1 **Title:** Characterization of *C. difficile* strains isolated from companion animals and the associated

2 changes in the host fecal microbiota

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14 **Journal:** *AEM*

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1 **Keywords:** *C. difficile*, *C. hiranonis*, microbiome, ribotype, antibiotic resistance, animal, canine,

2 equine, feline

3 **Abstract**

4 **Background**

5 *Clostridioides difficile* is an enteric pathogen historically known to cause hospital associated

6 (HA)-infections in humans. A major risk factor for CDI in humans is antibiotic usage as it alters

7 the gut microbiota and there is a loss of colonization resistance against *C. difficile*. In recent

8 years there has been an increase in community associated (CA)-*C. difficile* infection that does

9 not have the same risk factors as HA-CDI. Potential sources of CA-CDI have been proposed

10 and include animals, food, water, and the environment, however these sources remain poorly

11 investigated. Here, we define the prevalence of *C. difficile* strains found in different companion

12 animals (canines, felines, and equines) to investigate a potential zoonotic link. *C. difficile* strains

13 were identified by toxin gene profiling, fluorescent PCR ribotyping, and antimicrobial

14 susceptibility testing. 16s rRNA gene sequencing was done on animal feces to investigate the

15 relationship between the presence of *C. difficile* and the gut microbiota in different hosts.

16 **Results**

17 Here, we show that *C. difficile* was recovered from 20.9% of samples (42/201), which included

18 33 canines, 2 felines, and 7 equines. Over 69% (29/42) of the isolates were toxigenic and

19 belonged to 14 different ribotypes, with overlap between HA- and CA-CDI cases in humans. The

20 presence of *C. difficile* results in a shift in the fecal microbial community structure in both

21 canines and equines. Commensal Clostridia *C. hiranonis* was negatively associated with *C.*

22 *difficile* in canines. Further experimentation showed a clear antagonistic relationship between

23 the two strains *in vitro*, suggesting that commensal *Clostridia* might play a role in colonization

24 resistance against *C. difficile* in different hosts.

25 **Conclusions**

1   In this study we investigated a potentially important source of *C. difficile* transmission: the

2   companion animal population. *C. difficile* carriage was common in dogs, cats, and horses. *C.*

3   *difficile* isolates from companion animals included many of the same ribotypes known to cause

4   HA- and CA-CDI in humans, and had similar antimicrobial resistance profiles as those isolated

5   from human populations. These data contribute to our understanding of non-hospital exposure

6   to *C. difficile* in the human population and suggest new avenues for reducing *C. difficile*

7   prevalence in companion animals and, perhaps, thereby reducing CA-CDI in humans.

8   **Introduction**

9   *Clostridioides difficile* is the most common cause of hospital-acquired and antibiotic-associated

10 diarrhea in the United States, resulting in an estimated 29,000 deaths and over $4.8 billion

11 dollars in medical expenses each year [1, 2]. *C. difficile* was first identified as a primary

12 infectious cause of antibiotic-associated pseudomembraneous colitis and fatal colonic disease

13 in humans in the late 1970s [3, 4]. The widespread use of broad-spectrum antimicrobials

14 contributed to *C. difficile* infection (CDI) becoming a significant hospital-acquired (HA) disease in

15 the subsequent decades [3, 5-7]. It is now understood that many cases of CDI are community-

16 associated (CA-CDI) [8-10]. The epidemiological definition of CA-CDI is broad, meaning no

17 documented overnight stay in a healthcare facility in the prior 12 weeks [2]. In a population-

18 based study from Minnesota, CA-CDI accounted for 41% of all CDI cases [11]. Another study

19 from North Carolina reported that CA-CDI occurred in 21 and 46 per 100,000 person-years in

20 Veterans Affairs (VA) outpatients and Durham County populations, respectively [12]. Traditional

21 risk factors seem to be less important for the new population of CA-CDI that is emerging in

22 younger patients with lower rates of antibiotic exposure [11-14]. The prevalence of CA-CDI

23 cases threatens to undermine the progress being made in controlling CDI.

24   The sources from which humans acquire CA-CDI are not yet well understood, but

25 companion animals are a strong candidate. Companion animals carry toxigenic and non-

1   toxigenic *C. difficile*, and recent studies show considerable genetic overlap between animal and

2   human *C. difficile* strains [13, 15-21]. *C. difficile* prevalence ranges from 2-40% in the feces of

3   clinically normal and diarrheic dogs and cats [15, 22-26], although its clinical relevance to the

4   animals remains unclear. The close contact between pets and their owners in the 38.4% of US

5   households with dogs and 25.4% with cats [27] makes cross-species transmission far more

6   likely. Only 1.5% of Americans households own a horse, but *C. difficile* is an established cause

7   of diarrhea and colitis in horses [28] with a prevalence of 0-8% in healthy horses [29-33] and 12-

8   90% in diarrheic horses [31, 34, 35] suggesting that *C. difficile* in horses may be more likely to

9   be pathogenic. Even if we assume the lowest reported prevalence rates in dogs, cats and

10   horses, these numbers still indicate that millions of Americans closely interact with companion

11   animals carrying *C. difficile* each year. Other proposed sources of CA-CDI include farm animals,

12   food, water, and the environment, however these potential sources all remain poorly

13   investigated at this time [15-18, 36-39].

14   The risk factors for CDI or *C. difficile* carriage in companion animals are not well

15   understood. Risk factors associated with CDI in humans have been extensively investigated and

16   include antimicrobial therapy, hospitalization, increasing age, and immunosuppression. It is

17   known that mice, hamsters, and guinea pigs that are used in animal models rapidly develop CDI

18   after antibiotic treatment [40-42]. Some studies in horses show a strong association between

19   antimicrobial therapy and CDI [43, 44], while others indicate no distinct predictors for the

20   disease [45]. Literature on prerequisites for *C. difficile* colonization and infection in dogs and

21   cats mostly show no association with antibiotic therapy [23, 46, 47] while one study showed that

22   treatment with antibiotics was a risk factor for hospital acquired colonization [48].

23   There is substantial evidence to support the role of shifts in the gut microbiota, especially

24   shifts caused by antibiotics, in the pathogenesis of CDI in humans and animal models [49-51].

25   The exact molecular mechanisms by which the gut microbiota and their metabolites confer

4

1   protection are currently being investigated. Several mechanisms such as bile acid

2   transformations [50, 52], competitive exclusion [53] antimicrobial peptides [54], and activation of

3   host immune signaling [55] have been proposed. Therefore, there is growing interest in

4   understanding the interactions between *C. difficile* and the intestinal microbiome. However,

5   taxonomical shifts and microbial interactions associated with *C. difficile* colonization in

6   companion animals have not been explored. Since companion animals show significant genetic

7   overlap of *C. difficile* strains to that of humans, characterization of the *C. difficile* strains

8   circulating in companion animals and identifying their risk factors will help us better understand

9   the epidemiology of this pathogen. Furthermore, defining the fecal microbiota and its interaction

10   with *C. difficile* may identify new taxa associated with protection against *C. difficile* colonization

11   in different hosts. By defining the burden and strains of *C. difficile* prevalent in companion

12   animals we will investigate an understudied source that may contribute to CA-CDI in humans

13   and gastrointestinal disease in companion animals.

14   In this study, we collected and analyzed 201 fecal samples, from a tertiary hospital, from

15   three companion animals --- canines (dogs), felines (cats), equines (horses) --- and 5 samples

16   from ovines (sheep) as an example farm animal. We determined the prevalence of *C. difficile* in

17   these populations, and *C. difficile* strains isolated from animal stool were characterized in detail

18   by toxin gene profiling, toxin activity testing, ribotyping, and antibiotic susceptibility profiling.

19   Our study revealed widespread prevalence of toxigenic *C. difficile* in companion animals, with

20   significant overlap of ribotypes known to cause HA- and CA-CDI in humans. Using metadata on

21   age, gender, use of antibiotics, and gastrointestinal (GI) health status we determined risk

22   factors associated with *C. difficile* carriage in companion animals. As expected the presence of

23   *C. difficile* correlated with worse GI health status in equines, but not canines.

24   Fecal microbiota analysis revealed that the commensal *Clostridia Clostridium hiranonis*

25   was negatively correlated to *C. difficile* in canines before and after controlling for antibiotic

5

1   usage, GI health status, gender, and age. Competition studies with *C. hiranonis* and *C. difficile*

2   show a clear antagonistic relationship between the two strains *in vitro*. We developed further

3   epidemiological and experimental evidence of an interaction between *C. hiranonis* and *C.*

4   *difficile* in the canine gut, suggesting that commensal *Clostridia* may play a role in colonization

5   resistance against *C. difficile* in different hosts. Our study provides the broadest and most

6   detailed description to date of *C. difficile* across the companion animal species most contacted

7   by Americans. Our results contribute to our understanding of non-hospital exposure to *C.*

8   *difficile* in the human population and suggest new avenues for reducing *C. difficile* prevalence

9   in companion animals and, perhaps, thereby reducing CA-CDI in humans.

