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Prevalence of *Clostridium perfringens* Encoding the *netF* Toxin Gene in Dogs with Acute and Chronic Gastrointestinal Diseases

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Abstract: Several recent studies have reported a significantly greater prevalence of *Clostridium perfringens* encoding the novel pore-forming *netF* toxin gene in dogs with acute hemorrhagic diarrhea syndrome. However, the presence of *netF* in other canine diarrheal diseases remains poorly characterized. This retrospective, cross-sectional study aimed to describe the prevalence and abundance of *netF*-positive *C. perfringens* in fecal samples from 352 dogs with acute and chronic gastrointestinal diseases. Dogs were divided into five groups: acute hemorrhagic diarrhea syndrome (AHDS), acute diarrhea (AD), chronic enteropathy (CE), exocrine pancreatic insufficiency (EPI), and healthy controls (HCs). The abundances of *C. perfringens* 16S rRNA, the *C. perfringens* enterotoxin gene and the *C. perfringens netF* gene in fecal samples were analyzed by quantitative polymerase chain reaction. In total, 7 of 15 (46.7%) dogs with AHDS, 10 of 75 (13.3%) dogs with AD, 2 of 120 (1.7%) dogs with CE, 1 of 12 (8.3%) dogs with EPI, and 1 of 130 (0.8%) HC dogs tested positive for *netF*. This study provides further evidence that NetF may be a significant contributor to the etiology of AHDS and potentially to a subset of acute nonhemorrhagic diarrhea cases, while it was only rarely detected in chronic gastrointestinal disease phenotypes.

Keywords: *Clostridium perfringens*; pore-forming toxin; *netF*; canine; chronic enteropathy

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1. Introduction

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming bacterium ubiquitously found in the environment and is a commensal in the gastrointestinal tract of humans and animals [1,2]. The species, which was originally classified into five toxin types (A–E) on the basis of the production of four principal toxins (CPA, CPB, ETX, and ITX), has the ability to produce a diverse array of additional toxins. These toxins include the recently detected pore-forming toxins NetE, NetF, and NetG, which belong to the leukocidin/hemolysin superfamily [3].

Numerous predominantly enteric diseases in veterinary medicine, ranging from mild self-limiting diarrhea to fatal necrohemorrhagic enteritis (NE), are linked to overgrowth and toxin release by *C. perfringens*, potentially exacerbated by synergistic toxin interactions, coinfections, diet, and intestinal dysbiosis [4–6].

Nevertheless, the exact role of *C. perfringens* as an enteropathogen in dogs is not yet fully understood, given its presence in both diarrheic and healthy individuals [5]. However,

current evidence indicates that *C. perfringens* Type A strains encoding the novel pore-forming *netF* toxin gene are highly implicated in the pathogenesis of acute hemorrhagic diarrhea syndrome (AHDS) in dogs [7–9]. AHDS is characterized by an acute to peracute onset of hemorrhagic, watery diarrhea, often accompanied by vomiting as the initial clinical sign and severe fluid loss into the intestinal lumen [10]. Histological changes include severe necrosis of the intestinal mucosa and neutrophilic enterocolitis. Furthermore, clostridial antigens on the necrotic mucosal surface have been identified by immunohistochemistry, and in all five cases where *C. perfringens* was isolated, cultured, and subjected to PCR testing, the *netF* toxin gene was detected [11].

NetF-producing *C. perfringens* was first identified in 2015 from isolates linked to clinical cases of canine AHDS and foal necrotizing enteritis [12]. This discovery coincided with the identification of two additional novel pore-forming toxin genes, designated *netE* and *netG*. Notably, only *netF* exhibited a pronounced cytotoxic effect in vitro on an equine ovarian cell line and was found alongside the *netE* gene and *C. perfringens* enterotoxin gene (*cpe*) in all the cytotoxic isolates. Several previous studies support the hypothesis that pore-forming toxins play a significant role in the pathogenesis of AHDS in dogs, revealing a markedly greater prevalence of *C. perfringens* encoding NetF in fecal samples from dogs with AHDS than in those from canine parvovirus (CPV)-infected dogs and healthy control dogs [13–15]. However, there is a complete lack of data on the prevalence of *C. perfringens* encoding NetF in dogs with chronic gastrointestinal (GI) diseases, and only limited information is available for dogs with acute nonhemorrhagic diarrhea [16,17].

