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Diagnostic value of fecal cultures in dogs with chronic diarrhea

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Abstract

Background: Culture-based assessment of the fecal microbiome using fecal culture profiles frequently is performed in dogs with chronic diarrhea, but the diagnostic value of this approach has not been determined.

Objectives: To compare the reported results of fecal culture profiles and the polymerase chain reaction-based dysbiosis index (DI) between dogs with chronic diarrhea and healthy dogs; to assess interlaboratory variability in bacterial and fungal cultures among 3 veterinary diagnostic laboratories (diagnostic laboratory 1 [L1], diagnostic laboratory 2 [L2], diagnostic laboratory 3 [L3]); and to compare the reported interpretation of culture profiles (normobiosis versus dysbiosis) with those of the DI.

Animals: Eighteen dogs with chronic diarrhea (CDG) and 18 healthy control dogs (HG).

Methods: In this prospective, case-control study, fecal samples were submitted to 3 commercial laboratories for fecal culture. The microbiota was assessed using PCR assays. Dogs receiving antimicrobials were excluded.

Results: Dysbiosis index was significantly increased in CDG (mean, 0.9; SD, 3.8; 95% confidence interval [CI], −1.0; 2.8) compared to HG (mean, −3.0; SD, 2.8; CI, −4.3; −1.6; P = .0002), whereas cultures from all laboratories failed to detect significant differences (P = .66, .18, and .66, respectively). Hemolytic *Escherichia coli* was the only potential enteropathogen on culture, but no significant difference was found between CDG and HG. For diagnosis of dysbiosis, culture showed no agreement with DI (L1, κ = −0.21; CI, −0.44; −0.02; L2, κ = −0.33; CI, −0.58; −0.08; L3, κ = −0.25; CI, −0.39; −0.11). Furthermore, variability among the 3 laboratories was high (L1/L2, κ = 0.15; CI, −0.05; 0.35; L1/L3, κ = −0.08; CI, −0.01; −0.16; L2/L3, κ = −0.06; CI, −0.33; −0.20).

Conclusions and clinical importance: Fecal cultures failed to distinguish between diseased and healthy dogs, and a high level of interlaboratory variation for culture was found.

KEYWORDS
antibiotic, canine, chronic enteropathy*Escherichia coli*, interlaboratory

Abbreviations: CDG, chronic diarrhea group; DI, dysbiosis index; HG, healthy group; L1, diagnostic laboratory 1; L2, diagnostic laboratory 2; L3, diagnostic laboratory 3.

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1 | INTRODUCTION

Culture-based assessment of feces is a diagnostic tool that should be used to identify specific or opportunistic enterpathogenic bacteria (eg, *Salmonella* spp., *Campylobacter jejuni*, specific enteropathogenic *Escherichia coli* strains, *Yersinia* spp., *Clostridium perfringens*, *Clostridium difficile*) and fungi in animals showing clinical signs associated with infectious acute or chronic diarrhea.[1-3] Several commercial veterinary diagnostic laboratories offer fecal culture as a tool to assess microbial composition (ie, growth of gram-negative and gram-positive flora) and, furthermore, to provide treatment recommendations based on their own interpretation of normobiosis and dysbiosis. Doing so is problematic, because aerobic culture-based methods do not adequately represent the mostly anaerobic intestinal microbiota. Several limitations are associated using fecal culture to diagnose the cause of diarrhea, such as lack of standardization with regard to sampling technique (eg, amount of feces), shipping (eg, chilled vs. room temperature), and methodology (eg, culture media, subsampling, dilution error, method used to count colonies) among different laboratories. However, it should be implicit that submission of a fecal sample to a diagnostic laboratory is a request to confirm the diagnosis of an infectious disease. Fecal cultures should not be submitted to infer what constitutes normal versus abnormal feces, especially in dogs with chronic diarrhea. In addition, the diagnostic value of fecal cultures in dogs with chronic diarrhea without signs of systemic inflammation is questionable,[4] especially because putative bacterial enteropathogens frequently are isolated from healthy dogs.[4-7] Thus, the clinical utility of this method for identifying potentially enteropathogenic bacteria in dogs with chronic diarrhea is unclear.

Novel molecular genetic-based tests have been developed to assess the microbiota and have identified complex bacterial communities in the intestine of dogs. Compositional changes in the microbiota have been documented in dogs with chronic enteropathies and may play a role in the pathogenesis of the disease.[8,9] Some of these molecular genetic-based tests for dysbiosis are rapid PCR-based methods and represent promising tools for assessment of dysbiosis in dogs with chronic diarrhea.[10,11]

Chronic diarrhea in dogs has been defined as having a duration of ≥3 weeks.[12] The first stage of the diagnostic evaluation usually aims to rule out extraintestinal diseases and parasites.[12,13] Although imaging and histopathological evaluations frequently are restricted to patients with severe clinical signs, gastrointestinal protein loss, or suspicion of neoplastic infiltration or invasive infectious agents, fecal cultures sometimes are included in the first routine evaluation of these cases. More specifically, clinicians frequently submit fecal samples for cost-intensive "fecal culture profiles", although studies evaluating the diagnostic utility of fecal cultures in dogs with chronic diarrhea are lacking.[4,14]

We hypothesize that the diagnostic value of fecal culture profiles in dogs with chronic diarrhea is questionable. Thus, we aimed to (1) compare the results of fecal cultures and the interpretations provided by 3 diagnostic laboratories in dogs with chronic diarrhea and healthy control dogs; (2) compare these results to the interpretation of the PCR-based dysbiosis index (DI) to verify dysbiosis; and (3) to assess interlaboratory variability in bacterial and fungal cultures among 3 commercial laboratories (diagnostic laboratory 1 [L1], diagnostic laboratory 2 [L2], diagnostic laboratory 3 [L3]).

