FECAL LACTATE CONCENTRATIONS IN DOGS WITH GASTROINTESTINAL DISEASE

A Thesis

by

AMANDA BELLE BLAKE

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Chair of Committee,	Jan Suchodolski
Committee Members,	Jörg Steiner
	Jonathan Lidbury
	Aline Rodrigues Hoffmann
Head of Department,	Jonathan Levine

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ABSTRACT

Lactate concentrations in the blood or serum are currently used as prognostic indicators of certain diseases in human and veterinary medicine. Lactate concentrations in the feces are of interest because it is a metabolic product of fermentation by the intestinal microbiota. However, no cost effective method to quantify the D- and L-isoforms of lactate in canine feces is currently available. Therefore, the main objectives of this study were to modify and validate an enzymatic assay for the quantification of D-, L-, and total lactate in canine feces, and to characterize fecal lactate concentrations and bacterial abundances in healthy dogs and dogs with gastrointestinal diseases.

The enzymatic assay was validated with surplus homogenized fecal samples by determination of dilutional parallelism, spiking recovery, and intra- and inter-assay variability. Fecal samples were collected from healthy dogs (n=34), dogs with acute hemorrhagic diarrhea (AHD; n=20), dogs with chronic enteropathy (CE; n=15), and dogs with exocrine pancreatic insufficiency (EPI; n=34). Fecal lactate was measured with the new enzymatic assay and 11 bacterial groups were quantified with qPCR.

A canine fecal lactate reference interval was established from 34 healthy dogs and was 0.7-1.4 mM, 0.3-6.0 mM, and 1.0-7.0 mM for D-, L-, and total lactate, respectively. The assay for measurement of D-, L-, and total lactate in canine fecal samples was linear, accurate, precise, and reproducible. Significant increases in fecal lactate concentrations were observed in dogs with acute hemorrhagic diarrhea, dogs with chronic enteropathy (D-lactate only), and dogs with exocrine pancreatic insufficiency.

Blautia spp. and *Clostridium hiranonis* abundances were decreased in all diseased groups of dogs compared to healthy dogs. Dogs with EPI that were receiving enzyme replacement therapy had an increased abundance of *Lactobacillus* spp. and *Bifidobacterium* spp., and all dogs with EPI had an increased Dysbiosis Index compared to healthy dogs.

In conclusion, further studies are necessary to determine the clinical utility of lactate quantification in canine feces. Though lactate by itself may not be a good indicator of dysbiosis, bacterial metabolites together with bacterial abundances are promising targets for further elucidating the role of the microbiota in health and disease.

DEDICATION

Dedicated to my parents, Greg and Cindy Blake.

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NOMENCLATURE

AHD	acute hemorrhagic diarrhea
ATP	adenosine triphosphate
CE	chronic enteropathy
cTLI	canine trypsin-like immunoreactivity
EPI	exocrine pancreatic insufficiency
GC-MS	gas chromatography coupled to mass spectrometry
GI	gastrointestinal
GSH	glutathione
HPLC	high-pressure liquid chromatography
IBD	inflammatory bowel disease
LAB	lactic acid bacteria
LDH	lactate dehydrogenase
LLOD	lower limit of detection
LLOQ	lower limit of quantification
MCT	monocarboxylic acid transporter
MG	methylglyoxal
MM	master mix
NAD	nicotinamide adenine dinucleotide
NADH	reduced form of NAD
OE%	observed-to-expected ratio

qPCR	quantitative polymerase chain reaction
SBS	short bowel syndrome
SCFAs	short chain fatty acids
SMCT	Na^+-coupled monocarboxylic acid transporters
%CV	coefficient of variation

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1. INTRODUCTION

1.1 Lactate Production, Absorption, Clearance

Lactate is present in the body in two isoforms: D- and L-lactate. L-lactate is produced from pyruvate via lactate dehydrogenase (LDH) primarily during anaerobic glycolysis or intense exercise, when cell respiration alone cannot keep up with demands for nicotinamide adenine dinucleotide (NAD). Glycolysis is the first step in glucose metabolism and produces pyruvate, which, under aerobic conditions, is then transferred into the mitochondria. Here pyruvate undergoes oxidative decarboxylation and enters the Krebs Cycle producing approximately 30 molecules of adenosine triphosphate (ATP) per molecule of glucose (Berg et al., 2002). However, in anaerobic conditions, pyruvate cannot undergo oxidative decarboxylation and, therefore, needs to be converted to lactate in order to continue production of ATP. Production of lactate generates NAD, which is then recycled for use in more glycolysis reactions ending with a net production of only two ATP molecules per molecule of glucose (Figure 1). Some cells that lack mitochondria, such as erythrocytes, use anaerobic glycolysis as their main source of energy (De Backer, 2003). Although all cells will produce L-lactate, the majority of production in humans is attributable to skeletal muscle, erythrocytes, brain cells, and the renal medulla (Fall and Szerlip, 2005).

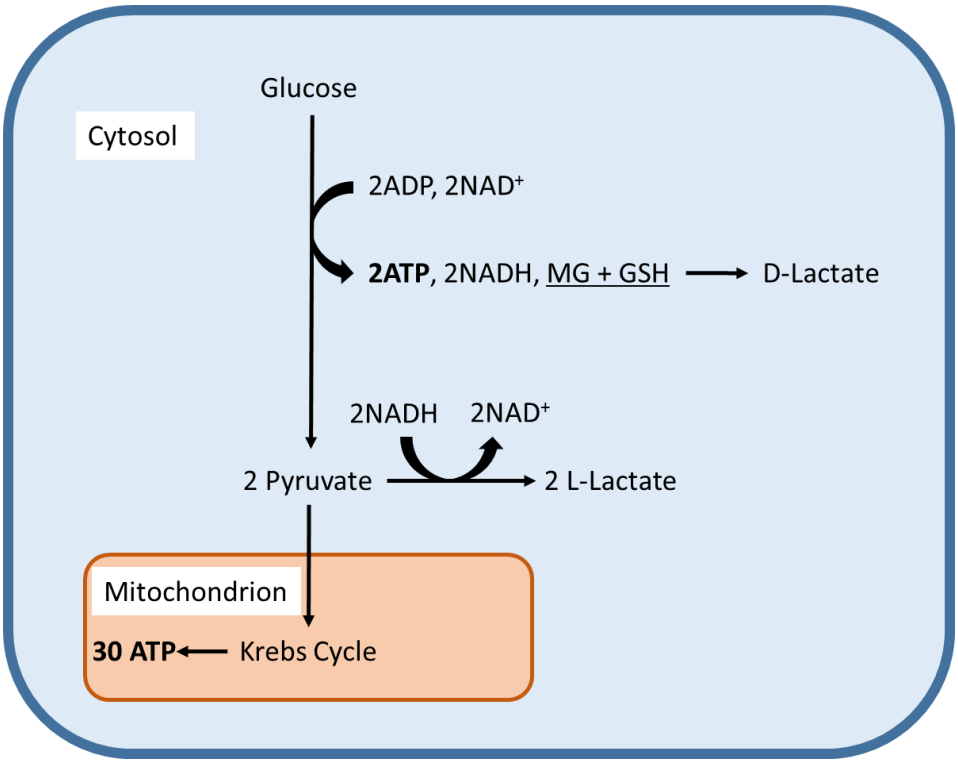

Figure 1. D- and L-lactate formation in the cell. Pyruvate is formed from glycolysis reactions and subsequently converted to L-lactate under anaerobic conditions. Methylglyoxal (MG) is formed by fragmentation of intermediates in the glycolysis pathway, and glutathione (GSH) is formed in conjunction with intermediates entering the pentose phosphate pathway (Allaman et al., 2015). GSH works with glyoxalase I and II enzymes to detoxify MG into D-lactate.

D-lactate is produced in even smaller amounts by the host via methylglyoxal (MG) metabolism (Figure 1). MG is present in all cells and is a by-product of glucose, protein, and fatty-acid metabolism (Allaman et al., 2015). It is highly reactive with nucleic acids and proteins and, therefore, needs to be degraded to protect cell integrity (Thornalley, 1996). MG is also a potent glycating agent that can react with protein residues to form advanced glycation end products (Bélanger et al., 2011). The glyoxalase

system uses the enzymes glyoxalase I and II to detoxify MG into D-lactate (Thornalley, 1993).

The intestinal microbiota is the collection of microorganisms that reside within the gastrointestinal (GI) tract and interact with each other and with host cells. Many of these microorganisms, specifically bacteria, have fermentative capabilities that allow them to produce organic acids that are then absorbed and utilized by the host. Bacterial groups cumulatively referred to as lactic acid bacteria (LAB) also produce lactate within the host GI tract. LAB are defined as bacteria that form lactate as a predominant product of carbohydrate fermentation (Liu, 2003). The lactate isomer produced depends on the genus, species, and sometimes the strain of bacteria (Table 1). The genus *Bifidobacterium* also produce lactate but are not considered LAB because they produce lactate and acetate in a 2:3 ratio (Stiles and Holzapfel, 1997).

Another source of D- and L-lactate in the GI tract is from dietary intake of fermented foods, such as yogurt and sauerkraut (Uribarri et al., 1998). Lactate-producing bacteria are often used in these foods as well as probiotics because they can have beneficial properties in the gut, such as lowering luminal pH and acting against pathogenic bacteria (Gilliland, 1990; Swanson et al., 2002a). Nutritional studies often aim to increase lactate concentrations or LAB abundance within the GI tract by supplementing fiber in the diet. Fructooligosaccharide supplementation in dogs produced minor increases in fecal lactate and LAB populations and improved some indicators of intestinal immune function (Swanson et al., 2002a; Swanson et al., 2002b). However, results from human studies contradicted these findings, showing that resistant starch

Table 1. Lactate isomers produced by bacteria in the gastrointestinal tract.

D-lactate	Both	L-lactate	Reference
Leuconostoc, *Oenococcus*	*Lactobacillus*, *Pediococcus*, *Weissella*	*Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactococcus*, *Tetragenococcus*, *Streptococcus*, *Vagococcus*	Liu, 2003
Leuconostoc	*Lactobacillus*, *Pediococcus* (except *L.dextrinicus*)	*Bifidobacterium*, *Lactococcus*, *Enterococcus*	Stiles and Holzapfel, 1997
	Enterococcus faecalis (primarily D-lactate), *Streptococcus sanguinis* (primarily D-lactate), *Escherichia coli* (primarily L-lactate)		Sheedy et al., 2009

supplementation and LAB probiotics failed to increase fecal lactate concentrations (Phillips et al., 1995; Matsumoto and Benno, 2004). Lowering of intestinal pH may have a larger role in the effectiveness of these dietary supplements, but Phillips et al. (1995) suggested that increased production of short chain fatty acids (SCFAs) and not lactate was responsible for this acidification. Furthermore, Edwards et al. (1985) found that changes in pH modified metabolic activities of bacteria without changing the bacterial population. Studies by Jiang and Savaiano (1997) showed that, in an acidic environment (pH 6.2), more SCFAs were produced, and there was a significant reduction in the amount of D-lactate. It was long thought that humans do not readily metabolize D-

lactate, which led to the World Health Organization recommending a limited intake of the D-lactate isomer (Jehanno et al., 1992). Consequently, attempts have been made to decrease the proportion of D-lactate producing organisms in fermented foods and L-lactate producers have become favorable (Liu, 2003). However, experiments by de Vrese et al. (1990) showed that long-term ingestion of D-lactate did not produce an accumulation of lactate in the body.

