A dysbiosis index to assess microbial changes in fecal samples of dogs with chronic inflammatory enteropathy

MK AlShawaqfeh¹,², B Wajid¹,³, Y Minamoto¹, M Markel¹, JA Lidbury¹, JM Steiner¹, E Serpedin² and JS Suchodolski¹,*

¹Gastrointestinal Laboratory, Texas A&M University, College Station, TX 77843-4474, USA, ²Department of Electrical and Computer Engineering, Texas A&M University, College Station, TX 77843-4474, USA and ³Department of Electrical Engineering, University of Engineering and Technology, 54890 Lahore, Pakistan

∗Corresponding author: Gastrointestinal Laboratory, Department of Small Animal Clinical Sciences, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4474, USA. Tel: +979-458 0933; E-mail: jsuchodolski@cvm.tamu.edu

One sentence summary: The aim was to develop a rapid PCR-based dysbiosis index to assess microbiota changes in dogs.

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ABSTRACT

Recent studies have identified various bacterial groups that are altered in dogs with chronic inflammatory enteropathies (CE) compared to healthy dogs. The study aim was to use quantitative PCR (qPCR) assays to confirm these findings in a larger number of dogs, and to build a mathematical algorithm to report these microbiota changes as a dysbiosis index (DI). Fecal DNA from 95 healthy dogs and 106 dogs with histologically confirmed CE was analyzed. Samples were grouped into a training set and a validation set. Various mathematical models and combination of qPCR assays were evaluated to find a model with highest discriminatory power. The final qPCR panel consisted of eight bacterial groups: total bacteria, Faecalibacterium, Turicibacter, Escherichia coli, Streptococcus, Blautia, Fusobacterium and Clostridium hiranonis. The qPCR-based DI was built based on the nearest centroid classifier, and reports the degree of dysbiosis in a single numerical value that measures the closeness in the $l_2$ – norm of the test sample to the mean prototype of each class. A negative DI indicates normobiosis, whereas a positive DI indicates dysbiosis. For a threshold of 0, the DI based on the combined dataset achieved 74% sensitivity and 95% specificity to separate healthy and CE dogs.

Keywords: microbiota; dysbiosis index; dogs; IBD

INTRODUCTION

Increasing evidence has associated imbalances in the intestinal microbial ecosystem to various diseases including inflammatory bowel disease (IBD), irritable bowel syndrome, obesity and diabetes in humans and animal models (Kelsen and Wu 2012; Handl et al. 2013; Gevers et al. 2014; Cox and Blaser 2015; Guard and Suchodolski 2016). The advancements in high-throughput sequencing in parallel with their decreasing costs allow the sampling of diverse microbial communities at reasonable rates. Therefore, numerous studies are currently underway to characterize the microbial communities inhabiting our world.

Chronic inflammatory enteropathy (CE) in dogs is defined as a syndrome in which dogs show chronic (duration >3 weeks) gastrointestinal signs, and in which common causes (e.g. parasites, enteropathogens) have been excluded. Similar to human IBD, the cause of canine CE is likely a combination of genetic predisposition, environmental factors (e.g. dietary...
antigens) and the intestinal microbiota that together induce an aberrant immune response in the host. Canine CE can be, based on response to treatment, further characterized as food-responsive enteropathy, antibiotic-responsive enteropathy or idiopathic IBD when immunosuppressant treatment is needed (Dandrieux 2016). There is an overlap in clinical signs between these enteropathies, and no clear biomarkers for differentiation have been recognized to date. The histological evaluation of intestinal biopsies may reveal a diffuse or multifocal inflammation, with the most commonly identified cell infiltration being of lymphoplasmacytic, followed by eosinophilic and neutrophilic type. Changes in the architecture of the intestinal mucosa such as villus atrophy are also commonly observed (Day et al. 2008).