2 | MATERIAL AND METHODS

2.1 | Animals

Our study was designed as a prospective, case-control (1:1) trial and was approved by the animal care and use committee (Ethikkommission) of the Centre for Clinical Veterinary Medicine LMU, Munich (reference 156-07-02-2019).Two groups of 18 dogs each were included in the study, 1 consisted of dogs with chronic diarrhea (CDG) and 1 of dogs without clinical signs serving as controls (HG). All dogs were recruited between February and November 2019 by the same clinician (MW). Dogs of either sex, neuter status, body weight, and at least 1 year of age were included. The HG included dogs presented for vaccination or annual health checkups and these dogs had no clinically relevant history or findings on physical examination. Exclusion criteria for HG were gastrointestinal signs or administration of antimicrobials or probiotics during the last 4 weeks before presentation. Only dogs with diarrhea of a minimum duration of 3 weeks were enrolled into the CDG. Exclusion criteria for CDG were administration of antimicrobials or probiotics during the 4 weeks before to presentation, clinical, or laboratory findings suggesting the necessity of antimicrobial treatment (rectal temperature > 39.0°C [102.2 °F], white blood cell count $<5 \times 10^9$/L or $>20 \times 10^9$/L, and band neutrophils $>1.5 \times 10^9$/L), suspicion or documentation of neoplastic infiltration of the intestine, and moderate to severe hypoalbuminemia (<2.3 g/dL). The clinical history of all dogs (HG and CDG) was recorded by using a standardized protocol with specific questions regarding the onset of diarrhea (CDG), fecal quality, number of defecations per day, vomiting, appetite, current treatment and previous treatment, other concurrent diseases, diet, and current dietary changes. The diagnostic evaluation in the CDG consisted of a CBC (in all dogs), serum biochemistry profile (ie, alanine aminotransferase, alkaline phosphatase, creatinine, urea nitrogen, total protein, albumin, glucose, sodium, potassium, chloride, phosphate, total calcium; all dogs), serum cobalamin and folate concentrations (14/18 dogs), basal serum cortisol concentration or adrenocorticotropin hormone stimulation test (14/18 dogs), fecal flotation (17/18 dogs), *Giardia* spp. ELISA (16/18 dogs), and abdominal ultrasound examination (16/18 dogs).

2.2 | Sample collection

Naturally passed feces from each dog were collected by the owner on the day of presentation. Fecal samples were mixed and immediately divided into 4 equally sized aliquots for fecal culture at 3 different commercial reference laboratories as well as microbiota analysis by quantitative PCR (qPCR), respectively. Fecal samples for culture were submitted to the 3 laboratories according to their instructions:

samples were sent on the same day to L1 and L2 by courier, and to L3 by regular mail. The aliquots for molecular genetics-based microbiota analysis were frozen at −80°C and were sent as a batch on dry ice to the Gastrointestinal Laboratory at Texas A&M University at the end of the study period. No information about the dogs' history was provided to any of the laboratories at the time of submission.

2.3 | Fecal culture

Each laboratory offered its unique test panel. Thus, the included tests and microbiological methods varied among the laboratories and were not standardized. The following diagnostic tests were offered at all 3 laboratories as part of their routinely offered "fecal culture profile": bacteriology (aerobically incubated), mycology, and specific testing for obligate and facultative pathogenic bacteria (*Salmonella*, *Campylobacter* spp., and *Yersinia* spp.). Laboratory 1 additionally performed a clostridial culture as part of its fecal profile. All 3 laboratories provided their own interpretation of test results (eg, "presence of abnormal flora" or "detection of pathogenic isolates" or both). Laboratory 1 and L2 subdivided the interpretation of the results into gram + and gram - spectrum of bacteria based on general microbiological nomenclature. Table S2 summarizes all results of tests performed for each individual dog.

2.4 | Microbiota analysis

2.4.1 | DNA extraction

The DNA was extracted from an aliquot of 100 mg of each fecal sample using a MoBio Power soil DNA isolation kit (MoBio Laboratories, Carlsbad, California) according to the manufacturer's instructions. The bead-beating step was performed on a homogenizer (FastPrep-24; MP Biomedicals, Santa Ana, California) at a speed of 4 m/s for 60 seconds. Fecal DNA was frozen at −80°C until further analysis.