10   **Materials and Methods**

11   **Collection of animal fecal samples and clinical data.** Animal fecal samples submitted for

12   routine microbiological/parasitology diagnostic evaluations to the NC State Veterinary Hospital

13   Microbiology & Molecular Diagnostic Laboratory (MMDL) were randomly selected and collected

14   from December 2016 to October 2017. The included samples were not necessarily

15   representative of GI disease, nor suspected of CDI. The animal species and the fecal sample

16   analysis workflow are presented in Figure 1. A total of 201 samples from canines (n=107),

17   felines (n=17), equines (n= 72), and ovines (n=5) were analyzed. Fecal samples were kept

18   under refrigeration for less than 24 hr, before they were aliquoted, and stored at -80°C until

19   further processing. Metadata on species, breed, age, sex, current antimicrobial therapy (if

20   available), and visit purpose were collected from patient records and discharge comments.

21   **Spore enrichment from feces and *C. difficile* isolation.** *C. difficile* spores were recovered

22   from animal feces as previously described [56]. Fecal samples were thawed and resuspended

23   in 500 µL of pre-reduced phosphate buffer, of which 100 µL were transferred into 5 mL

24   selective taurocholate (Sigma-Aldrich, MO, USA) cycloserine (Fisher Scientific, PA, USA)

25   cefoxitin (Sigma-Aldrich, MO, USA) fructose (Fisher Scientific, PA, USA) broth (TCCFB) for

1   spore enrichment, followed by incubation at 37°C for 24-48 hr anaerobically. The inoculated

2   broth was then inspected for growth by visual turbidity. If turbid, the samples were streaked on

3   a taurocholate cycloserine cefoxitin fructose agar (TCCFA) plate and incubated at 37°C for 24-

4   48 hr anaerobically. The TCCFA plates were inspected for *C. difficile* colony morphology (large

5   flat colonies with appearance of ground glass). The recovered presumptive colonies were

6   further confirmed by direct colony PCR and 16S rRNA gene sequencing (See protocol below).

7   Glycerol stocks (15%) of the confirmed isolates were made and stored at -80°C for downstream

8   analysis. If the colonies present on the TCCFA plates were confluent, and not isolated the

9   colonies were re-streaked onto fresh TCCFA plates and incubated at 37°C for 24-48 hr

10 anaerobically to get isolated colonies. Two to three passages were often needed to obtain pure

11 culture.

12 **Confirmation of *C. difficile* by direct colony PCR and 16S rRNA gene sequencing.** The

13 protocol was adapted from a procedure commonly used for the family Lachnospiraceae [57].

14 PCR was performed directly on colonies isolated from the TCCFA plates without DNA

15 extraction. The colonies were added to a PCR reaction mixture containing 25 µL of 2X Taq

16 polymerase master mix, universal 16S rRNA primers (Table 1, 1 µL of 5 µM forward primer, 1

17 µL of 5 µM reverse primer), and Milli-Q water up to 22 µL. The PCR program consisted of 95°C

18 for 5 min; 24 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min; and final extension

19 at 72°C for 7 min. Positive and negative water controls were used in all runs. The amplified

20 PCR products were analyzed by electrophoresis in a 1.6% agarose gel stained with GelRed.

21 The gels were visualized under UV light, using a Molecular Imager GelDoc ™ XR+ (Bio-Rad).

22 The amplicons were analyzed for nucleotide detection by sanger sequencing. The 16S rRNA

23 gene sequences obtained were processed in BLASTn [58], and analyzed for 100% sequence

24 identity with *C. difficile.*

1   **Antimicrobial susceptibility testing.** *C. difficile* frozen stocks were cultured on TBHI (Brain

2   heart infusion, Fisher Scientific, PA, USA) supplemented with 100 mg/L L-cysteine (Fisher

3   Scientific, PA, USA) and 0.1% taurocholate (TCA) plates at 37˚C for 24 hr. Susceptibility of the

4   *C. difficile* isolates to antibiotics were analyzed using an Etest (bioMerieux, Marcy-l'Étoile,

5   France) as per manufacturer instructions. Briefly an inoculum of 10^5 cfu/ml of each isolate was

6   applied on a prereduced Brucella agar supplemented with Vitamin K1 and hemin (Hardy

7   Diagnostics, Santa Maria, CA) using a sterile cotton swab. Strips (bioMerieux, Marcy-l'Étoile,

8   France) of cefotaxime (cephalosporin), clindamycin (lincosamides), ciprofloxacin

9   (Fluoroquinolones), levofloxacin (Fluoroquinolones), vancomycin and metronidazole were

10   applied to each plate and incubated at 37˚C for 24 hr anaerobically. The test results were

11   interpreted using human CLSI (The Clinical and Laboratory Standards Institute) breakpoints

12   shown in table 5 [59].

13   **Toxin profiling using Multiplex PCR to identify toxin genes.** A multiplex PCR assay

14   described in Presson et al. [60] was used for the detection of *C. difficile* toxin genes A (*tcdA*),

15   and B (*tcdB*), as well as binary toxins (*cdtA* and *cdtB*), with 16S rRNA genes used as an

16   internal PCR control. Additionally, the following controls were used in each run i) *C. difficile*

17   R20291 (a toxigenic strain with a toxin gene profile of *tcdA* positive, *tcdB* positive, *cdtA/B*

18   positive), *C. difficile* F200 a nontoxigenic strain with a toxin gene profile *tcdA* negative, *tcdB*

19   negative, *cdtA/B* negative [61], and water control. The PCR reaction mixture contained 25 μL of

20   2X Taq polymerase master mix (New England Biolabs, MA, USA; 1X containing 10 mM Tris-

21   HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5% Glycerol, 0.08% IGEPAL® CA-630,

22   0.05% Tween® 20, 25 U/ml Taq DNA Polymerase), 5 μL of genomic DNA, and 12 primers

23   used at the concentration described in Table 1. Genomic DNA was prepared from *C. difficile*

24   grown overnight on BHI plates. A loop full of cells was harvested from the plates, and

25   subsequently DNA was extracted using a DNeasy UltraClean Microbial Kit (Qiagen Valencia,

1   CA) as per the manufacturer's instructions. Thermocycler conditions used were 10 min at 94°C,

2   followed by 35 cycles of 50 sec at 94°C, 40 sec at 54°C and 50 sec at 72°C, and a final

3   extension of 3 min at 72°C. The reaction products were separated on a 1.5% agarose (Fisher

4   Scientific, PA, USA) gel and detected by GelRed (VWR International PA, USA), staining.

5   Images were captured using Molecular Imager GelDoc ™ XR+ (Bio-Rad).

6   **Strain typing using fluorescent PCR ribotyping.** Ribotyping was done using fluorescent

7   PCR ribotyping of the 16S and the 23S rRNA intergenic spacer sequence as described

8   previously [62]. *C. difficile* colonies for PCR ribotyping were picked from TBHI plates incubated

9   at 37˚C for 24 hr anaerobically. Isolated colonies were sub-cultured into 5 ml BHI broth and

10   additionally incubated overnight at 37˚C anaerobically. A 1:10 dilution of this culture was

11   heated at 95°C for 15 min and stored at 4°C until later use as template. The PCR reaction

12   consisted of a 25 µL volume that included 12 µL of Promega PCR Master Mix (Fisher Scientific,

13   PA, USA), 0.5 µL forward primer (GTGCGGCTGGATCACCTCCT) and 0.5 µL 6-

14   carboxyfluorescein (FAM)-labeled reverse primer (56

15   FAM/CCCTGCACCCTTAATAACTTGACC) that were both adjusted to 10 pmol/µL, 10.5 µL of

16   nuclease free water, and 1 µL of DNA template. The PCR cycling conditions were as follows:

17   95°C for 10 min followed by 35 cycles of (denaturation – 95°C for 0.5 min, annealing – 55°C for

18   0.5 min, and elongation – 72°C for 1.5 min), and final elongation of 72°C for 10 min. The

19   fluorescent PCR amplicons obtained were diluted 1:1000 with sterile DNase/RNase free water.

20   The templates were then loaded on to a capillary electrophoresis plate containing a 1:240 ratio

21   of ROX 1000 size standard and Hi-Di Formamide. The resulting chromatograms were analyzed

22   using Applied Biosystems Peak Scanner Software (v. 1.0). The distribution of peaks were then

23   analyzed against a database characterized and validated by Martinson et al., [62]

24   (https://thewalklab.com/tools/) that matches with known ribotypes with the same peaks.

9

1   **Vero cell cytotoxicity assay of *C. difficile* positive fecal samples.** Fecal *C. difficile* toxin

2   activity of the samples that tested positive for *C. difficile* was measured using a Vero cell

3   cytotoxicity assay [63, 64]. Briefly Vero cells were grown and maintained in DMEM media

4   (Gibco Laboratories, MD, USA) with 10% fetal bovine serum (Gibco Laboratories, MD, USA)

5   and 1% Penicillin streptomycin solution (Gibco Laboratories, MD, USA) in a cell culture

6   incubator (37°C and 5% CO_2). Trypsin-EDTA (0.25%; Gibco Laboratories, MD, USA) was

7   added to the cells with a contact time of 2-3 min. Cells that came off the flask surface were

8   gently washed with 1X DMEM media and harvested by centrifugation 1,000 RPM for 5 min.