In various previous studies targeting the 16S rRNA gene by either clone libraries, next generation sequencing or quantitative PCR, we have identified alterations in specific bacterial groups within the gut microbiota between healthy dogs and dogs with acute diarrhea and CE (Honneffer, Minamoto and Suchodolski 2014; Alshawaqfeh et al. 2016). Commonly altered bacterial groups include increases in Proteobacteria (especially Escherichia coli), and decreases in Firmicutes, especially Faecalibacterium, Ruminococcus and Blautia. Most of these previous studies evaluated a small number of animals. One study with the largest number of dogs evaluated so far (85 healthy dogs and 65 dogs with CE) used a 16S rRNA sequencing approach, and reported that microbiota signatures can separate dogs with IBD from healthy dogs with high discriminatory power (Vazquez-Baeza et al. 2016). While sequencing-based approaches are very informative, they have some drawbacks. The first disadvantage is relatively high cost associated with the sequencing process. The second drawback is the relatively longer turnaround time required to receive sequencing results, and the bioinformatic tools required to process and report data. In this study, we aimed to evaluate a panel of quantitative PCR (qPCR) assays that may be useful to differentiate the fecal microbiota from healthy dogs and dogs with chronic enteropathies.

MATERIAL AND METHODS

Sample population

Naturally passed fecal samples were analyzed from 95 healthy dogs and 106 dogs with chronic signs of gastrointestinal disease and confirmed inflammatory changes on histopathology. All dogs participated in clinical studies evaluating biomarkers for chronic enteropathies. The protocol for sample collection was approved by the Texas A&M University Institutional Animal Care and Use Committee (AUP #2012–83). All dogs were pets living in various home environments and were all on a variety of commercial pet foods. Dogs receiving antibiotics within the past 3 months of sample collection were excluded from this study. Fecal samples were collected at home, and the owners were instructed to immediately freeze samples and ship them on ice overnight to the laboratory. Dogs were classified as having CE due to their chronic signs of gastrointestinal (GI) disease (>3 weeks duration) and histopathologic evidence of mucosal inflammation, classified by the WSAVA histopathologic criteria (Day et al. 2008). The information for the animals (i.e. age, weight, gender, breed) was obtained from clinical records. For each dog, the canine chronic enteropathy activity index (CCEAI) was calculated that describes disease severity (Allenspach et al. 2007). A dietary history was obtained by asking owners for the diet fed at time of sample collection. The dietary macronutrients protein and fat of these diets were obtained from analyses data provided directly by the company manufacturing the diet. The signalment of the dogs is summarized in Table S1 (Supporting Information).

Quantitative PCR panel

DNA was extracted from each fecal sample (100 mg) using the MoBio Power soil DNA isolation kit (MoBio Laboratories, USA) according to the manufacturer’s instructions. The qPCR assays were designed and evaluated previously (Suchodolski et al. 2012b; Panasevich et al. 2014; Minamoto et al. 2015; White et al. 2017). Briefly, qPCR reactions were performed using SYBR green-based reaction mixtures. The final total reaction volume was 10 μL. The final mix consisted of 5 μl SsoFast EvaGreen® supermix (Bio-Rad Laboratories, CA, USA), 0.4 μl each of a forward and reverse primer (final concentration: 400 nM), 2.6 μl of PCR water and 2 μl of normalized DNA (final concentration: 5 ng/μl). The PCR conditions for PCR were as follows: initial denaturation at 98°C for 2 min, then 40 cycles with denaturation at 98°C for 3 s and annealing for 3 s (see Table 1 for specific annealing temperatures for the final qPCR panel). Melt curve analysis was performed post-amplification using these conditions: 95°C for 1 min, 55°C for 1 min and increasing incremental steps of 0.5°C for 80 cycles for 5 s each. All samples were run in duplicate fashion. The qPCR data were expressed as the log amount of DNA (fg) for each particular bacterial group/10 ng of isolated total DNA (Suchodolski et al. 2012b; Panasevich et al. 2014; White et al. 2017).

To test specificity of the used primers, 16S rRNA gene clone libraries were constructed from the PCR amplicons for each primer set as described previously (Ritchie, Steiner and Suchodolski 2008; Ritchie et al. 2010). Briefly, fecal DNA was amplified with each primer set. The PCR amplicons were then ligated into pCR4-TOPO® vectors and transformed into competent DH5α™-T1R Escherichia coli by heat shock following the manufacturer’s instructions (Invitrogen, Carlsbad, CA). For each primer set, 20 clones were picked, plasmids were purified and the amplified sequence sequenced by Sanger sequencing as described (Ritchie, Steiner and Suchodolski 2008; Ritchie et al. 2010).