Under normal conditions, the intestinal microbiota has the capacity to further metabolize D- and L-lactate into other SCFAs that can be beneficial to the host (Halperin and Kamel, 1996). Human fecal microbiota cultures have been shown to convert D- and L-lactate to butyrate, acetate and propionate (Duncan et al., 2004; Bourriaud et al., 2005; Morrison et al., 2006; Belenguer et al., 2007). Specific bacterial groups implicated in these processes include *Veillonella parvula* and *Propionibacterium freudenreichii* (Seeliger et al., 2002), which convert lactate to acetate and propionate, and *Megasphaera elsdenii* (Counotte et al., 1981; Hashizume et al., 2003), which converts lactate to butyrate. More recently, Duncan et al. (2004) identified strains of *Eubacterium hallii* and *Anaerostipes caccae* as well as a new species within the *Clostridium* cluster XIVa that utilize lactate to form butyrate as an end product. Although the intestinal microbiota can lower lactate concentrations in the GI tract by utilizing it, they generally do not metabolize all of it. Healthy humans have a fecal lactate concentration of less than 3 mmol/L (Duncan et al., 2007), indicating that any lactate unused by bacteria is either absorbed by the host, or excreted in the feces.

Multiple studies in cows (Preston and Noller, 1974; Wolffram et al., 1988), sheep (Ding and Xu, 2003), and rats (Ogihara et al., 2000) have shown that lactate is absorbed through the intestinal epithelium. There are three mechanisms for lactate absorption or transport across cell membranes: carrier-mediated transport by monocarboxylic acid transporter (MCT), exchange with inorganic anions, and passive diffusion (Poole and Halestrap, 1993; Ding and Xu, 2003; Allen and Holm, 2008). Absorption is somewhat concentration-dependent suggesting the presence of saturable and nonsaturable mechanisms (Ogihara et al., 2000). Tamai et al. (1995) demonstrated the presence of H^+-coupled monocarboxylate transporter MCT1 in rat intestinal epithelial cells and that the transporter had a higher affinity for L-lactate with an uptake coefficient twice that of D-lactate. D- and L-lactate uptake by MCT1 is inhibited by acetate, propionate, butyrate, benzoic acid, nicotinic acid, pravastatin, and valproic acid (Wolffram et al., 1988; Tamai et al., 1995). Furthermore, monocarboxylic acid uptake by MCT1 increases in acidic pH, and uptake of L-lactate is stereoselectively inhibited by ibuprofen, a monocarboxylic drug (Tamai et al., 1995; Tachikawa et al., 2011).

A second class of MCTs that contribute to the absorption of lactate from the intestine are Na^+-coupled monocarboxylic acid transporters (SMCT). SMCT1 (SLC5A8) is present on the luminal surface of intestinal epithelial cells and facilitates absorption of SCFAs and lactate (Poole and Halestrap, 1993; Ganapathy et al., 2008). Similar to the H^+-coupled MCT1, SMCT1 exhibits a higher affinity for L-lactate compared to D-lactate (Miyauchi et al., 2004; Martin et al., 2006). Ibuprofen and other non-steroidal anti-

inflammatory drugs also block uptake of other substrates by SMCT1 (Ganapathy et al., 2008).

Lactate produced by host cells through anaerobic glycolysis and methylglyoxal metabolism, and lactate absorbed from the intestine, ends up in the blood. Several physiological mechanisms are present in the host that regulate the amount of lactate in the blood to prevent accumulation and disturbance of acid-base homeostasis. The liver and kidneys are the main organs responsible for clearing lactate from the blood and are able to clear 50-70% and 20-30%, respectively, of blood lactate (Yudkin and Cohen, 1975; Madias, 1986; Pang and Boysen, 2007; Allen and Holm, 2008; Vernon and LeTourneau, 2010). One of the ways these organs clear lactate is by metabolizing it to pyruvate and glucose. The enzymes implicated in this process are L-lactate dehydrogenase, D-lactate dehydrogenase, and d-2-hydroxyacid-dehydrogenase, all of which have been isolated from mammalian liver and kidney (Tubbs, 1965; Cammack, 1969; de Bari et al., 2002; Flick and Konieczny, 2002; Ewaschuk et al., 2005).

L-lactate dehydrogenase, like L-lactate, is more abundant than D-lactate dehydrogenase, and it is widespread in multiple tissue types, including skeletal muscle (Flick and Konieczny, 2002). It was historically thought that mammals lacked the ability to efficiently metabolize D-lactate. Then studies in the 1960s described d-2-hydroxyacid-dehydrogenase in the liver and kidney of mammals that metabolizes D-lactate to pyruvate, albeit at a much slower rate than L-lactate dehydrogenase metabolizes L-lactate (Tubbs, 1965; Cammack, 1969). Experiments by de Vrese et al. (1990) confirmed presence and activity of d-2-hydroxyacid-dehydrogenase in humans.

In the early 2000s, D-lactate dehydrogenase was isolated in rodents and humans (de Bari et al., 2002; Flick and Konieczny, 2002). It is localized mainly within mitochondria of the liver and kidney but is also present in mitochondria of other tissues as well (de Bari et al., 2002; Flick and Konieczny, 2002). Using rat liver mitochondria, de Bari et al. (2002) identified three translocators that move D-lactate across the mitochondrial membrane: the D-lactate/H^+ symporter, and the D-lactate/oxoacid or malate antiporters. Once D-lactate is in the mitochondria, it is oxidized into pyruvate, which can then either enter the Krebs cycle to produce ATP or be converted into glucose via gluconeogenesis. Different types of tissue oxidize D- and L-lactate at different rates. Studies of bovine tissues revealed that rates of D- and L-lactate oxidation were greatest in the kidney, followed by heart, liver, and muscle tissue (Harmon et al., 1984). Brandt et al. (1984) used radiolabeled lactate in rat tissues and found that brain and kidney tissue oxidized L-lactate more efficiently than D-lactate, whereas the opposite was true in liver tissue.

Na^+-coupled MCTs are also present in the kidney epithelia, where they actively reabsorb lactate (Ganapathy et al., 2008). L-lactate is reabsorbed in the proximal convoluted tubule more readily than D-lactate and the isomers exhibit mutual interference (Oh et al., 1985; Halperin and Kamel, 1996). Passive diffusion along a lactate concentration gradient created by removal of lactate by oxidation within the kidney cells might contribute to reabsorption (Hohmann et al., 1974).

Aside from metabolism of lactate by oxidation, the kidneys also contribute to lactate clearance through excretion in the urine. Estimates of lactate elimination through renal excretion vary from less than 2% to almost 20% with excretion of D-lactate being

greater than excretion of L-lactate (Connor et al., 1983; Oh et al., 1985; de Vrese et al., 1990). However, renal excretion increases with increased blood lactate concentration and in metabolic acidosis (Harmon et al., 1984; Allen and Holm, 2008), which could be the reason behind some of the variation noted above. Some of the variation may be attributable to species differences as well. Giesecke et al. (1981) suggested that rats and rabbits may differ in their renal threshold values for excretion of D-lactate, and Oh et al. (2010) compared his data in humans to that of dogs and suggested that humans do not reabsorb D-lactate in the kidneys as efficiently as dogs.

1.2 Blood Lactate Concentrations

Normal lactate concentration in whole blood of healthy humans is less than 2 mmol/L with the L- isomer comprising about 98% (Huckabee, 1961; Allen and Holm, 2008; Vernon and LeTourneau, 2010). D-lactate is present in plasma of healthy adults only in small amounts ranging from 0.01 to 0.07 mmol/L (Brandt et al., 1980; de Vrese and Barth, 1991; McLellan et al., 1992). This is possibly due to the kidney preferentially excreting D-lactate and preferentially reabsorbing L-lactate (Oh et al., 1985). Similar to humans, plasma lactate concentrations of dogs and cats are generally less than 2 mmol/L (Pang and Boysen, 2007). In healthy adult dogs, plasma lactate ranges from 0.3 to 3.6 mmol/L, but there is a slightly higher range in puppies indicating the need to be cautious when interpreting values from younger animals (Evans, 1987; Hughes et al., 1999; McMichael et al., 2005). Hughes et al. (1999) also found that small differences in plasma lactate concentrations can be caused by different sample collection sites and

repeated sampling. Any differences due to these variables can be avoided by using the same procedure in a clinical setting as was used to obtain the reference interval. Though small, these differences should be kept in mind when comparing data between studies that used different sample collection or handling techniques. Rand et al. (2002) also found that struggling in cats can increase plasma lactate concentrations by up to ten-fold. This should be considered when interpreting clinical data against the resting reference interval.

When there is a malfunction of one or more of the systems involved in lactate production or clearance, a condition called lactic acidosis can occur. Lactic acidosis is defined as having a blood lactate concentration ≥ 5 mmol/L associated with a decrease in blood pH, whereas hyperlactatemia occurs without change in blood pH and an increase to only 2 to 5 mmol/L blood lactate (Mizock and Falk, 1992). Uribarri et al. (1998) defined D-lactic acidosis specifically as having metabolic acidosis accompanied by ≥ 3 mmol/L serum D-lactate. L-lactic acidosis is typically caused by tissue hypoxia or underlying diseases, such as sepsis, liver disease, diabetes mellitus, or respiratory failure (Ewaschuk et al., 2005; Sharkey and Wellman, 2015). L-lactic acidosis is more common in humans and animals than D-lactic acidosis, and therefore this isoform is more often measured than D-lactate in the clinical setting (Ewaschuk et al., 2005; Sharkey and Wellman, 2015).

In cases of D-lactic acidosis, the excess lactate originates from bacterial production of this isoform in the intestinal lumen. Unlike L-lactic acidosis, D-lactic acidosis is characterized by episodes of encephalopathy that are often worsened in

conjunction with food intake (Uribarri et al., 1998; Ewaschuk et al., 2005). Furthermore, D-lactic acidosis has been reported in diseases associated with alterations in the intestinal microbiota, such as short bowel syndrome (SBS) in humans (Kowlgi and Chhabra, 2015), diarrhea in calves (Lorenz, 2004), and exocrine pancreatic insufficiency (EPI) in a cat (Packer et al., 2005). D-lactic acidosis has also been tentatively associated with antibiotic (Coronado et al., 1995) and probiotic use (Munakata et al., 2010) in human patients with SBS. However, these were single case reports and more comprehensive studies are needed to examine the association between antibiotic and probiotic use and acidosis. Serial measurements of whole blood or plasma lactate concentrations in critically ill dogs are used to predict patient outcome, determine the severity of disease, and assess treatment response (Nel et al., 2004; Mooney et al., 2014; Cortellini et al., 2015; Sharkey and Wellman, 2015; Eichenberger et al., 2016). Though plasma or blood lactate concentration is useful in a clinical setting, we can gain a better understanding of the role of lactate in the GI tract by measuring it closer to the source, in the feces.

1.3 Microbiota and Lactate in Health and Disease

There is mounting evidence of the relationship between the intestinal microbiota, the metabolites it produces (such as lactate), and health and disease (Blake and Suchodolski, 2016). In humans, it has been implicated that certain metabolites produced by the intestinal microbiota may influence host metabolism (Morrison and Preston, 2016). Methods to characterize the intestinal microbiota have evolved from traditional

bacterial culture methods to high-throughput sequencing of the entire metagenome. However, quantitative polymerase chain reaction (qPCR) is often employed to measure abundance of specific bacterial groups. An extensive review of the role of the intestinal microbiota in dogs and cats is available elsewhere (Blake and Suchodolski, 2016).

With lactate being a major metabolite of bacterial origin, examining the lactate concentrations in the feces should provide us with an idea of disturbances to the GI microbiota. Lactate does not usually accumulate in the GI tract in a healthy state, as denoted by a fecal lactate concentration in healthy humans of 0 to 3 mmol/L (Duncan et al., 2007). However, in a disease state, fecal lactate can become increased (Bustos et al., 1994; Sato and Koiwa, 2008; Mayeur et al., 2013). There are several theories why lactate accumulates in the GI tract. Ewaschuk et al. (2005) describes the process in SBS as a series of events: poor carbohydrate digestion results in sugars being delivered to the colon, where the pH is decreased by organic acid production through fermentation, finally resulting in acid-resistant *Lactobacillus* spp. growing preferentially. In vitro studies by Belenguer et al. (2007) point to a different reason for lactate accumulation; their work suggests that once the intraluminal pH decreases past a certain point, lactate production will be maintained but lactate utilization will decrease. Regardless of why the accumulation occurs, it is of interest to determine the metabolic and ecological consequences of excess lactate in the intestinal lumen. This is important to gain a better understanding of the role that the microbiota plays in the various disease processes involving lactate accumulation.

