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Changes in the fecal microbiota in dogs with acute hemorrhagic diarrhea during an outbreak in Norway

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Abstract

Background: A severe form of acute hemorrhagic diarrhea syndrome (AHDS) occurred in dogs in the Oslo region of Norway during autumn 2019.

Objectives: To characterize the fecal microbiota of dogs with AHDS during the outbreak and compare it to that of healthy dogs from the same period and before the outbreak.

Animals: Dogs with AHDS ($n = 50$), dogs with nonhemorrhagic diarrhea ($n = 3$), and healthy dogs ($n = 11$) were sampled during the outbreak. In addition, 78 healthy dogs from the same region were sampled before the outbreak between 2017 and 2018.

Methods: Retrospective case-control study. The fecal microbiotas were characterized using 16S rRNA gene amplicon sequencing.

Results: Dogs with AHDS had significantly different microbiota composition ($R^2 = .07$, $P < .001$) and decreased intestinal diversity relative to healthy dogs from the outbreak period (median, 2.7; range, 0.9–3.5 vs median, 3.2; range, 2.6–4.0; $P < .001$). The microbiota in dogs with AHDS was characterized by a decrease of Firmicutes and an outgrowth of Proteobacteria, with increased numbers of *Clostridium perfringens* and *Providencia* spp. Among the *Providencia* spp., 1 showed 100% sequence identity with a *Providencia alcalifaciens* strain that was cultivated and isolated from the same outbreak. No *Providencia* spp. was found in healthy dogs sampled before the outbreak.

Conclusions and Clinical Importance: Dogs with AHDS had marked changes in fecal microbiota including increased numbers of *Providencia* spp. and *C. perfringens*, which may have contributed to the severity of this illness.

KEYWORDS

canine, dysbiosis, hemorrhagic diarrhea, intestinal microbiota, Norway

Abbreviations: AHDS, acute hemorrhagic diarrhea syndrome; ASV, amplicon sequence variants; GI, gastrointestinal; NMBU, Norwegian University of Life Sciences; NSC, Norwegian Sequencing Centre.

Kristin Herstad and Pål Trosvik contributed equally as first authors. Eric J. de Muinck and Ellen Skancke contributed equally as last authors.

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1 | INTRODUCTION

Idiopathic acute hemorrhagic diarrhea syndrome (AHDS) in dogs is characterized by acute onset of hemorrhagic diarrhea, often associated with vomiting, leading to severe hemoconcentration and lethargy.^{1,2}

In contrast to humans, where syndromes are clearly described, the syndrome of AHDS in dogs is poorly defined. This severe form of necrotizing enteritis is of unknown etiology, but dietary components and bacterial toxins are considered important contributors.³ The peracute clinical signs in conjunction with *Clostridium perfringens* proliferation have led to the assumption that toxins, presumably produced by these bacteria, are involved.⁴ However, these toxins are not detected in feces in all cases, and they also are found in healthy dogs. These observations cast doubt on the importance of these toxins as a direct cause of AHDS in dogs.⁵⁻⁷ Susceptibility to AHDS appears to differ among dogs, including dogs that live in similar environments. Thus, it is likely that other factors, such as properties of the intestinal microbiota, influence disease development.⁸⁻¹⁰ A few reports, based on bacterial culture, have described the presence of *Providencia alcalifaciens* in dogs with diarrhea.^{11,12} Previous studies using targeted quantitative polymerase chain reaction (qPCR) methods have shown that a dysbiotic intestinal microbiota in dogs with acute diarrhea and AHDS is characterized by changes such as higher abundance of Proteobacteria, *C. perfringens*, *Fusobacterium*, and *Turibacter* and lower abundance of Ruminococcaceae, *Faecalibacterium*, *Prevotella*, *Blautia*, *Eubacterium*, Lachnospiraceae, *Sutterella*, and *Bifidobacterium* spp.⁸⁻¹⁰ These studies, however, did not find increased abundance of *Providencia* spp. in dogs with AHDS.

Between August and November 2019, an unusually high number of dogs with acute hemorrhagic diarrhea was observed in the Oslo region of Norway. Dogs presented with acute clinical signs including vomiting, lethargy, anorexia, and profuse watery, hemorrhagic diarrhea. Because of the severity of clinical signs, a comprehensive outbreak investigation was undertaken to identify underlying causes.¹³ Clinical examinations and diagnostic testing identified no obvious underlying cause, leading to the presumptive diagnosis of idiopathic AHDS. However, a main finding was positive fecal culture results for *P. alcalifaciens*.

We used 16S rRNA gene amplicon sequencing to characterize the fecal microbiota in dogs with AHDS during an outbreak and compared it to the fecal microbiota of healthy dogs from the same time period (2019) and healthy dogs before the outbreak (2017-2018). We hypothesized that dogs with AHDS would have fecal dysbiosis and a distinct profile of gut microbes, including increased abundance of *Providencia* spp., which would not be apparent in healthy dogs.