Dysbiosis index development and validation

Samples were grouped into two sets. The first set was used as a training set and consisted of 36 healthy and 51 diseased samples. The second set was used as a validation set and consisted of samples obtained from a distinct group of 59 healthy and 51 diseased dogs. Various mathematical models (e.g. 1norm, true difference, Degree centrality weight, Eigen centrality weight) and combination of PCR assays were evaluated to find the final panel with highest discriminatory power. PCR assays were initially used on the training set to measure the abundances of selected bacterial taxa that have been shown to be altered in dogs with gastrointestinal disease in previous studies, either based on qPCR assays or 16S rRNA gene-based sequencing studies (Xenoulis et al. 2008; Suchodolski et al. 2010, 2012a;b; Rossi et al. 2014; Honnefer, Minamoto and Suchodolski 2014; Minamoto et al. 2014, 2015; Guard et al. 2015; Vazquez-Baeza et al. 2016; Alshawaqfeh et al. 2017; Isaiah et al. 2017). Based on this literature review, the following assays were chosen: total bacteria, Proteobacteria, Firmicutes, Fusobacteria, Bacteroidetes, Ruminococcaceae, Bifidobacterium spp., Blautia spp., Faecalibacterium spp., Turicibacter spp., Lactobacillus spp., Clostridium perfringens, C. hiranonis and E. coli. First, an exhaustive search wrapper feature selection method was employed to identify a smaller subset of PCR assays with high classification power between healthy and
### Table 1. Oligonucleotides primers/probes used in this study.

<table>
<thead>
<tr>
<th>qPCR primers/probe</th>
<th>Sequence (5'-3')</th>
<th>Target</th>
<th>Annealing (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>GAAGCGGCCTACTGGGCAC</td>
<td>Faecalibacterium</td>
<td>60</td>
</tr>
<tr>
<td>Reverse</td>
<td>GTGCAAGCGAGTTGCAAGCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>KGGGCTCAACMCMTATTGCGT</td>
<td>Fusobacteria</td>
<td>51</td>
</tr>
<tr>
<td>Reverse</td>
<td>TCGGTTAGCTTGGGCGTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>TCTGATGTGAAAGGCTGGGCTTA</td>
<td>Blautia</td>
<td>56</td>
</tr>
<tr>
<td>Reverse</td>
<td>GGCTTAGCCACCCGACACCTAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CCTACGGGGAGCCAGCAGT</td>
<td>Universal Bacteria</td>
<td>59</td>
</tr>
<tr>
<td>Reverse</td>
<td>ATTACCGCGGGCTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>CAGACCCGGCAACGATTGGA</td>
<td>Turicibacter</td>
<td>63</td>
</tr>
<tr>
<td>Reverse</td>
<td>TACCATCGCTGGTGGTAIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GTAATACCTTTGCTCATGGA</td>
<td>E. coli</td>
<td>55</td>
</tr>
<tr>
<td>Reverse</td>
<td>ACCAGGTATCTAATCTTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>AGTAAAGCTCTCTATCTGCTCT</td>
<td>C. hiranonis</td>
<td>50</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGGGAAGGGAGGATTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>TTATTTGAAAGGGAATGCTG</td>
<td>Streptococcus</td>
<td>54</td>
</tr>
<tr>
<td>Reverse</td>
<td>GTGAACTTTCCACTCTCACAC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Summary statistics for qPCR results and final DI.