As mentioned previously, increased serum lactate is associated with D-lactic acidosis in maldigestive disease processes such as SBS in humans (Kowlgi and Chhabra, 2015), diarrhea in calves (Lorenz, 2004), and EPI in a cat (Packer et al., 2005). Interestingly, in human patients with SBS, fecal D-lactate concentrations are increased even when serum lactate concentrations are normal (Bustos et al., 1994). Another study by Hove and Mortensen (1995) showed that when large amounts of lactulose were fed to healthy individuals, fecal D-lactate increased to 13.6 mmol/ L while plasma and urine lactate concentrations remained the same. Together, these studies suggest that other mechanisms are involved in the development of acidosis, such as increased absorption or impaired metabolism of D-lactate. As the focus lies more on changes within the GI tract during disease, our focus should then be on lactate concentrations in the feces.

To obtain a more comprehensive understanding of the role of lactate in GI disease, it may be useful to examine fecal lactate concentrations in a variety of diseases that have different characteristics. Profound alterations to the intestinal microbiome have been identified in dogs with acute hemorrhagic diarrhea (AHD), including decreased *Lactobacillus, Faecalibacterium, Turicibacter*, and *Streptococcus*, and increased *Escherichia coli* and *Clostridium perfringens* (Markel et al., 2012; Suchodolski et al., 2012). Unterer et al. (2014) endoscopically visualized mucosal lesions in the intestines of dogs with AHD and isolated *C. perfringens* on the small intestinal mucosa of two thirds of cases. Dogs with chronic enteropathies (CE), such as inflammatory bowel disease, also had alterations to their intestinal microbiome, including decreases in *Faecalibacterium* spp. and Fusobacteria, during times of clinically active disease

(Suchodolski et al., 2012). Preliminary studies using untargeted fecal metabolomics showed an abundance of fecal lactate in dogs with CE (Honneffer et al., 2015). However, further studies are warranted to determine the clinical utility of fecal lactate concentrations as a marker of dysbiosis. Exocrine pancreatic insufficiency (EPI), much like other diseases characterized by maldigestion (i.e., SBS), causes changes to the intestinal microbiota. Simpson et al. (1990) reported increased *Lactobacillus* and *Streptococcus* in the duodenum of dogs with EPI before dogs underwent treatment with pancreatic enzyme replacement. Similarly, Westermarck et al. (1993) reported increased total counts of bacteria in the small intestine of dogs with EPI, regardless of whether they had received therapy. The authors also noted that oral administration of the antibiotic tylosin decreased these counts to normal levels.

1.4 Measuring Lactate Concentrations

Many of the methods used to measure lactate involve enzymes and are dependent upon the following reaction (Rosenberg and Rush, 1966):

$$\text{Lactate} + \text{NAD}^+ \xleftrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH} + \text{H}^+$$

The production of NADH, or the reduced form of NAD, can be measured by spectrophotometry at 340nm wavelength (Olson, 1962). However, the reaction favors production of lactate. Therefore, a pyruvate trapping reaction will allow it to proceed in the forward direction and the amount of NADH formed will be directly proportional to the amount of lactate in the original sample (Goodall and Byers, 1978). When measuring lactate enzymatically in biological samples, it is necessary to deproteinize the samples

first to remove potential competitors for NAD^+, other enzymes present in the sample, and stabilize the lactate molecules (Goodall and Byers, 1978). Both isomers of lactate can be measured by utilizing stereospecific forms of LDH, D-LDH and L-LDH (Brandt et al., 1980).

Because L-lactate was shown to be useful clinically early on, methods to measure it in a clinical setting evolved. Allen et al. (2008) summarizes the clinical utility of lactate in veterinary patients, usually utilized as a prognostic indicator in varying diseases from colic to gastric dilatation volvulus. Most hand-held blood lactate analyzers used today are based on enzymatic amperometry and measure L-lactate only (Pang and Boysen, 2007). The principle behind amperometry is this: lactate oxidase coats an electrode and reacts with lactate generating hydrogen peroxide, which then generates an electrical current that is proportional to the amount of lactate in the sample (Pang and Boysen, 2007; Allen and Holm, 2008). This method is convenient for use in the clinical setting and can generate results within minutes (Allen and Holm, 2008). However, many of the hand-held lactate analyzers are ill suited to modification for use in other biological samples due to their optimization for use with whole blood. For instance, the Lactate Scout Plus by EKF Diagnostics makes automatic compensations for hematocrit and measures lactate concentrations from 0.5-25 mmol/L (Lactate Scout+, SensLab GmbH, Leipzig, Germany).

Another way of measuring lactate concentrations involves the use of chromatography, such as gas chromatography coupled to mass spectrometry (GC-MS) or high-pressure liquid chromatography (HPLC). Both of these methods require the use of

expensive laboratory equipment that is often available only to reference laboratories. GC-MS techniques generally cannot differentiate between D- and L-lactate isomers, unless they are multidimensional or coupled to dual mass spectrometers (Heil et al., 1998). HPLC can be used for stereospecific measurements of lactate (Omole et al., 1999), but it is more expensive and has a longer turnaround time than GC-MS. Both GC-MS and HPLC methods have been used to measure lactate in biological samples such as urine and serum (Hoffmann et al., 1989; Heil et al., 1998; Omole et al., 1999; Allen and Holm, 2008; Packer et al., 2012). However, their limited use with feces is somewhat due to the inherent molecular contamination within feces that requires more steps in sample preparation and can damage the column.

Enzymatic spectrophotometry assays are best suited for use with measuring lactate in feces. Many studies have utilized these methods previously in human feces (Bustos et al., 1994; Mayeur et al., 2013), cow feces (Shimomura and Sato, 2006; Sato and Koiwa, 2008), and murine feces (Rul et al., 2011). Swanson et al. (2002a) measured lactate concentrations in canine feces using the spectrophotometric method described by Barker and Summerson (1941). However, this method is not able to distinguish D- and L-lactate.

1.5 Hypothesis and Specific Objectives

Based on the information presented in the literature, our hypothesis is that fecal lactate concentrations are increased in dogs with GI disease. Therefore, the aims of the present study were: 1) to modify and validate an enzymatic assay for the quantification

of fecal D-, L-, and total lactate in canine feces; 2) to characterize fecal lactate concentrations in healthy dogs and dogs with various gastrointestinal diseases; and 3) to quantify abundance of lactic acid bacteria in feces by qPCR and to compare to fecal lactate concentrations and dysbiosis in dogs with GI disease.

2. MATERIALS AND METHODS

2.1 Samples

Healthy control dogs (n=34) had no signs of GI disease. Fecal lactate concentrations from all dogs were used to calculate the healthy reference interval. However, due to limited amount of feces, bacterial abundances were quantified in 18 dogs. These dogs (n=18) were used as the control group for comparisons of lactate and bacterial abundances between healthy and diseased dogs.

Dogs with acute hemorrhagic diarrhea (AHD; n=20) underwent diagnostic workup to exclude other causes of disease. Acute was defined as duration of diarrhea less than three days. Feces were collected before any treatment was started.

Dogs with chronic enteropathy (CE; n=15) had GI signs for more than 3 weeks and had histopathologic findings consistent with CE. Feces were collected prior to endoscopy and bowel cleanse.

Dogs with exocrine pancreatic insufficiency (EPI; n=34) were diagnosed by a serum trypsin-like immunoreactivity (cTLI) of less than 2.5 µg/L. Dogs were separated into two groups; those that were currently receiving pancreatic enzyme replacement therapy (treated EPI, n=29) and those that had not yet received therapy (untreated EPI, n=5). Duration of enzyme replacement therapy was obtained for a subset of the dogs treated for EPI (n=12).

All dogs included in our analysis were client owned, and all feces collected were naturally voided and so Institutional Animal Care and Use Committee approval was not

needed. Dogs were at least one year of age and had not received antibiotics for at least three weeks prior to sample collection. All fecal samples were received frozen or cool on dry ice or ice packs. Once received at the laboratory, the feces were either immediately aliquoted then stored at -80°C (treated EPI samples; n=12), or stored at -80°C for aliquoting at a later date (all other samples). Please refer to Table 2 below for collection information and shipping conditions on the various samples.

Table 2. Fecal sample collection details.

Group and Number	Collection	Storage and Shipping Conditions
healthy (n=34)	single TP (n=16), 3 consecutive bowel movements, pooled (n=18)	-80°C, ice packs overnight shipping
AHD (n=20)	single TP	-80°C, dry ice
CE (n=15)	single TP	-80°C, dry ice and ice packs overnight shipping
treated EPI (n=29)	single TP (n=12), 3 consecutive bowel movements, pooled (n=17)	-80°C, ice packs, overnight shipping
untreated EPI (n=5)	3 consecutive bowel movements, pooled	-80°C, ice packs, overnight shipping

TP = time point, AHD = acute hemorrhagic diarrhea, CE = chronic enteropathy, treated EPI = dogs with exocrine pancreatic insufficiency receiving enzyme replacement therapy, untreated EPI = dogs with exocrine pancreatic insufficiency not receiving enzyme replacement therapy

2.2 Materials

The following materials were used:

6N Trichloroacetic acid Sigma-Aldrich, St. Louis, MO, USA

D-/L-lactate Enzymatic Kit	R-Biopharm Inc., Washington, MO, USA
PowerSoil® DNA Isolation Kit	MOBIO Laboratories, Inc., Carlsbad, CA, USA
SsoFast™ EvaGreen® Supermix	Bio-Rad Laboratories, CA, USA
Triethanolamine	Sigma-Aldrich, St. Louis, MO, USA

2.2.1 Instruments and Machines

The following instruments and machines were used:

AutoRep E pipette	Rainin, Woburn, MA, USA
Centrifuge 5424R	Eppendorf AG, Hamburg, Germany
Centrifuge rotor FA-45-24-11	Eppendorf AG, Hamburg, Germany
Centrifuge, Galaxy Mini	VWR, West Chester, PA, USA
epBlue v10.09.0000 software	Eppendorf AG, Hamburg, Germany
epMotion 5075 Vacuum TMX	Eppendorf AG, Hamburg, Germany
Gen5 2.07 plate reader software	BioTek® Instruments, Inc., Winooski, VT, USA
Lyophilizer - FreeZone 2.5Plus	LABCONCO Corporation, Kansas City, MO, USA
Nanodrop 1000 software v3.8.1	Thermo Scientific, Rockford, IL, USA
pH-meter - Orion Star A211	Thermo Scientific, Rockford, IL, USA
Pipetman® P-2, P-20, P-100, P-200, P-1000	Rainin, Woburn, MA, USA
Pipette E4XLS P-20, P-100, P-300, P-1000	Rainin, Woburn, MA, USA
Plate incubator - Stat Fax - 2200	Awareness Technology Inc., Palm City, FL, USA
PURELAB® Ultra Water Purification System, ELGA LabWater	VWR, West Chester, PA, USA
qPCR thermal cycler - CFX96TM	Bio-Rad Laboratories, CA, USA
Scale - Voyager Pro	Ohaus, Parsippany, NJ, USA
Scale - XS6002S	Mettler Toledo, Columbus, OH, USA
Shaker - Micromix 5	DPC Cirrus Inc., Flanders, NJ, USA
Spectrophotometer, Nanodrop 1000	Thermo Scientific, Rockford, IL, USA
Standard Stirrer	VWR, West Chester, PA, USA

Statistical software package Prism 5.0	GraphPad software Inc., San Diego, CA, USA
Synergy2 multi-mode plate reader	BioTek® Instruments, Inc., Winooski, VT, USA
Vortex mixer	Fisher Scientific, Pittsburg, PA, USA

2.2.2 Disposable Materials

The following disposable materials were used:

