	-0.00011
Val	CHOL	0.483	0.0003	+++++	4.75E-05
Cysth	PHOS	0.4718	0.0005	+++++	0.00011
Trp	ALB	0.4619	0.0006	+++++	0.000172
Car	BUN	0.4583	0.0007	+++++	0.000234
Cysth	CALA	0.4564	0.0008	+++++	0.000297
Glu	ALT	0.4509	0.0009	+++++	0.000459
Phser	HS	0.4453	0.0011	++++	0.000521
Hypro	Cobalamin	-0.4416	0.0012	----	0.000684
Ile	CHOL	0.4358	0.0014	++++	0.000846
Asp	Folate	0.4316	0.0016	++++	0.000908
Val	Total WBC	0.4323	0.0017	++++	0.00087
Hypro	BUN	0.4297	0.0017	++++	0.000833
Hypro	ALP	0.429	0.0017	++++	0.000995
Ethan	Folate	0.4244	0.0019	++++	0.001157
Arg	PHOS	0.4213	0.0021	++++	0.00142
Asp	MCHC	-0.4193	0.0024	----	0.001582
Leu	Total WBC	0.4166	0.0026	++++	0.001644
Hypro	CHOL	0.4117	0.0027	++++	0.001706
Asp	HS	0.4104	0.0028	++++	0.001769
Ethan	ALT	0.4093	0.0029	++++	0.001831
Homocys	TPRO	0.4076	0.003	++++	0.002193
Lys	TBILI	-0.4028	0.0034	----	0.002256
Taur	RDW	0.4053	0.0035	++++	0.002218

Table A-14. Continued.

Variable	by Variable	Spearman ρ	p-value	direction	q-value
Homocys	TRIG	0.401	0.0035	++++	0.00258
Homocys	PP	0.401	0.0039	++++	0.002743
Citr	TRIG	0.3948	0.0041	++++	0.002905
Taur	PCT	0.3969	0.0043	++++	0.002967
Ethan	CALA	0.3923	0.0044	++++	0.003029
1-Mhis	PHOS	0.3919	0.0045	++++	0.002992
His	TLI	0.3911	0.0045	++++	0.003054
Gly	CREA	0.3905	0.0046	++++	0.003716
Cysth	PLI	-0.3844	0.0053	----	0.003979
3-Mhis	age	-0.3822	0.0056	----	0.004341
Aaba	HS	-0.3796	0.006	----	0.004303
1-Mhis	age	-0.3796	0.006	----	0.004265
Pro	Folate	0.38	0.006	++++	0.004528
Amm	BCS	0.4249	0.0063	++++	0.00459
Hylys	CREA	0.3769	0.0064	++++	0.004552
Arg	CALA	0.377	0.0064	++++	0.004615
Asp	TRIG	0.3761	0.0065	++++	0.005077
Met	TLI	0.3731	0.007	++++	0.005239
Taur	TRIG	0.3717	0.0072	++++	0.005202
Taur	PLT	0.4593	0.0072	+++++	0.005164
Cysth	MCHC	-0.3757	0.0072	----	0.005426
Phser	HGB	0.3738	0.0075	++++	0.005388
Glu	TRIG	0.37	0.0075	++++	0.006051
Taur	TPRO	0.3661	0.0082	++++	0.006213
Gln	BCS	0.4109	0.0084	++++	0.006775
Citr	MCHC	-0.3657	0.009	----	0.006838
Tyr	PLI	0.3619	0.0091	++++	0.0069
Homocys	age	0.3615	0.0092	++++	0.007462
Homocys	MPV	-0.362	0.0098	----	0.008324
Phe	HS	-0.3545	0.0107	----	0.008587
Gly	Cobalamin	-0.3531	0.011	----	0.008949
AAAA	TPRO	0.3516	0.0114	++++	0.009111
Hypro	CALA	0.351	0.0116	++++	0.009474
Thr	Cobalamin	-0.3494	0.012	---	0.009536
Ser	TBILI	-0.349	0.0121	---	0.009498
Citr	PP	0.3524	0.0121	++++	0.00956
Pea	ALT	-0.3487	0.0122	---	0.009523
Gly	MCHC	-0.3518	0.0122	----	0.009585

Table A-14. Continued.