Thus, the objective of this study was to assess and compare the prevalence and abundance of *C. perfringens*, specifically strains encoding *netF* and enterotoxin genes, in fecal samples from dogs presenting with acute and chronic gastrointestinal diseases.

2. Materials and Methods

2.1. Study Design

This was a retrospective, cross-sectional study that utilized data and materials from dogs with four different gastrointestinal disease phenotypes, which were prospectively collected for previous studies. However, the data analyzed in this study have not been previously reported. Fecal samples were collected from several veterinary hospitals in the USA, Germany, and Switzerland between 2012 and 2024.

2.2. Study Population

2.2.1. Dogs with GI Disease

A total of 222 dogs with acute and chronic gastrointestinal diseases were included in the disease group. Dogs were eligible for inclusion if they could be classified into the following groups on the basis of their medical history, clinical signs, and diagnostic work-up: dogs with acute hemorrhagic diarrhea syndrome (AHDS), dogs with acute diarrhea (AD), dogs with chronic enteropathy (CE), and dogs with exocrine pancreatic insufficiency (EPI).

Inclusion in the AHDS group followed previously defined criteria [15,18,19], namely, dogs of any breed, age, or sex with acute hemorrhagic diarrhea lasting less than three days. Additionally, serum biochemistry, a complete blood count, an abdominal ultrasound, and a fecal examination were performed in all dogs to exclude cases with underlying diseases potentially causing hemorrhagic diarrhea (e.g., pancreatitis, intoxication, endoparasites, renal or liver failure, parvovirus infection, or foreign bodies).

Dogs were included in the AD group if they presented with acute uncomplicated nonhemorrhagic diarrhea lasting less than five days without any signs of systemic inflammation, sepsis, severe illness, or significant dehydration [16,17,20].

The dogs in the CE group were diagnosed on the basis of the presence of chronic gastrointestinal signs lasting longer than three weeks after other potential extraintestinal diseases causing GI signs were ruled out [21,22].

Dogs included in the EPI group were diagnosed on the basis of evaluations of medical records and a serum canine trypsin-like immunoreactivity (cTLI) concentration below the reference range.

2.2.2. Healthy Control Dogs

A total of 130 clinically healthy, privately owned dogs that were presented for routine health care services or dogs that belonged to students or employees of the veterinary hospitals were included in the healthy control group. Dogs were excluded if they had a recent history of gastrointestinal signs in the last six months.

2.3. Sample Handling

Archival fecal samples remaining from prior routine fecal examinations or diagnostic workups were examined for *C. perfringens* 16S rRNA, the *C. perfringens* enterotoxin gene, and the *netF* toxin gene by qPCR. Naturally passed fecal samples were collected from each dog and stored in Eppendorf tubes. The samples were frozen at −80 °C until analysis. They were then placed on dry ice and subsequently sent to the Gastrointestinal Laboratory at Texas A&M University for microbiota analysis.

2.4. Quantitative PCR (qPCR) Assay

The fecal samples were analyzed by qPCR for the abundance of the *C. perfringens* 16S rRNA gene, *C. perfringens* enterotoxin gene, and *C. perfringens netF* toxin gene using previously published oligonucleotide primers and assays (CPerf16SF, CPerf269R, CPerf1875, cpe F, cpe R, and netF-F) [12,23,24].

The PCR conditions were as follows: incubation at 95 °C for 20 s, followed by 40 cycles at 95 °C for 5 s and 10 s at the optimal annealing temperature. When conducting probe-based assays, the master mix contains a total of 10 µL of TaqMan reaction mixture consisting of 5 µL of TaqMan Fast Universal Master Mix (2×), No AmpErase UNG from Applied Biosystems(Waltham, Massachusetts, USA), 0.4 µL of each primer (400 nM final concentration), 1 µL of water, 0.2 µL of the probe (200 nM final concentration), 1 µL of 1% bovine serum albumin (BSA, 0.1% concentration), and 2 µL of DNA (1:10 or 1:100 dilution). When performing SYBR-based assays, SYBR-based reaction mixtures with a total volume of 10 µL contained 5 µL of SsoFast EvaGreen supermix from Bio-Rad Laboratories(Hercules, California, USA), 1.6 µL of water, 0.4 µL of each primer (400 nM final concentration), 1 µL of 1% BSA (0.1% final concentration), and 2 µL of DNA (1:10 or 1:100 dilution).