2.4.2 | Quantitative PCR

The abundances of total bacteria and 7 bacterial taxa (ie, *Faecalibacterium* spp., *Turicibacter* spp., *Streptococcus* spp., *E. coli*, *Blautia* spp., *Fusobacterium* spp., and *Clostridium hiranonis*), which had been identified as being altered in dogs with gastrointestinal disease in previous studies, were quantified by specific qPCR assays. The results were used to calculate the previously described DI using a mathematical algorithm.[10] The technique, containing the oligonucleotide sequence of the primers and the annealing temperatures, has already been described in detail elsewhere.[10] A DI < 0 indicates normobiosis, whereas a DI ≥2 indicates dysbiosis, and values between 0 and 2 are considered equivocal. The abundances of *C. perfringens* 16S rRNA and *C. perfringens* enterotoxin genes in feces were quantified by qPCR assays using previously reported oligonucleotide primers and assay conditions.[15] The PCR conditions were 95°C for 20 seconds, 40 cycles at 95°C for 5 seconds, and 10 seconds at the optimized annealing temperature. For probe-based assays, the master mix consisted of 10 μL of TaqMan reaction mixtures, consisting of 5 μL of TaqMan Fast Universal PCR master mix (2×), No AmpErase UNG (Applied Biosystems, Foster City, California), 0.4 μL of each primer (concentration, 400 nM), 0.2 μL of the probe (concentration, 200 nM), 1 μL of 1% bovine serum albumin (BSA; concentration, 0.1%), 1 μL of water, and 2 μL of DNA (1:10 or 1:100 dilution). For *N′*,*N′*-dimethyl-*N*-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-*N*-propylpropane-1,3-diamine-based (SYBR) assays, PCR procedures ran at 95°C for 2 minutes, 40 cycles at 95°C for 5 seconds, and 10 seconds at the optimized annealing temperature with 10 μL of SYBR-based reaction mixtures consisting of 5 μL of SsoFast EvaGreen supermix (Biorad Laboratories, Hercules, California), 0.4 μL of each primer (concentration, 400 nM), 1 μL of 1% BSA (concentration, 0.1%), 1.6 μL of water, and 2 μL of DNA (1:10 or 1:100 dilution). The oligonucleotide sequences of the primers, probes, and the annealing temperatures are presented in Table S1.

2.5 | Statistical analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Prism c7.0, GraphPad Software, San Diego, California) and a web-based program for calculating Cohen's kappa and weighted kappa values (https://www.graphpad.com/quickcalcs/kappa1/). The distribution of data was tested using the D'Agonisto-Pearson omnibus normality test. The association of categorical variables (ie, sex, gram-positive or gram-negative bacteria, or hemolytic or mucoid growing *E. coli*, *Proteus mirabilis*, *Klebsiella* spp., *Acinetobacter johnsonii*, alpha-hemolytic *Streptococcus* spp., aerobic spore-forming bacteria, *Enterococcus* spp., fungal, or *Clostridia* spp. between CDG and HG) and group (CDG or HG) were assessed using Fisher's exact test. Differences in continuous variables (ie, age, weight, DI, abundances of *Faecalibacterium* spp., *Turicibacter* spp., *Streptococcus* spp., *E. coli*, *Blautia* spp., *Fusobacterium* spp., *C. hiranonis*, total bacteria, *C. perfringens*, and *C. perfringens* enterotoxin gene) between CDG and HG were evaluated using an unpaired *t* test or the Mann-Whitney *U* test depending on their distribution.

The agreement in classifying the fecal microbiota as normobiotic and equivocal or dysbiotic by the DI and the 3 laboratories each (ie, abnormal vs normal microbiota, presence of growing gram-negative/gram-positive microbiota, hemolytic *E. coli*) was evaluated using Cohen's kappa (κ) coefficient.[16] For culture results with >2 categories (eg, mycology) a weighted kappa was calculated. The interpretation of Cohen's kappa coefficient can be found in the legend of Table 2.

3 | RESULTS

3.1 | Study population

Thirty-six dogs (CDG, n = 18; HG, n = 18) were included in the study. Frequency of breed, sex, body weight, and age did not differ between

TABLE 1 Demographics of dogs with chronic diarrhea and healthy control dogs

	CDG (n = 18)		HG (n = 18)		*P* value
Sex	10 male, 8 females		6 male, 12 females		.31
Neutered/intact	5/13		9/9		.31
Breeds	Mixed breed (4), Australian Shepard dog (1), Bracke (1), Cavalier King Charles Spaniel (1), Chow-Chow (1), Dalmatian (1), German Shepard dog (1), Magyar Vizsla (1), Maltese (1), Miniature Pinscher (1), Pug (1), Small Muensterlaender (1), Standard Poodle (1), Standard Schnauzer (1), Whippet (1)		Mixed breed (4), Labrador Retriever (3), Border Collie (2), Dachshund (2), Dobermann Pinscher (2), Beagle (1), Bearded Collie (1), Chihuahua (1), German Shepard Dog (1), Goldendoodle (1)		
	Median	*Range*	*Median*	*Range*	
Body weight (kg)	14.5	2.7-30	20.6	3.5-33.3	.27
Age (years)	5.0	1.0-12.0	3.0	1.0-10.0	.16

Abbreviations: CDG, chronic diarrhea group; HG, healthy group; n, number of dogs.

TABLE 2 Comparison of agreement of microbiota analysis and fecal cultures between individual laboratories

Laboratory	Laboratory	Level of agreement	CI	Level of agreement
DI	L1	$\kappa = -0.21$	−0.44; −0.02	Disagreement
DI	L2	$\kappa = -0.33$	−0.58; −0.08	Disagreement
DI	L3	$\kappa = -0.25$	−0.39; −0.11	Disagreement
L1	L2	$\kappa = 0.15$	−0.05; 0.35	Poor agreement
L2	L3	$\kappa = -0.06$	−0.33; 0.20	Disagreement
L1	L3	$\kappa = 0.08$	−0.01; 0.16	Poor agreement

Note: Interpretation of Cohen's kappa (κ) value: $\kappa < 0$: disagreement; $0 \leq \kappa \geq 0.4$: poor agreement; $0.4 < \kappa > 0.75$: fair to good agreement; $\kappa \geq 0.75$: strong agreement.
Abbreviations: CI, confidence interval; DI, dysbiosis index; L1, diagnostic laboratory 1; L2, diagnostic laboratory 2; L3, diagnostic laboratory 3.