Variable	by Variable	Spearman ρ	p-value	direction	q-value
AAAA	PP	0.3517	0.0123	++++	0.009947
Citr	BUN	0.3467	0.0127	+++	0.01021
Ala	TPRO	0.3457	0.013	+++	0.010172
3-Mhis	ALP	0.3457	0.013	+++	0.010334
Gln	MPV	0.3482	0.0132	+++	0.010297
Ala	ALB	0.345	0.0132	+++	0.010559
Hypro	MPV	-0.3471	0.0135	---	0.011721
Gly	CHOL	0.3399	0.0147	+++	0.011983
Pea	HS	0.3387	0.015	+++	0.012246
Car	PHOS	0.3378	0.0153	+++	0.013008
Phser	GLU	0.3354	0.0161	+++	0.01317
Phser	HCT	0.3383	0.0163	+++	0.013433
Pro	PP	0.3373	0.0166	+++	0.013595
Taur	PP	0.3367	0.0168	+++	0.014057
Ans	ALB	0.3321	0.0173	+++	0.014019
Hypro	MCHC	-0.3354	0.0173	---	0.014482
Gln	fecal score	-0.3339	0.0178	---	0.014444
Orn	age	0.3307	0.0178	+++	0.014506
Citr	CHOL	0.3302	0.0179	+++	0.014569
Glu	Folate	0.3301	0.018	+++	0.014931
Urea	CREA	0.3289	0.0184	+++	0.014893
Pro	MCHC	-0.3322	0.0184	---	0.015156
Gly	CALA	0.3282	0.0187	+++	0.015718
His	BCS	0.3684	0.0193	++++	0.01588
Cysth	PP	0.3293	0.0195	+++	0.016142
Gln	PCT	-0.3285	0.0198	---	0.016405
Ethan	ALP	0.3247	0.0201	+++	0.016867
Ans	PLT	-0.4015	0.0206	----	0.017629
Ethan	ALB	0.3215	0.0214	+++	0.017792
Thr	HGB	-0.3244	0.0216	---	0.018254
Hypro	CREA	0.3199	0.0221	+++	0.018616
Aaba	MPV	0.3222	0.0225	+++	0.018778
Aaba	PLT	-0.3955	0.0227	----	0.018741
Hypro	age	-0.3185	0.0227	---	0.019103
3-Mhis	PHOS	0.3177	0.0231	+++	0.019265
Citr	MPV	-0.3203	0.0233	---	0.019728
3-Mhis	Total WBC	0.3192	0.0238	+++	0.01989
1-Mhis	Folate	0.3157	0.024	+++	0.019952

Table A-14. Continued.

Variable	by Variable	Spearman ρ	p-value	direction	q-value
Glu	CREA	-0.3156	0.0241	---	0.020014
Homocys	BCS	0.3559	0.0242	++++	0.020277
Ile	GLU	-0.3148	0.0245	---	0.020539
Thr	PLI	0.3141	0.0248	+++	0.020901
Leu	PLI	0.3132	0.0252	+++	0.021664
Hypro	RBC	-0.3148	0.026	---	0.022026
Cys	CREA	0.3109	0.0264	+++	0.022588
Car	CREA	0.3097	0.027	+++	0.022651
Asp	PP	0.3126	0.0271	+++	0.022913
Urea	RBC	-0.312	0.0274	---	0.023175
Urea	TLI	0.3083	0.0277	+++	0.023637
Orn	TLI	0.3074	0.0282	+++	0.0237
Gly	BUN	0.3072	0.0283	+++	0.024662
Orn	TRIG	0.3053	0.0293	+++	0.024924
Pea	PHOS	0.305	0.0296	+++	0.025387
Urea	PLI	0.3041	0.0301	+++	0.025749
Pro	BCS	0.3426	0.0305	+++	0.027111
Citr	TPRO	0.301	0.0319	+++	0.027073
Pro	CHOL	0.301	0.0319	+++	0.027136
Thr	CHOL	0.3007	0.032	+++	0.027298
Orn	PLT	0.3735	0.0322	++++	0.02746
Asp	PHOS	0.3001	0.0324	+++	0.028223
Thr	HCT	-0.3017	0.0332	---	0.028385
Pea	ALP	-0.2985	0.0334	---	0.028747
3-Mhis	BUN	0.2978	0.0338	+++	0.02961
Glu	TLI	0.2965	0.0347	+++	0.029572
Amm	TLI	0.2964	0.0347	+++	0.030334
Taur	fecal score	0.2981	0.0355	+++	0.030396
Met	BCS	0.3333	0.0356	+++	0.030459
Val	PP	0.2977	0.0357	+++	0.031521
Phe	PLI	0.2932	0.0368	+++	0.032483
Leu	GLU	-0.2917	0.0378	---	0.033646
Cysth	MPV	-0.2929	0.039	---	0.034008
1-Mhis	BUN	0.2895	0.0394	+++	0.03427
Orn	TBILI	-0.289	0.0397	---	0.034532
Pea	RBC	-0.2915	0.04	---	0.034995
Ala	age	0.2878	0.0405	+++	0.035857
Taur	TLI	0.2867	0.0414	+++	0.036919

Table A-14. Continued.