BioClean™ pipette tips in Green-Pak™ (20, 1000 µL)	Rainin, Woburn, MA, USA
Blue Max™ Jr., polypropylene tube, 15 mL	Falcon, Franklin Lakes, NJ, USA
Costar® Microcentrifuge tube (1.7 mL)	Corning Inc., Corning, NY, USA
Innoculation loops w/ needle, sterile, 10 µL, Globe Scientific	Fisher Scientific, Hampton, NH, USA
Magnetic micro-stir bar	VWR, West Chester, PA, USA
Magnetic stir bar	VWR, West Chester, PA, USA
Microcentrifuge tube, seal-rite® (1.5 mL)	USA Scientific, Orlando, FL, USA
Microcentrifuge tube, seal-rite® (2.0 mL)	USA Scientific, Orlando, FL, USA
Microplates, 96-well, PS, F-bottom, clear	Greiner Bio-One GmbH, Germany
Microseal® B Adhesive Sealer	Bio-Rad Laboratories, CA, USA
Multiplate® PCR Plates™, 96-well, clear, unskirted	Bio-Rad Laboratories, CA, USA
Parafilm®	VWR, West Chester, PA, USA
Rainin® pipette tips (10, 250, 1000 µL)	Rainin, Woburn, MA, USA
Screw cap Micro tube, 2 mL	Sarstedt, Sarstedtstraße, Germany
Slef-standing centrifugal polypropylene tubes, 50 mL	Corning Inc., Corning, NY, USA

2.3 Quantification of Fecal Lactate

2.3.1 Deproteinization of Feces

Frozen samples were thawed at room temperature for aliquoting. An aliquot of 125 ± 5 mg feces was made for each fecal sample and placed into 1.5 mL microcentrifuge tubes. Aliquots were then stored at -80°C until deproteinization. Deproteinization of fecal samples was achieved by using the protocol of Rul et al. (2011) with modifications. Briefly, 750 µL of 0.1 M triethanolamine buffer (pH 9.15) was added to each fecal aliquot. The tubes were vortexed and placed in the refrigerator (~4°C) for three hours, vortexing every hour to ensure thorough mixing. The samples were then centrifuged at 13,000 x g for 5 minutes at 4°C. Next, 495 µL of the supernatant was carefully pipetted off into a new 1.5 mL microcentrifuge tube. These supernate aliquots were stored at -80°C overnight to provide ample time for the next processing steps. From empirical experience, the extra freeze-thaw cycles favorably increased protein pellet size while not affecting lactate concentrations. The next morning, samples were thawed at room temperature for 20-40 minutes. Once thawed, 10 µL of 6 M trichloroacetic acid was added to each sample. The samples were vortexed for 10 seconds and placed in an ice bath for 20 minutes. Then samples were vortexed for a few seconds and centrifuged at 4,500 x g for 20 minutes at 4°C. After centrifugation, a protein pellet was noted in the bottom of each tube. Next, 400 µL of supernatant was pipetted off into a 2 mL microcentrifuge tube and 1600 µL 0.1 M triethanolamine buffer (pH 9.15) added to achieve a neutral or alkaline pH (between 7 and 10). These

deproteinized fecal extracts were either used immediately for lactate analysis or stored in -80°C for later use.

2.3.2 Spectrophotometric Analysis

Ultraviolet spectrophotometric analysis of fecal lactate was performed using a commercially available enzymatic kit (D-/L-Lactate Enzymatic Kit, R-Biopharm Inc.) with modifications to the manufacturer protocol for use with a 96-well plate format. The concentrations of D- and L-lactate were determined by measuring the sequential formation of NADH by the increase in absorption at 340 nm wavelength following addition of stereospecific D- and L-LDH (Supplemental material; manufacturer protocol for D-/L-lactic acid kit). The protocol was modified for use with a 96-well plate format by dividing all volumes by a factor of ten. Standard dilutions were made for D- and L-lactate by the addition of ultra-pure water (PURELAB® Ultra Water Purification System, ELGA LabWater; Table 3). Master mix (MM) solution was made on an as needed basis depending on the number of samples and consisted of solution 1 (100 µL for each sample), 2 (20 µL for each sample), and 3 (2 µL for each sample) mixed in a 15 or 50 mL tube (Table 4). Blanks, standards, and samples were pipetted in duplicate onto a 96-well plate (Table 5) and placed on a plate shaker for one minute. After a 15-minute incubation at 25°C, the first absorbance (A1) was read at 340 nm wavelength. Then 2 µL D-LDH was added to each well, shook for one minute, and incubated at 25°C for 30 minutes. The second absorbance (A2) reading was taken and then 2 µL L-LDH added to each well, shook and incubated at 25°C for 30 minutes. The third absorbance (A3)

Table 3. Standard dilutions of D- and L-lactate.

Standard	Dilution	Concentration (g/L)
1	1:4	0.05175
2	1:10	0.0207
3	1:20	0.01035
4	1:40	0.005175
5	1:80	0.0025875

Stock concentration = 0.207 g/L

Table 4. Description of solutions included in enzymatic kit.

Solution Number	Description*
1	approx. 30 ml solution, consisting of: glycylglycine buffer, pH approx. 10.0; L-glutamic acid, approx. 440 mg
2	approx. 210 mg NAD, lyophilizate, reconstituted in 6 ml redist. Water
3	approx. 0.7 ml glutamate-pyruvate transaminase suspension, approx. 1100 U
4	approx. 0.7 ml D-lactate dehydrogenase solution, approx. 3800 U
5	approx. 0.7 ml L-lactate dehydrogenase solution, approx. 3800 U

*Descriptions obtained from manufacturer protocol

Table 5. Samples and reagent volumes.

Well type	Volumes
blanks	122 µL MM, 100 µL water
standards (D- and L-lactate)	122 µL MM, 100 µL standards 1-5
unknown samples	122 µL MM, 100 µL fecal extract

MM = master mix

reading was taken and then data processing was performed. A path length adjustment setting was implemented on the plate reader software (Gen5 v. 2.07, BioTek® Instruments, Inc.) to account for using a protocol originally designed for use with 1 cm diameter cuvettes. Standard curve and lactate concentrations were calculated. The standard curve was set to be quadratic and lactate concentrations were adjusted by subtracting the absorbance difference of the blank. Then, if D- or L-lactate concentrations were below their respective lower limits of quantification (g/L), concentrations were adjusted to 0.002 g/L for D-lactate or 0.0007 g/L for L-lactate. Final lactate concentrations were also adjusted based on starting weight of the feces, dilution factor, and dry matter content (Supplemental material; Excel file with interactive formulas and manufacturer protocol for L-lactic acid kit).

2.4 Analytical Validation of the Assay for Measurement of Fecal Lactate

Surplus homogenized canine fecal samples from seven dogs were used for analytical validation. Validation variables tested were lower limit of detection, lower limit of quantification, dilutional parallelism, spiking recovery, and intra- and inter-assay variability. Deproteinized fecal extract stability was evaluated by measuring D- and L-lactate in seven sample extracts at baseline, 24 hours of storage at 4°C, and 28 days of storage at -80°C. The reference intervals for canine fecal lactate concentrations were calculated from the central 95^{th} percentile from 34 healthy dogs. These dogs were determined to be healthy based on evaluations of the owner questionnaires. Dogs had no signs of disease and were at least 1 year of age.

Lower limit of detection (LLOD) and lower limit of quantification (LLOQ) were assessed by measuring ten duplicates of the blank and calculating the mean and standard deviation of the absorbance differences, A2-A1 (D-lactate) and A3-A2 (L-lactate). The analytical sensitivity (S) at the lower end of the standard curve was calculated by:

$$S = \frac{\Delta \text{ concentration}}{\Delta \text{ intensity}}$$

where Δ concentration = (concentration standard 4) – (concentration standard 5) = 0.00259 g/L, and Δ intensity = (ΔA standard 4- ΔA standard 5)-ΔA_{blank}. (ΔA standards calculated from average of 8 runs). Next LLOD and LLOQ were calculated by the following equations:

$$LLOD = ks_{bl}S$$

$$LLOQ = ks_{bl}S$$

where k is chosen based on desired level of confidence (k=3 for LLOD and k=10 for LLOQ) and s_{bl} is the standard deviation of the blank.

Assay linearity was evaluated by assessing dilutional parallelism for seven different fecal samples at dilutions of 1, 1:2, 1:4, 1:10, 1:20, 1:40, and 1:80 for each sample. Dilutions were performed by the addition of ultra-pure water (PURELAB® Ultra Water Purification System, ELGA LabWater). The accuracy of the assay was measured by mixing previously quantified extracts of four samples in a 1:1 ratio. The percentage of lactate recovery was calculated as the observed-to-expected ratio (OE%):

$$OE\% = \left[\frac{\text{observed value (mM)}}{\text{expected value (mM)}}\right] \times 100$$

To evaluate precision of the assay, four different fecal samples that spanned the working range of the assay were analyzed 8 times within the same assay run on one single plate. The intra-assay coefficient of variation (%CV) was calculated as:

$$\%\text{CV} = \left(\frac{\text{standard deviation}}{\text{mean}}\right) \times 100$$

The reproducibility of the assay was evaluated by analyzing seven different fecal samples in 8 separate assay runs on different days, followed by calculation of inter-assay %CVs. All fecal validation samples were run in duplicate and at dilutions that allowed them to fall within the working range of the assay.

2.5 Quantification of Bacterial Groups in Feces

A 600-1200 mg aliquot of feces was lyophilized for each sample and dry matter weights were obtained. DNA was extracted from the lyophilized fecal samples with a commercially available kit (PowerSoil® DNA Isolation Kit, MOBIO Laboratories, Inc., Carlsbad, CA, USA) and DNA concentration measured (Spectrophotometer, Nanodrop 1000, Thermo Scientific, Rockford, IL, USA). DNA was normalized for concentration on a 96-well plate using a pipetting robot (epMotion 5075 Vacuum TMX, Eppendorf AG, Hamburg, Germany). Separate real-time quantitative polymerase chain reaction (qPCR) assays were used to amplify and quantify DNA from eleven different bacterial groups (Universal, *Faecalibacterium* spp., *Turicibacter* spp., *Streptococcus* spp., *Escherichia coli*, *Blautia* spp., *Fusobacterium* spp., *Clostridium hiranonis*, *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp.) using protocols and primers described in Table 6. SYBR-based reaction mixtures (total 10 µl) contained 5 µl

SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, CA, USA), 2.2 µl water, 0.4 µl of each primer (final concentration: 400 nM), and 2 µl of normalized DNA (final concentration: 5 ng/µl). A melt curve analysis was performed after the amplification cycles as follows: increments of 0.5°C from 65°C to 95°C for 5 seconds each. Samples were analyzed in duplicate fashion, and a commercially available qPCR thermal cycler (CFX96TM, Bio-Rad Laboratories, CA, USA) was used for all qPCR assays.

The abundance of bacterial DNA for each bacterial group was compared between diseased groups and healthy controls. Additionally, microbiota data was expressed as a previously described Dysbiosis Index (AlShawaqfew et al., 2016; Suchodolski, 2016), where a Dysbiosis Index below zero is indicative of a healthy microbiota.

Table 6. Primers and cycling conditions used in qPCRs.