9   Cells were plated at 1×10^4 cells per well in a 96-well flat bottom microtiter plate (Corning, NY,

10 USA), and incubated overnight at 37°C / 5% CO_2. Fecal samples were defrosted on ice and a

11 1:10 dilution was made using 1X PBS. The samples were then centrifuged at 1,750 RPM for 5

12 min, and the supernatants were collected from each sample and sterilized by passing them

13 through a single 0.22-μm filter. The sterilized samples were then diluted by 10-fold to a

14 maximum of 10^{-6} using 1X PBS. Sample dilutions were incubated 1:1 with PBS or antitoxin

15 (TechLabs, TX, USA) for 40 min at room temperature after which it was added to the Vero

16 cells. Control containing purified *C. difficile* toxins (A and B; List Biological Labs, CA, USA) and

17 antitoxin were included. Plates were viewed under 200X magnification for Vero cell rounding

18 after an overnight incubation at 37°C / 5% CO_2. The cytotoxic titer was defined as the

19 reciprocal of the highest dilution that produced rounding in 80% of Vero cells for each sample.

20 **16S rRNA-based bacterial community sequencing using Illumina MiSeq platform.** Fecal

21 samples (195) were subjected to community 16S rRNA gene sequencing; 6 samples (2

22 canines, 2 felines, and 2 equines were excluded due to not having enough sample available or

23 poor quality of the sequencing run. Microbial DNA was extracted from the fecal samples using

24 the Mag Attract Power Microbiome kit (Mo Bio Laboratories, Inc.). A dual-indexing sequencing

25 strategy was used to amplify the V4 region of the 16S rRNA gene [65]. Each 20-μl PCR mixture

10

1   contained 2 μl of 10X Accuprime PCR buffer II (Life Technologies, CA, USA), 0.15 μl of

2   Accuprime high-fidelity polymerase (Life Technologies, CA, USA), 5 μl of a 4.0 μM primer set, 1

3   μl DNA, and 11.85 μl sterile nuclease free water. The template DNA concentration was 1 to 10

4   ng/μl for a high bacterial DNA/host DNA ratio. The PCR conditions were as follows: 2 min at

5   95°C, followed by 30 cycles of 95°C for 20 sec, 55°C for 15 sec, and 72°C for 5 min, followed

6   by 72°C for 10 min. Libraries were normalized using a Life Technologies SequalPrep

7   normalization plate kit as per manufacturer's instructions for sequential elution. The

8   concentration of the pooled samples was determined using the Kapa Biosystems library

9   quantification kit for Illumina platforms (Kapa Biosystems, MA, USA). Agilent Bioanalyzer high-

10 sensitivity DNA analysis kit (Agilent CA, USA) was used to determine the sizes of the

11 amplicons in the library. The final library consisted of equal molar amounts from each of the

12 plates, normalized to the pooled plate at the lowest concentration. Sequencing was done on

13 the Illumina MiSeq platform, using a MiSeq reagent kit V2 (Ilumina, CA, USA) with 500 cycles

14 according to the manufacturer's instructions, with modifications [65]. Sequencing libraries were

15 prepared according to Illumina's protocol for preparing libraries for sequencing on the MiSeq

16 (Ilumina, CA, USA) for 2 or 4 nM libraries. PhiX and genomes were added in 16S amplicon

17 sequencing to add diversity. Sequencing reagents were prepared according to the Schloss

18 SOP (https://www.mothur.org/wiki/MiSeq_SOP#Getting_started), and custom read 1, read 2

19 and index primers were added to the reagent cartridge. FASTQ files were generated for paired

20 end reads.

21 **Community-sequencing bioinformatic analysis.** Analysis of the V4 region of the 16S rRNA

22 gene was done in the statistical programming environment R [66] using the package DADA2

23 [67]. Version 1.8 of the DADA2 tutorial workflow (https://benjjneb.github.io/dada2/tutorial.html)

24 was followed to process the MiSeq data. Forward/reverse read pairs were trimmed and filtered,

25 with forward reads truncated at 230 nt and reverse reads at 160 nt, no ambiguous bases were

11

1   allowed, and each read required to have less than two expected errors based on their quality

2   scores. Error corrected ASVs were independently inferred for the forward and reverse reads of

3   each sample and then read pairs were merged to obtain amplicon sequence variants (ASVs).

4   Chimeric ASVs were identified and removed. For taxonomic assignment ASVs were compared

5   to the Silva v132 database using the implementation of the RDP naive Bayesian classifier

6   available in the DADA2 R package [68, 69]. A BLASTn web search [58] matched ASV4 to the

7   NCBI 16S reference sequence NR_028611.1 for *C. hiranonis* strain TO-931. ASVs with at least

8   97% identity to this *C. hiranonis* 16S sequence were then identified using the BLASTn

9   command-line tool [58]. One canine sample and one equine sample received fewer than 30

10   reads after running DADA2 and so were excluded from subsequent analysis of microbiome

11   profiles. The read depth of the remaining samples ranged from 9338 to 60009 reads.

12   **C. difficile and C. hiranonis co-culture assay.**

13   Competition assays were developed to test the interactions between *C. difficile* strain R20291

14   and *C. hiranonis* TO-931T. Overnight cultures of both strains were grown individually in BHI

15   supplemented with 100 mg/L L-cysteine for *C. difficile,* and additionally supplemented with 2 uM

16   hemin for *C. hiranonis*. The cultures were grown anaerobically at 37°C. After 14 h of growth,

17   both cultures were subcultured to 1:10 and 1:5 into BHI plus L-cysteine and hemin, and allowed

18   to grow for 3-4 h. Once both the cultures doubled they were back diluted in fresh BHI plus L-

19   cysteine and hemin media to obtain a concentration of 1×10^6 CFU/mL for 1x, 1×10^7 CFU/mL for

20   10x, and 1×10^8 CFU/ mL for 100x. Competition controls included monocultures of 1x *C. difficile,*

21   1x *C. hiranonis,* 10x *C. hiranonis,* and 100x *C. hiranonis. C. difficile* and *C. hiranonis* were mixed

22   at 1:1, 1:10, and 1:100 ratios respectively. Appropriate dilutions were plated after 24 h of

23   incubation of all treatments anaerobically at 37 °C. Bacterial enumeration was performed and

24   expressed as Log CFU/mL of culture.

12

1   **Statistical analysis.** Fisher's exact test for significance, odds ratios, and confidence intervals

2   for the host metadata-*C. difficile* associations reported in Table 3 were evaluated using Prism

3   version 7.0 for Windows (GraphPad Software, La Jolla, CA, United States). Statistical

4   significance was set at a *p*-value of <0.05 for all analyses (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$,

5   ****$p < 0.0001$). All statistical analysis of the microbiota profiles was performed in R; code and

6   data are available on GitHub at https://github.com/rthanis/animal-cdiff. The phyloseq and vegan

7   R packages were used to obtain diversity indices and ordination plots [70, 71]. Associations of

8   animal type and *C. difficile* with alpha diversity were measured by a two-sided Wilcoxon rank

9   sum test, and associations with Bray-Curtis beta diversity were done by permanova using the

10 adonis2 function from the vegan package. Differential-abundance analysis was performed

11 using the ALDEx2 R package [72] and visualized with the ggplot2 R package [73]. Logistic

12 regression of *C. difficile* presence in canines against *C. hiranonis* and other host variables was

13 performed using the brms R package [74, 75] interface to the Bayesian statistical inference

14 software Stan [76].

15 **Results**

16 ***C. difficile* prevalence, ribotype, and toxin gene profiles vary by animal species.**

17 Forty-two *C. difficile* strains were isolated from a total of 201 animal fecal samples submitted to

18 the NC State University Veterinary Hospital MMDL. *C. difficile* was recovered from 30.8%

19 (33/107) of canines, 11.8% (2/17) of felines, 9.7% (7/72) of equines, and no ovines (0/5). To

20 determine the genetic diversity of the *C. difficile* strains circulating in animals we characterized

21 the toxin gene profiles and PCR ribotypes of the isolates. *C. difficile* isolates obtained from all

22 animal groups were subjected to multiplex PCR for detection of genes that encode for toxins A

23 (*tcdA*), B (*tcdB)*, and binary toxins (*cdtA* and *cdtB*). In total, 69% (29/42) of the isolates were

24 positive for at least one of the toxin genes tested, and the rest were non-toxigenic. Five

25 different toxin gene profiles resulted that included 2.4% (1/42) *tcdA tcdB cdtA cdtB,* 52.4%

13

1   (22/42) *tcdA tcdB,* 9.5% (4/42) *tcdA,* 4.7% (2/42) *tcdB* and 30.9% (13/42) non-toxigenic isolates

2   (Table 1). The gene profile *tcdA tcdB* was the most prevalent accounting for 17/33 of canine,

3   1/2 of feline, and 4/7 of equine *C. difficile* isolates. Isolates from canines had toxin variant

4   genotypes in addition to the non-variant genotypes. Overall, 18 different known ribotypes were

5   observed and three *C. difficile* isolates had patterns that did not match the established

6   database (Table 2). Different ribotypes were shared between different animal groups. The most

7   frequent canine ribotypes were F014-020 (7/33) and F106 (7/33), followed by FP310 (5/33).