CDG and HG (Table 1). In CDG, the median duration of diarrhea was 13.5 (range, 1-96) months. Owners described soft stool quality in 9/18 (50%), watery diarrhea in 9/18 (50%), and intermittent hematochezia in 6/18 (33%) dogs. The number of defecations per day was increased (>3 times per day) in 11/18 (61%) dogs. Seven of 18 (39%) dogs had additional chronic vomiting and 4/18 (22%) had decreased appetite. Six of 18 (33%) dogs received a commercial diet, 5/18 (28%) a hydrolyzed protein diet, 4/18 (22%) a commercial single protein/single carbohydrate diet, and 3/18 (17%) a home-cooked diet. Six of 18 dogs (33%) received medication at the date of presentation (levothyroxine PO [n = 2], pancreatic enzymes PO [n = 1], pantoprazole PO [n = 1], prednisolone PO [n = 1], oclacitinib PO [n = 1], omeprazole PO [n = 1], metamizole PO [n = 1], cobalamin supplement PO [n = 1]). Drugs and dietary supplements (excluding antimicrobials) that the patients received in the 2 years before presentation included: fenbendazole PO, toltrazuril PO, probiotic PO, metamizole PO, omeprazole PO, maropitant PO, prednisolone PO, pantoprazole PO, tramadol PO, meloxicam PO, and cyclosporin PO. Antimicrobials that were administered to the dogs at least 2 months before presentation were: metronidazole PO (9/18 in total: 1/18, 2 months; 2/18, 3 months; 3/18, 4 months; 1/18, 7 months; 1/18, 12 months; and 1/18 24 months before sample collection), amoxicillin-clavulanic acid PO (2/18; 2 and 10 months previously), and trimethoprim-sulfonamide PO (1/18; 3 months previously). Concurrent diseases in CDG included dermatological disorders (2/18), hypothyroidism (2/18), and orthopedic problems (1/18).

3.2 | Comparison of microbiota analysis by qPCR between CDG and HG

The DI was significantly higher ($P = .0002$) in CDG (mean, 0.9; SD, 3.8; 95% CI, −1.0; 2.8) in comparison to HG (mean, −3.0; SD, 2.8; CI, −4.3; −1.6; Figure 1). An increased DI (> 2) was found in 44% (8/18) of the dogs of the CDG and in 6% (1/18) of the HG. The abundance of *Faecalibacterium* ($P = .01$) and *Fusobacterium* ($P = .03$) was significantly decreased in the CDG in comparison to the HG (Figure 1), whereas abundances of *Turicibacter*, *Streptococcus*, *E. coli*, *Blautia*, and *C. hiranonis* were not significantly different between CDG and HG. All dogs with a decreased abundance of *C. hiranonis* in both groups had a DI > 2. Only 1 of the CDG dogs with DI > 2 had a normal abundance of *C. hiranonis*, but in this dog *Streptococcus* was increased (Figure 1). Polymerase chain reaction was performed for *C. perfringens* and *C. perfringens* enterotoxins genes, but no difference between CDG and HG could be identified. *Clostridium perfringens* was found in 16/18 (89%) dogs of CDG and in all dogs (100%) of the HG.