Target	Primer sequences (5' - 3')	Initial denaturing temp(°C), time	# of cycles	Denaturing temp(°C), time	Annealing temp(°C), time	Reference
Universal	F-CCTACGGGAGGCAGCAGT R-ATTACCGCGGCTGCTGG	98, 2 min	35	98, 5 sec	59, 5 sec	Lubbs et al., 2009
Faecalibacterium spp.	F-GAAGGCGGCCTACTGGGCAC R-GTGCAGGCGAGTTGCAGCCT	98, 2 min	40	98, 5 sec	60, 5 sec	Garcia-Mazcorro et al., 2012
Turicibacter spp.	F-CAGACGGGGACAACGATTGGA R-TACGCATCGTCGCCTTGGTA	98, 2 min	40	98, 3 sec	57, 3 sec	Suchodolski et al., 2012
Streptococcus spp.	F-TTATTTGAAAGGGGCAATTGCT R-GTGAACTTTCCACTCTCACAC	95, 2 min	40	95, 5 sec	54, 10 sec	Furet et al., 2004
Escherichia coli	F-GTTAATACCTTTGCTCATTGA R-ACCAGGGTATCTAATCCTGTT	98, 2 min	40	98, 3 sec	55, 3 sec	Malinen et al., 2005
Blautia spp.	F-TCTGATGTGAAAGGCTGGGGCTTA R-GGCTTAGCCACCCGACACCTA	98, 2 min	40	98, 4 sec	56, 4 sec	Suchodolski et al., 2012
Fusobacterium spp.	F-KGGGCTCAACMCMGTATTGCGT R-TCGCGTTAGCTTGGGCGCTG	98, 2 min	40	98, 4 sec	50.5, 4 sec	Suchodolski et al., 2012
Clostridium hiranonis	F-AGTAAGCTCCTGATACTGTCT R-AGGGAAAGAGGAGATTAGTCC	95, 3 min	40	95, 30 sec	59, 5 sec	Kitahara et al., 2001
Lactobacillus spp.	F-AGCAGTAGGGAATCTTCCA* R-CACCGCTACACATGGAG**	95, 2 min	40	95, 5 sec	58, 10 sec	Malinen et al., 2005
Bifidobacterium spp.	F-TCGCGTCYGGTGTGAAAG R-CCACATCCAGCRTCCAC	98, 2 min	40	98, 3 sec	60, 3 sec	Rinttila et al., 2004
Enterococcus spp.	F-CCCTTATTGTTAGTTGCCATCATT R-ACTCGTTGTACTTCCCATTGT	98, 3 min	40	98, 3 sec	61, 3 sec	Malinen et al., 2005

*Originally described by Walter et al., 2001. **Originally described by Heilig et al., 2002

2.6 Statistical Analysis of Results

All statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, La Jolla, California, USA) or JMP® Pro 12.2.0 (64-bit, SAS Institute Inc.). Shapiro-Wilk test for normality was used to determine nonparametric distribution of data. Kruskal-Wallis tests were used to evaluate differences in variables between diseased and healthy groups. For post hoc analysis, we chose to use Steel-Dwass test for multiple comparisons with control (healthy group) test to independently compare ranks of each group to healthy dogs. Spearman's rank correlation analysis was performed on the subset of dogs with EPI for which duration of enzyme therapy was known (n=12). Fisher's exact test was used to compare proportions of dogs with a Dysbiosis index above zero and below zero.

3. RESULTS

3.1 Analytical Validation of Assay for Measurement of Fecal Lactate

The lower limit of detection for D- and L-lactate concentrations were 0.0006 and 0.0002 g/L, respectively. The lower limit of quantification for D- and L-lactate concentrations were 0.0021 and 0.0008 g/L, respectively. Observed-to-expected ratios for dilutional parallelism ranged from 92% to 111% for D-lactate, 89% to 109% for L-lactate, and 88% to 104% for total lactate (Table 7). Recovery for spiking sample extracts ranged from 96% to 103% for D-lactate, 96% to 119% for L-lactate, and 98% to 113% for total lactate (Table 8). Average intra-assay coefficients of variation for D-, L-, and total lactate were 5%, 5%, and 4%, respectively (Table 9). Average inter-assay %CVs for D-, L-, and total lactate were 24%, 20%, and 19%, respectively (Table 10). Fecal lactate was stable in deproteinized fecal extracts for 24 hours of storage at 4°C (average %CV: 9, 4, 4, for D-, L-, and total lactate, respectively; Table 11) and 28 days of storage at -80°C (average %CV: 4, 4, 3, for D-, L-, and total lactate, respectively; Table 12). Canine fecal lactate reference interval was 0.7-1.4 mM, 0.3-6.0 mM, and 1.0-7.0 mM for D-, L-, and total lactate, respectively.

Table 7. Dilutional parallelism of seven fecal samples. Observed-to-expected ratios are in bold and the minimum, maximum, mean, and standard deviation of those observed-to-expected ratios are provided in the box at the end of the table.

	D-lactate			L-lactate			total lactate		
Sample 1	O	E	**OE%**	O	E	**OE%**	O	E	**OE%**
S1	HIGH			HIGH					
S1 1:2	HIGH	N/A	N/A	HIGH	N/A	N/A	N/A	N/A	N/A
S1 1:4	0.047	N/A	N/A	HIGH	N/A	N/A	N/A	N/A	N/A
S1 1:10	0.021	0.019	**112**	HIGH	N/A	N/A	N/A	N/A	N/A
S1 1:20	0.011	0.009	**112**	0.033	N/A	N/A	0.043	N/A	N/A
S1 1:40	0.005	0.005	**102**	0.014	0.016	**87**	0.019	0.022	**88**
S1 1:80	LOW	0.002	N/A	0.007	0.008	**91**	N/A	N/A	N/A
Sample 2									
S2	HIGH			HIGH					
S2 1:2	HIGH	N/A	N/A	HIGH	N/A	N/A	N/A	N/A	N/A
S2 1:4	0.030	N/A	N/A	HIGH	N/A	N/A	N/A	N/A	N/A
S2 1:10	0.013	0.012	**106**	HIGH	N/A	N/A	N/A	N/A	N/A
S2 1:20	0.005	0.006	**89**	0.028	N/A	N/A	0.033	N/A	N/A
S2 1:40	0.003	0.003	**92**	0.014	0.014	**104**	0.017	0.017	**104**
S2 1:80	LOW	0.002	N/A	0.007	0.007	**100**	N/A	N/A	N/A
Sample 3									
S3	HIGH			HIGH					
S3 1:2	0.033	N/A	N/A	HIGH	N/A	N/A	N/A	N/A	N/A
S3 1:4	0.017	0.016	**103**	HIGH	N/A	N/A	N/A	N/A	N/A
S3 1:10	0.007	0.007	**107**	0.032	N/A	N/A	0.039	N/A	N/A
S3 1:20	0.003	0.003	**93**	0.017	0.016	**105**	0.020	0.020	**102**
S3 1:40	LOW	0.002	N/A	0.009	0.008	**109**	N/A	N/A	N/A
S3 1:80	LOW	0.001	N/A	0.005	0.004	**112**	N/A	N/A	N/A
Sample 4									
S4	HIGH			HIGH					
S4 1:2	0.048	N/A	N/A	HIGH	N/A	N/A	N/A	N/A	N/A
S4 1:4	0.028	0.024	**117**	HIGH	N/A	N/A	N/A	N/A	N/A
S4 1:10	0.011	0.010	**117**	0.028	N/A	N/A	0.039	N/A	N/A
S4 1:20	0.005	0.005	**99**	0.013	0.014	**96**	0.018	0.019	**93**
S4 1:40	LOW	0.002	N/A	0.007	0.007	**102**	N/A	N/A	N/A
S4 1:80	LOW	0.001	N/A	0.003	0.003	**101**	N/A	N/A	N/A

Table 7 Continued

	D-lactate			L-lactate			total lactate		
Sample 5	O	E	**OE%**	O	E	**OE%**	O	E	**OE%**
S5	0.044			HIGH					
S5 1:2	0.020	0.022	**89**	0.044	N/A	N/A	0.064	N/A	N/A
S5 1:4	0.011	0.011	**96**	0.020	0.022	**91**	0.031	0.032	**96**
S5 1:10	LOW	0.004	N/A	0.008	0.009	**89**	N/A	N/A	N/A
S5 1:20	LOW	0.002	N/A	0.004	0.004	**96**	N/A	N/A	N/A
S5 1:40	LOW	0.001	N/A	LOW	0.002	N/A	N/A	N/A	N/A
S5 1:80	LOW	0.001	N/A	LOW	0.001	N/A	N/A	N/A	N/A
Sample 6									
S6	0.007			0.027			0.034		
S6 1:2	0.003	0.004	**93**	0.014	0.013	**102**	0.017	0.017	**101**
S6 1:4	LOW	0.002	N/A	0.007	0.007	**111**	N/A	N/A	N/A
S6 1:10	LOW	0.001	N/A	0.003	0.003	**109**	N/A	N/A	N/A
S6 1:20	LOW	0.000	N/A	LOW	0.001	N/A	N/A	N/A	N/A
S6 1:40	LOW	0.000	N/A	LOW	0.001	N/A	N/A	N/A	N/A
S6 1:80	LOW	0.000	N/A	LOW	0.000	N/A	N/A	N/A	N/A
Sample 7									
S7	0.011			0.020			0.031		
S7 1:2	0.005	0.005	**92**	0.010	0.010	**101**	0.015	0.015	**98**
S7 1:4	LOW	0.003	N/A	0.005	0.005	**100**	N/A	N/A	N/A
S7 1:10	LOW	0.001	N/A	LOW	0.002	N/A	N/A	N/A	N/A
S7 1:20	LOW	0.001	N/A	LOW	0.001	N/A	N/A	N/A	N/A
S7 1:40	LOW	0.000	N/A	LOW	0.000	N/A	N/A	N/A	N/A
S7 1:80	LOW	0.000	N/A	LOW	0.000	N/A	N/A	N/A	N/A

	D-lactate	**L-lactate**	**total lactate**
min	92	89	88
max	111	109	104
mean	99	100	97
SD	7	7	5

O = observed lactate concentration (g/L), E = expected lactate concentration (g/L), OE% = observed-to-expected ratio.

Table 8. Spiking recovery of four canine fecal samples. Observed-to-expected ratios are in bold and the minimum, maximum, mean, and standard deviation of those observed-to-expected ratios are provided in the box at the end of the table.

	D-lactate (mM)			**L-lactate (mM)**			**total lactate (mM)**		
Sample A	102			237			339		
Sample B	27			66			92		
Sample C	13			27			40		
Sample C	4			6			10		
	D-lactate			L-lactate			total lactate		
	O	E	**OE%**	O	E	**OE%**	O	E	**OE%**
A+B	62	64	**96**	155	151	**102**	216	215	**100**
A+C	58	58	**101**	127	132	**96**	185	189	**98**
A+D	53	53	**100**	121	122	**100**	174	174	**100**
B+A	62	64	**96**	155	151	**102**	216	215	**100**
B+C	20	20	**100**	55	46	**119**	75	66	**113**
B+D	15	15	**98**	40	36	**113**	55	51	**108**
C+A	58	58	**101**	127	132	**96**	185	189	**98**
C+B	20	20	**100**	55	46	**119**	75	66	**113**
C+D	9	9	**103**	17	16	**100**	25	25	**101**
D+A	53	53	**100**	121	122	**100**	174	174	**100**
D+B	15	15	**98**	40	36	**113**	55	51	**108**
D+C	9	9	**103**	17	16	**100**	25	25	**101**
D-lactate				**L-lactate**			**total lactate**		
min	96			96			98		
max	103			119			113		
mean	100			105			103		
SD	2			8			5		

O = observed lactate concentration (mM), E = expected lactate concentration (mM), OE% = observed-to-expected ratio, SD = standard deviation.

Table 9. Intra-assay variability of four canine fecal samples. Coefficients of variation are in bold and the minimum, maximum, mean, and standard deviation of these coefficients of variation are provided in the box at the end of the table.

sample	1	2	3	4
number of repeats	8	8	8	8
	D-lactate			
mean (mM)	109	22	14	6
standard deviation (mM)	6	1	1	0
% CV	**6**	**4**	**7**	**4**
	L-lactate			
mean (mM)	217	123	25	10
standard deviation (mM)	16	7	1	0
% CV	**7**	**6**	**4**	**3**
	total lactate			
mean (mM)	327	145	39	16
standard deviation (mM)	19	7	1	0
% CV	**6**	**5**	**3**	**3**

	D-lactate	**L-lactate**	**total lactate**
min	4	3	3
max	7	7	6
mean	5	5	4
SD	1	2	1

%CV = coefficient of variation, SD = standard deviation.