2.5. Statistical Analysis

The data analysis was performed using a commercially available statistical software (GraphPad Prism Version 10.2.3., San Diego, CA, USA). Data for the qPCR assays and signalment were assessed for normality via the D'Agostino–Pearson Omnibus normality test. For comparisons of signalment, Fisher's exact test (sex) and Kruskal–Wallis test (age, weight) were used. Fisher's exact test was used to compare the proportions of dogs positive for *C. perfringens* 16S rRNA or the *cpe* and *netF* toxin genes among the groups. Multiple comparisons were performed using Dunn's multiple comparison test. A Bonferroni correction was applied to adjust the *p* values. Spearman's rank correlation was used to evaluate the correlation between the abundance of *C. perfringens* 16S rRNA and the presence of the toxin genes. The odds ratio for the presence of *C. perfringens* encoding the *netF* toxin gene in dogs with gastrointestinal disease phenotypes compared with healthy

controls was tested using the Baptista–Pike method. The results were considered significant if $p < 0.05$.

3. Results

A total of 352 dogs were included in the study; 15 had AHDS, 75 had AD, 120 had CE, 12 had EPI, and 130 dogs were clinically healthy.

3.1. Signalment

There was no significant difference in age ($p = 0.204$) or sex ($p = 0.202$) between the GI disease phenotype groups. However, a significant difference in body weight ($p = 0.015$) was detected among the study population (Table 1). No information regarding weight, age, or breed was available for 27 dogs.

Table 1. Comparison of signalment (age, weight, and sex) among 352 dogs with different gastrointestinal disease phenotypes: acute hemorrhagic diarrhea syndrome (AHDS), acute diarrhea (AD), chronic enteropathy (CE), exocrine pancreatic insufficiency (EPI), and healthy controls (HC).

	HC	AHDS	AD	CE	EPI
n	130	15	75	120	12
Age (years)	4.5 (0.3–13.0) [a]	2.4 (0.5–10.0) [a]	5.0 (0.5–15.5) [a]	5.0 (0.7–13.0) [a]	6.5 (2.0–12.0) [a]
Weight (kg)	18.9 (1.6–75.0) [a]	14.3 (3.4–44.4) [ab]	15.0 (2.5–41.6) [a]	14.3 (1.7–72.6) [a]	31.4 (14.5–42.3) [b]
Sex					
Male	60 (13 intact) [a]	8 (1 intact) [a]	39 (21 intact) [a]	61 (24 intact) [a]	2 (1 intact) [a]
Female	70 (14 intact) [a]	7 (2 intact) [a]	36 (7 intact) [a]	59 (11 intact) [a]	10 (all spayed) [a]

Continuous data are presented as median (range). Rows that do not share a common superscript letter differ significantly from each other ($p < 0.05$).

3.1.1. Dogs with Acute Hemorrhagic Diarrhea Syndrome

The median age of the 15 dogs that presented with AHDS was 2.4 years (range: 0.5–10.0 years). The most frequently represented breeds were mixed-breeds ($n = 4$), Labrador Retrievers ($n = 2$), and Dachshunds ($n = 2$). Eight dogs were male (one intact and seven castrated) and seven dogs were female (two intact and five spayed).

3.1.2. Dogs with Acute Diarrhea

The median age of the 75 AD-affected dogs was 5.0 years (range: 0.5–15.5 years). The dogs belonged to 33 different breeds, with mixed-breed dogs being the most common ($n = 25$). The most common purebred dogs were Labrador Retrievers ($n = 7$), followed by Maltese ($n = 3$) and Golden Retrievers ($n = 3$). In this group, 39 dogs were male (21 intact and 18 castrated) and 36 dogs were female (7 intact and 29 spayed).

3.1.3. Dogs with Chronic Enteropathy

Among the 120 dogs diagnosed with CE, the median age was 5.0 years (range: 0.7–13.0 years). A wide variety of breeds were represented in this study population. The five most prevalent breeds were mixed breeds ($n = 22$), German Shepherds ($n = 6$), Cavalier King Charles Spaniels ($n = 6$), Australian Shepherds ($n = 5$), and Poodles ($n = 5$). Sixty-one dogs were male (24 intact and 37 castrated) and 59 dogs were female (11 intact and 48 spayed).

3.1.4. Dogs with Exocrine Pancreatic Insufficiency

In the group of 12 dogs with EPI, the median age was 6.5 years (range: 2.0–12.0 years). The most common breed was German Shepherd ($n = 8$), followed by Labrador Retriever,

Rough Collie, Chihuahua, and Spanish Water Dog ($n < 2$ dogs each). In this group, 2 dogs were male (1 intact and 1 castrated) and 10 dogs were female (all spayed).