8   The two feline *C. difficile* isolates belonged to two different ribotypes, FP310 and FP501. All

9   seven equine *C. difficile* isolates were also from different ribotypes. One equine isolate,

10 belonging to the ribotype 078-126 encoded all four-toxin genes. Ribotypes associated with

11 human CDI were also detected in some canines and equines, F014-020, F106, 078-126, and

12 F002.

13 **Most animals carrying toxigenic *C. difficile* had no detectable toxin activity in their feces.**

14 Isolation of toxigenic strains of *C. difficile* from the feces of animals led us to test if there was

15 detectable toxin activity in the fecal samples using a Vero cell cytotoxicity assay. Toxin activity

16 was below the limit of detection in 39 of the 42 fecal samples that were positive for *C. difficile*

17 carriage. In two samples the toxin activity levels were low and only resulted in 50% rounding in

18 the highest concentration (data not shown). These samples contained *C. difficile* isolates

19 belonging to ribotype F106 or F014-020 and belonged to canines, which were treated for non-

20 gastrointestinal related issues. The other sample had a titer of 2 Log_{10} reciprocal dilution toxin

21 per 100µL/mL fecal sample, and contained *C. difficile* belonging to an unidentified ribotype.

22 This sample also belonged to a canine, which was treated for anorexia and anemia.

23 ***C. difficile* prevalence in canines was not significantly associated with key demographic**

24 **factors.**

14

1   The demographic data of canines and equines, which had the most samples, was analyzed

2   further. The key demographic variables including age, gender, gastrointestinal (GI) health

3   status, and antibiotic use in canines and their association with *C. difficile* prevalence are

4   presented in Table 3. The overall proportion of canines with reported GI disease at the time of

5   sampling was 46.3% (48/103). Animals on antibiotics during the time of treatment for which

6   medication data was available was 56.7% (59/104). The study included males (58.6%; 61/104)

7   and females (41.3%; 43/104). A total of 44 different dog breeds with an age range of 3 months

8   to 13 years (median 8 years) were grouped into 4 age categories: <1 yr (6.4%; 5/77), 1-4 yr

9   (18.1%; 14/77), 5-9 yr (38.9%; 30/77), ≥ 10 yr (36.3%; 28/77). A two-sided Fisher's exact test

10   between *C. difficile* presence and gender, GI health status, or concurrent use of antibiotics in

11   canines all yielded p-values above 0.05 (Table 3). Antibiotic intake appeared moderately

12   positively associated, with an odds ratio of 1.84 (95% confidence interval (0.80, -4.26); p = 0.2).

13   In an exploratory analysis that considered age as a continuous variable, we did observe a

14   higher prevalence of *C. difficile* in dogs between 5 and 11 years and lower in younger and older

15   dogs (Supplementary Figure 1).

16   ***C. difficile* prevalence in equines is associated with GI health status.**

17   The demographic variables and their association with *C. difficile* prevalence in equines are

18   presented in Table 4. In equines, 53.8% (35/65) had symptoms indicative of a GI disorder and

19   62.1% (36/58) of the reported cases were known to be on antibiotics. The sampled population

20   included males (60.9%; 39/64) and females (39%; 25/64). Equines were grouped into three age

21   categories <2 (9.6%; 5/52), 2-10 (38.5%; 20/52), >10 (51.9; 27/52) and included 25 different

22   breeds. Age was not tested for significance because of the low number of *C. difficile* positives

23   under each category. GI health status was positively associated with *C. difficile* presence

24   (p=0.01 by two-sided Fisher's exact test; odds ratio 95% confidence interval of (1.66, ∞). All

1   equine samples from which *C. difficile* was recovered belonged to animals that had colic or

2   other GI related issues.

3   **C. difficile isolates from companion animals were resistant to antibiotics commonly**

4   **used in clinical settings, but not front line antibiotics used to treat CDI.**

5   To further characterize the *C. difficile* strains isolated from animals, we tested the frequency of

6   resistance to antibiotics used to treat CDI (vancomycin and metronidazole), and those

7   considered as risk factors (cefotaxime, clindamycin, ciprofloxacin, and levofloxacin) for human

8   CDI. All isolates were resistant to cefotaxime and the majority (97%) of them were resistant to

9   ciprofloxacin, whereas resistance to clindamycin and levofloxacin varied (23% and 59%

10 respectively) (Table 5). Only one toxigenic isolate (2.5%, ribotype F014-020) from canine was

11 resistant to metronidazole with a MIC of 512 µg/ml. In all other isolates the MIC for

12 metronidazole remained low (0.38 to 1.5 µg/ml). All *C. difficile* isolates characterized were

13 susceptible to vancomycin.

14 **C. difficile can be detected from animal feces using Illumina 16S rRNA gene sequencing.**

15 We sequenced the V4 region of the 16S rRNA gene and processed the resulting sequencing

16 reads with DADA2 [67] to obtain amplicon sequence variants (ASVs). In contrast to traditional

17 97% sequence similarity OTUs, ASVs have single-nucleotide resolution, which allows species-

18 level classification for some species by exact matching of V4 amplicon sequences [77]. The

19 DADA2 species-classification algorithm identified three *Clostridioides difficile* ASVs (ASV82,

20 ASV1962, and ASV2073) that exactly matched reference *C. difficile* 16S sequences in the Silva

21 database. Two equine samples contained all three ASVs, while all other samples with *C.*

22 *difficile* contained only ASV82. According to the rrnDB [78] *C. difficile* has 12 copies of the 16S

23 gene and so it is possible that the *C. difficile* strain found in these two equine samples contains

24 all three ASVs at different 16S copies. An additional *Clostridioides* ASV (ASV843) with

16

1   unknown species identification had 2 nucleotide mismatches from ASV82 and was detected in

2   3/5 ovine samples and no other animals.

3   We compared the detection of *C. difficile* via community 16S sequencing to that from the

4   spore-enrichment lab assay. 16S sequencing had good positive predictive value for the results

5   of the lab assay, especially in canines where 19/21 of the samples in which *C. difficile* was

6   detected by 16S sequencing were also positive for *C. difficile* presence in the lab assay. The

7   lab assay detected more *C. difficile* positive samples overall (39 vs. 31) and particularly in

8   canines (31 vs. 21), but each assay detected *C. difficile* in some samples the other assay did

9   not (Table 6). For all subsequent analyses of the effect of *C. difficile* presence on the

10   microbiota, we therefore define samples as *C. difficile* positive if *C. difficile* was detected by the

11   laboratory testing or if ASV82 was detected in the 16S rRNA data.

12   **Differences in the fecal microbiota were associated with animal type and *C. difficile***

13   **prevalence.**

14   We measured alpha diversity of fecal microbiota samples using inverse Simpson index and

15   evaluated differences between animal type and differences between *C. difficile* positive and

16   negative cohorts within animal types. The distributions of the inverse Simpson index at three

17   microbial taxonomic ranks (Family, Genus, and ASV) are presented in Figure 2A. At each

18   taxonomic rank, canine and feline samples had similar diversities to each other, as did equine

19   and ovine samples, with canine and feline samples having lower diversities than equine and

20   ovine samples. We considered whether *C. difficile* presence was associated with alpha

21   diversity within a host animal type, for the two animals (canines and equines) with larger

22   sample sizes (Supplementary Figure 2). *C. difficile* presence was not associated with genus-

23   level inverse-Simpson diversity in canines (p = 0.84, two-sided Wilcoxon rank sum test; non-

24   parametric 95% confidence interval for the median effect of *C. difficile* presence (-1.1, 1.2) but

25   was associated with lower genus-level inverse Simpson diversity in equines (p = 0.019, two-

17

1 sided Wilcoxon rank sum test; non-parametric 95% confidence interval for the median effect of

2 *C. difficile* presence (-7.0, -0.75), with similar patterns observed for family- and ASV-level

3 diversity (Supplemental Figure 2).

4   To elucidate factors related to the differences and similarities between fecal microbial

5 community structures (β diversity) we performed non-metric multidimensional scaling (NMDS)

6 based on Bray-Curtis dissimilarities between samples at the ASV level. The samples clustered

7 based on animal group (Figure 2B). The first axis separates the canines and felines from the

8 equines and ovines, and the second axis separates the ovines from the equines. No effect of

9 *C. difficile* presence was visually apparent in this cross-species NMDS plot. The presence of *C.*

10 *difficile* resulted in a statistically significant but weak shift in the fecal microbial community

11 structure in both canines (permonova, *p* = 0.0021, R^2 = 0.026) and equines (permonova, *p* =

12 0.00028, R^2 = 0.030). This result indicates that the degree to which the structure of the whole

13 fecal microbial community could be explained by *C. difficile* presence or absence was low.