Table 10. Inter-assay variability of seven canine fecal samples. Coefficients of variation are in bold and the minimum, maximum, mean, and standard deviation of these coefficients of variation are provided in the box at the end of the table.

sample	1	2	3	4	5	6	7
number of repeats	8	7*	8	8	8	8	8
	D-lactate						
mean (mM)	75	106	20	29	15	3	4
standard deviation (mM)	4	6	7	8	1	2	1
% CV	**6**	**5**	**32**	**29**	**5**	**56**	**31**
	L-lactate						
mean (mM)	233	224	143	76	30	7	7
standard deviation (mM)	12	21	19	15	3	4	2
% CV	**5**	**10**	**13**	**20**	**11**	**55**	**28**
	total lactate						
mean (mM)	309	331	163	105	45	10	12
standard deviation (mM)	16	24	18	23	4	5	3
% CV	**5**	**7**	**11**	**22**	**8**	**54**	**28**

	D-lactate	**L-lactate**	**total lactate**
min	5	5	5
max	56	55	54
mean	23	20	19
SD	18	16	16

*one repeat was thrown out due to pipetting error
%CV = coefficient of variation, SD = standard deviation.

Table 11. Stability of deproteinized fecal extracts for seven canine fecal samples at 4°C for 24 hours. Coefficients of variation are in bold and the mean of these coefficients of variation for D-, L-, and total lactate are provided.

D-lactate					
sample	inter-assay mean (mM)	initial (mM)	24h fridge (mM)	% change	**%CV**
1	75	81	81	-2	**0**
2	106	117	100	5	**8**
3	20	10	15	0	**22**
4	29	12	18	6	**19**
5	15	16	14	6	**9**
6	3	3	3	-1	**1**
7	4	3	3	7	**0**
				Mean %CV = 9	
L-lactate					
1	233	252	240	1	**2**
2	224	242	239	-1	**1**
3	143	160	167	-5	**2**
4	76	46	43	14	**3**
5	30	34	29	4	**8**
6	7	8	9	-10	**7**
7	7	7	6	9	**7**
				Mean %CV = 4	
total lactate					
1	309	333	321	0	**2**
2	331	360	339	1	**3**
3	163	169	182	-5	**3**
4	105	58	61	12	**3**
5	45	50	43	5	**8**
6	10	11	12	-8	**5**
7	12	10	9	8	**5**
				Mean %CV = 4	

%CV = coefficient of variation.

Table 12. Stability of deproteinized fecal extracts for seven canine fecal samples at -80°C for 4 weeks. Coefficients of variation are in bold and the mean of these coefficients of variation for D-, L-, and total lactate are provided.

D-lactate					
sample	inter-assay mean (mM)	initial (mM)	4wk freezer (mM)	% change	**%CV**
1	75	71	72	1	**1**
2	106	102	114	-5	**5**
3	20	16	14	12	**8**
4	29	24	25	2	**3**
5	15	15	18	-10	**9**
6	3	4	4	-8	**2**
7	4	3	2	13	**1**
				Mean %CV = 4	
L-lactate					
1	233	219	182	10	**9**
2	224	185	191	3	**2**
3	143	120	122	4	**1**
4	76	64	66	3	**2**
5	30	23	21	10	**4**
6	7	10	9	-1	**7**
7	7	5	4	13	**2**
				Mean %CV = 4	
total lactate					
1	309	290	253	8	**7**
2	331	287	305	1	**3**
3	163	136	136	5	**0**
4	105	87	91	3	**2**
5	45	38	39	3	**1**
6	10	14	13	-3	**5**
7	12	7	7	13	**2**
				Mean %CV = 3	

%CV = coefficient of variation.

3.2 Quantification of Lactate and Bacterial Groups in Canine Feces

Metadata was available for a portion of dogs included in the study and is presented in Table 13. Graphical representations of results are presented in Figures 2 through 5.

3.2.1 Dogs with AHD v Healthy Control Dogs

D-, L-, and total fecal lactate concentrations were significantly increased in dogs with AHD compared to healthy control dogs (Table 14). *Turicibacter*, *Blautia*, *C. hiranonis*, and *Enterococcus* were significantly decreased in dogs with AHD compared to healthy control dogs (Table 14).

3.2.2 Dogs with CE v Healthy Control Dogs

D-lactate concentrations in feces were significantly increased in dogs with CE compared to healthy control dogs (Table 14). *Faecalibacterium*, *Blautia*, and *C. hiranonis* were significantly decreased in dogs with CE compared to healthy control dogs (Table 14).

3.2.3 Dogs with EPI v Healthy Control Dogs

Dogs with untreated and treated EPI had significantly increased D-, L-, and total fecal lactate concentrations (Table 14). Dogs with untreated and treated EPI also had significantly less abundance of *Blautia* and *C. hiranonis* when compared to healthy control dogs (Table 14). Dogs treated for EPI uniquely had increased abundance of

Lactobacillus and *Bifidobacterium* compared to healthy control dogs (Table 14). Both untreated and treated dogs with EPI had an increased Dysbiosis Index (Table 14). A subset of dogs treated for EPI (n=12), had received pancreatic enzyme replacement therapy for 0.08-9.50 years (median 4.45 years), and duration was negatively correlated with *Turicibacter* abundance (Spearman's ρ= -0.634; p=0.0268).

Table 13. Metadata for dogs included in the study, if available.

	Healthy	**AHD**	**CE**	**Untreated EPI**	**Treated EPI**
Age (years; median, range)	6.5, 1-12	N/A	6, 3-11	2, 1.1-4	3.75 1-14
Gender (female/male)	10/8	N/A	2/4	2/3	21/7
Breed (top 3, n)	GSD (7), Miniature Schnauzer (3), Mixed breed (4)	N/A	Mixed breed (3), Other (11)	GSD (3), Chihuahua (1), Pembroke Corgi (1)	GSD (17), Mixed breed (2), Other (11)

Table 14. D-, L-, and total fecal lactate concentrations (median [min-max] mM), abundance of bacterial groups (median [min-max] LogSQ) and Dysbiosis Index (median [min-max]) in disease dogs compared to healthy control dogs with Steel-Dwass test. If no p-value is listed, $p \geq 0.05$.

	Healthy	AHD	CE	Untreated EPI	Treated EPI
D-lactate	0.8 [0.7-1.3]	0.8 [0.5-47.1]; p=0.0428	0.9 [0.7-22.5]; p=0.0416	31.2 [13.1-57.3]; p=0.0033	4.1 [0.7-53.3]; p=0.0002
L-lactate	0.6 [0.3-5.7]	5.4 [0.3-78.1]; p=0.0038	1.4 [0.3-52.6]	68.5 [61.6-124.2]; p=0.0035	9.3 [0.3-184.7]; p=0.0005
Total lactate	1.3 [1.1-6.6]	6.5 [1.1 -125.2]; p=0.0028	2.2 [1.1-75.0]	103.1 [81.5-181.4]; p=0.0035	13.9 [1.0-233.6]; p=0.0006
Universal	11.60 [9.20-12.08]	11.56 [10.67-12.14]	11.60 [11.19-12.05]	11.67 [9.20-11.91]	11.81 [9.20-12.46]
Faecalibacterium	6.80 [4.12-7.71]	5.77 [4.28-7.62]	4.64 [2.82-7.80]; p=0.0256	4.88 [3.82-7.25]	6.03 [4.05-7.76]
Turicibacter	6.27 [4.37-8.83]	4.40 [3.20-6.88]; p=0.0004	5.20 [2.58-7.23]	4.73 [2.67-7.79]	7.50 [4.09-8.39]
Streptococcus	5.48 [3.80-8.43]	4.58 [3.25-7.71]	4.87 [3.80-8.75]	4.77 [3.80-9.04]	7.96 [3.80-9.49]
Escherichia coli	6.63 [4.27-10.61]	6.53 [3.72-8.72]	6.78 [3.32-8.85]	10.44 [3.32-11.36]	8.32 [4.89-11.94]
Blautia	10.72 [8.41-11.15]	7.80 [6.31-9.58]; p<0.0001	9.70 [8.22-10.58]; p=0.0084	8.79 [6.74-9.94]; p=0.0135	9.15 [6.74-10.99]; p=0.0020
Fusobacterium	9.25 [7.23-10.14]	9.43 [7.23-10.41]	8.50 [6.68-10.48]	7.23 [7.23-9.87]	8.38 [7.23-10.54]
Clostridium hiranonis	6.16 [0.90-6.70]	4.89 [0.90-6.53]; p=0.0097	5.26 [0.90-6.02]; p=0.0095	0.90 [0.90-0.90]; p=0.0060	5.35 [0.90-6.68]; p=0.0071
Dysbiosis Index	-3.8 [-7.6 to 3.3]	-2.2 [-7.2 to 3.3]	0.1 [-7.2 to 7.4]	4.7 [-0.7 to 8.4]; p=0.0120	-0.1 [-7.3 to 8.2]; p=0.0064

Table 14 Continued

	Healthy	AHD	CE	Untreated EPI	Treated EPI
Lactobacillus	3.68 [3.68-8.43]	4.72 [3.68-7.69]	4.68 [3.68-6.51]	5.29 [3.68-8.65]	6.71 [3.82-8.66]; p=0.0001
Bifidobacterium	3.93 [3.20-7.30]	3.94 [3.20-6.06]	3.86 [3.20-6.54]	6.97 [3.20-8.12]	6.84 [3.20-8.64]; p<0.0001
Enterococcus	4.30 [1.68-6.78]	3.69 [1.66-5.23]; p=0.0070	5.46 [1.72-7.22]	5.54 [1.68-8.11]	5.14 [2.79-7.13]

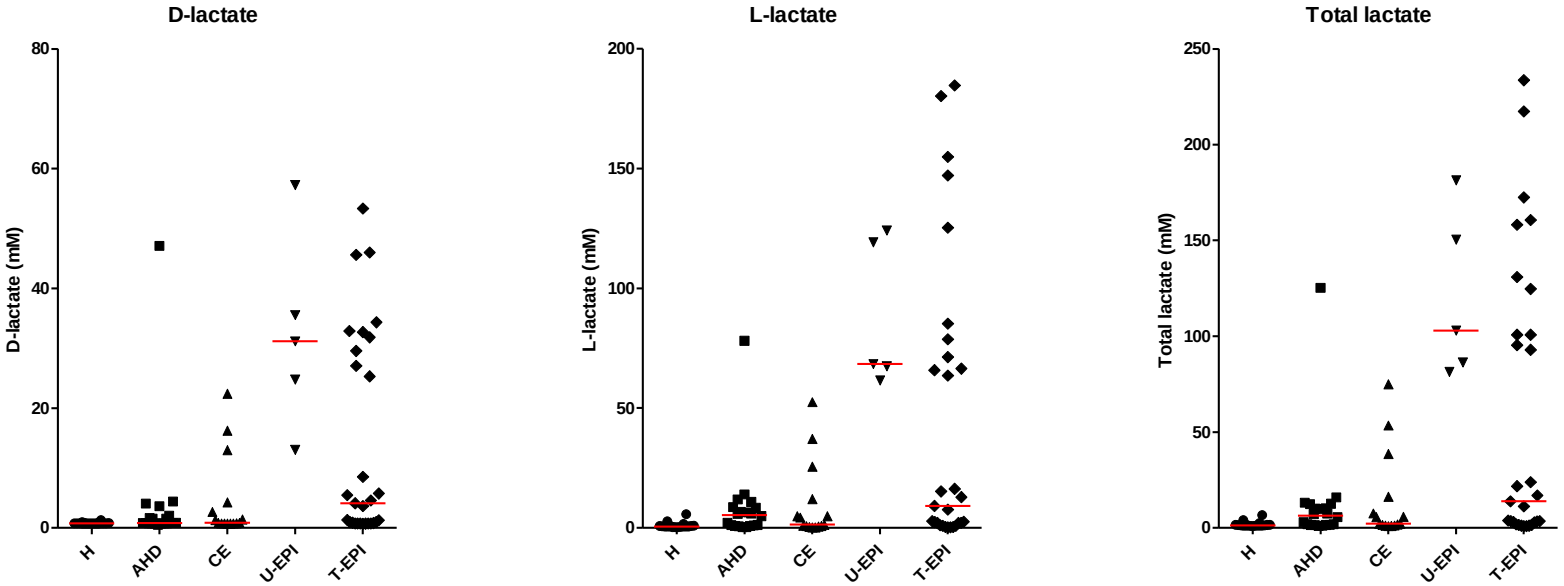

Figure 2. Lactate concentrations (mM) in the feces of healthy and diseased dogs. The groups on the x-axis are defined as follows: H = healthy dogs, AHD = dogs with acute hemorrhagic diarrhea, CE = dogs with chronic enteropathy, U-EPI = dogs with exocrine pancreatic insufficiency not receiving enzyme replacement therapy, T-EPI = dogs with exocrine pancreatic insufficiency receiving enzyme replacement therapy.