3.1.5. Healthy Control Dogs

The median age of the 130 HC dogs was 4.5 years (range: 0.3–13.0 years). Overall, 34 breeds were represented, with the 3 most common breeds being mixed breeds ($n = 66$), Labrador Retrievers ($n = 11$), and Miniature Schnauzers ($n = 7$). Among the healthy control dogs, 60 were male (13 intact and 47 castrated) and 70 were female (14 intact and 56 spayed).

3.2. Comparison of the Prevalence of the C. perfringens 16S rRNA Gene Between Dogs with Different GI Disease Phenotypes

Significant differences in the prevalence (Table 2) and abundance (Table 3) were observed among the study population with different gastrointestinal disease phenotypes and healthy controls. *C. perfringens* 16S rRNA was detected in most of the fecal samples from dogs with acute ($n = 72/75$; 96.0%) and chronic gastrointestinal diseases ($n = 103/120$; 85.8%), dogs with AHDS ($n = 15/15$; 100.0%), dogs with EPI ($n = 11/12$; 91.7%), and healthy control dogs ($n = 99/130$; 76.2%). However, there was a significantly greater prevalence ($p < 0.001$) in dogs with AD compared with HCs.

Table 2. Prevalence of *C. perfringens* 16S rRNA, *C. perfringens* enterotoxin gene, and *C. perfringens* encoding the *netF* toxin gene in feces from healthy control dogs and dogs with acute hemorrhagic diarrhea syndrome, acute diarrhea, chronic enteropathy, and exocrine pancreatic insufficiency.

	HC (*n* = 130)	**AHDS (*n* = 15)**	**AD (*n* = 75)**	**CE (*n* = 120)**	**EPI (*n* = 12)**
C. perfringens 16S rRNA	99/130 (76.2%) ^{a}	15/15 (100.0%) ^{ab}	72/75 (96.0%) ^{b}	103/120 (85.8%) ^{ab}	11/12 (91.7%) ^{ab}
C. perfringens enterotoxin	38/130 (29.2%) ^{a}	5/15 (33.3%) ^{ab}	40/75 (53.3%) ^{b}	25/120 (20.8%) ^{a}	4/12 (33.3%) ^{ab}
netF toxin gene	1/130 (0.8%) ^{a}	7/15 (46.7%) ^{b}	10/75 (13.3%) ^{b}	2/120 (1.7%) ^{a}	1/12 (8.3%) ^{ab}
netF and *cpe* toxin genes	0/130 (0.0%) ^{a}	3/15 (20.0%) ^{b}	9/75 (12.0%) ^{b}	1/120 (0.8%) ^{a}	1/12 (8.3%) ^{ab}

Rows that do not share a common superscript letter differ significantly from each other ($p < 0.05$).

Table 3. Abundance of *C. perfringens* 16S rRNA, *C. perfringens* encoding the *cpe* gene, and *C. perfringens* encoding the *netF* toxin gene in dogs with acute and chronic gastrointestinal diseases and healthy controls. Values represent median (range) log DNA/g feces.

	***C. perfringens* 16S rRNA**		***cpe* Gene**		***netF* Toxin Gene**	
	Median	**Range**	**Median**	**Range**	**Median**	**Range**
HC	4.21	(1.13–7.90) ^{b}	1.84	(1.84–9.13) ^{b}	0.01	(0.01–1.39) ^{b}
AHDS	7.62	(5.00–8.82) ^{a}	2.74	(1.80–8.70) ^{ab}	0.10	(0.01–6.90) ^{a}
AD	6.51	(1.13–8.47) ^{a}	3.70	(1.80–9.08) ^{a}	0.10	(0.01–6.19) ^{a}
CE	4.39	(1.10–8.00) ^{b}	1.84	(1.80–7.50) ^{b}	0.10	(0.01–2.93) ^{a}
EPI	5.85	(1.13–7.87) ^{ab}	2.90	(1.84–8.74) ^{ab}	0.01	(0.01–3.52) ^{b}

Superscript letters indicate significant difference ($p < 0.05$) between gastrointestinal disease phenotypes and healthy controls.

There was no significant difference in the abundance of *C. perfringens* 16S rRNA between dogs with CE ($p > 0.999$) and those with EPI ($p = 0.073$) and healthy controls.

Nevertheless, a significantly greater abundance was observed in the AD and AHDS groups than in the healthy control and CE groups ($p < 0.001$) (Figure 1).