2 | MATERIALS AND METHODS

2.1 | Animals and samples

Ours was a retrospective case control study. Dogs with AHDS (n = 50) were client-owned dogs that presented to the University Hospital at the Norwegian University of Life Sciences (NMBU) between August 1, 2019 and October 31, 2019. Inclusion criteria were dogs with acute hemorrhagic diarrhea for which no underlying cause based on history and clinical investigations was found. The clinical investigation was determined based on the veterinarian's discretion and included tests such as routine hematology and biochemistry, diagnostic imaging (radiology or ultrasound examination of abdomen or both), coagulation tests,

fecal examination for parasites, fecal culture for *Salmonellae* and *Campylobacter*, fecal PCR tests for *C. perfringens* A/B and *NetE/NetF* enterotoxin genes with or without fecal PCR testing for parvovirus. The diagnostic evaluation of these dogs was conducted in collaboration with the National Veterinary Institute and the Norwegian Food Safety Authority, in part to determine the cause of the outbreak. Fecal samples were collected from dogs to characterize the microbiota as described here. Written consent was given by dog owners for using collected fecal samples for investigative and research purposes.

In addition to the dogs with AHDS, 3 dogs with nonhemorrhagic diarrhea were included. These dogs were examined and treated during the same period as the dogs with AHDS. In these dogs, history and clinical investigation did not indicate AHDS (Figure S1).

Healthy dogs (n = 11) were staff-owned dogs without evidence of gastrointestinal (GI) diseases, no prior history of previous GI diseases, and had not been given any medications. Samples from these dogs were collected during the outbreak.

Fecal samples were collected after natural defecation from dogs as soon as possible, and no later than 2 days after they presented to NMBU. Samples were frozen at -80°C before processing.

We also obtained fecal samples from 78 healthy female and male dogs from a range of breeds and ages, collected during the period from October 2017 to August 2018 (healthy dogs preoutbreak). These samples were obtained by consenting dog owners who collected approximately 2 g of fresh feces into collection tubes containing 3 mL 96% ethanol to stop any further bacterial replication. Samples subsequently were sent by mail to the University of Oslo, where they were stored at -20°C pending processing. These samples were collected as part of another study and are included here only for comparison with the outbreak cohort. Sequence data can be made available upon request. A flow diagram shows the different groups of dogs included in the study (Figure 1).

2.2 | 16S rRNA sequencing

Fecal DNA was extracted using the MagAttract PowerSoil DNA KF kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

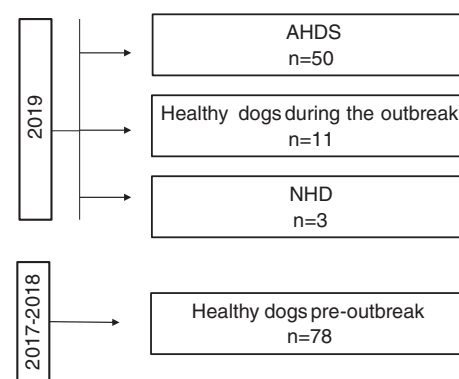


FIGURE 1 The flow diagram shows the different groups of dogs included in this study. AHDS, acute hemorrhagic diarrhea syndrome; NHD, nonhemorrhagic diarrhea

Library preparation for DNA sequencing was carried out as previously described,¹⁴ targeting the V4 region of the 16S rRNA gene with the 515f-805r primer pair. Sequencing of the 2 × 300 pair-end reads was performed using the MiSeq platform at the Norwegian Sequencing Centre (NSC).

Sequence read demultiplexing was carried out using a custom routine developed at NSC (https://github.com/nsc-norway/triple_index-demultiplexing). Primer sequences were trimmed using the “fastx_truncate” function in the USEARCH software suite.¹⁵ Further sequence data processing was performed using the Divisive Amplicon Denoising Algorithm as implemented in the dada2 v1.16 R-package.¹⁶ Taxonomic classification of amplicon sequence variants (ASVs) was done using the Ribosomal Database Project v16 training set.¹⁷

2.3 | Sequence comparison among ASVs classified as *Providencia* with *P. alcalifaciens* outbreak isolates

For a comparative study as part of the outbreak investigation,¹⁸ 8 *P. alcalifaciens* isolates from dogs with AHDS (Figure S2) were genome sequenced using a combination of the Nanopore and Illumina sequencing platforms. These DNA sequences were used to generate hybrid assemblies for each isolate. Then, all 16S rRNA gene sequences from the 8 isolate assemblies were extracted using CBS Feature extractor tool (<http://www.cbs.dtu.dk/services/FeatureExtract/>, October 2020). Each of the 8 isolates had 7 copies of the 16S rRNA gene.

All 56 rRNA gene V4 sequences from these 8 strains were aligned, with default settings, using an online implementation of the MUSCLE alignment algorithm (<https://www.ebi.ac.uk/Tools/msa/muscle/>, October 2020). We identified and included all 7 V4 16S rRNA regions of *P. alcalifaciens*, as found in 3 annotated isolates (strains FDAARGOS_408, NCTC10286, 1701003) using Geneious Prime (version 11.0.3 + 7). In Geneious, we aligned these 21 sequences with the sequences from the 8 isolates, and the 6 ASV 16S rRNA amplicon sequences from our study. For those isolates that had ≥2 16S rRNA sequences with a pairwise 100% sequence similarity, we used a reference sequence in the alignment to minimize redundancy. The resulting alignment was used to create an unrooted phylogenetic tree of the V4 region using 21 16S rRNA genes from outbreak isolates, reference strains, and 6 amplicon sequences. The tree was generated in Geneious Prime using the Neighbor-joining algorithm with the Jukes-Cantor distance model and 1000 replicates. This alignment was used to compare the ASVs with the corresponding region from the isolated strains.