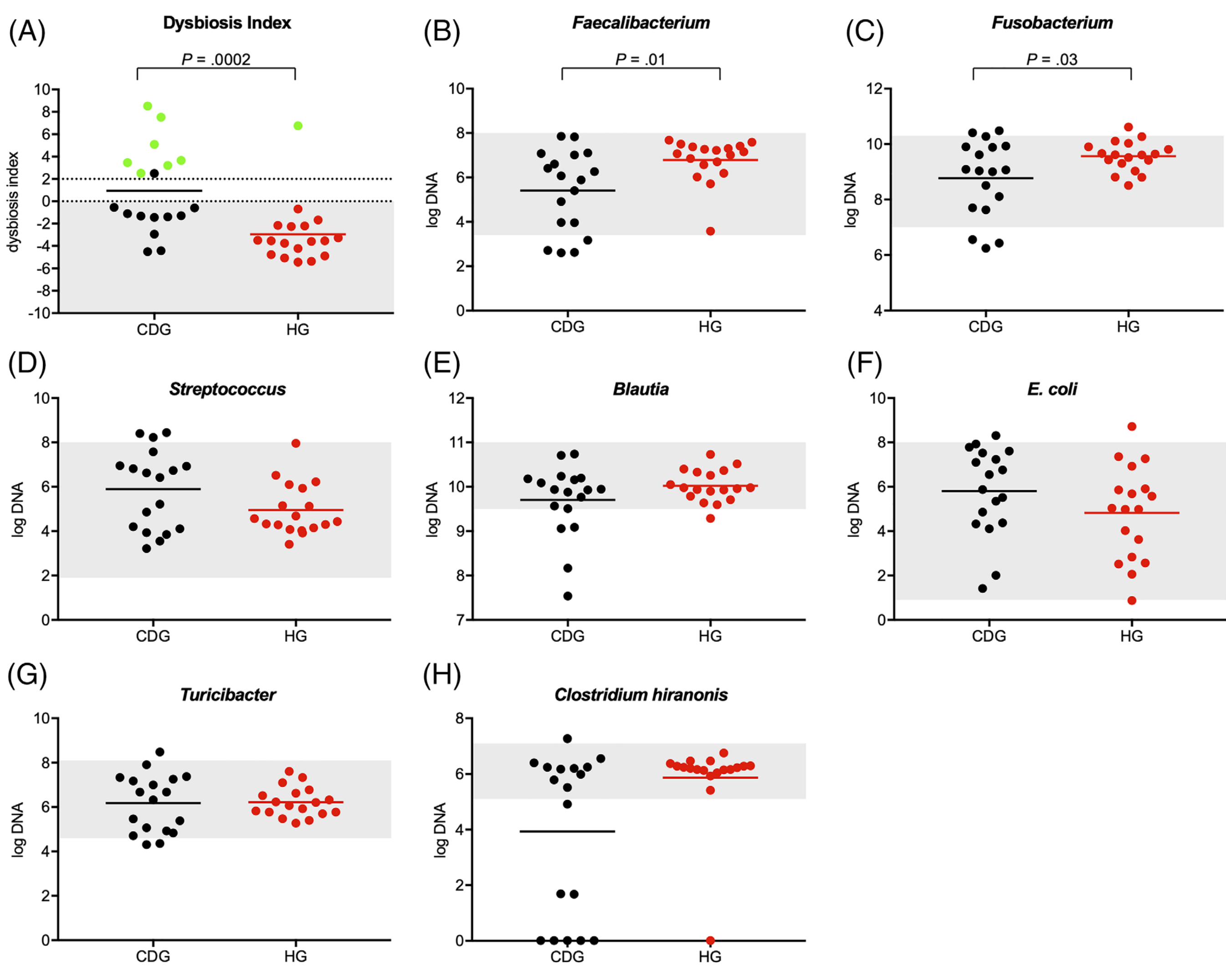

FIGURE 1 Dysbiosis index. This figure shows the dysbiosis index, A, and the abundances of *Faecalibacterium*, B, *Fusobacterium*, C, *Streptococcus*, D, *Blautia*, E, *Escherichia coli*, F, *Turicibacter*, G, and *Clostridium hiranonis*, H, in dogs with chronic diarrhea (CDG) and healthy control dogs (HG). Dots show individual dogs, bars show the means for each group. The reference intervals are shaded in grey. In A, green dots represent dogs with a decreased abundance of *Clostridium hiranonis*. A dysbiosis index <0 indicates normobiosis. A dysbiosis index above 2 indicates dysbiosis. The interval between 0 and 2 is defined as equivocal

3.3 | Comparison of fecal cultures between CDG and HG

Fecal culture results from all 3 laboratories were not significantly different between CDG and HG. The tests offered, the results, and their interpretation differed among the 3 laboratories and, therefore, main findings are presented for each laboratory separately. The term "normal or abnormal microbiota" was used as interpretation by the laboratories themselves when the results of cultures were reported.

Laboratory 1 (L1) reported "abnormal microbiota" in 14/18 dogs of CDG and in 16/18 dogs of HG (*P* = .66; Figure 2). Gram-negative bacteria were not found in 2 dogs of each group. Gram-positive bacteria could not be cultured in 3 dogs from each group. The only identified bacteria considered as potential enteropathogen by the laboratory was hemolytic *E. coli* in 4/18 CDG and 8/18 HG dogs (*P* = .29; Figure 3). Only L1 tested for anaerobic bacterial growth. *Clostridium* spp. (>1 million colony forming units/g) was found in 8/18 of CDG and in 10/18 of HG. All reported bacterial groups are summarized in Table 3. Unrequested susceptibility testing for antimicrobials was routinely provided for 6 different bacterial isolates (ie, *Acinetobacter* spp., *Buttiauxella ferragutiae*, hemolytic *E. coli* [not in cases with a low growth rate], mucoid growing *E. coli*, *Enterobacter cloacae*, and *Klebsiella variicola*).

Laboratory 2 (L2) interpreted the bacterial microbiota as "abnormal" in 5/18 dogs of CDG and 10/18 dogs of HG (*P* = .18; Figure 2). Gram-negative bacteria were present in 16/18 dogs of each group. Gram-positive bacteria were cultured in 17/18 dogs of CDG and 12/18 dogs of HG. Hemolytic growing *E. coli* were documented in 2/18 dogs of CDG and 4/18 dogs of HG (*P* = .66; Figure 3). No other potential enteropathogens were isolated from any of the samples.

Laboratory 3 (L3) reported "abnormal microbiota" in 4/18 dogs of CDG and 2/18 dogs of HG (Figure 2; *P* = .66) Hemolytic *E. coli* were

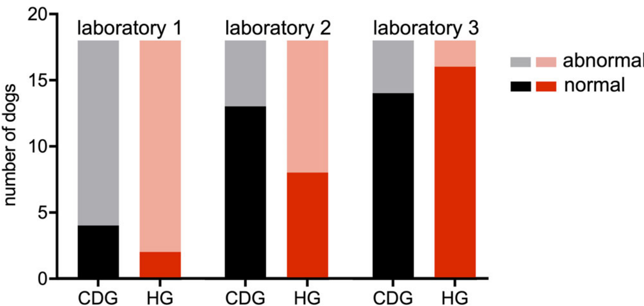

FIGURE 2 Fecal culture interpretation. Interpretations of the fecal culture results for dysbiosis given by the 3 commercial laboratories in dogs with chronic diarrhea (CDG) and healthy control dogs (HG). There was no significant association between the interpretation provided by each laboratory and group (CDG or HG)