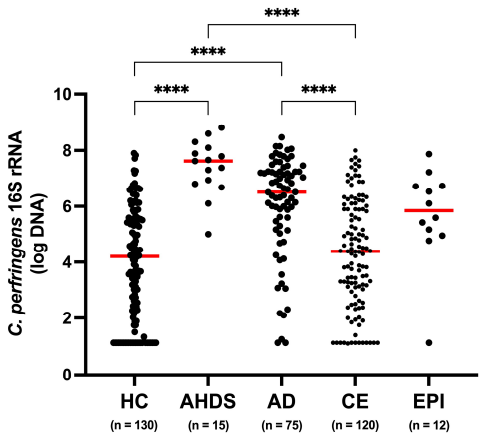

Figure 1. The figure shows the abundance of *C. perfringens* 16S rRNA in dogs with acute and chronic gastrointestinal diseases and healthy control dogs. The red horizontal lines indicate the median. Asterisks represent statistical significance (**** $p < 0.0001$).

3.3. Comparison of the Prevalence of C. perfringens Carrying the Enterotoxin Gene Between Dogs with Different GI Disease Phenotypes

The highest prevalence of enterotoxin-positive *C. perfringens* was observed in dogs with acute diarrhea, with 40 out of 75 (53.3%) testing positive for the *cpe* gene. This prevalence was significantly greater than that in healthy control dogs (29.2%; $p = 0.001$) and dogs with CE (20.8%; $p < 0.001$). In contrast, *C. perfringens* carrying the enterotoxin gene were less prevalent among the other GI disease phenotypes (AHDS 33.3%, EPI 33.3%), with no significant difference compared with healthy control dogs.

The abundance of enterotoxin-positive *C. perfringens* was significantly greater in dogs with acute diarrhea than in those with CE ($p < 0.001$) and HCs ($p = 0.028$), whereas no significant difference was detected between the other groups (Figure 2). No strong correlation was observed between the abundance of *C. perfringens* 16S rRNA and the presence of the enterotoxin gene, except in the groups of healthy and CE dogs, where a mild-to-moderate, but significant, correlation was found (Table S1).

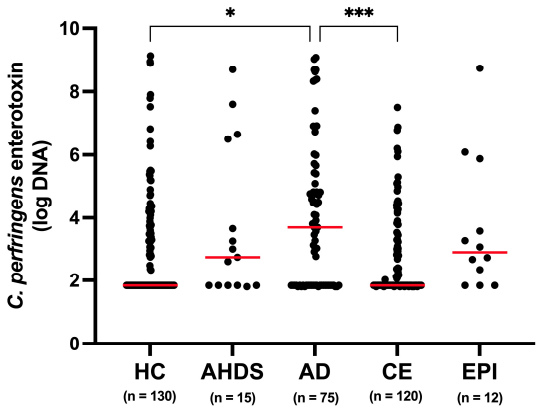

Figure 2. The figure shows the abundance of *C. perfringens* enterotoxin gene in dogs with acute and chronic gastrointestinal diseases and healthy control dogs. The red horizontal lines indicate the median. Asterisks represent statistical significance (* $p < 0.05$; *** $p < 0.001$).

3.4. Comparison of the Prevalence of C. perfringens Carrying the netF Toxin Gene Between Dogs with Different GI Disease Phenotypes

A significant difference ($p < 0.001$) in the presence of the *netF* toxin gene was detected in the fecal samples across the different GI disease phenotypes. The prevalence of the *netF* toxin gene was significantly greater in dogs with AHDS ($p < 0.001$) and AD ($p < 0.001$) than in healthy controls ($n = 1/130$; 0.8%). While a greater frequency of *netF* toxin gene-positive cases was detected in the AHDS group ($n = 7/15$; 46.7%) than in the AD group ($n = 10/75$; 13.3%), this difference was not statistically significant ($p = 0.067$).

The abundance of the *netF* toxin gene was significantly greater in dogs with AHDS ($p < 0.001$), AD ($p < 0.001$), and CE ($p < 0.001$) than in healthy controls. The abundance of the *netF* toxin gene was significantly lower in dogs with EPI than in those with AHDS ($p = 0.001$), CE ($p = 0.044$) and AD ($p = 0.032$), but no statistically significant difference was detected compared with the HC ($p > 0.999$) group (Figure 3). No strong correlation was observed between the abundance of *C. perfringens* 16S rRNA and the presence of the *netF* toxin gene (Table S2).