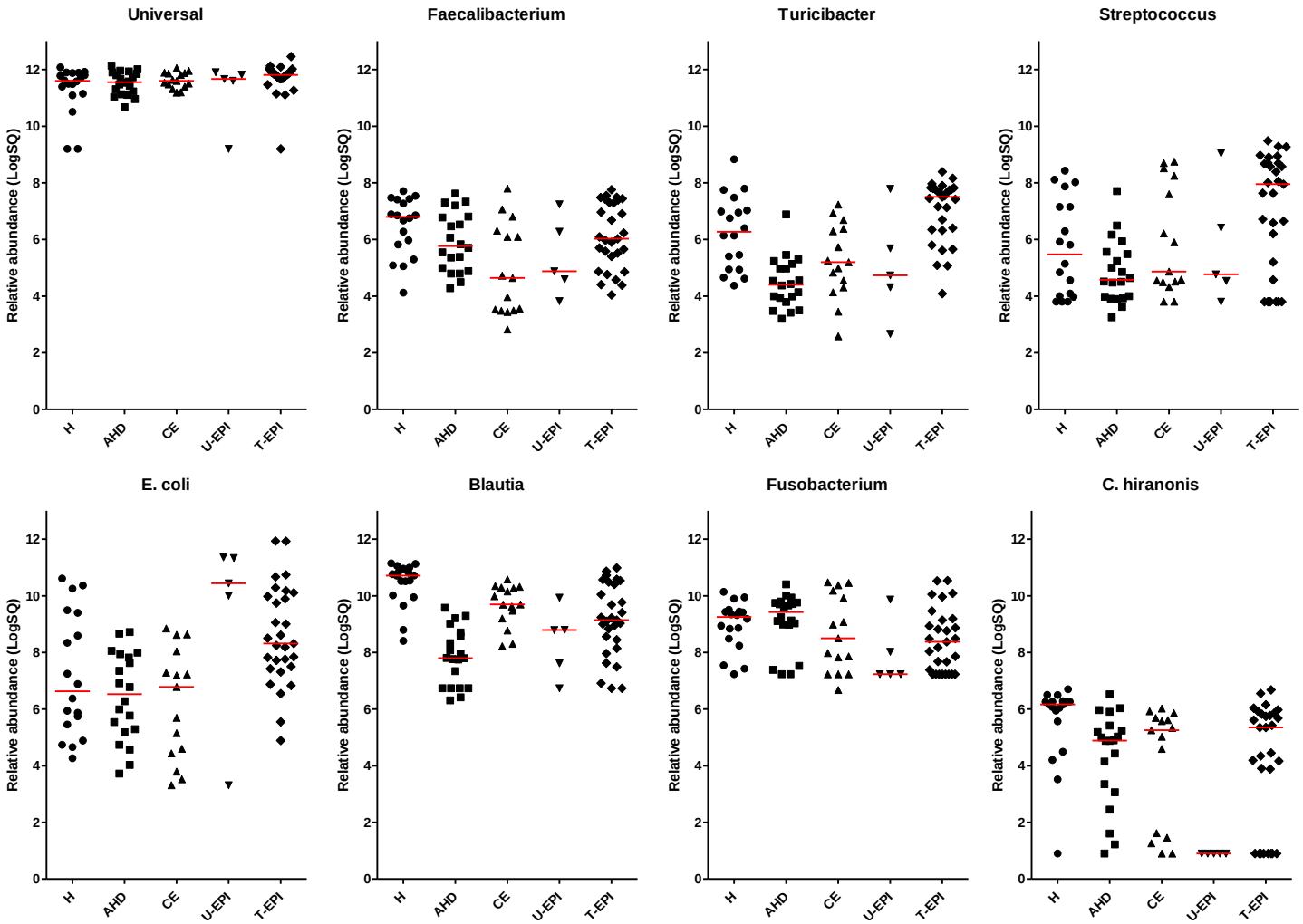

Figure 3. Bacterial abundances of those bacterial groups used to calculate Dysbiosis Index in the feces of healthy and diseased dogs.

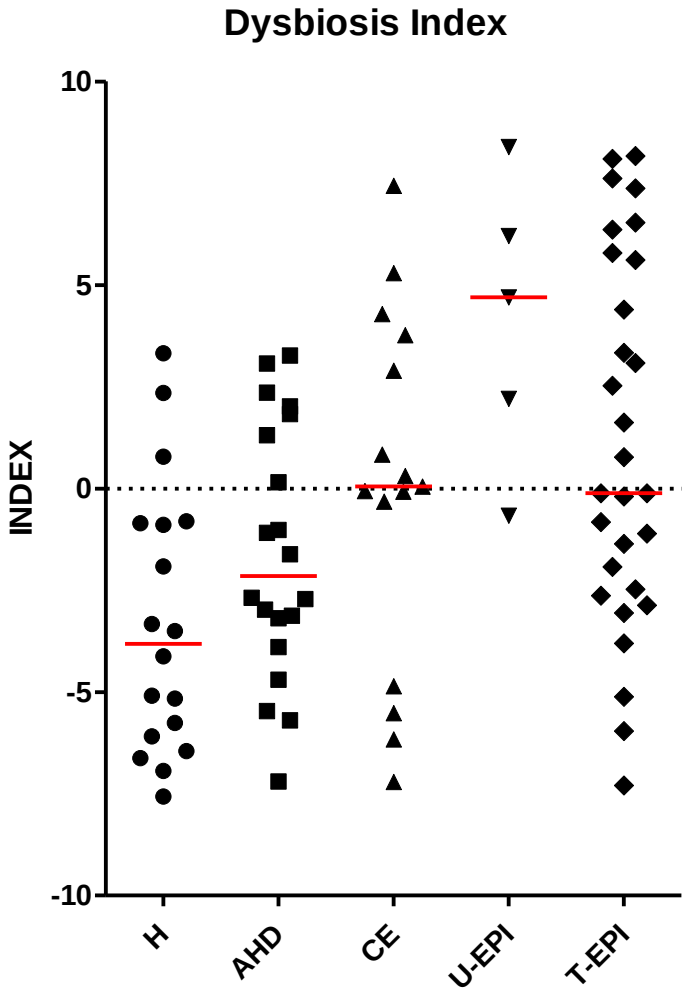

Figure 4. Dysbiosis Index for healthy and diseased dogs.

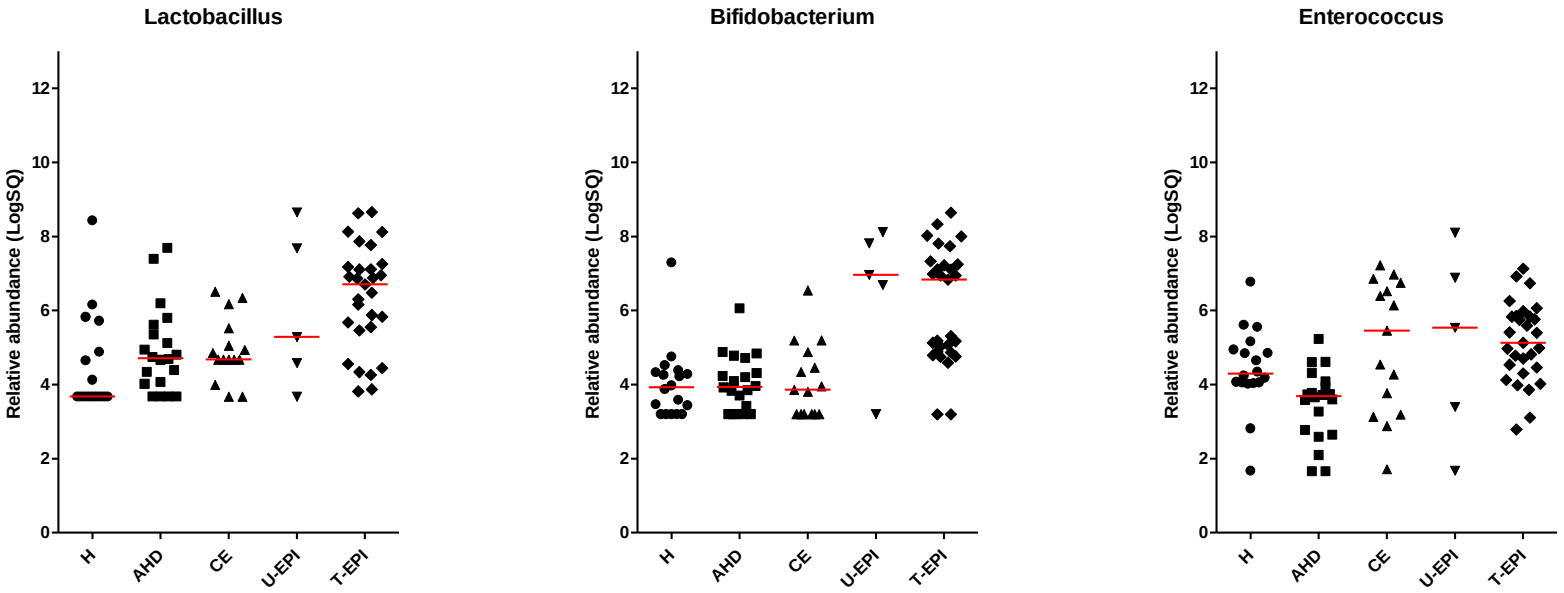

Figure 5. Abundance of specific lactate-producing bacterial groups in healthy and diseased dogs.

4. DISCUSSION

4.1 Analytical Validation of an Assay for Measurement of Fecal Lactate

An enzymatic assay for the measurement of D-, L-, and total lactate in canine feces was successfully established for use in a 96-well plate format. Generally acceptable observed-to-expected ratios for dilutional parallelism and spiking recovery fall between 80% and 120%. Observed-to-expected ratios for dilutional parallelism ranged from 92% to 111% for D-lactate, 89% to 109% for L-lactate, and 88% to 104% for total lactate (Table 7), which suggests the assay is linear. Observed-to-expected ratios for spiking recovery ranged from 96% to 103% for D-lactate, 96% to 119% for L-lactate, and 98%-113% for total lactate (Table 8), which suggests the assay is accurate. Generally acceptable coefficients of variation for intra- and inter-assay variability differ for different types of assays, but are best under 15%. Average intra-assay coefficients of variation for D-, L-, and total lactate were 5%, 5%, and 4%, respectively (Table 9), which suggests the assay is precise. Average inter-assay coefficients of variation for D-, L-, and total lactate were 24%, 20%, and 19%, respectively (Table 10). Some of this inter-assay variation can be explained by the methodology used for validation. Separate fecal aliquots were made for each sample to be analyzed in inter-assay variation. Therefore, potential variation could be also due to some differences in the distribution of lactate in feces. More studies need to be performed to determine the variation of lactate within stool samples. However, our analysis suggests that the assay is reproducible to differentiate lactate concentration across the disease populations.

Lactate was stable in deproteinized fecal extracts for 24 hours of storage at 4°C (average %CV: 9, 4, 4, for D-, L-, and total lactate, respectively; Table 11) and 28 days of storage at -80°C (average %CV: 4, 4, 3, for D-, L-, and total lactate, respectively; Table 12).

The reference interval for canine fecal lactate (n=34) was 0.7-1.4 mM, 0.3-6.0 mM, and 1.0-7.0 mM for D-, L-, and total lactate, respectively. The assay has a working range of 0.007 to 0.581 mM for D-lactate, and 0.003 to 0.581 mM for L-lactate, as determined by the lower limit of detection and highest standard concentration. After dilution that occurred during the deproteinization procedure, all reference samples fell below or within this range.