2.4 | Statistical analysis of data

Tests for ASV enrichment among groups were done using the test for differential expression based on the negative binomial distribution, as implemented in the DESeq2 v1.28.1 R-package.¹⁹ All *P* values reported from these tests were subjected to Benjamini-Hochberg correction for multiple hypothesis testing. Multivariate analysis of variance with permutation (PERMANOVA) was carried out with the “adonis” function in the vegan v.2.5.6 R-package (<https://CRAN.R->

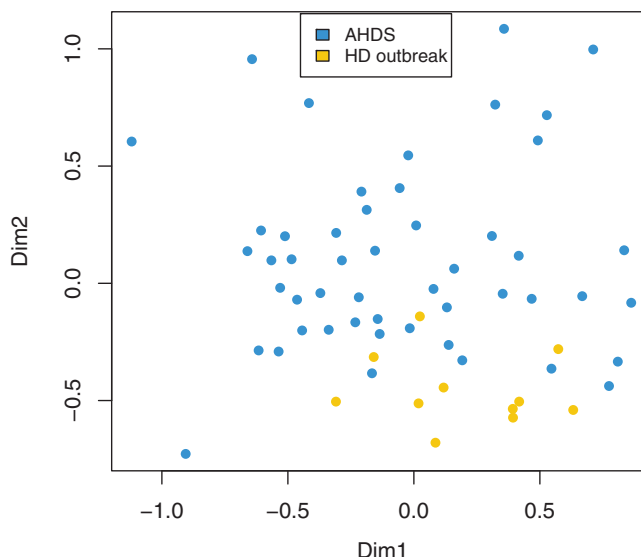


FIGURE 2 Nonmetric multidimensional scaling plot based on Bray-Curtis distances for the acute hemorrhagic diarrhea syndrome (AHDS) and healthy dogs (HD) from the outbreak. The microbiota composition in the dogs with AHDS ($n = 50$) was significantly different from the healthy dogs ($n = 11$; PERMANOVA, $R^2 = 0.07$, $P < .001$)

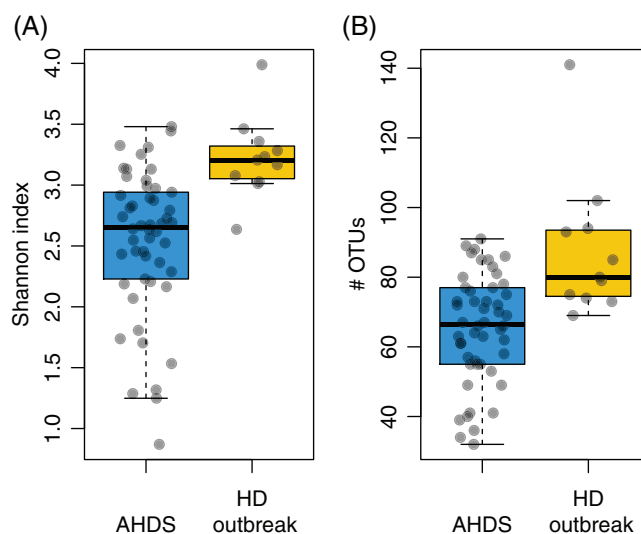


FIGURE 3 A,B, Comparison of gut bacterial diversity between AHDS and healthy dogs (HD) from the outbreak. A, Diversity as represented by the Shannon index was significantly reduced in dogs with acute hemorrhagic diarrhea syndrome (AHDS) ($P < .001$, Wilcoxon rank sum test; median, 2.7; range, 0.9–3.5) relative to healthy dogs (median, 3.2; range 2.6–4.0). B, Dogs with AHDS had reduced observed species richness ($P < .001$; median, 67, median range 32–91) relative to healthy dogs (median, 80; range, 69–141)

project.org/package=vegan). Two-sided, unpaired Wilcoxon rank sum tests were carried out using the “wilcox.test” function. The logistic regression model used to test for any relationship between health condition (dogs with AHDS vs healthy dogs during the outbreak) and