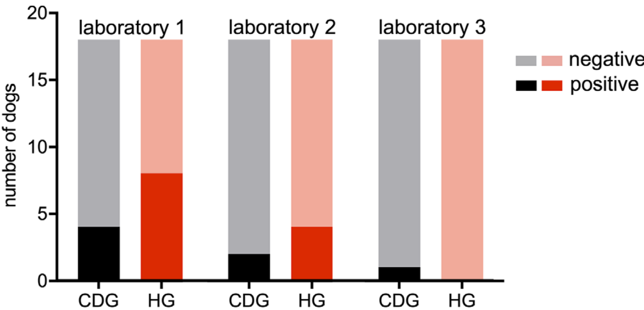

FIGURE 3 Growth of hemolytic *Escherichia coli*. Comparison of growth of fecal hemolytic *E. coli* reported by 3 different commercial laboratories in dogs with chronic diarrhea (CDG) and healthy control dogs (HG). There was no significant association between the growth of hemolytic *E. coli* as reported by each laboratory and group (CDG or HG)

TABLE 3 Bacterial isolates reported by laboratory 1 in dogs with chronic diarrhea and healthy control dogs

n (CDG)	n (HG)	Bacterial isolate	*P* value
0	2	*Acinetobacter* spp.	.49
5	6	Aerobic, spore-forming bacteria	>.99
6	7	Alpha-hemolytic *Streptococcus* spp.	>.99
1	0	*Bacillus cereus*	>.99
1	0	*Buttiauxella ferraguti*	>.99
8	10	*Clostridium* spp.	.74
1	0	*Enterobacter cloacae*	>.99
8	7	*Enterococcus* spp.	>.99
16	16	*Escherichia coli*	>.99
4	8	Hemolytic *E. coli*	.29
1	1	Mucoid growing *E. coli*	>.99
1	1	*Klebsiella* spp.	>.99
2	1	*Proteus mirabilis*	>.99
1	0	*Pseudomonas* spp.	>.99
0	1	*Staphylococcus epidermidis*	>.99

Abbreviations: CDG, chronic diarrhea group; HG, healthy group.

found in none of the HG and only in 1 dog of CDG (*P* > .99) and for this strain a sensitivity testing for antimicrobials was provided (Figure 3). As was the case for L2, no other enteropathogens were found by L3.

Fungal culture showed no significant difference in any variable between HG and CDG for any of the 3 laboratories. Fungal organisms were cultured in 4/18 (CDG) and 1/18 (HG) by L1 (*P* = .34), none of the samples by L2 (*P* > .99), and 3/18 (CDG) and 1/18 (HG) by L3 (*P* = .60; Table 4).

3.4 | Comparison of interpretation of microbiota analysis and fecal cultures among individual laboratories

For the following evaluation, all dogs in both groups were analyzed collectively. The overall assessment of "abnormal intestinal microbiota" for any of the 3 laboratories did not agree with the DI. Overall levels of agreement between each laboratory and the DI are shown in Table 2.

Agreement between L1 and L2 for the growth of gram-negative bacteria was fair to good (κ = 0.44; CI, −0.02; 0.89), but there was a disagreement on the growth of gram-positive bacteria (κ = −0.22; CI, −0.34; −0.10). Laboratory 3 provided no results on the growth of gram-positive or gram-negative bacteria.

Agreement on growth of hemolytic *E. coli* results was fair to good between L1 and L2 (κ = 0.57; CI, 0.29; 0.85), poor between L1 and L3 (κ = 0.11; CI, −0.09; 0.31), and there was overall disagreement between L2 and L3 (κ = −0.05; CI, −0.14; −0.04).

Assessment of fungal cultures showed disagreement or poor agreement among the 3 laboratories (L1 and L2: κ = 0.00; CI, 0.00; 0.00; L1 and L3: κ = 0.28; CI, −0.07; 0.63; L2 and L3: κ = 0.00; CI, 0.00; 0.00), and there was also poor agreement for the presence of *Candida* spp. (L1 and L2: weighted κ = 0.00; L1 and L3: weighted κ = 0.33; L2 and L3: weighted κ = 0.00).

4 | DISCUSSION

The objective of our prospective clinical trial was to compare the results of fecal cultures with the DI in dogs with chronic diarrhea. An important aspect is that the DI assesses the composition of the microbiota, whereas culture as performed and interpreted by the 3 laboratories attempted to both assess the microbiota as well as document the presence of enteropathogenic bacteria. Assessing microbiota composition based on culture should, however, not be the diagnostic method of choice to assess dysbiosis because of the limitations stated in the introduction. Nevertheless, offering fecal culture for this purpose is still common practice in several veterinary diagnostic laboratories. Our study did not detect any significant differences in commercial fecal culture results in dogs with chronic diarrhea compared to healthy dogs. Furthermore, agreement on the interpretation of normobiosis vs dysbiosis was found to be poor among the 3 laboratories. No putative

TABLE 4 Results of fungal cultures of dogs with chronic diarrhea and healthy control dogs

	Laboratory 1		Laboratory 2		Laboratory 3	
Isolate	n (CDG)	n (HG)	n (CDG)	n (HG)	n (CDG)	n (HG)
No growth	14	17	18	18	15	17
Candida spp. (mild)	2	1	-	-	-	-
Candida spp. (moderate)	1	-	-	-	1	1
Candida spp. (severe)	1	-	-	-	1	-
Geotrichophythum sp.	-	-	-	-	1	-

Abbreviations: CDG, chronic diarrhea group; HG, healthy group; n, number of dogs.

bacterial pathogens could be detected in any dog except hemolytic *E. coli* and the clinical relevance of this finding must be questioned because of the similar and often higher isolation rates in dogs of the HG. These findings raise the question as to whether routine fecal culture testing is of any use in dogs with chronic diarrhea.