In summary, the assay for measurement of D-, L-, and total lactate in canine fecal samples was linear, accurate, precise, and reproducible.

4.2 Quantification of Lactate and Bacterial Groups in Canine Feces

This study quantified D-, L-, and total lactate as well as major bacterial groups in feces of healthy dogs and dogs with various gastrointestinal diseases. Significant differences were observed in concentrations of fecal lactate between diseased and healthy dogs (Table 14, Figure 2). Dogs with exocrine pancreatic insufficiency had the highest fecal lactate concentrations followed by dogs with acute hemorrhagic diarrhea. Dogs with chronic enteropathy had a significant increase in D-lactate concentrations, however, L-lactate and total lactate did not reach the level of significance. *Blautia* and *Clostridium hiranonis* were significantly decreased in all diseased groups compared to

healthy dogs (Table 14, Figure 3). Dogs with chronic enteropathy also had decreased *Faecalibacterium* spp. and dogs with acute hemorrhagic diarrhea had decreased *Turicibacter* (Table 14, Figure 3). The Dysbiosis Index tended to increase in dogs with AHD (p=0.3256) and CE (p=0.0745), however, it did not reach the level of significance (Figure 4). Although the proportion of dogs with a Dysbiosis Index above zero was also higher in dogs with AHD (35%) or CE (53%) compared to healthy dogs (17%), these comparisons did not reach the level of significance (p=0.2778 and p=0.0613, respectively). Three additional lactate-producing bacterial groups were quantified in the feces; *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp. The taxa *Enterococcus* spp. were uniquely decreased in dogs with acute hemorrhagic diarrhea, and *Lactobacillus* spp. and *Bifidobacterium* spp. were uniquely decreased in dogs receiving enzyme replacement therapy for EPI. Both treated and untreated dogs with EPI had a dysbiosis as indicated by the significantly increased DI (Table 14, Figure 4).

Statistical differences were not found for abundances of lactate-producing bacteria in dogs not receiving enzyme replacement therapy for EPI, most likely due to the small number of animals analyzed in this group (n=5). For ethical reasons, it is difficult to obtain fecal samples from dogs diagnosed with EPI that have not received treatment. The diagnostic test for EPI is a serum trypsin-like immunoreactivity of less than 2.5 µg/L. Once diagnosed, treatment should not be delayed for the sole purpose of collecting feces for analysis.

It was previously mentioned that nutritional studies often aim to increase lactate concentrations or lactic acid bacteria abundance within the GI tract. However, these

dietary interventions produce only minor changes relative the changes observed in this study. Fructooligosaccharide supplementation in dogs increased mean counts of *Bifidobacterium* and *Lactobacillus* by 0.58 and 0.66 cfu $\log_{10}$/g DM, respectively, and increased mean fecal lactate by 0.053 mmol (Swanson et al., 2002a). Similarly in humans, fructooligosaccharide supplementation increased fecal *Bifidobacterium* and *Lactobacillus* by less than 0.5 $\log_{10}$/g wet feces and increased fecal lactate by 0.019 mmol/g dry feces (Ten Bruggencate et al., 2006). In contrast, dogs with EPI (treated) in this study showed increases in *Bifidobacterium* and *Lactobacillus* of 2.91 and 3.03 LogSQ, respectively, and increased fecal lactate concentrations by 12.6 mmol/L. Minor increases in lactate or lactate-producing bacteria in the GI tract may have some beneficial effects, but the GI disease processes shown here are likely to have a much more profound impact on any changes observed in the microbiota or their metabolites. Moreover, other factors, such as pH, may influence the metabolic activities of the microbiota without actually changing the abundance of any bacterial groups (Edwards et al., 1985).

The results of this study are in agreement with previous studies that examine the fecal microbiota composition of dogs with GI disease. In one study using 454-pyrosequencing and qPCR analysis, dogs with acute hemorrhagic diarrhea had decreases in *Blautia*, *Faecalibacterium*, and *Turicibacter* spp. when compared to healthy dogs (Suchodolski et al., 2012). Decreased proportions of Bacteroidetes, *Faecalibacterium*, and an unclassified genus within Ruminococcaceae were also observed in dogs with hemorrhagic and non-hemorrhagic acute diarrhea, and no differences were identified in

Bifidobacterium, *Lactobacillus*, or *E. coli* (Guard et al., 2015). Similarly, this study observed significant decreases in *Blautia* and *Turicibacter* abundance and no difference in *Bifidobacterium*, *Lactobacillus*, or *E. coli* when dogs with acute hemorrhagic diarrhea were compared to healthy dogs.

Dysbiosis is typically associated with alterations in the predominant bacterial groups, such as the eight groups tested here, and has recently been linked to alterations in bacterial metabolites, such as SCFAs, secondary bile acids, and other immunomodulatory metabolites (Suchodolski, 2016). Accordingly, in dogs with acute diarrhea, decreased abundances of *Faecalibacterium* spp. were correlated with increases in butyrate and decreases in propionate concentrations in the feces (Guard et al., 2015). Furthermore, alterations of the microbiota in dogs with inflammatory bowel disease (IBD), a type of chronic enteropathy, were associated with changes in the functional gene content and serum metabolites (Minamoto et al., 2015). Microbiota changes shown by qPCR abundances in feces of dogs with IBD included significantly decreased *Blautia*, *Faecalibacterium*, and *Turicibacter* spp. (Minamoto et al., 2015), which agrees with our results. However, in the group of dogs with chronic enteropathy presented here, there was no significant decrease in abundance of *Turicibacter*. This could be attributed in part to individual variation in the microbiota composition, as described elsewhere (Blake and Suchodolski, 2016).

A recent study identified differences in the microbiota and functional gene content of dogs with EPI using next generation sequencing and PICRUSt (Isaiah et al., 2017). Again, in agreement with our findings, decreases in *Faecalibacterium* and

Blautia and increases in *Lactobacillus* and *Bifidobacterium* were found in dogs with EPI compared to healthy dogs (Isaiah et al., 2017). Additionally, *Coprococcus*, *Ruminococcus*, *Eubacterium*, *Bacteroides*, *Slackia*, and *Fusobacterium* were decreased and *Enterococcus* were increased in dogs with EPI (Isaiah et al., 2017). Dogs with EPI often have a concurrent overgrowth of bacteria in their small intestine (Westermarck et al., 1993), and it is unclear whether bacterial quantification in the feces is directly representative of bacteria present in the small intestine. Recently, Honneffer et al. (2017) reported that the microbiota and microbial metabolites vary along the gastrointestinal tract in healthy dogs. However, in dogs with IBD, similar changes in the microbiota were observed in the small intestine and fecal samples, suggesting that dysbiosis originating in the small intestine can still be detected in the feces (Suchodolski, 2016).

Antibiotics are often administered to dogs with EPI to combat the overgrowth of bacteria in the small intestine, despite evidence of pancreatic enzyme replacement therapy modulating bacteria levels alone (German, 2012). Alterations in the microbiota have been described in association with antibiotic usage (Minamoto et al., 2015), and a recent study has suggested that dysbiosis can be induced by antibiotic administration in healthy dogs (Suchodolski et al., 2016). For these reasons, we included only dogs that had not received antibiotics for at least three weeks prior to feces collection.

4.3 Strengths and Limitations

In addition to the small sample sizes obtained for some of the diseased groups, this study was limited by the lack of background information available for dogs included

in the study, especially lack of information on antibiotic usage. Although no dogs were administered antibiotics in the three weeks prior to fecal collection, full prior history of antibiotic usage was not available for some dogs. Many of the samples used were left over from other studies and this information could not be obtained retrospectively from owners. However, some dogs included in our study had not received antibiotics for at least 6 months prior to collection and differences in these dogs were similar to the rest of their prospective disease groups. Furthermore, none of the dogs with AHD had a known history of antibiotic usage. Recognizing that type and duration of antibiotic administration may result in different changes to the microbiota, this information is imperative to future studies. Studies of antibiotic administration to healthy humans has suggested that severe shifts in the microbial populations are induced, and certain bacterial groups may not return to normal for up to 12 months (Dethlefsen et al., 2008; Rashid et al., 2015). Ideally, future studies should be performed on animals with no history of antibiotic usage. However, this may be unrealistic because antibiotics are often used as a first or second line of defense when companion animals are experiencing diarrhea or vomiting. In addition, there is anecdotal evidence that animals improve clinically with antibiotics.

The validated enzymatic assay for the quantification of lactate in canine feces has some strengths and limitations of its own. The 96-well plate format allows up to 37 samples to be analyzed at once and is cost efficient compared to other methods of analysis. The narrow working range of the assay may be considered a limitation; however, samples above the working range can be diluted easily. The healthy reference

interval for lactate does not exceed the upper limit of the working range. Therefore, we can deduce that samples with lactate concentrations above the working range of the assay are abnormal.

4.4 Applications, Implications, and Outlook

The assay for measurement of lactate in canine feces has made available the opportunity to obtain concentrations of fecal lactate in future studies on gastrointestinal diseases in companion animals. We have directly measured lactate concentrations as well as bacterial abundances of groups thought to produce lactate in the feces of dogs. These findings support the theory of lactate accumulation described by Ewaschuk et al. (2005), where lactate accumulates in association with increased lactic acid bacteria such as *Lactobacillus* spp. However, this theory might not explain lactate accumulation in all cases, as shown by the accumulation of lactate in feces of dogs with acute hemorrhagic diarrhea and chronic enteropathy without concurrent increases in lactate-producing bacterial groups. Future work could examine lactate consuming bacterial groups or look at the microbial metabolites in a broader sense to understand the metabolic changes occurring in diseased states. In perspective of the current knowledge, our findings do not support the theory that D-lactic acidosis is caused solely by overproduction of lactate by bacterial groups in the gastrointestinal tract, as an increase in luminal D-lactate appears to occur frequently in GI disease. Overproduction of lactate may be one component in the development of acidosis, but other components, such as abnormal intestinal barrier function, could also potentially be involved. There was no prevalence of obvious

neurologic signs in dogs included in this study despite all disease groups having a significantly increased concentration of D-lactate in the feces compared to healthy dogs based on owner questionnaires. Other mechanisms, such as increased absorption or impaired metabolism of D-lactate, should be examined in the development of lactic acidosis.

Our findings raise several new questions that could be addressed in future work. Fecal lactate may influence serum lactate concentrations on a subclinical level and could be clinically useful information in certain disease processes such as shock and sepsis. This study did not identify the origin of the lactate molecules present in the feces, and since lactate is also produced by host cells (albeit in small amounts), one question that needs to be answered is ‘where is the lactate coming from?’. It is possible that in certain disease processes where the intestinal epithelium undergoes oxidative stress (i.e. inflammation), epithelial cells will produce and excrete higher amounts of lactate into the intestinal lumen.

5. CONCLUSION

An enzymatic assay for the measurement of D-, L-, and total lactate in canine feces was successfully established for use in a 96-well plate format. The assay was linear, accurate, precise, and reproducible. We then quantified D-, L-, and total lactate as well as major bacterial groups in feces of healthy dogs and dogs with various gastrointestinal diseases. Significant increases in fecal lactate concentrations were observed in dogs with acute hemorrhagic diarrhea, dogs with chronic enteropathy (D-lactate only), and dogs with exocrine pancreatic insufficiency. *Blautia* and *Clostridium hiranonis* were significantly decreased in all diseased groups compared to healthy dogs. While alterations in other bacterial groups were present in the various diseases studied, dogs with exocrine pancreatic insufficiency had the most profound alterations as evidenced by their significantly increased Dysbiosis Index.

In conclusion, further studies are necessary to determine the clinical utility of lactate quantification in canine feces. Though lactate by itself may not be a good indicator of dysbiosis, bacterial metabolites together with bacterial abundances are promising targets for further elucidating the role of the microbiota in health and disease.

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