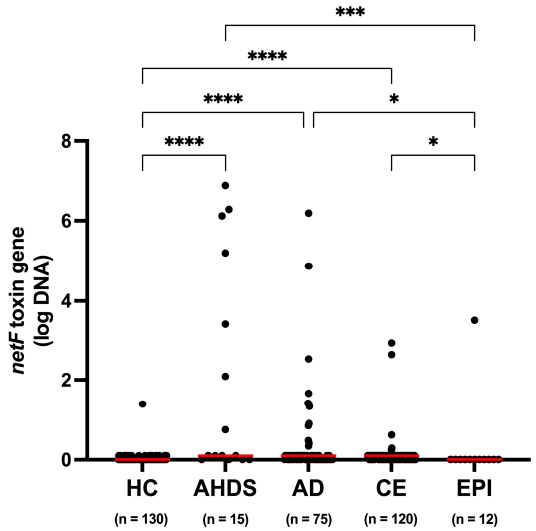

Figure 3. The figure shows the abundance of *C. perfringens* encoding the *netF* toxin gene in dogs with acute and chronic gastrointestinal diseases and healthy control dogs. The red horizontal lines indicate the median. Asterisks represent statistical significance (* $p < 0.05$; *** $p < 0.001$; **** $p < 0.0001$).

The odds ratios for identifying gastrointestinal disease phenotypes associated with the presence of the *netF* toxin gene are summarized in Table 4. Compared with healthy control dogs, dogs with acute hemorrhagic diarrhea syndrome (OR: 112.9; 95% confidence interval [CI]: 15.0–1270.0; $p < 0.001$) and those with acute diarrhea (OR: 19.9; [3.3–217.3]; $p < 0.001$) were significantly more likely to test positive for the *netF* toxin gene.

Table 4. Odds ratio for the presence of *C. perfringens* encoding the *netF* toxin gene in feces from dogs with acute and chronic gastrointestinal diseases compared to healthy control dogs. Significance was set a $p < 0.05$.

GI Phenotype	Odds Ratio	95% CI	*p* Value
AHDS	112.9	15.0–1270.0	<0.001
AD	19.9	3.3–217.3	<0.001
CE	2.2	0.3–31.9	0.609
EPI	11.7	0.6–223.5	0.162

4. Discussion

Over time, increasing evidence indicates that the presence of *C. perfringens*, encoding the novel pore-forming toxin NetF, which was first identified in 2015 [12], is strongly associated with acute hemorrhagic diarrhea syndrome (AHDS) in dogs, with reported prevalence rates ranging from 48 to 67% [13–15].

However, to date, there are no published data regarding the prevalence of *netF* toxin gene-positive *C. perfringens* in dogs with chronic gastrointestinal diseases, such as chronic enteropathy and exocrine pancreatic insufficiency. Furthermore, only limited information is available concerning the *netF* toxin gene in dogs with acute nonhemorrhagic diarrhea. This is the first study to assess and compare the prevalence of the *netF* toxin gene in both acute and chronic gastrointestinal diseases. Additionally, the prevalence of *C. perfringens* 16S rRNA and *C. perfringens* encoding the enterotoxin gene was investigated.

The results of our study indicate that *C. perfringens*, encoding the *netF* toxin gene, is significantly more prevalent in dogs with AHDS than in those with other gastrointestinal disease phenotypes. Nearly half of the dogs tested positive for the *netF* toxin gene (47%), which aligns with findings from recent other studies. This finding supports the hypothesis that NetF is a major virulence factor responsible for AHDS. However, the present study also revealed a significantly greater prevalence of the *netF* toxin gene in dogs with acute diarrhea (AD) than in those with chronic gastrointestinal diseases and healthy controls. These findings are consistent with two previous studies reporting prevalences of 4% and 13% in dogs with acute nonhemorrhagic diarrhea [16,17]. Although the association was statistically significant, the low number of positive cases suggests that NetF is unlikely to be a frequent cause of acute diarrhea but may contribute to a mild form of acute nonhemorrhagic diarrhea in a subset of dogs.