<table>
<thead>
<tr>
<th></th>
<th>Healthy&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CE&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mann-Whitney P-value</th>
<th>AUC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Universal Bacteria</td>
<td>13.5 (10.9–15.1)</td>
<td>12.4 (10.5–15.0)</td>
<td>&lt;0.0001</td>
<td>0.69 (0.61–0.75)</td>
</tr>
<tr>
<td>Faecalibacterium</td>
<td>6.2 (2.8–8.0)</td>
<td>4.4 (1.1–7.5)</td>
<td>&lt;0.0001</td>
<td>0.78 (0.72–0.84)</td>
</tr>
<tr>
<td>Turicibacter</td>
<td>6.1 (1.9–9.1)</td>
<td>4.3 (1.1–8.2)</td>
<td>&lt;0.0001</td>
<td>0.71 (0.64–0.78)</td>
</tr>
<tr>
<td>Streptococcus</td>
<td>4.4 (3.5–9.1)</td>
<td>6.8 (3.5–9.5)</td>
<td>&lt;0.0001</td>
<td>0.72 (0.65–0.78)</td>
</tr>
<tr>
<td>E. coli</td>
<td>4.8 (1.1–9.1)</td>
<td>6.8 (1.1–9.8)</td>
<td>&lt;0.0001</td>
<td>0.70 (0.63–0.78)</td>
</tr>
<tr>
<td>Blautia</td>
<td>9.7 (7.2–11.7)</td>
<td>9.4 (5.6–11.6)</td>
<td>&lt;0.0001</td>
<td>0.68 (0.60–0.75)</td>
</tr>
<tr>
<td>Fusobacterium</td>
<td>8.0 (4.7–10.7)</td>
<td>6.2 (3.3–9.9)</td>
<td>&lt;0.0001</td>
<td>0.80 (0.74–0.86)</td>
</tr>
<tr>
<td>C. hiranonis</td>
<td>6.4 (0.7–6.9)</td>
<td>3.1 (0.4–6.9)</td>
<td>&lt;0.0001</td>
<td>0.86 (0.80–0.91)</td>
</tr>
<tr>
<td>Dysbiosis index</td>
<td>−4.8 (−9.1 to 1.7)</td>
<td>3.3 (−6.2 to 9.3)</td>
<td>&lt;0.0001</td>
<td>0.93 (0.89–0.96)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data expressed as median (minimum—maximum) log DNA/gram of feces.

CE = chronic enteropathy.

AUC = area under ROC curve (95% confidence interval).

diseased subjects (Kohavi and John 1997). Wrappers represent a broad family of feature selection algorithms, in which optimal features are identified with respect to a particular classifier subset of features that was employed for spanning the space of possible subsets (Kohavi and John 1997). The operation of a wrapper algorithm consisted of two steps: (i) search the space of possible subsets and (ii) evaluate the quality of each candidate subset by running the classifier on the subset and measuring its performance. The nearest centroid classifier was employed as the learning algorithm and the brute force, an exhaustive search technique that enumerates all possible subsets of features, was employed for spanning the space of possible subsets (Kohavi and John 1997). The brute force search showed that a subset of only seven taxa (Faecalibacterium, Turicibacter, Streptococcus, E. coli, Blautia, Fusobacterium and C. hiranonis) attained best specificity and sensitivity performance. Therefore, only these seven bacteria were used to construct the model. To overcome the variability between samples, test and validation samples were normalized by the abundance of universal bacteria. The model was built using the nearest prototype (centroid) classifier. The nearest centroid classifier (NCC) first trains the model with labeled data in order to determine the centroid of each healthy ($\mu_{CH}$) and diseased ($\mu_{CD}$) class. Second, NCC assigns the test sample to the class whose centroid is closest. Geometrically, the centroid of each class can be considered as a point in a space with dimensions equal to the number of variables, and the NCC measures the distance between the test sample and these centroids in order to assign it to the healthy class ($C_h$) or diseased class ($C_d$). The model employs Euclidean distance as measure of closeness.

The degree of dysbiosis was quantified as a single numerical value, called the dysbiosis index (DI), that measures the closeness (in the $l_2 \text{-- norm}$) of the test sample to the mean (prototype) of each class. More formally, DI is defined as the difference between (Euclidean distance between the test sample and the healthy class centroid) and the (Euclidean distance between the
Figure 1. Results of the final eight qPCR assays and the final DI. All assays were significantly different between healthy dogs (H) and dogs with chronic inflammatory enteropathy (CE) ($P < 0.001$).

test sample and the diseased class centroid). Mathematically, the DI of a test sample $z$ is defined as

$$DI(z; \mu_{CD}, \mu_{CH}) = \|z - \mu_{CD}\|_2^2 - \|z - \mu_{CH}\|_2^2,$$

where $\mu_{CD}$ and $\mu_{CH}$ stand for the centroid of the diseased and healthy samples in the training set, respectively. A value of zero means that the test sample lies at equal distance from the center of both classes. The higher the DI, the more deviation of the sample from normobiosis. For example, a sample with $DI = 8$ is farther away from the normobiotic reference than a sample with $DI = 2$; thus, the first sample is more dysbiotic than the second sample.

To evaluate the efficiency of the DI model, 5-fold stratified cross validation was conducted over the training set. To mitigate...
Figure 2. Scatter plots of the DI and ROC curves for healthy dogs and dogs with chronic inflammatory enteropathy (CE) in the training set and validation set ($P < 0.001$ for both datasets).

the variance in the generalization error due to small sample size, this cross validation experiment was repeated 100 times (Braga-Neto and Dougherty 2004). To assess the diagnostic performance of the DI and its capacity to track the imbalance in bacterial population, the trained DI was validated using the independent validation dataset (i.e. 59 healthy samples and 51 diseased samples).