14 **_C. hiranonis_ is negatively correlated with _C. difficile_ presence in canines.**

15 Next, we sought to determine whether the presence of *C. difficile* was associated with specific

16 changes in canine fecal microbial communities. We focused on canines due to the high sample

17 number and the high *C. difficile* prevalence. We performed a compositional principle-

18 components analysis (PCA) [71] to visualize the variation in community composition among

19 canine samples and the ASVs driving this variation. The resulting sample ordination shows a

20 weak but apparent association of *C. difficile* presence with overall community structure (Figure

21 3A). To determine which ASVs drive this association, we performed a differential-abundance

22 analysis against *C. difficile* presence with the ALDEx2 R package [79]. For each ASV, ALDEx2

23 reports an effect size estimating the difference in the taxon's centered-log-ratio (a measure of

24 relative abundance) between groups (*C. difficile* positive vs. negative samples) divided by the

25 difference within groups. Figure 3B shows the ASVs with the largest ALDEx2 effect sizes on the

1   taxa PCA ordination that match the sample ordination of Figure 3A. Visualizing the relative

2   abundances of the top positive and negatively associated ASVs indicates that many of the top

3   associations are largely driven by differential prevalence (presence/absence) (Figure 3C).

4   The largest negative effect size reported by ALDEx2 is that of ASV4. Although this ASV

5   was not assigned to a species from the Silva database, we used BLAST to determine that it

6   exactly matches the 16S V4 region of [*Clostridium*] *hiranonis* strain TO-931. We therefore

7   sought to further understand the relationship between [*Clostridium] hiranonis* with *C. difficile* in

8   our samples. There are 23 ASVs with >99% similarity to *C. hiranonis* strain TO-931; these are

9   ASV4 and 22 additional ASVs that differ from ASV4 by 1 bp. These 22 ASVs have lower

10   prevalence than ASV4 and always appear alongside but have lower abundance than ASV4,

11   suggesting a variety of strains of *C. hiranonis* that carry ASV4 and these other variants at

12   additional copies of the 16S gene. Considering these other ASVs allowed us to identify a

13   possible strain of *C. hiranonis* that may be particularly negatively associated with *C. difficile*.

14   ASV62 appears in 19/52 (27%) of *C. difficile* negative samples but 0/33 of *C. difficile* positive

15   samples (Figure 3C). Bayesian logistic regression of *C. difficile* presence against the presence

16   of ASV4 with or without ASV62 along with sample variables demonstrates that the negative

17   association of *C. difficile* with *C. hiranonis* remains after controlling for antibiotics usage, GI

18   health status, gender, and age and that *C. hiranonis* carrying ASV62 potentially has a much

19   stronger negative effect on *C. difficile* presence than other *C. hiranonis* strains (ASV4 alone:

20   odds-ratio has a 90% credible interval of 0.17–0.57; ASV4 with ASV62: odds-ratio has 90%

21   credible interval of 0.005–0.12). *C. hiranonis* in felines was also negatively associated with *C.*

22   *difficile* (p = 0.029 by two-sided Fisher's exact test; 0 of 12 felines with *C. hiranonis* and 2 of 3

23   felines without *C. hiranonis* are *C. difficile* positive). *C. hiranonis* was only detected in 1 equine

24   sample.

19

1   ***C. difficile* suppresses *C. hiranonis* growth in a concentration dependent manner in co-**

2   **culture *in vitro*.**

3   To test the hypothesis that there is an exclusionary relationship between *C. difficile* and *C.*

4   *hiranonis* in the canine gut, we developed a co-culture *in vitro* assay in rich medium. *C. difficile*

5   and *C. hiranonis* were grown alone (monoculture) and in concert at different concentrations over

6   a 24 hour period in BHI medium (Figure 4). *C. difficile* growth over a 24 hour period was not

7   inhibited by *C. hiranonis* cells at the 1:1 concentration and even when *C. hiranonis* outnumbered

8   *C. difficile* by a factor of 10 (*C. difficile* to *C. hiranonis* ratio), whereas *C. hiranonis* growth was

9   significantly inhibited by 24 hours (Figure 4A and B). It was only when *C. hiranonis* outnumbered

10 *C. difficile* cells by a factor of 100 that *C. hiranonis* growth was not affected at 24 hours (Figure

11 4C). This provides further evidence that there is a relationship between these two strains, but

12 the mechanism of their interaction still requires further study.

13 **Discussion**

14 The prevalence of *C. difficile* in the dogs, cats, and horses we studied was substantial, and

15 supports the possibility that millions of Americans may be exposed to *C. difficile* each year

16 through contact with companion animals. *C. difficile* was detected in 31% of the canines in our

17 study, which is within the previously reported range (2-40%), although slightly higher than the

18 range (10.5-18.4%) reported from other parts of the U.S, perhaps because our study population

19 was recruited from a tertiary animal hospital [16, 17, 23, 80]. Felines were colonized at 11.8%

20 (2/17) in our study, which is within the previously reported range (2-30%) [16, 22, 24, 26, 48,

21 81], as was the 9.7% *C. difficile* carriage rate of equines in our study given the varying age and

22 GI health status of the studied population [16, 31, 32]. Significant variation exists in *C. difficile*

23 prevalence rates in companion animals reported by different studies, likely due to various

24 factors such as differences in the type of isolation technique, geographic location, and study

1   population. Nevertheless, the mounting body of evidence is consistent with a large interface

2   between humans and companion animals carrying *C. difficile*.

3   The *C. difficile* strains isolated from companion animal feces in this study, included

4   ribotypes associated with CDI epidemics in humans. Eighteen different *C. difficile* ribotypes

5   were identified in this study. The most common ribotypes recovered were F014-020 and F106

6   from both canines and equines. Interestingly, a recent epidemiological survey of human *C.*

7   *difficile* isolates conducted in the U.S. between 2011 and 2017 reported ribotype 106 as the new

8   dominant ribotype, and the most common isolated from CA-CDI. Ribotype 014-020 was the

9   second most common ribotype among HA-CDI [82]. We also isolated from an equine the 078-

10   126 ribotype that is a known epidemic strain in humans often linked to CA-CDI [82]. The

11   nationwide gradual change in circulating ribotypes in humans and the interspecies sharing of the

12   predominant ribotypes warrant increased utilization of a one health framework that can account

13   for potential zoonotic transmission to understand the changing epidemiology of *C. difficile*.

14   The clinical relevance of *C. difficile* carriage in dogs and cats remains unclear, but

15   asymptomatic carriage does not preclude transmission to susceptible hosts. In this study, most

16   animals carrying toxigenic *C. difficile* had no detectable toxin activity in their feces via a Vero cell

17   cytotoxicity assay. This could possibly be explained by toxin break down since the fecal samples

18   were not frozen immediately after collection, but is also consistent with largely asymptomatic

19   carriage in dogs and cats. For example, the three dogs that had detectable toxin activity in the

20   feces did not present any atypical fecal consistency or GI-related issues. Asymptomatic toxin

21   carriage has been previously recognized in canines by Weese *et al.* [46], but this does not

22   preclude the possibility of symptomatic CDI developing later or after transmission to a different

23   host species. Asymptomatic carriage where *C. difficile* is able to colonize, proliferate, and

24   produce toxin is also reported in humans, and its role in disease transmission and the

25   mechanism of protection is currently under active investigation [83].

1 Antimicrobial resistance plays a major role in driving the emergence of epidemic isolates

2 and the associated changes in the epidemiology of *C. difficile* [84]. The low prevalence in our

3 study of resistance to vancomycin and metronidazole, the front-line antibiotics used to treat CDI

4 in humans, is similar to previous reports in humans and animals [32, 82, 85], although there are

5 reports of emerging resistance to metronidazole from other parts of the world [86]. Historically

6 clindamycin, later cephalosporins, and more recently fluoroquinolones are recognized as risk

7 agents for CDI in humans. Resistance to clindamycin was found in 23% of the *C. difficile*

8 isolates in our study. All isolates in our study were resistant to ciprofloxacin a second-generation

9 fluoroquinolone, consistent with patterns in human isolates [87]. Resistance to cefotaxime, a

10 third-generation cephalosporin, was common among all isolates, similar to human studies that

11 report 100% resistance [87, 88]. However, most isolates were sensitive to levofloxacin, a newer

12 third-generation fluoroquinolone. The acquisition of fluoroquinolone resistance by certain

13 epidemic strains (027/BI/NAP1) was the key event linked to their rapid emergence and

14 dissemination in North America [89].

15 Alterations in the fecal microbiota associated with *C. difficile* carriage in dogs and horses

16 were limited. In canines the alpha diversity was similar between *C. difficile* positive and negative

17 cohorts. However, in equines *C. difficile* prevalence was associated with lower genus-level

18 inverse Simpson diversity very similar to that reported in humans during CDI [51, 90] and

19 asymptomatic carriage [90]. Permanova analysis revealed weak, but significant, differences in

20 community structures between *C. difficile* positive and negative cohorts in canines and equines.

21 Alterations in the fecal microbiota, specifically a decrease in the abundance of specific species

22 and overall diversity, is associated with CDI in humans [51], yet how the gut microbiota of

23 domestic animals changes in the presence of *C. difficile* remains unclear.