The DI is a qPCR-based tool and has been used to assess fecal dysbiosis in dogs with chronic enteropathy,[10,17] dogs with acute diarrhea,[18,19] and healthy dogs after antimicrobial administration.[10,20,21] As shown in previous studies, a significant difference in the occurrence of dysbiosis was found between dogs in the CDG and the HG, with 8/18 dogs in the CDG having a DI > 2.

The etiology of chronic diarrhea in dogs is poorly understood but is presumed to be multifactorial. Several studies suggest a critical role of the intestinal microbiota,[8,22] and dysbiosis has been described in human and canine patients with chronic enteropathies (CE).[23,24] Documentation of changes in the intestinal microbiota is important, because dysbiosis is considered a factor in the pathogenesis of CE.[25] Alterations in the intestinal microbiota can lead to functional changes, such as a decrease in short-chain fatty acids[17] and abnormal bile acid metabolism.[18,20,26] For example, secondary bile acids have local and systemic anti-inflammatory properties and are an important driver of a healthy gut metabolism.[27] In the colon, primary bile acids are converted to secondary bile acids. The main converter of bile acids in dogs is *C. hiranonis*.[18,20,26] Decreased abundance of *C. hiranonis* leads to lack of conversion of primary to secondary bile acids, which is associated with an increased DI.[10,18,20,26] Decreased numbers of *C. hiranonis* also were associated with a lower fecal concentration of secondary bile acids and with an increased abundance of *E. coli*.[27] In our study, all dogs with decreased abundance of *C. hiranonis* had a DI > 2, including 8/18 in CDG and 1/18 in the HG. The latter dog had no signs of gastrointestinal disease and no medication history before sample collection. This finding suggests that a small subset of clinically healthy dogs can have subclinical dysbiosis. Interestingly, after 1 year this dog developed chronic diarrhea. Therefore, long-term studies are warranted to evaluate the effect of dysbiosis on developing chronic gastrointestinal signs and the potential role of DI as an early marker for chronic gastrointestinal disease.

Abundances of *Faecalibacterium* and *Fusobacterium* were decreased in some of the dogs of CDG. Decreased *Faecalibacterium* is a consistent finding in dogs and people with CE and gained attention because of their ability to secrete anti-inflammatory peptides in in vitro studies.[28,29] Although, most of the time, the causal relationship between dysbiosis and disease remains unclear, it seems important to recognize intestinal dysbiosis so as to incorporate this information into individual treatment strategies. Besides treatment of the underlying disease process in dogs with CE (eg, food-responsive disease, immune-mediated inflammation), restoration of the normal microbiota might be useful as an adjunctive treatment.

The culture results of all 3 laboratories failed to detect any difference between CDG and HG. The definition of an "abnormal microbiota" varied broadly among the laboratories and none of the laboratories provided information about their diagnostic criteria, thus emphasizing that a clear consensus is lacking when using fecal culture. Laboratory 1 routinely gave unrequested detailed information about the isolates identified and suggested a dysbiotic state in all but 2 of the HG dogs and in all but 4 of the CDG dogs. In comparison, L2 and L3 concluded dysbiosis less frequently in both groups of dogs. Surprisingly, more dogs with an interpretation of "abnormal microbiota" were reported in the HG compared to the CDG by both L1 and L2. This finding is in contrast to the results of the DI and to observations from studies showing that changes in the microbiota are associated with CE but usually not present in healthy individuals. Hemolytic *E. coli* were the only identified bacteria considered as facultative pathogens. Historical data suggest that the ability of *E. coli* to hemolyze erythrocytes is linked to different virulence factors, and these isolates can be the causative agent in extraintestinal diseases (eg, urinary tract infections, wound infections).[30,31] However, hemolytic *E. coli* are part of the normal intestinal microbiota of healthy individuals and the pathogenic role of hemolytic *E. coli* strains in CE is not clear.[32] Moreover, none of the laboratories provided information on what specific hemolytic *E. coli* strain was present and whether it was a pathogenic isolate or not. Other classic enteropathogens, such as *Salmonella* spp., thermophilic *Campylobacter* spp., and *Yersinia enterocolica* were not found in any of the samples, which support the idea that enteropathogens do not play a major role in dogs with CE.

Laboratory 1 and L3 provided unrequested antimicrobial susceptibility testing for hemolytic *E. coli* as part of the fecal panel. Sensitivity testing was based only on selected isolates found on the agar plates. Thus, it does not reflect the resistance pattern of all (hemolytic) *E. coli* in the intestines. Unjustified antibiotic usage can lead to a higher proportion of resistant *E. coli* isolates in canine feces.[19] By providing sensitivity testing of facultative enteropathogens in dogs with chronic diarrhea, veterinarians might get the impression that antibiotics are indicated in these cases. However, antibiotic treatment should not be

based on fecal culture findings. Specialists in veterinary gastroenterology strongly suggested in a recent proposal for rational antibacterial use in dogs with chronic diarrhea that antibiotics should be reserved for those dogs with evidence of true infection (ie, signs of systemic inflammatory response syndrome or evidence of adherent-invasive bacteria in intestinal biopsy samples).[33] Untargeted use of antibiotics based on fecal culture results likely contributes to the spreading of resistant bacteria and stands in contrast to principles of responsible antibiotic stewardship.[34,35]