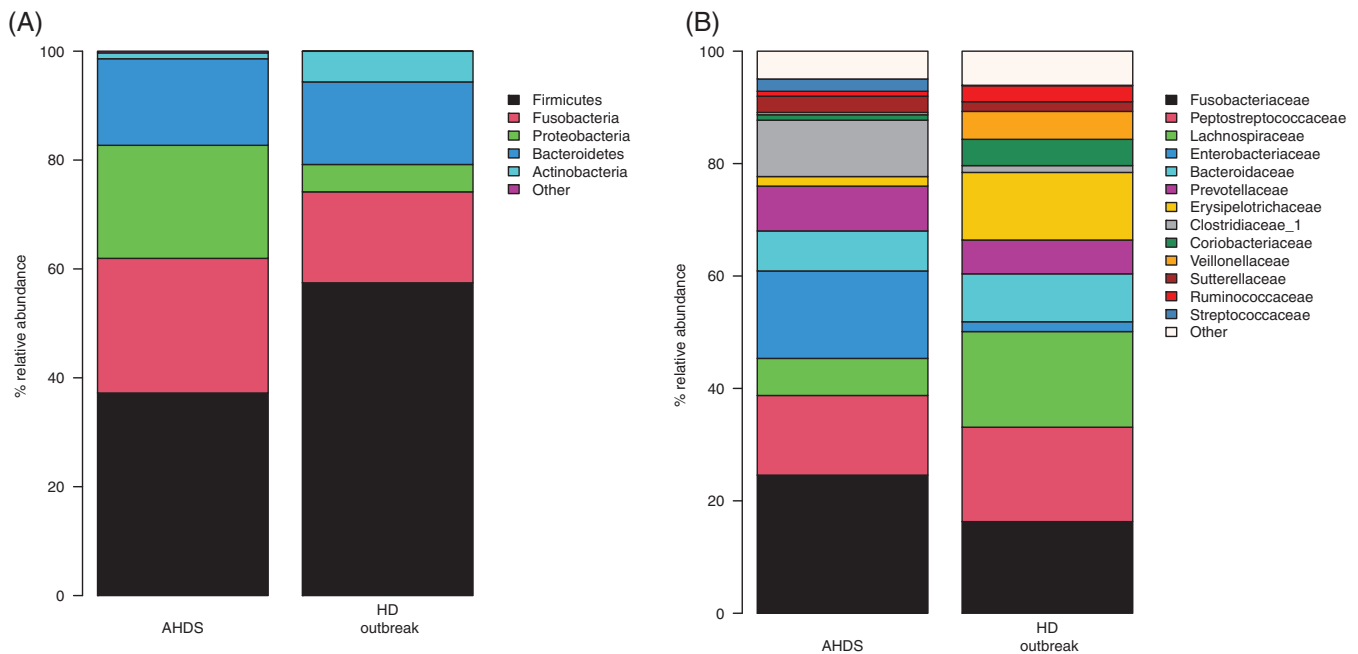


FIGURE 4 A,B, Mean relative abundances of microbiota composition at the phylum and family level in dogs with acute hemorrhagic diarrhea syndrome (AHDS) ($n = 50$) and healthy dogs (HD) from the outbreak ($n = 11$) dogs

sex was done using the “glm” function with binomial errors. All statistical tests were carried out in R v.4.0.2.

2.4.1 | Data accessibility

The sequence data have been deposited in the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) with accession number PRJNA725169.

3 | RESULTS

The 50 dogs with AHDS were between 4 months and 14 years with a median age of 6.5 years. Various breeds were represented, with 30% (17/50) of the dogs being of a miniature breed (<10 kg). Healthy dogs during the outbreak ($n = 11$) were between 4 months and 15 years of age with a median of 4 years, and none of the dogs in this group was of a miniature dog breed (Figure S1). No specific breed was overrepresented in the cohort. No significant difference in age ($P > .30$, Wilcoxon rank sum test) or sex ($P > .15$, logistic regression model) distribution was found between the dogs with AHDS and the healthy dogs during the outbreak. The healthy dogs pre-outbreak consisted of various breeds and ages of both sexes.

3.1 | Microbial analysis

3.1.1 | Sequencing analysis

After quality filtering, pair merging and chimera removal, the outbreak sequence data consisted of 2 697 402 reads, with a per sample mean of

42 147 ($\pm 13\ 030$ SD). The total number of observed ASVs was 790. To account for differences in sampling depth, we used common scaling²⁰ to the lowest sample read number (13 056 reads). This read number appears to capture the main diversity in the fecal samples (Figure S3).

3.1.2 | Fecal microbiota composition—alpha and beta diversity in dogs with AHDS vs healthy dogs during the outbreak

The overall fecal microbiota composition in dogs with AHDS was significantly different from that of healthy dogs ($R^2 = .07$, $P < .001$, PERMANOVA; Figure 2).

Dogs with AHDS had significantly decreased Shannon index (median, 2.7; range, 0.9-3.5 vs median, 3.2; range, 2.6-4.0; $P < .001$, Wilcoxon rank sum test; Figure 3A) and species richness (median, 67; range, 32-91 vs median, 80; range, 69-141; $P < .001$, Wilcoxon rank sum test; Figure 3B) relative to the healthy dogs.

3.1.3 | Differences in microbiota populations in dogs with AHDS vs dogs with nonhemorrhagic diarrhea and healthy dogs sampled during the outbreak

Figure 4A,B shows the most abundant phyla and families in dogs with AHDS and healthy dogs during the outbreak, respectively.

Significant phylum-level differences were identified in the microbiota composition of dogs with AHDS compared to healthy dogs. The dogs with AHDS showed, on average, a 4-fold increase in relative abundance of Proteobacteria ($P < .001$, Wilcoxon rank sum test), whereas Firmicutes was decreased by 20% ($P = .017$) and

TABLE 1 The ratio of log₂ fold change of bacterial taxa (ASVs) in healthy dogs from the outbreak period and AHDS dogs as detected and filtered by DESeq2