24 The largest negative association between any detected taxa and *C. difficile* was with *C.*

25 *hiranonis* in dogs and cats. This corroborates the recent report of a similar negative association

22

1   between *C. difficile* and *C. hiranonis* in opportunistically collected canine fecal samples [91]. *C.*

2   *hiranonis* is a commensal bacterium that encodes the bile acid inducible (*bai*) operon and is

3   capable of 7α-dehydroxylation of primary bile acids into secondary bile acids. Microbial

4   mediated secondary bile acids are known to play a key role in inhibiting the *C. difficile* life cycle

5   *in vitro* [92, 93]. Secondary bile acid synthesis is regulated by 7α-dehydroxylating gut bacteria

6   from the *Clostridium* cluster XIVa [94], which includes *C. hiranonis* and *C. scindens*. *C. scindens*

7   was associated with partial colonization resistance against *C. difficile* in a mouse model [50],

8   however very little is known about the 7α-dehydroxylating capacity of *C. hiranonis* in the canine

9   gut [95] and needs to be further investigated [96].

10   The presence of *C. difficile* in the co-culture significantly inhibited *C. hiranonis* growth in a

11   concentration dependent manner *in vitro* using a co-culture system in rich medium. The

12   mechanisms for this suppression are unknown currently, but we hypothesize that competition of

13   nutrients might play a role via the Stickland reaction [95] or *C. difficile* could be making an

14   inhibitory product that inhibits other commensals. Recently, Kang *et al.* found that closely related

15   *C. scindens* produces a tryptophan-derived antibiotic that is able to inhibit *C. difficile* [97]. *C.*

16   *difficile* was also able to secrete cyclic dipeptides that inhibited *C. scindens*. More work needs to

17   be done using *C. hiranonis* strains isolated from canines, as we were unsuccessful with this

18   isolation, in a medium that mimics the canine gut environment. Nevertheless, the commensal

19   *Clostridium* strains could be a promising probiotic to prevent *C. difficile* in canines and humans.

20   **Conclusions**

21   In this study we investigated a potentially important source of *C. difficile* transmission: the

22   companion animal population. *C. difficile* carriage was common in dogs, cats, and horses. *C.*

23   *difficile* isolates from companion animals included many of the same ribotypes known to cause

24   HA- and CA-CDI in humans, and had similar antimicrobial resistance profiles as those isolated

25   from human populations. The large amount of contact between people and companion animals

1   carrying *C. difficile*, as well as the similarities between *C. difficile* isolates from humans and

2   companion animals, are consistent with the possibility of zoonotic transmission. However, it will

3   be critical going forward to develop new studies that can rigorously establish that zoonotic

4   transmission of *C. difficile* is a reality and not just a possibility. Understanding how *C. difficile* is

5   able to colonize different companion animals and what factors are able to prevent colonization

6   will be important for developing novel therapeutics to eradicate *C. difficile* in the animal

7   population, and hopefully thereby decreasing CA-CDI cases in humans.

8   **Ethics approval and consent to participate.**

9   Not applicable.

10   **Consent for publication.**

11   Not applicable.

12   **Availability of data and material.**

13   Raw sequences have been deposited in the Sequence Read Archive (SRA) with SRA accession

14   number: PRJNA562547.

15   **Competing interests.**

16   CMT is a scientific advisor to Locus Biosciences, a company engaged in the development of

17   antimicrobial technologies. CMT is a consultant for Vedanta Biosciences.

18   **Funding.**

21   **Authors' contributions.**

22   RT, AR, ADR, and TB designed and performed the experiments. RT, MRM, NSB, JAW, MJ,

23   BJC and CMT analyzed and interpreted the data. RT, MRM, NSB, and BJC analyzed and

24   displayed the microbiome data. RT, MRM, MJ, BJC and CMT contributed to writing the paper.

25   All authors edited the manuscript.

24

1 **Acknowledgements.**

2 None to report.

3 **Figure legends.**

4 **Figure 1: Animal fecal samples analysis workflow.**

5 Schematic presenting the workflow of fecal sample collection, overall *C. difficile* prevalence

6 determination, *C. difficile* isolate characterization, and fecal microbiome analysis.

7 **Figure 2: The fecal microbiota associated with *C. difficile* prevalence in animals.**

8 A) Alpha diversity in *C. difficile* positive (circles) and negative (open triangle) samples in

9 canines, felines, ovines, and equines. The distribution of inverse Simpson index is presented for

10 different animal groups at the family, genus, and ASV levels. B) Evaluation of beta-diversity in

11 different animal groups that were *C. difficile* positive or negative. Using unsupervised clustering

12 the nonmetric multidimensional scaling (NMDS) illustrates the dissimilarity indices via Bray-

13 Curtis distances between the bacterial communities from animal feces.

14 **Figure 3: Community composition differences between *C. difficile* positive and negative**

15 **canine samples using a centered-log-ratio (CLR) transform of the ASV abundances.**

16 Community differences were analyzed by principal components analysis (PCA) and differential-

17 abundance analysis (with ALDEx2) of the CLR-transformed abundances. A) and B) show the

18 first two principal components (PCs), which explain 15% and 7% of the variance. Panel A shows

19 the samples and Panel B shows the top ten positive and negatively associated taxa detected by

20 ALDEx2. C) shows the CLR relative abundance for a subset of differentially abundant ASVs in

21 *C. difficile* positive and negative samples. These ASVs are the 4 with the largest positive effect

22 size (ASV82, ASV233, ASV331, and ASV37), the 4 with the largest negative effect size (ASV4,

23 ASV10, ASV20, and ASV112), and the lower-prevalence *C. hiranonis* ASV (ASV62). Violin plots

24 show the estimated distribution of the CLR values in *C. difficile* positive and negative samples,

25 accounting for uncertainty in each sample due to multinomial sampling error during sequencing.

25

1   Points indicate the mean CLR value for each sample. The numbers to the right of the violin plots

2   indicate the prevalence of the ASV.

3   **Figure 4: *C. difficile* inhibits *C. hiranonis* growth in co-culture.**

4   Co-culture assays in Brain heart infusion media supplemented with 100 mg/L L-cysteine

5   between *C. difficile* and *C. hiranonis*, where cultures were mixed in the ratio of 1:1 (A), 1:10 (B),

6   or 1:100 (C). Colonies were enumerated and expressed as CFU/mL of culture at time points 0

7   and 24 h for the competition controls (monocultures of *C. difficile* and *C. hiranonis*) and the co-

8   cultures (1:1, 1:10, and 1:100). Data presented represents mean ± SD of at least four

9   experiments in A and B and triplicate experiments in C. Statistical significance between 24 h

10   monoculture and the respective co-culture treatments was determined by Student's parametric

11   t-test with Welch's correction (**, $p < 0.01$; ***, $p < 0.001$).

12   **Supplementary Figure 1: *C. difficile* presence by lab assay versus age in canines.** Each

13   point denotes a sample that was positive via the lab assay or negative. Canine age group in

14   years is listed by circle color.

15   **Supplementary Figure 2: Alpha diversity versus *C. difficile* presence in canines (A) and**

16   **equines (B).** Points indicate the inverse Simpson diversity in each sample for each taxonomic

17   rank (Family, Genus, and ASV), with the same color and shape as in Figure 2. Crosses indicate

18   the median and inter-quartile range for that group.

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52   Supplementary Table 1. Primer sequences used for colony PCR and 5-plex PCR

PCR	Gene target	Primer	Target sequence 5'-3'	Product size (bp)
Colony	16S rRNA	16S rRNA For 16S rRNA Rev	AGAGTTTGATCCTGGCTCAG ACGGCTACCTTGTTACGACTT	1465
5-plex	*tcdA*	tcdA-F3345 tcdA-R3969	GCATGATAAGGCAACTTCAGTGGTA AGTTCCTCCTGCTCCATCAAATG	629
5-plex	*tcdB*	tcdB-F5670 tcdB-R6079A tcdB-R6079B	GCATTTCTCCATTCTCAGCAAAGTA GCATTTCTCCATTCTCAGCAAAGTA GCATTTCTCCGTTTTCAGCAAAGTA	410
5-plex	*cdtA*	cdtA-F739A cdtA-F739B cdtA-R958	GGGAAGCACTATATTAAAGCAGAAGC GGGAAACATTATATTAAAGCAGAAGC CTGGGTTAGGATTATTTACTGGACCA	221
5-plex	*ctdB*	ctdB-F617 cdtB-R878	TTGACCCAAAGTTGATGTCTGATTG CGGATCTCTTGCTTCAGTCTTTATAG	262
5-plex	16S rDNA	PS13 PS14	GGAGGCAGCAGTGGGGAATA TGACGGGCGGTGTGTACAAG	1062

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Table 1. Toxin gene profiles of *C. difficile* isolated from different animals

Toxin gene profile	Animal groups			
	Canine	Feline	Equine	Total (%)
tcdA, *tcdB*, *cdtA*, *cdtB*	0	0	1	1 (2.4)
tcdA, *tcdB*	17	1	4	22 (52.4)
tcdA	4	0	0	4 (9.5)
tcdB	2	0	0	2 (4.7)
None	10	1	2	13 (30.9)
Total	33	2	7	42

Table 2. *C. difficile* PCR ribotypes isolated from different animals

PCR ribotypes	Animal groups			
	Canine	Feline	Equine	Total
F014-020*	7	0	1	8
F106*	7	0	1	8
FP310	5	1	0	6
FP313	3	0	0	3
F010	2	0	1	3
FP415	1	0	0	1
F087	1	0	0	1
FP418	1	0	0	1
FP484	1	0	0	1
FP499	1	0	0	1
FP407	0	0	1	1
078-126*	0	0	1	1
F002*	0	0	1	1
FP501	0	1	0	1
Unnamed ribotypes	2	0	1	3
# of different ribotypes	10	2	6	18
Total	31	2	7	40

C. difficile ribotype reported to be associated with acute *C. difficile* infection and relapses in humans. Ribotypes separated by hyphen represent similar strains and may belong to either ribotype.