Reproducibility of DI

To evaluate the reproducibility of the qPCR panel, fresh fecal samples were obtained from five dogs. The feces were homogenized using a stomacher for 90 s at normal speed (Seward Stomacher® 80 Biomaster, Seward, Norfolk, UK). Five aliquots were prepared from each fecal sample, DNA was extracted and the final qPCR assays were performed separately for each aliquot. The variability was determined by calculating the coefficient of variations for each sample.

Statistical analysis

The data for the qPCR assays and signalment (i.e. age, weight) were tested for normal distribution using D’Agostino & Pearson omnibus normality tests. Group comparisons were performed using either Student’s t-test or Mann–Whitney tests as appropriate. Correlations were performed using Spearman r. Significance was set at $P < 0.05$. Receiver-operating characteristic (ROC) curve analysis was performed for sensitivity and specificity calculations and calculation of the area under the curve (AUC) under the ROC curve. The ROC curve allows to graphically visualize the discrimination power of the binary predictive model by plotting the true positive rate against the false positive rate under various threshold settings. The AUC summarizes the performance
of the binary predictor over the entire range of threshold values. Larger values (maximum is 1) indicate higher accuracy. All statistics were performed in GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, USA).

RESULTS
Signalement of dogs
A total of 95 healthy dogs and 106 dogs with CE were analyzed. The median (range) age in the healthy dogs was 4.8 years (1–11.7 years) and in the diseased group was 6.1 years (1–13.2 years), which was not significantly different between groups (P = 0.260). The median body weights were 18.0 kg (0.9–50.0) in healthy vs 21.5 kg (1.7–72.6) in the diseased group (P = 0.127).

A wide variety of breeds were represented. The most common breeds in the healthy control group were mixed breed (n = 33), Labrador Retrievers (n = 10), Australian Shepherd (n = 5) and Miniature Schnauzer (n = 3). The most common breeds in the diseased group were mixed breeds (n = 14), German Shepherd dogs (n = 11), Boxer (n = 7), Labrador Retrievers (n = 6) and Cavalier King Charles Spaniel (n = 5). There were no significant differences in the proportions of male and female dogs in each group (Fisher’s exact test; P = 0.283). The clinical disease was scored using the CCEAI in the diseased group with a mean (SD) of 7.7 (4.3). Reported histological changes varied widely across dogs, and were predominantly of lymphoplasmacytic infiltrates, with a subset of dogs also showing eosinophilic and/or neutrophilic components. Predominant clinical signs were reported as large bowel diarrhea in 36 dogs, small bowel diarrhea in 27 dogs and of mixed origin in 14 dogs. No clear information was obtainable for the remaining dogs.

DI in healthy vs diseased dogs
All eight bacterial targets that were part of the final qPCR panel were significantly different (P < 0.001) between the healthy and diseased group. Total bacteria, Faecalibacterium, Turicibacter, Blautia, Fusobacterium and C. hiranonis were significantly lower in the disease group. In contrast, E. coli and Streptococcus were significantly higher in the diseased compared to healthy dogs (Table 2, Fig. 1). The AUC to separate healthy from diseased dogs ranged from 0.69 for total bacteria to 0.86 for C. hiranonis (Table 2). The DI (Fig. 2), which summarizes the results from all eight PCR assays in one number, achieved the best separation, with an AUC of 0.93 (95% confidence interval: 0.89–0.96) on the combined dataset. Table 2 and Fig. 3 show the AUC values and the ROC curves separately for the training set and the validation set. The performance in terms of sensitivity and specificity for varying threshold values is shown in Table 3 for the combined dataset and separately for the training set and the validation set. In the combined dataset, for a threshold value of 0, DI yielded a 82% sensitivity and 91% specificity. The performance of DI over the entire range of the ROC curve is depicted in Fig. 2 for the training set and validation set.