Genus/Species	ASV no.	log ₂ fold change	P value
<i>Providencia</i>	Asv39	23,90	<.001
<i>Escherichia/Shigella</i>	Asv38	23,42	<.001
<i>Alloprevotella</i>	Asv59	23,12	<.001
<i>Clostridium_sensu_stricto</i>	Asv40	22,79	<.001
<i>Escherichia/Shigella</i>	Asv64	22,71	<.001
<i>Providencia</i>	Asv71	22,64	<.001
Enterobacteriaceae ^a	Asv61	22,40	<.001
<i>Fusobacterium</i>	Asv66	22,29	<.001
<i>Clostridium_XIVa</i>	Asv94	22,23	<.001
<i>Clostridium_XIVb</i>	Asv79	21,82	<.001
<i>Streptococcus</i>	Asv37	21,72	<.001
<i>Paraprevotella</i>	Asv47	21,62	<.001
<i>Clostridium_XI</i>	Asv85	21,13	<.001
<i>Fusobacterium</i>	Asv52	21,00	<.001
<i>Clostridium_XI</i>	Asv57	20,61	<.001
<i>Clostridium_XI</i>	Asv119	20,50	<.001
<i>Haemophilus haemoglobinophilus</i>	Asv95	20,19	<.001
<i>Bacteroides</i>	Asv121	18,97	<.001
<i>Sutterella</i>	Asv138	18,96	<.001
<i>Sutterella</i>	Asv68	6,80	<.01
<i>Streptococcus</i>	Asv18	4,98	<.01
<i>Escherichia/Shigella</i>	Asv5	4,59	<.001
<i>Providencia</i>	Asv6	3,49	<.001
<i>Clostridium_sensu_stricto</i> ^b	Asv3	2,64	<.01
Lachnospiraceae ^a	Asv14	-2,91	<.001
<i>Blautia</i>	Asv32	-3,02	<.01
<i>Faecalibacterium prausnitzii</i>	Asv25	-3,35	<.001
<i>Catenibacterium mitsuokai</i>	Asv20	-5,97	<.001
<i>Coprobacillus</i>	Asv271	-11,28	<.01
<i>Fusobacterium</i>	Asv169	-24,39	<.001
<i>Cetobacterium</i>	Asv157	-25,84	<.001
<i>Collinsella</i>	Asv284	-27,54	<.001

Note: A log₂ fold change with a positive value indicates increased presence of the given bacterial taxa in AHDS dogs relative to the healthy dogs, whereas a negative value indicates reduced presence of the given bacterial taxa.

Abbreviations: AHDS, acute hemorrhagic diarrhea syndrome; ASV, amplicon sequence variants.

^aClassified to family as lowest level.

^bAccording to BLAST, this ASV was classified with 100% identity to *Clostridium perfringens*. The other ASVs classified as *Clostridium_sensu_stricto* were also implemented in a BLAST search, but did not reveal similar high identity.

Actinobacteria was decreased by a factor of 5 ($P < .001$). On the family level, dogs with AHDS were depleted for Lachnospiraceae, Ruminococcaceae, Erysipelotrichaceae and Veillonellaceae ($P < .001$ for all comparisons, Wilcoxon rank sum test), whereas they showed significantly increased abundance of Enterobacteriaceae ($P < .001$) and Clostridiaceae_1 ($P = .03$).

Analysis using DESeq2 identified 32 ASVs that had a significant differential abundance in dogs with AHDS compared with healthy

dogs (DESeq2, Table 1). Of these, 24 ASVs were more abundant in dogs with AHDS, including *Escherichia* spp./*Shigella* spp. ($P < .001$), *Haemophilus haemoglobinophilus* ($P < .001$), *Sutterella* spp. ($P < .01$), as well as 3 ASVs affiliated with *Providencia* spp., of which 1 (ASV number 6) was particularly prevalent ($P < .001$; Figure 5A and DESeq2 Table 1). Furthermore, of the 25 ASVs classified as *Clostridium sensu stricto*, 1 ASV (ASV number 3) showed 100% sequence identity to *C. perfringens* (Figures S4 and S5). This ASV was highly enriched in

dogs with AHDS ($P < .001$, DESeq2, Table 1 and Figure 5B), and we henceforth refer to it as *C. perfringens*.

Eight ASVs, including *Faecalibacterium prausnitzii*, *Blautia* spp. and *Collinsella*, were significantly more abundant in healthy dogs ($P < .01$, DESeq2, Table 1). The 3 dogs with nonhemorrhagic diarrhea also had evidence of fecal dysbiosis with lower abundance of Actinobacteria and an increase in *C. perfringens*, but with levels of *Providencia* similar to the healthy dogs during the outbreak (Figures S6 and S7). Because of the small number of samples in this category, these are anecdotal observations without statistical significance.

3.1.4 | *Providencia* spp. in dogs with AHDS vs healthy dogs from the outbreak period

We found 6 ASVs classified as the genus *Providencia*, of which 3 were significantly enriched in the dogs with AHDS (ASV numbers 6, 39 and 70; DESeq2, Table 1). In dogs with AHDS, ASV number 6 was found in all but 1 sample and had a mean relative abundance of 5.6%. In contrast, its abundance was 1.2% in healthy dogs from the outbreak period. On the other hand, ASV number 39 and ASV number 71 were present at lower abundances, representing 0.49% and 0.31% of the mean relative abundances in dogs with AHDS, respectively. These 2 ASVs were not observed in healthy dogs.