Consistent with our findings, these studies also reported a greater abundance of *C. perfringens* 16S rRNA and *C. perfringens* encoding the enterotoxin gene in dogs with AD at the time of presentation. However, since the current study found no strong correlation between the abundance of *C. perfringens* 16S rRNA and the presence of both the *netF* and enterotoxin genes, we conclude that the toxins in question may not play a significant role in the disease process. These findings suggest that the increased abundance of *C. perfringens* 16S rRNA represents transient dysbiosis independent of the underlying cause of diarrhea. The normalization of the abundance of different clostridial strains typically occurs within one week in most dogs with acute, uncomplicated diarrhea, regardless of therapy. For example, Stübing et al. [17] reported increases in the abundance of *C. perfringens* 16S rRNA and *C. perfringens* enterotoxin genes in 35% and 25% of dogs with acute diarrhea, respectively. However, by Day 6, these elevated clostridial strains were no longer detectable, and their concentrations had decreased to the reference interval in all dogs, regardless of the therapeutic approach. Similarly, a study by Werner et al. [16] reported a decrease in the abundance of clostridial strains in dogs with uncomplicated diarrhea by Day 6, with no significant difference between the placebo group and the group treated with antimicrobial agents.

Notably, when considering dogs with AHDS and the presence of enterotoxin-positive *C. perfringens*, our study demonstrated that the prevalence of *cpe* was similar in dogs with AHDS (33%) and healthy dogs (29%). This contrasts with *C. perfringens* encoding the *netF* toxin gene, which is significantly more prevalent in dogs with AHDS than in healthy dogs. These findings suggest that the *cpe* gene plays only a minor to negligible role in the pathogenesis of AHDS and is not useful for diagnostic purposes, given its presence in healthy individuals as well. Moreover, earlier findings demonstrated that even when CPE was detected, it had no effect on clinical severity, hospitalization duration, or mortality rates in dogs with AHDS [18,19].

In summary, we support the hypothesis that *C. perfringens* carrying various toxins are part of the normal intestinal microbiota in dogs. Under certain conditions, these strains can proliferate, produce toxins, and lead to acute intestinal mucosal injury. The differing degrees of overgrowth and types of toxin-producing *C. perfringens*, along with the amounts of toxins released, are likely responsible for the varying severity of acute diarrhea. For example, in cases of mild, uncomplicated diarrhea, a transient increase in clostridial strain numbers is commonly observed. This likely reflects short-term dysbiotic changes driven by acute intestinal injury.

In acute hemorrhagic diarrhea syndrome, the overgrowth of clostridial strains, particularly more toxigenic strains such as those encoding the NetF toxin, is more pronounced, resulting in significant mucosal lesions compared with those in dogs with uncomplicated nonhemorrhagic diarrhea. However, within the AHDS group, there was considerable variation in disease activity and intestinal lesions. Some dogs may exhibit an uncomplicated course with lesions primarily in the colon and milder effects in the small intestine, whereas others may experience severe disease characterized by a high clinical activity index, significant protein loss, loss of intestinal barrier function, sepsis, and widespread clostridial overgrowth throughout the gastrointestinal tract [10,11,15]. In fatal cases, clostridial strains and lesions may even be present in the stomach [25]. Thus, the proliferation of toxigenic *C. perfringens* may represent a continuum, ranging from mild dysbiosis in acute uncomplicated nonhemorrhagic diarrhea to severe nonhemorrhagic diarrhea, with increasing toxigenic influence on uncomplicated hemorrhagic diarrhea and severe AHDS. Importantly, *C. perfringens* is not a transmissible infection in dogs with intestinal disorders; its overgrowth is self-limiting and often resolves without antibiotics. This highlights the need of responsible antibiotic use in managing suspected *C. perfringens*-associated enteric diseases in dogs, as antibiotics may not be necessary in most cases [15–17,26,27].

The reasons why some dogs are more severely affected remain unclear. It is possible that predisposing factors create a suitable environment for the proliferation and colonization of *C. perfringens* encoding the NetF toxin, similar to observations in broiler chickens with necrotic enteritis (NE). Known predisposing factors for NE include coccidial infection, immunosuppression, high dietary protein levels, mycotoxin contamination in feed, and environmental stressors such as heat and cold [28,29]. The combination of NetF with other toxins, virulence factors, and specific risk factors likely contributes to the development of acute hemorrhagic diarrhea syndrome. However, the underlying factors involved in this condition are not fully understood, highlighting the need for further research to identify the primary predisposing factors.