There was a significant correlation between DI and CCEAI scores (r = 0.682; P < 0.001), but there was no significant difference in the DI between dogs with predominantly small intestinal bowel signs and dogs with predominantly large bowel signs (P = 0.416). There was no significant correlation between DI and age or weight. Dietary information was available from 42 healthy dogs and 55 diseased dogs. Median and range protein content in the diet of healthy dogs was significantly higher (70.5; 40.1–87.5 g protein/1000 kcal ME) compared to the diet-fed diseased dogs (56.2; 40.1–71.0; P < 0.01). However, there was no correlation between the DI and protein content in healthy (r = −0.043; P = 0.789) or in diseased dogs (r = 0.048; P = 0.594). Median fat content of diets was not significantly different between healthy (38.3; 25–92.2 g fat/1000 kcal ME) and diseased dogs (34.0; 18.6–48.4; P = 0.1649). There was also no correlation between the DI and fat content in the diet (r = −0.049; P = 0.75). There was no significant difference in the crude fiber content in the diets fed to healthy (5.0; 1.8–8.4 g/1000 kcal ME) vs diseased dogs 4.6; 2.0–9.0; P = 0.679) and no correlation with the DI (r = −0.014; P = 0.885).

Specificity of primers and reproducibility of the DI assay
The sequence analysis of the PCR amplicons confirmed the specificity of each primer set for the targeted bacterial groups. The coefficients of variations (CV%) for the five aliquots for each of the five fecal samples were 2.9%, 7.1%, 8.9%, 9.2% and 10.5%, respectively. Larger values (maximum is 1) indicate higher accuracy. All statistics were performed in GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, USA).

DISCUSSION
In this study, we have shown that the abundance of key bacterial groups as measured by qPCR is altered between healthy dogs and dogs with CE. Furthermore, combining the results using a mathematical algorithm yields a DI that shows better
separation between the groups than the individual assays alone. Common analysis conducted in microbiome studies is to evaluate the within-sample diversity ($\alpha$-diversity) and between-sample diversity ($\beta$-diversity), and identify specific bacterial taxa that may act as potential biomarkers for the biological process at hand. Several mathematical and statistical tools have been employed in these studies such as standard measurements of specific bacterial taxa and/or reporting of ratios, supervised and unsupervised learning including clustering, principle component analysis and principle coordinate analysis. These studies have revealed a dysbiosis of gut microbiota in patients with intestinal inflammation, although there is an overlap in fecal microbiota between the healthy and diseased populations (Gevers et al. 2014; Vazquez-Baeza et al. 2016). For example, one study reported a reduction in Faecalibacterium prausnitzii and a Firmicutes/Bacteroidetes ratio as consistent changes in IBD patients, and suggested using $F$. prausnitzii as potential biomarker for IBD (Sokol et al. 2009). In one study, dysbiosis associated with IBD in humans was characterized by a decrease in the ratio between $F$. prausnitzii and E. coli (Duboc et al. 2013). Another study proposed a sequencing-based microbial DI, which is defined as the logarithm of the ratio between the total abundance in organisms increased in Crohn’s Disease (CD) (e.g. Enterobacteriaceae, Pasteurellaceae, Veillonellaceae and Fusobacteriaceae) and the total abundance of organisms decreased in CD (Erysipelotrichales, Bacteroidiales and Clostridiales) (Gevers et al. 2014). Another recently developed diagnostic test for fecal dysbiosis in humans, the ‘GA-map test’, utilizes 54 DNA probes for the measurement of bacteria at various taxonomic levels (Casen et al. 2015), and showed dysbiosis in 70% of patients with treatment-naïve IBD, in 80% of IBD in remission patients and in 16% of healthy individuals. Recently, a sequencing-based DI was also reported for dogs with IBD, showing separation between diseased and healthy dogs with an AUC of 0.93 (Vazquez-Baeza et al. 2016). A current limitation of sequence-based analysis of the microbiota is the relative long turnaround time for results, especially when compared to qPCR assays, which as reported here, can be performed within one single day. Such rapid turnaround assays may prove useful to identify and track the fecal dysbiosis due to CEs in dogs over time and in response to treatments. Furthermore, even in indices developed based on sequencing techniques, only certain bacterial taxa are included in the final dysbiosis model (Gevers et al. 2014; Vazquez-Baeza et al. 2016) The bacterial taxa included in the final canine qPCR-based DI have all been shown to be altered in previous sequencing and qPCR-based studies between healthy dogs and dogs with intestinal inflammation. For example, Faecalibacterium and Turicibacter were shown to be decreased in dogs with IBD (Suchodolski et al. 2012b; Rossi et al. 2014; Minamoto et al. 2015; Vazquez-Baeza et al. 2016). Fusobacterium was decreased in dogs with IBD, although interestingly it is typically increased in fecal samples of humans with IBD (Minamoto et al. 2014; Vazquez-Baeza et al. 2016; Alshawafqeh et al. 2017). Bifidobacterium was significantly decreased in dogs with IBD and chronic diarrhea (Minamoto et al. 2014; Vazquez-Baeza et al. 2016). Escherichia coli was shown to be increased in dogs with IBD (Minamoto et al. 2014). Streptococcus was increased in dogs with IBD (Vazquez-Baeza et al. 2016; White et al. 2017). Clostridium hiranonis was also decreased in dogs with IBD (Vazquez-Baeza et al. 2016; Alshawafqeh et al. 2017). The latter bacterial group is of interest, as it has bile acid 7 alpha-dehydroxylating activity, and a decrease in $C$. hiranonis may suggest bile acid dysmetabolism, which has been shown to be present in humans with IBD (Duboc et al. 2013) and also in dogs in pilot studies (Guard and Suchodolski 2016; Kent et al. 2016). While all of the bacterial taxa analyzed were significantly different between healthy dogs and dogs with CE, the combined results expressed as DI had the highest discriminatory power. This is explainable due to the known individuality in the abundances of specific bacteria taxa between dogs (Guard and Suchodolski 2016), and measurement of the abundance of one single taxon cannot distinguish between health and disease with high accuracy.