Laboratory 1 and L2 separated between gram-positive and gram-negative bacteria based on general microbiological nomenclature in their results. Interestingly, both laboratories found no gram-negative bacteria in 8 dogs, but were in agreement only in 2 of these dogs. Laboratory 1 failed to detect any gram-positive bacteria in 7 dogs, and L2 in 6 dogs. Both laboratories only agreed for 1 dog. Two dogs in L2 and 1 dog in L1 had neither a gram-positive nor a gram-negative cultivatable microbiota (all belonged to the HG). Primarily aerobically growing bacteria (eg, *Enterococcus* as part of the gram-positive microbiota and Enterobacteriaceae, such as *E. coli*, as part of the gram-negative microbiota) are cultivated in routine fecal cultures.[36-38] However because of sequencing techniques, it is known that strictly anaerobic bacteria predominate in the intestinal microbiota. Thus, failing to culture anaerobic bacteria from fecal cultures leads to an inadequate representation of the overall composition of the intestinal microbiota.[39-41] Our results emphasize that the absence or presence of cultivable microbiota impedes assessment of the composition of the intestinal microbiota.

Laboratory 1 identified increased growth of *Clostridium* spp. in 50% of the samples, which was defined as "abnormal" by the laboratory itself. According to literature, this percentage is considered low, because with appropriate sample handling and culture techniques *C. perfringens* can be identified in feces of 80% healthy dogs.[42,43] *Clostridium* spp. are characterized by anaerobic growth.[43] Under aerobic conditions, which are usually present during transportation and shipping of fecal samples, certain clostridial strains can form spores within minutes, which can require specialized culture methods to induce germination. Thus, the presence of *Clostridia* spp. might be underestimated by culture.[44,45] There was no difference in *Clostridium*-positive samples between CDG and HG. The relevance of clostridial growth in dogs with intestinal disease is questionable, because it is not the presence of clostridial species, but the presence of certain enterotoxins that likely plays a pathogenic role (eg, in acute hemorrhagic diarrhea syndrome), and is associated with clinical signs.[46-48]

In comparison, PCR detected the *C. perfringens* 16S rRNA gene in all except 2 samples. Samples from dogs of the CDG did not have a higher abundance of the gene compared to HG. Thus, our findings further support the notion that clostridial strains, in general, and *C. perfringens*, in particular, are considered unlikely to have played a role in the pathogenesis of chronic diarrhea in the present study population.

Aerobic fungi were only isolated from a few samples, with the majority consisting of *Candida* spp, and no difference was found between CGD and HG. This finding is consistent with recent molecular genetic-based investigations that documented a similar abundance of *Candida* spp. in fecal samples from dogs with acute diarrhea and healthy dogs.[49] Studies in humans showed that the fungal microbiota might play a role in CE[50] and thus the inability of fungal culture to discern a difference between groups in our study is concerning.

Significant disagreement was found among laboratories in the interpretation of abnormal microbiota. Factors that explain differences could include random errors, a systematic bias of the analytical procedure, application errors within the laboratories, interpretation errors, and preanalytical (including transport) errors.[51,52] Transportation of samples differed and was based on the instructions provided by the individual laboratories. Moreover, bias could be caused by different subsampling methods, which can either take place in the clinic by dividing samples or in the laboratory.[55] Furthermore, differences in culture methods among laboratories potentially could have a substantial impact on culture results.

Establishing guidelines for adequate sample handling and shipping conditions would be essential for reproducible results. However, even if these procedures were to be standardized, definition of dysbiosis based on culture methods is not defined and primarily based on individual subjective interpretation. Consequently systematic bias could occur among different microbiologists.

Agreement between fecal culture results and DI generally was poor. The DI was significantly different between healthy and diseased individuals, whereas in contrast, fecal cultures did not show such a difference.

Our study had some important limitations. First, it was not possible to assess the laboratories' agreement on the assessment of the presence of enteropathogenic bacteria other than hemolytic *E. coli*, because these organisms were not found in any of the samples. However, the results do support that classic enteropathogens do not play an important role in dogs with CE. Second, information about the microbiological methods of the 3 commercial laboratories was not available. It can be assumed that methodological differences among the laboratories existed, which limits the direct comparability of results. However, an aim of the study was to assess the agreement of culture results among different laboratories from a clinical perspective, independent of their methods. Our findings show that clinicians might receive different results depending on the laboratory they choose. Third, it is difficult to define a gold standard for the evaluation of dysbiosis. However, recent studies have indicated that the results of the DI agreed well with more comprehensive sequencing methods.[18,20] Moreover, our results suggest that the DI is a relevant tool to assess dysbiosis in dogs with CE and healthy dogs, comparable to findings of previous studies.

Our results indicate that fecal cultures are not useful for identifying dysbiosis of dogs with CE. In fact, interpretation of culture results and routinely provided sensitivity testing for antibiotics can even be misleading and result in unnecessary antibiotic treatment. Fecal culture should be reserved for detecting enteropathogens without giving any recommendations on their treatment.

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CONFLICT OF INTEREST

Drs Suchodolski, Lidbury, and Steiner are employees of the Gastrointestinal Laboratory, which performs diagnostic testing, including the dysbiosis index, on a fee-for-service basis.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Prospective collection and analysis of canine fecal samples was approved by the Ethics Committee of the Centre of Veterinary Medicine, LMU, Germany (reference 156-07-02-2019).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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