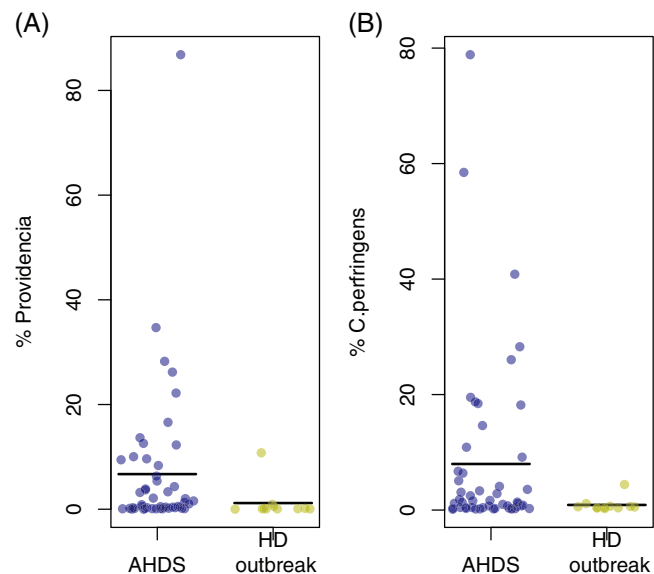


FIGURE 5 A,B. Mean relative abundances of *Providencia* spp. and *Clostridium perfringens* in AHDS dogs (6 ASVs) were significantly more abundant in dogs with AHDS. The figure shows the combined relative abundance of 6 ASVs classified as this genus. Three of these were significantly enriched in dogs with AHDS (DESeq2, Table 1) B. *Clostridium perfringens* in dogs with AHDS and healthy dogs. The figure shows data for a single ASV (ASV no. 3) that showed 100% identity to known *C. perfringens* isolates, and that was significantly more abundant in dogs with AHDS (DESeq2, Table 1). AHDS, acute hemorrhagic diarrhea syndrome; ASV, amplicon sequence variants

We compared the 16S rRNA V4 regions from the 6 *Providencia* spp. ASVs with the corresponding sequences from 8 *P. alcalifaciens* isolates obtained from dogs suffering from AHDS during the outbreak, as well as 3 reference strains (Figure 6). We found that only ASV number 6 matched these isolates with 100% sequence identity, demonstrating that ASV number 6 is closely related to the *P. alcalifaciens* strains implicated in the AHDS outbreak.

The other 5 ASVs classified as *Providencia* showed nucleotide differences with all of the 16S rRNA gene V4 region sequences to which they were compared. This finding indicates that these represent different *P. alcalifaciens* strains, or possibly other species.

3.1.5 | *Providencia* in healthy dogs sampled before the outbreak

To determine if *Providencia* is part of the normal microbiota of dogs in the region, we compared our sequencing results from dogs with AHDS ($n = 50$) and healthy dogs during the outbreak ($n = 11$) with sequencing results from healthy dogs before the outbreak ($n = 78$). In healthy dogs before the outbreak, 5 818 558 16S rRNA gene amplicon sequences were generated, from which we failed to identify a single ASV classified as *Providencia*. For comparison, an ASV identical to *C. perfringens* (ASV number 3) was found in 77 of the 78 samples at a mean relative abundance of 0.7% (range, 0%-11%). Small but significant differences ($R^2 = .05$, $P = .01$, PERMANOVA) were found between healthy dogs before and during the outbreak. When comparing dogs with AHDS and healthy dogs preoutbreak, a significant difference was found in microbiota composition (PERMANOVA, $R^2 = .06$, $P < .01$; Figure S8).

4 | DISCUSSION

We report increased abundance of *Providencia* spp. and *C. perfringens* in dogs with AHDS compared to healthy dogs. Furthermore, we found widespread fecal dysbiosis in dogs with AHDS, with a general overgrowth of *Proteobacteria* and depletion of putatively beneficial species. One ASV classified as *Providencia* (ASV number 6) showed 100% sequence identity to 16S rRNA gene V4 sequences of *P. alcalifaciens* isolates from dogs with AHDS during the outbreak. We also report that *Providencia* spp. were not part of the normal dog microbiota in the time before the AHDS outbreak in 2019. However, the small but significant difference between healthy dogs before and during the outbreak may indicate that *Providencia* spp. entered the population in the time before the outbreak. However, whether this bacterium was the actual cause of the outbreak, or whether its presence was favored by the altered microbiota, remains unknown, and the question is beyond the scope of our study.

Although proliferation of *C. perfringens* and presence of enterotoxin genes have been associated with AHDS in dogs in several studies,^{5,6,21,22} other factors may be involved in the pathogenesis. *Providencia* spp. also may have pathogenic properties. *Providencia alcalifaciens* previously has been linked with enteritis in dogs,^{11,23} although others have claimed its