Variable	by Variable	Spearman ρ	p-value	direction	q-value
Lys	AST	-0.2852	0.0425	---	0.037382
Glu	AST	0.2845	0.043	+++	0.038944
Gln	TPRO	-0.2824	0.0446	---	0.039206
Trp	age	0.2821	0.0449	+++	0.039268
Taur	Folate	0.282	0.045	+++	0.039831
Pro	TPRO	0.2813	0.0456	+++	0.040193
Car	Total WBC	0.2835	0.046	+++	0.041455
Amm	age	0.2791	0.0473	+++	0.041518
Ala	PP	0.2818	0.0474	+++	0.04228
Taur	age	0.278	0.0482	+++	0.044442
Tyr	TLI	0.2755	0.0504	+++	0.044405
3-Mhis	BCS	-0.3115	0.0504	---	0.044667
Pea	CALA	-0.2751	0.0507	---	0.044729
Gly	ALP	0.275	0.0508	+++	0.045091
3-Mhis	TBILI	0.2745	0.0512	+++	0.045654
Citr	PHOS	0.2739	0.0518	+++	0.046816
Orn	MCHC	-0.2752	0.053	---	0.047078
Ala	TLI	0.2723	0.0533	+++	0.047341
Gln	PP	-0.2747	0.0536	---	0.047603
Gly	age	-0.2715	0.0539	---	0.048065
Phe	CALA	0.271	0.0544	+++	0.048527
Asp	age	0.2704	0.0549	+++	0.04949
Gly	HGB	-0.2721	0.0559	---	0.049852

Table A-15. Spearman's correlation of plasma amino acids. This table shows spearman's rank and q-values for each correlation of individual amino acid concentrations in plasma to serum biochemistry panel (blue), complete blood count (orange), clinical data (body condition score, BCS; fecal score; age of the dog; green), GI panel (purple), and hemolysis score (red). Only significant (q<0.05) comparisons are shown.

Variable	by Variable	Spearman ρ	p-value	direction	q-value
Urea	BUN	0.9638	<.0001	++++++++++	2.5484E-05
Gly	PHOS	0.5547	<.0001	++++++	-1.1773E-05
Hylys	PHOS	0.5604	<.0001	++++++	-4.9031E-05
3-Mhis	CREA	0.6608	<.0001	+++++++	-8.6289E-05
Hypro	PHOS	0.6053	<.0001	++++++	-1.2355E-04
Citr	ALP	0.5089	0.0001	+++++	-1.6080E-04
Citr	CALA	0.5102	0.0001	+++++	-9.8063E-05
Glu	age	0.4995	0.0002	+++++	-1.3532E-04
Car	BUN	0.4955	0.0002	+++++	-7.2578E-05
Asp	HS	0.49	0.0003	+++++	-1.0984E-04
Cysth	PHOS	0.4823	0.0003	+++++	-1.4709E-04
Ile	Total WBC	0.495	0.0003	+++++	-1.8435E-04
3-Mhis	CALA	0.4893	0.0003	+++++	-1.2161E-04
Glu	ALT	0.4789	0.0004	+++++	-1.5887E-04
Ethan	CREA	0.4773	0.0004	+++++	-9.6125E-05
Val	CHOL	0.4678	0.0005	+++++	-3.3383E-05
Arg	PHOS	0.4664	0.0006	+++++	2.9359E-05
Leu	CHOL	0.4601	0.0007	+++++	9.2101E-05
Asp	Folate	0.4559	0.0008	+++++	3.5484E-04
Trp	ALB	0.4445	0.0011	++++	8.1759E-04
Gly	CREA	0.4309	0.0016	++++	7.8033E-04
Val	Total WBC	0.4354	0.0016	++++	7.4307E-04
Ethan	PLT	-0.5274	0.0016	-----	9.0581E-04
Cysth	CALA	0.4275	0.0018	++++	1.0686E-03
Hypro	CHOL	0.4225	0.002	++++	1.0313E-03
Hypro	ALP	0.4227	0.002	++++	1.3940E-03
3-Mhis	age	-0.4161	0.0024	----	1.4568E-03
Ethan	PCT	-0.4189	0.0025	----	1.5195E-03
Met	TLI	0.4124	0.0026	++++	1.4823E-03
1-Mhis	PHOS	0.4134	0.0026	++++	1.5450E-03
Amm	BCS	0.4623	0.0027	+++++	1.7077E-03
Ile	CHOL	0.4082	0.0029	++++	1.7705E-03
Leu	Total WBC	0.4112	0.003	++++	1.8332E-03