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Table 3. Associations between clinical characteristics and *C. difficile* prevalence outcomes in canines

Demographic Factors	N (%)	Toxigenic	Non-toxigenic	Negative	Percent prevalence	*P*-value	Odds ratio*	95% CI
Age in years								
<1	5 (6.4)	1	0	4	20			
1-4	14 (18.1)	0	1	13	7.1			
5-9	30 (38.9)	10	2	18	40			
≥10	28 (36.3)	7	3	18	35.7			
Not known	30	9	0	21	30			
Gender								
M	61 (58.6)	18	3	40	34.4	0.39	1.53	0.66-3.56
F	43(41.3)	8	3	32	25.5			
Not known	3	1	0	2	33.3			
Gastrointestinal Status								
GI condition	48 (46.6)	11	4	33	31.3	>0.99	1.02	
Healthy	55 (53.3)	15	2	38	30.9			0.44-2.31
Not known	4	1	0	3	25.0			
Antibiotics								
Treated	59 (56.7)	18	4	37	37.3	0.20	1.84	
Non-treated	45 (43.2)	10	1	34	24.4			0.80-4.26
Not known	3	0	0	3	0			

101 *Odds ratio was calculated for *C. difficile* prevalence in males to females, GI condition to healthy, and antibiotic treated to non-
102 treated samples.

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107 Table 4. Association between clinical characteristics and *C. difficile* prevalence outcomes in equines

Demographic Factors	N (%)	*C. difficile* prevalence				*P*- value	Odds ratio*	95% CI
		Toxigenic	Non-toxigenic	Negative	Percent prevalence			
Age in years								
<2	5 (9.6)	1	1	3	40			
2-10	20 (38.5)	0	1	19	5			
≥10	27 (51.9)	1	0	26	3.6			
Not known	20	3	0	17	15			
Gender						0.42	0.44	0.10 – 1.78
M	39 (60.9)	3	0	36	7.6			
F	25 (39)	2	2	21	16			
Not known	8	0	0	8	0			
Gastrointestinal Status						0.01	∞	1.66 - ∞
GI condition	35 (53.8)	5	2	28	20			
Healthy	30 (46.2)	0	0	30	0			
Not known	7	0	0	7	0			
Antibiotics						0.70	1.61	0.29-8.66
Treated	36 (62.1)	4	1	31	13.9			
Non-treated	22 (37.9)	1	1	20	9.09			
Not known	14	0	0	14	0			

108 *Odds ratio was calculated for *C. difficile* prevalence in males to females, GI condition to healthy, and antibiotic treated to non-
109 treated samples.
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13   Table 5. *In vitro* antimicrobial susceptibility of *C. difficile* isolates

Antimicrobial (Breakpoint, µg/ml)	MIC range[a]	MIC$_{50}$[b]	MIC$_{90}$[c]	No. (%) of resistant isolates
Cefotaxime (≥64)	>32[d]	>32	>32	39 (100)
Clindamycin (≥8)	2->256	4	>256	9 (23)
Ciprofloxacin (≥8)	6->32	24	>32	38 (97)
Levofloxacin (≥8)	5->32	8	>32	23 (59)
Metronidazole (≥32)	0.38->256	1.0	1.5	1 (2.5)
Vancomycin (≥32)	0.38-1.0	0.75	0.75	0 (0)

20   [a]MIC range is the minimum inhibitory concentrations (expressed in µg/ml). [b]MIC$_{50}$ is the
21   minimum inhibitory concentration at which 50% of the isolates were inhibited. [c]MIC$_{90}$ is the
22   minimum inhibitory concentration at which 90% of the isolates were inhibited. [d]CLSI breakpoint
23   was 64 µg/ml, however the highest concentration on the commercially available test strip was 32
24   µg/ml. Therefore, any isolate found to be resistant at 32 µg/ml was marked resistant.

53 Table 6. Comparison between the detection of *C. difficile* via community 16S sequencing and
54 the spore-enrichment lab assay

		16S -	16S +	Total
Canine	Lab -	71	2	73
	Lab +	12	19	31
	Total	83	21	104
Equine	Lab -	56	6	62
	Lab +	4	3	7
	Total	60	9	69
Feline	Lab -	13	1	14
	Lab +	1	0	1
	Total	14	1	15
Ovine	Lab -	5	0	5
	Lab +	0	0	0
	Total	5	0	5
Total	Lab -	145	9	154
	Lab +	17	22	39
	Total	162	31	193

55 The grey cells denote samples for which *C. difficile* was detected by only one assay. Only
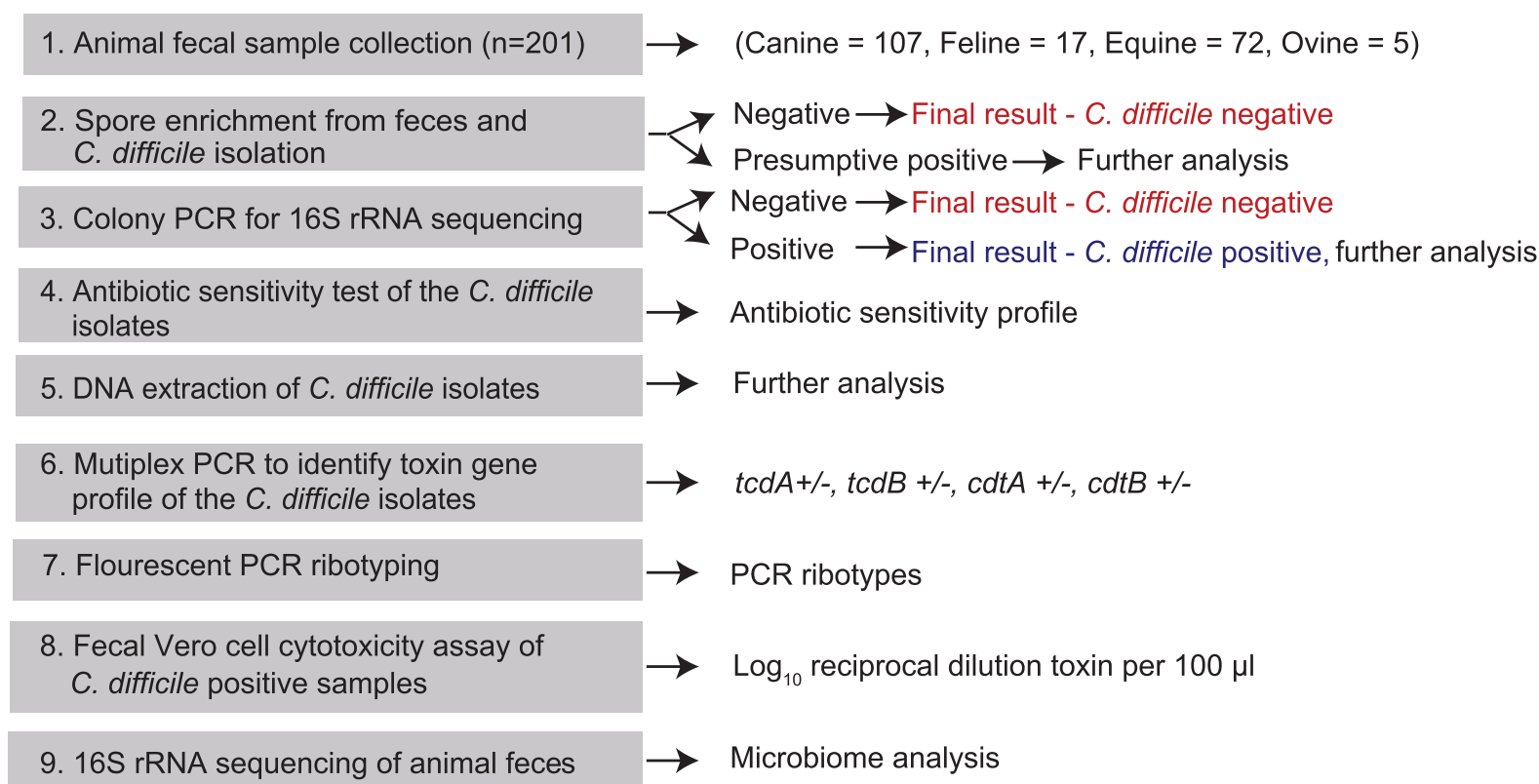56 animals for which 16S data is available are included.