Future studies will need to evaluate the clinical utility of the DI as an assessment tool for microbiota dysbiosis associated with CEs, and the usefulness of tracking microbiota over time and in response to treatment. It is well established that CEs involve a multifactorial disease, and the microbiota is just one of several factors in the pathogenesis of the disease. This is also likely the cause for the overlap in the DI seen between healthy and diseased dogs, which has also been reported in humans with IBD (Gevers et al. 2014). Therefore, the DI may be helpful to assess whether the microbiota is normal or dysbiotic in CE, as an optimal assessment of CE would encompass assessment of intestinal and systemic inflammation, tissue damage, immune function and microbiota dysbiosis. When more validated treatment options for dysbiosis (e.g. fecal microbiota transplantation) become available, using microbiota assessments tools such as the DI may help the clinician whether the microbiota returns to a normal state. This warrants further clinical studies.

The initial evaluation performed here shows that the DI is not affected by age, body weight and gender. Dietary history was only available from approximately half of the dogs. The initial data suggest that there was no impact of protein, fiber and fat content on the microbiota index. Although dogs with CE were fed diets with lower protein content, these were similar in the range to the protein content fed to the healthy dogs. Accordingly, there was no correlation with protein content and DI, as has also been shown in previous studies (Minamoto et al. 2015; Vazquez-Baeza et al. 2016). While high dietary protein intake may have an effect on the intestinal microbiota of dogs, this is typically only seen in diets that have a very high protein content, higher than fed in the majority of commercial diets (Herstad et al. 2017; Sandri et al. 2017). Furthermore, increase in protein typically leads to increase in $E$. coli and decrease in Faecalibacterium (Herstad et al. 2017; Sandri et al. 2017), which would be in contrast to the findings of our study. Therefore, consistent with our previous findings, diet did not appear to be a confounding factor for our results. However, it is possible that the study was not powered enough to detect diet as a confounder, and this is a limitation of the study. Some dietary factors cannot be assessed easily. For example, the quality of the dietary macronutrients (e.g. protein source) will affect how much protein reaches the colon and may serve as microbial substrate. Furthermore, since dogs were on a variety of different diets, it was also not possible to assess the effect of soluble vs non-soluble fiber content. Comparisons of healthy vs diseased dogs on the same diet would be ideal to assess the effect of diet as a confounding factor. Furthermore, breed and age-matched case controlled studies would also be useful to elucidate effects of these other environmental factors on the microbiota.

In conclusion, we report a rapid qPCR-based DI for the assessment of microbial dysbiosis in fecal samples of dogs. The DI may allow for tracking whether the microbiota normalizes in response to treatment (i.e. DI falls below 0).

SUPPLEMENTARY DATA
Supplementary data are available at FEMSEC online.
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Conflict of interest. None declared.

REFERENCES