Table A-15. Continued.

Variable	by Variable	Spearman ρ	p-value	direction	q-value
Hylys	MCHC	-0.4107	0.0031	----	0.00220
Hylys	Folate	0.4015	0.0035	++++	0.00226
Homocys	MPV	-0.4041	0.0036	----	0.00232
Leu	PLI	0.3993	0.0037	++++	0.00228
Tyr	PLI	0.3988	0.0037	++++	0.00265
Ans	TBILI	0.3948	0.0041	++++	0.00261
Hypro	BUN	0.3949	0.0041	++++	0.00297
1-Mhis	age	-0.3911	0.0045	----	0.00344
AAAA	PP	0.391	0.005	++++	0.00390
Hypro	Cobalamin	-0.3832	0.0055	----	0.00406
Citr	MCHC	-0.3852	0.0057	----	0.00412
Gln	BCS	0.4282	0.0058	++++	0.00489
Ser	TBILI	-0.3754	0.0066	----	0.00485
Pro	Folate	0.3758	0.0066	++++	0.00561
Citr	TRIG	0.3707	0.0074	++++	0.00557
Lys	TBILI	-0.3708	0.0074	----	0.00624
AAAA	TPRO	0.3667	0.0081	++++	0.00650
Cysth	PLI	-0.3654	0.0084	----	0.00696
Gly	Cobalamin	-0.3625	0.0089	----	0.00773
Pea	PHOS	0.3588	0.0097	++++	0.00809
Pro	MCHC	-0.3604	0.0101	----	0.00855
Hypro	RBC	-0.3583	0.0106	----	0.00861
Ethan	age	-0.3545	0.0107	----	0.00878
Hypro	CALA	0.3536	0.0109	++++	0.00924
Aaba	PLT	-0.4352	0.0114	----	0.00960
Asp	PHOS	0.3499	0.0118	+++	0.00956
Citr	BUN	0.3499	0.0118	+++	0.00963
Gln	fecal score	-0.3532	0.0119	----	0.00959
Homocys	TRIG	0.3495	0.0119	+++	0.00995
Gln	PCT	-0.3516	0.0123	----	0.01032
Phser	GLU	0.3465	0.0127	+++	0.01028
Hypro	age	-0.3465	0.0127	---	0.01064
Thr	Cobalamin	-0.3454	0.0131	---	0.01060
Phe	PLI	0.3453	0.0131	+++	0.01057
Hypro	HGB	-0.3487	0.0131	---	0.01073
Gln	MPV	0.3479	0.0133	+++	0.01079
Thr	PLI	0.3443	0.0134	+++	0.01085
Gly	CHOL	0.3438	0.0135	+++	0.01212

Table A-15. Continued.