C. perfringens encoding the *netF* toxin gene was detected in only 2/120 dogs with chronic enteropathy and in 1/12 dogs with exocrine pancreatic insufficiency. These findings suggest that pore-forming toxins do not play a role in the pathogenesis of these disease complexes. However, since AHDS has been identified as a risk factor for chronic diarrhea, and dogs with chronic gastrointestinal diseases can sometimes experience sudden worsening or flare-ups of their signs, it cannot be excluded that bacterial overgrowth or specific toxins contribute to this process. Therefore, we cannot state with complete certainty that NetF is not involved in chronic gastrointestinal diseases. Nevertheless, based on the findings of this study we consider its contribution very unlikely and conclude that identifying *netF*-producing *C. perfringens* in dogs with chronic enteropathies has little to no diagnostic value. In addition, it is important to note that, although a mild to moderate correlation was found between the abundance of *C. perfringens* 16S rRNA and the enterotoxin gene in dogs with CE, we believe that the pathogenesis of chronic enteropathies involves more complex and more relevant factors beyond the presence of *C. perfringens* and its toxins. This is supported by known alterations and functional changes in the fecal and serum

metabolome in dogs with CE, including short-chain fatty acids, bile acids, amino acids, and vitamins [30–32].

This study has several limitations, primarily due to its retrospective study design. Medical records were occasionally incomplete, resulting in potential gaps in the data and limiting the comprehensiveness of the analysis. In addition, the sample size across the different study groups was nonhomogeneous. We intentionally included only a small number of dogs with AHDS, as several studies have already been published on the prevalence of the *netF* toxin gene in dogs with this condition, and the present study specifically focused on dogs with chronic enteropathies. However, we unfortunately had a small sample size of dogs with exocrine pancreatic insufficiency, which may have led to suboptimal statistical power when comparing this group to other gastrointestinal diseases. Finally, no further fecal samples were available, so longitudinal data on the abundance of *C. perfringens* 16S rRNA, *cpe*, and *netF* across different GI disease phenotypes could not be evaluated. Consequently, we could not determine whether these strains decrease over time without the use of antibiotics or, if so, at what time point.

Nevertheless, two previous studies independently demonstrated that the number of *C. perfringens* carrying NetF decreased rapidly over time without the use of any specific treatment in dogs with AHDS. More specifically, in the study by Ziese et al. [14], where 57% of the dogs tested positive for NetF-toxin-producing *C. perfringens* on the day of presentation, only one dog remained positive on Day 7. In the study by Reisinger et al. [15], where nearly 70% of dogs with AHDS were positive for the *netF* toxin gene, the prevalence decreased by more than 50% by Day 7, with only 22% of the dogs remaining positive. On Day 21, *C. perfringens* carrying *netF* were no longer detectable in any of the dogs except for one. These findings imply that NetF declines rapidly and may go undetected if testing is not conducted immediately at the onset of the disease. This finding suggests that the actual prevalence of *netF*-positive *C. perfringens* in dogs with AHDS and other gastrointestinal diseases may be greater than that reported in this and previous studies. In addition, we hypothesize that the acute onset of AHDS or AD makes the involvement of NetF more likely compared to chronic diseases. Nevertheless, this study cannot rule out the possibility that NetF contributes to the etiology of chronic gastrointestinal diseases as well, due to its rapid decline and the long duration of the illness.

In conclusion, this study provides further evidence that the pore-forming toxin NetF may be a key virulence factor specifically involved in the etiology of AHDS and potentially in a subset of dogs with acute nonhemorrhagic diarrhea. Since it was rarely detected in chronic gastrointestinal diseases, we hypothesize that NetF does not play a significant role in the pathogenesis of phenotypes associated with chronic gastrointestinal diseases. Further research is needed to identify the main predisposing factors of AHDS, as understanding the risk factors can be crucial for developing effective prevention strategies for this condition.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pets2010009/s1, Table S1: Correlation Table Abundance of *C. perfringens* 16S rRNA and *cpe*; Table S2: Correlation Table Abundance of *C. perfringens* 16S rRNA and *netF*.

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Abbreviations

The following abbreviations are used in this manuscript:

AHDS	Acute hemorrhagic diarrhea syndrome
AD	Acute diarrhea
CE	Chronic enteropathy
EPI	Exocrine pancreatic insufficiency
HC	Healthy control
CPA	Alpha toxin
CPB	Beta toxin
ETX	Epsilon toxin
ITX	Iota toxin
NE	Necrohemorrhagic enteritis
CPE	Clostridium perfringens enterotoxin
cpe	Clostridium perfringens enterotoxin gene
cTLI	Canine trypsin-like immunoreactivity
qPCR	Quantitative polymerase chain reaction

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