1. Animal fecal sample collection (n=201)	→	(Canine = 107, Feline = 17, Equine = 72, Ovine = 5)

2. Spore enrichment from feces and *C. difficile* isolation	→	Negative → Final result - *C. difficile* negative Presumptive positive → Further analysis
3. Colony PCR for 16S rRNA sequencing	→	Negative → Final result - *C. difficile* negative Positive → Final result - *C. difficile* positive, further analysis
4. Antibiotic sensitivity test of the *C. difficile* isolates	→	Antibiotic sensitivity profile
5. DNA extraction of *C. difficile* isolates	→	Further analysis
6. Mutiplex PCR to identify toxin gene profile of the *C. difficile* isolates	→	*tcdA+/-, tcdB +/-, cdtA +/-, cdtB +/-*
7. Flourescent PCR ribotyping	→	PCR ribotypes
8. Fecal Vero cell cytotoxicity assay of *C. difficile* positive samples	→	Log_{10} reciprocal dilution toxin per 100 μl
9. 16S rRNA sequencing of animal feces	→	Microbiome analysis

Figure 1: Animal fecal sample analysis workflow.
Schematic presenting the workflow of fecal sample collection, overall *C. difficile* prevalence determination, *C. difficile* isolate characterization, and fecal microbiome analysis.
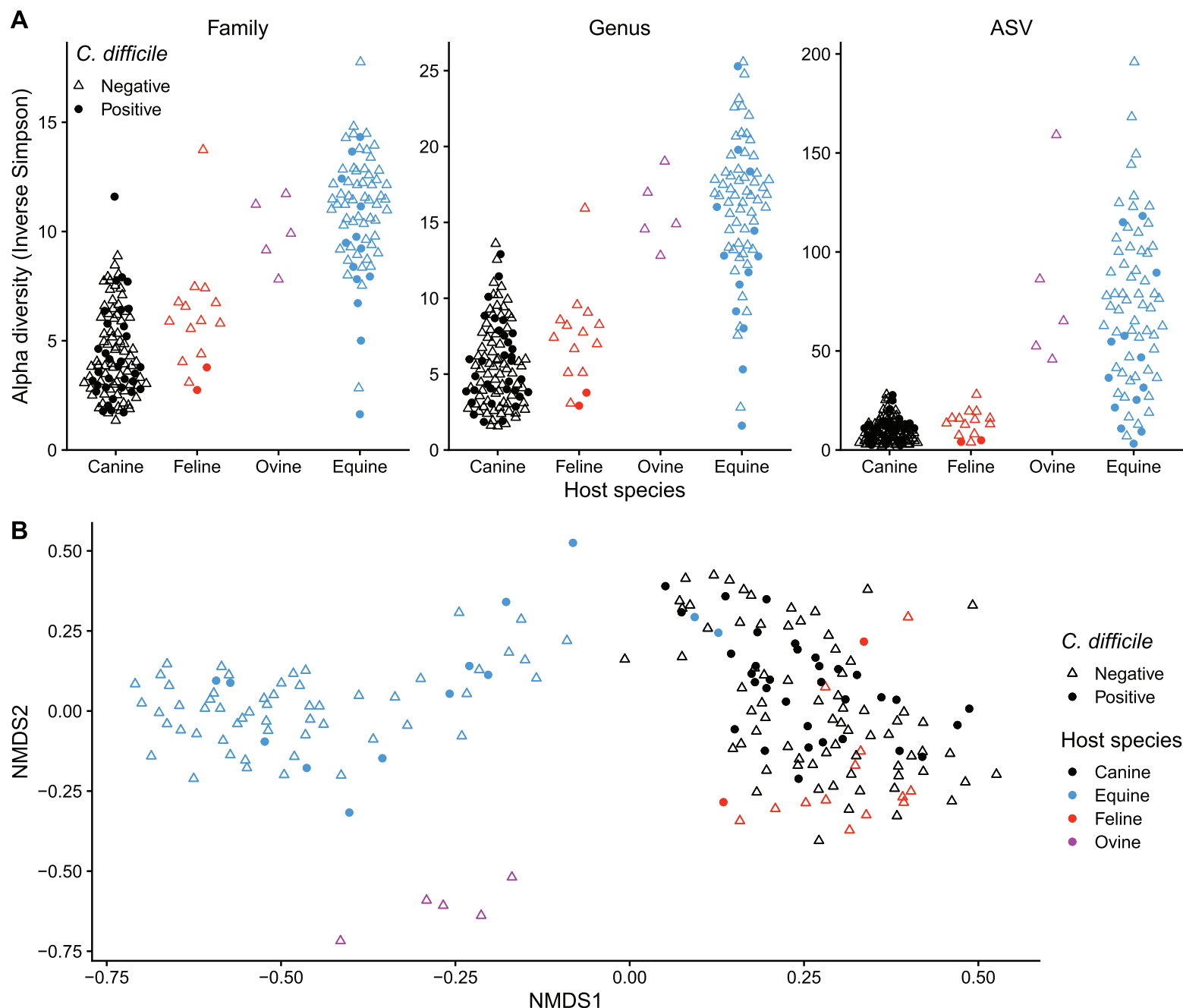
Figure 2: The fecal microbiota associated with *C. difficile* prevalence in animals.
A) Alpha diversity in *C. difficile* positive (circles) and negative (open triangle) samples in canines, felines, ovines, and equines. The distribution of inverse Simpson index is presented for different animal groups at the family, genus, and ASV levels. B) Evaluation of beta-diversity in different animal groups that were *C. difficile* positive or negative. Using unsupervised clustering the nonmetric multidimensional scaling (NMDS) illustrates the dissimilarity indices via Bray-Curtis distances between the bacterial communities from animal feces.
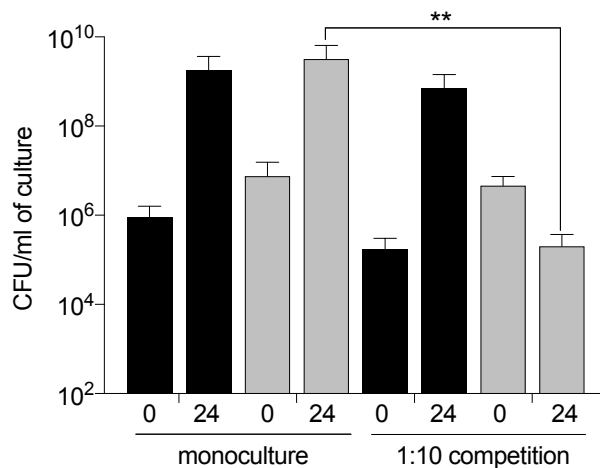
Figure 3: Community composition differences between *C. difficile* positive and negative canine samples using a centered-log-ratio (CLR) transform of the ASV abundances. Community differences were analyzed by principal components analysis (PCA) and differential-abundance analysis (with ALDEx2) of the CLR-transformed abundances. A) and B) show the first two principal components (PCs), which explain 15% and 7% of the variance. Panel A shows the samples and Panel B shows the top ten positive and negatively associated taxa detected by ALDEx2. C) shows the CLR relative abundance for a subset of differentially abundant ASVs in *C. difficile* positive and negative samples. These ASVs are the 4 with the largest positive effect size (ASV82, ASV233, ASV331, and ASV37), the 4 with the largest negative effect size (ASV4, ASV10, ASV20, and ASV112), and the lower-prevalence C. hiranonis ASV (ASV62). Violin plots show the estimated distribution of the CLR values in *C. difficile* positive and negative samples, accounting for uncertainty in each sample due to multinomial sampling error during sequencing. Points indicate the mean CLR value for each sample. The numbers to the right of the violin plots indicate the prevalence of the ASV.
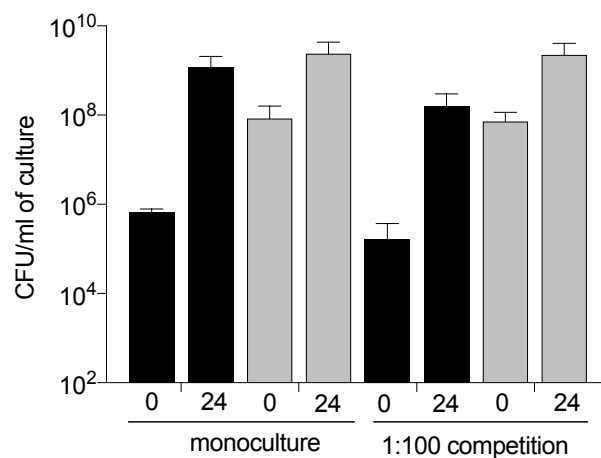
Figure 4: *C. difficile* inhibits *C. hiranonis* growth in co-culture.
Co-culture assays in Brain heart infusion media supplemented with 100 mg/L L-cysteine between *C. difficile* and *C. hiranonis*, where cultures were mixed in the ratio of 1:1 (A), 1:10 (B), or 1:100 (C). Colonies were enumerated and expressed as CFU/mL of culture at time points 0 and 24 h for the competition controls (monocultures of *C. difficile* and *C. hiranonis*) and the co-cultures (1:1, 1:10, and 1:100). Data presented represents mean ± SD of at least four experiments in A and B and triplicate experiments in C. Statistical significance between 24 h monoculture and the respective co-culture treatments was determined by Student's parametric t-test with Welch's correction (**, $p < 0.01$; ***, $p < 0.001$).