Variable	by Variable	Spearman ρ	p-value	direction	q-value
3-Mhis	Total WBC	0.3428	0.0148	+++	0.01248
Aaba	MPV	0.3415	0.0152	+++	0.01244
Cysth	MCHC	-0.3415	0.0152	---	0.01271
3-Mhis	ALP	0.3372	0.0155	+++	0.01387
Thr	HGB	-0.3371	0.0167	---	0.01493
Urea	CREA	0.3306	0.0178	+++	0.01489
Citr	CHOL	0.3306	0.0178	+++	0.01486
Cys	TRIG	0.3306	0.0178	+++	0.01492
Car	MCHC	-0.3335	0.0179	---	0.01508
Gly	MCHC	-0.3332	0.0181	---	0.01514
Citr	PP	0.3327	0.0182	+++	0.01521
Taur	TRIG	0.3293	0.0183	+++	0.01617
Gly	CALA	0.3267	0.0193	+++	0.01703
Phser	TLI	0.3245	0.0202	+++	0.01730
Met	PLI	0.3237	0.0205	+++	0.01786
Hylys	MPV	-0.3255	0.0211	---	0.01802
Taur	Folate	0.3218	0.0213	+++	0.01818
Cysth	PP	0.3245	0.0215	+++	0.01845
Gly	BUN	0.3206	0.0218	+++	0.01851
Asp	MCHC	-0.3235	0.0219	---	0.01857
His	TLI	0.3202	0.022	+++	0.01894
Arg	CALA	0.3193	0.0224	+++	0.01950
Urea	TLI	0.3179	0.023	+++	0.01996
AST	ALT	0.3168	0.0235	+++	0.02052
Hypro	MCHC	-0.3187	0.0241	---	0.02069
Hypro	HCT	-0.3183	0.0243	---	0.02075
Homocys	PP	0.318	0.0244	+++	0.02111
Ala	ALB	0.3141	0.0248	+++	0.02127
Orn	TBILI	-0.3138	0.025	---	0.02234
Orn	TLI	0.3115	0.0261	+++	0.02240
Ethan	MCH	0.3143	0.0262	+++	0.02246
Homocys	TPRO	0.3111	0.0263	+++	0.02293
Amm	fecal score	-0.3133	0.0268	---	0.02329
Thr	HCT	-0.3124	0.0272	---	0.02345
Car	CALA	0.3089	0.0274	+++	0.02431
Cys	CHOL	0.3072	0.0283	+++	0.02448
Car	CREA	0.3069	0.0285	+++	0.02504
Urea	RBC	-0.3089	0.0291	---	0.02500

Table A-15. Continued.

Variable	by Variable	Spearman ρ	p-value	direction	q-value
1-Mhis	Folate	0.3058	0.0291	+++	0.02526
Gln	TPRO	-0.3053	0.0294	---	0.02533
Hylys	TRIG	0.3051	0.0295	+++	0.02539
3-Mhis	BCS	-0.3443	0.0296	---	0.02555
3-Mhis	PHOS	0.3045	0.0298	+++	0.02642
Pea	GLU	-0.3029	0.0307	---	0.02638
Urea	PLI	0.3029	0.0307	+++	0.02644
Homocys	BCS	0.3419	0.0308	+++	0.02640
Ethan	TBILI	0.3028	0.0308	+++	0.02657
Pro	BCS	0.3415	0.031	+++	0.02683
Pro	PP	0.3049	0.0313	+++	0.02769
Aaba	HS	-0.3004	0.0322	---	0.02945
Gln	PP	-0.3004	0.034	---	0.02952
His	BCS	0.336	0.0341	+++	0.03088
Car	PHOS	0.2952	0.0355	+++	0.03144
Phser	PLI	0.2943	0.0361	+++	0.03261
Ser	Total WBC	0.2954	0.0373	+++	0.03267
Ser	Cobalamin	-0.2922	0.0374	---	0.03363
Pro	CHOL	0.2909	0.0384	+++	0.03369
Hypro	CREA	0.2906	0.0385	+++	0.03376
Citr	PHOS	0.2905	0.0386	+++	0.03402
Val	PLI	0.2901	0.0389	+++	0.03428
Ala	TPRO	0.2897	0.0392	+++	0.03424
Orn	PHOS	0.2897	0.0392	+++	0.03421
1-Mhis	BUN	0.2897	0.0392	+++	0.03427
Hypro	MPV	-0.2924	0.0393	---	0.03543
Pro	PHOS	0.288	0.0405	+++	0.03640
Citr	MPV	-0.2894	0.0415	---	0.03796
Pea	fecal score	0.2873	0.0431	+++	0.03802
Orn	MCHC	-0.2872	0.0432	---	0.03828
Glu	HS	0.2839	0.0435	+++	0.03855
Gly	age	-0.2835	0.0438	---	0.03871
Phe	HS	-0.2833	0.044	---	0.03897
Thr	CHOL	0.2829	0.0443	+++	0.04173
Gly	ALP	0.2794	0.0471	+++	0.04210
Glu	TRIG	0.2789	0.0475	+++	0.04246
Hylys	ALP	0.2785	0.0479	+++	0.04272
B-ala	BCS	0.3144	0.0482	+++	0.04289

Table A-15. Continued.