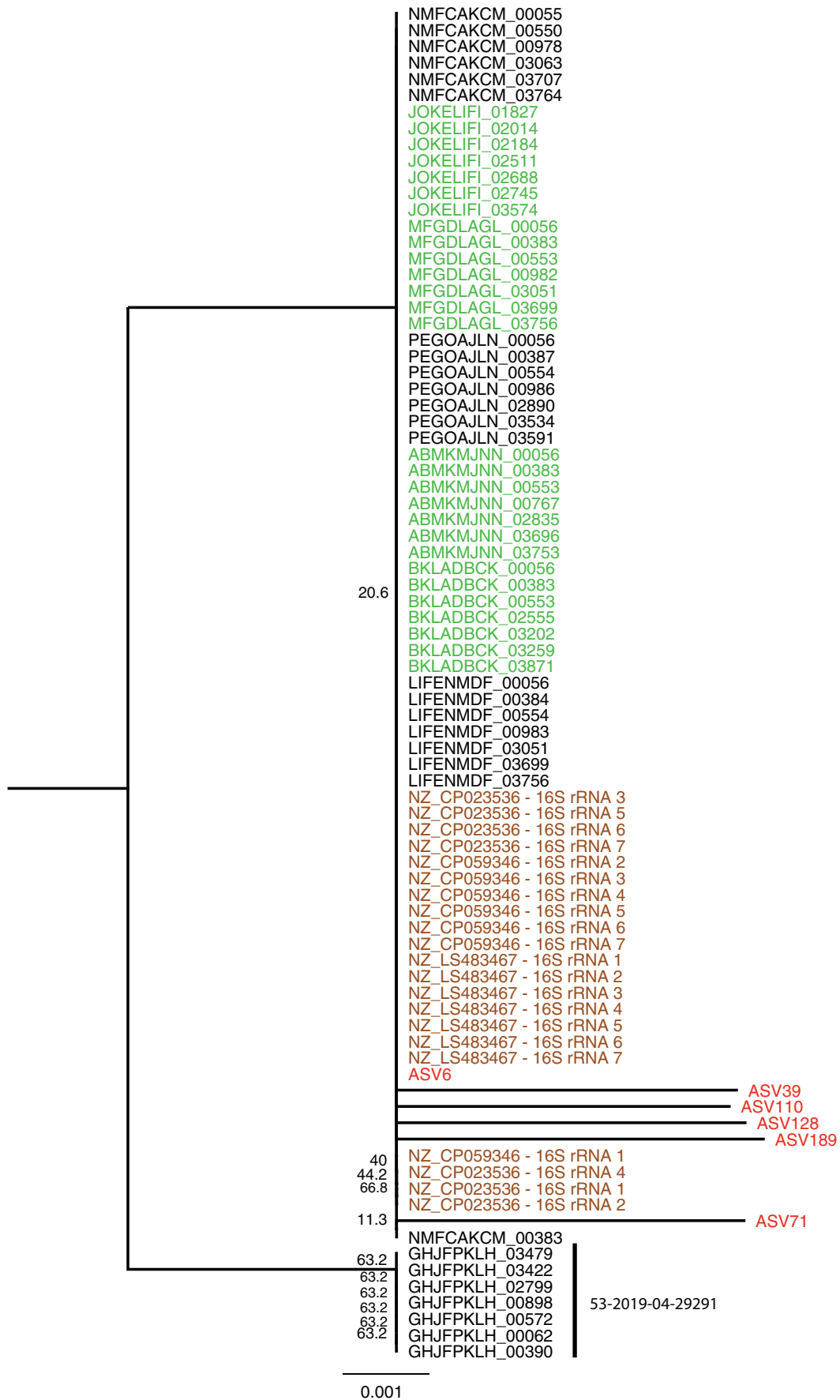


FIGURE 6 Legend on next page.

presence is a consequence of the altered intestinal microenvironment in dogs with gastroenteritis.¹² In humans, studies have found that *Providencia* spp., including *P. alcalifaciens*, are an important cause of traveler's diarrhea.²⁴ *Providencia alcalifaciens* also has been associated with diarrhea in children, but other enteric pathogens also were present in these cases.²⁵ In 2005, dogs from different areas in Oslo and nearby regions suffered from hemorrhagic diarrhea with unusually high numbers of *P. alcalifaciens*. *Providencia alcalifaciens* was isolated from 6 dogs, with 1 fatal outcome. Although it could not be determined whether or not this bacterium was the primary cause, the study demonstrated that it had the potential to invade mammalian epithelial cells in *in vitro* studies.²⁶ Its potential to invade cells also has been demonstrated in other *in vitro* studies.^{27,28} In addition to invasiveness, enterotoxins may be another contributing virulence mechanism, and cytolethal distending toxins produced by *P. alcalifaciens* and *Providencia rettgeri* have been associated with diarrhea in humans.²⁹ Moreover, *P. alcalifaciens* may produce manganese superoxide dismutase which enhances protection against phagocytosis.³⁰ Routine testing for enteropathogens usually does not include *Providencia* spp., because it is not recognized for being enteropathogenic and requires special culturing methods for identification.³¹ Thus, the presence of *Providencia* spp. in fecal samples from humans with diarrhea is likely not often detected. However, a study in humans found *Providencia* spp. in 7.5% of diarrhea samples.³² In dogs, the occurrence of *Providencia* spp. in diarrhea samples is unknown. Whether *Providencia* spp. should be regarded as enteropathogens and be investigated in dogs with diarrhea requires further study.

Members of the Firmicutes phylum such as *Faecalibacterium prausnitzii* and *Blautia* spp., as well as an ASV classified as family Lachnospiraceae, were depleted in dogs with AHDS, as has been reported in other studies.⁸⁻¹⁰ These bacteria belong to *Clostridium* clusters IV and XIVa, which contain efficient short chain fatty acid producers that are important for intestinal homeostasis.³³ Thus, their depletion may contribute to development of gastroenteritis.³⁴ Furthermore, these microbes contribute to the maintenance of mucosal integrity and protect against invasion.³⁵ Dysbiosis characterized by the loss of these bacteria may further enhance mucosal damage caused by pathogens.^{36,37} However, most knowledge about the effects of depletion of important bacterial groups in intestinal dysbiosis comes from studies in humans and mouse models of the human microbiome. The dog microbiome is very similar to that of humans, much more so than that of mice,³⁸ emphasizing the relevance of microbiome studies in dogs. Nevertheless, more studies focusing on dogs, including *in vitro* experiments,³⁹ are needed to determine the extent to which interactions among specific groups of intestinal microbes and the intestinal mucosa resemble those observed in humans.