Variable	by Variable	Spearman ρ	p-value	direction	q-value
Hylys	RBC	-0.2806	0.0484	---	0.04345
Citr	TPRO	0.2771	0.049	+++	0.04381
Thr	PHOS	0.2767	0.0494	+++	0.04377
Ile	PLI	0.2767	0.0494	+++	0.04394
3-Mhis	GGT	-0.2764	0.0496	---	0.04400
Pea	ALT	-0.2763	0.0497	---	0.04516
Homocys	MCHC	-0.2776	0.0509	---	0.04553
3-Mhis	PCT	-0.2773	0.0513	---	0.04559
Ans	PLT	-0.3419	0.0514	---	0.04585
3-Mhis	TBILI	0.274	0.0517	+++	0.04671
Amm	MPV	0.2757	0.0526	+++	0.04778
Ethan	MCV	0.2745	0.0537	+++	0.04834
Phser	Cobalamin	0.2711	0.0543	+++	0.04830
1-Mhis	MCHC	-0.2739	0.0543	---	0.04836
Phser	HGB	0.2738	0.0544	+++	0.04953
Arg	age	-0.2697	0.0556	---	0.04999
Asp	TRIG	0.2692	0.0561	+++	0.04995

Table A-16. Spearman's correlation of serum and fecal amino acid concentrations with clinical activity index (CCECAI) and histopathological scores of stomach, small intestine (SI) and colonic biopsies. Amino acid three letter codes preceded with an 'f' denote fecal amino acid. Only those comparisons that reached the level of significance prior to FDR correction are shown.

Variable	by Variable	Spearman ρ	P-value	Q-value
CCECAI	SI score	0.712	0.0003	0.063
SI score	Met	0.698	0.001	0.063
Val	CCECAI	0.474	0.003	0.181
colon score	Citr	0.617	0.004	0.192
Ala	CCECAI	0.450	0.005	0.192
stomach score	Met	0.584	0.007	0.237
stomach score	Pro	0.524	0.018	0.517
colon score	Asp	0.512	0.021	0.517
Ile	CCECAI	0.368	0.023	0.517
CCECAI	fASN	-0.463	0.026	0.517
SI score	Val	0.493	0.027	0.517
stomach score	Ala	0.486	0.030	0.523
SI score	fMET	-0.522	0.038	0.595
colon score	Amm	0.460	0.042	0.595
SI score	fLEU	-0.509	0.044	0.595
Hypro	CCECAI	-0.320	0.050	0.595

Table A-17. Instrument parameters for acquisition and target transitions.

Compound	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
Tyramine	138.1	77.1	28.1	30
	138.1	103	21.6	30
	138.1	121.1	10.2	30
Acetylcholine	146.4	58	46.5	39
	146.4	60	10.2	39
	146.4	87	12.9	39
Serotonin	177.1	115	28.2	30
	177.1	117	28.1	30
	177.1	160	10.2	30
Tryptamine	161.2	117.1	25	30
	161.2	128.9	10.2	30
	161.2	144	10.2	30
Tryptophan	205.4	118	25.1	36
	205.4	146	16.9	36
	205.4	188.1	10.2	36
L-Tryptophan (Indole D5)‡‡	210.3	122.1	27.6	36
	210.3	150	18.7	36
	210.3	192.1	10.2	36
Anthranilic acid	138.1	64.9	28.8	32
	138.1	92	21	32
	138.1	120	10.2	32
15N Anthranillic Acid‡‡	139.1	65	30.1	30
	139.1	93.1	22.3	30
	139.1	121	10.2	30
Indole-3-acetamide	175.1	77	42.9	39
	175.1	103	33.7	39
	175.1	130	14.4	39
Indole-3-lactic acid	206.1	118.1	21.6	49
	206.1	130	31.1	49
	206.1	188.1	10.2	49
Indole-3-acetic acid	176.2	130	16.1	47
	176.2	144	10.2	47
	176.2	158	10.2	47
Indole-3-acetic acid 2-2-d2‡	178.1	78	42.6	44
	178.1	105.1	32.1	44
	178.1	132	16.1	44

Table A-17. Continued.

Compound	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)
Indole-3-acetaldehyde	160.1	118.1	12.9	45
	160.1	130	29.9	45
	160.1	132	14.6	45
Indole-3-carboxaldehyde	146.1	65	39.2	47
	146.1	91	25.9	47
	146.1	118	14.9	47
2-C13 Indole[‡‡]	119.1	65	33.2	66
	119.1	89	43.2	66
	119.1	91	23.3	66
Indole	118.1	65	33.2	61
	118.1	89	40.9	61
	118.1	91	22.7	61

[‡]compound used as internal standard
[‡‡]compounds used as external standards for retention time verification and standard curves