In addition to depletion of putatively beneficial bacterial species, we also observed an enrichment of Proteobacteria, including *Escherichia coli/Shigella* and *Sutterella*. A previous study described a similar pattern of intestinal dysbiosis in dogs with diarrhea, including acute, hemorrhagic and chronic diarrhea, with dogs with AHDS experiencing the most marked microbiota alterations.¹⁰ Interestingly, we found that the 3 dogs with nonhemorrhagic diarrhea also had evidence of fecal dysbiosis with lower abundances of *Actinobacteria* and an increase in *C. perfringens*, yet with *Providencia* numbers similar to the healthy controls (Figures S6 and S7). Although this particular observation suggests a key role for *Providencia* in the etiology of AHDS, the small number of dogs with nonhemorrhagic diarrhea precludes any conclusion. However, the fact that we did not observe any *Providencia* sequences in a relatively large cohort sampled over 2 previous years indicates that these bacteria are not a normal part of the dog microbiota, at least not in the Oslo area. Thus, it is possible that they became established in the population during the period after the summer of 2018, eventually becoming widespread and highly abundant in many dogs. An epidemiological investigation of the outbreak has been performed,¹³ and further studies are underway that may provide an answer about the role of *Providencia* spp. in this outbreak.

Dogs with AHDS had increased relative abundances of *C. perfringens* compared to healthy dogs, which is consistent with previous observations.⁴ However, healthy dogs also may harbor relatively high numbers of *C. perfringens*,²² suggesting that this species is an opportunistic pathogen that can function as part of a healthy gut microbial system. Indeed, in our samples from 2017 to 2018 we found that this species is a normal and relatively abundant member of the healthy dog microbiota. A recent study found that the pore-forming toxin genes NetE and NetF, commonly associated with certain strains of *C. perfringens*, were more prevalent in dogs with AHDS than in healthy controls.⁵ This finding indicates a potential role for *C. perfringens* in the etiology of AHDS, while also suggesting that it is possible to develop the disease in the absence of toxin-producing strains, and that these strains can be found in healthy individuals. Although microbial factors likely influence the development of AHDS, their contribution may depend on the intestinal immune defenses of the individual dog, as reviewed previously.⁴⁰

One limitation of our study was the low number of healthy dogs included in the control group. Ideally, we would have included a larger cohort of breed- and age-matched control dogs to provide a more optimal comparison with dogs with AHDS. Furthermore, the 16S rRNA V4 gene fragment does not, in most cases, provide adequate information for accurate classification at the species level. Although the fact that ASV number 6 was identical in sequence to several outbreak isolates of *P. alcalifaciens* provides an indication that they are

FIGURE 6 Neighbor-joining tree of the V4 region of 83 16S rRNA genes from outbreak isolates, reference strains and 6 amplicon sequences. Sequences from each isolate with a pairwise 100% similarity of the V4 16S rRNA region were replaced by a representative sequence to reduce the redundancy. The number of combined sequences is indicated in brackets for each sequence. The amplicon sequence variants (ASVs) are marked in red, the reference strain sequences in brown, and the outbreak strains in green. In black are sequences from nonoutbreak isolates. The phylogeny shows that all *Providencia alcalifaciens* sequences cluster at the same node together with ASV6, except for the sequences of isolate 53-2019-04-29291, which is the outgroup here. The numbers at the nodes indicate bootstrap values higher than 20

very closely related, we were not able to provide classification beyond the genus level for the other *Providencia* ASVs. Lastly, we do not know if *Providencia* spp. disappear from the host or remain members of the microbiome. This question is beyond the scope of our study, but follow-up sampling of the dogs included in our study would provide valuable insight into potential effects on colonization dynamics. A previous study of dogs with AHDS suggested that changes in the intestinal microbiota might outlast GI inflammation.⁹ Thus, the environmental and ecological factors that led to the introduction and proliferation of *Providencia* in the population need to be further investigated.

In conclusion, we found increased abundance of *Providencia* spp. in dogs with AHDS relative to healthy dogs, offering a plausible explanation for the unexpectedly high numbers of dogs suffering from a severe form of AHDS during this outbreak. Our results indicate the complexity of the AHDS outbreak, where *Providencia* spp. may have contributed to this severe outbreak of diarrhea. The condition could be compounded by concurrent outgrowths of *C. perfringens* in a dysbiotic background microbiota. Additional studies are needed to learn more about the population genomic structure of *Providencia* spp. and identify species and strains with pathogenic potential. Genomic and functional studies also are needed to elucidate the mechanisms of pathogenicity.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The sequence data have been deposited in the National Centre for Biotechnology Information (NCBI) Sequence Read Archive (SRA) with accession number PRJNA725169.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Authors declare no IACUC or other approval was needed.

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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