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Prevalence of *Clostridium difficile* and *Clostridium perfringens* in Swiss horses with and without gastrointestinal disease and microbiota composition in relation to *Clostridium difficile* shedding

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ABSTRACT

Overgrowth of enteric clostridia in dysbiosis in horses with colic is presumed but scarcely investigated. The objective was to provide prevalence data of *Clostridium difficile* and *Clostridium perfringens* in horses with and without gastrointestinal disease in Switzerland, and investigate microbiota differences between *C. difficile* shedders and non-shedders.

Fecal samples were taken from healthy horses (n = 103), horses with colic (n = 98) and horses with diarrhea (n = 151). Colic horses were sampled on three days. Selective enrichment culture and molecular typing for *C. difficile* and *C. perfringens* was performed. Microbiota differences between horses with colic shedding (n = 7) and not shedding (n = 7) *C. difficile* were assessed using metagenomic sequencing.

The cumulative prevalence (19% *C. difficile*; 16% *C. perfringens)* was higher compared to single day samples (1–10% *C. difficile*; 3–8% *C. perfringens, all* $p < 0.003$). Horses with colic shed significantly more *C. difficile* ($p < 0.001$) but not *C. perfringens* ($p = 0.09$) compared to healthy horses. Prevalence in horses with diarrhea was 8% for both *Clostridium* species. There were no significant microbiota differences between *C. difficile* shedders and non-shedders with regards to relative abundance on any phylogenetic level, and alpha diversity. Limited differences were seen on LEfSE analysis and in beta diversity indices.

Multiple fecal samples should be taken when investigating shedding of enteric clostridia. As horses with colic shed more enteric clostridia compared to healthy horses special biosecurity protocols for horses with colic should be considered in hospitals. Differences in microbiota composition between *C. difficile* shedders and non-shedders were limited. Further studies on the role of dysbiosis in *C. difficile* are needed.

1. Introduction

Clostridium (*C.*) *difficile* and *Clostridium* (*C.*) *perfringens* are important enteric pathogens in human and veterinary medicine, but healthy animals also harbor these pathogens (Baverud, 2002).

C. difficile is considered an important cause of acute enterocolitis in horses and foals (Weese et al., 2001) and has also been suggested as a cause of duodenitis/proximal jejunitis (anterior enteritis) (Arroyo et al., 2006). Reported shedding rates of *C. difficile* based on fecal samples in healthy adult horses vary between 0%–25%, depending on geographical location and horse population sampled, particularly age and history of antimicrobial exposure (Ossiprandi et al., 2010; Weese et al., 2001). In most horses, this does not result in disease, and it is presumed that some degree of intestinal dysbiosis is required, allowing *C. difficile* to overgrow, produce toxins and cause disease. However, dysbiosis is a rather nebulous term, with no clear definition. While marked alterations in the fecal microbiota have been identified in people with recurrent *C. difficile* infection, studies of horses are scarce. The microbiota of five horses shedding *C. difficile* was not different to five control horses; however only three horses were affected by

Abbreviations: AMOVA, analysis of molecular variance; ANOSIM, analysis of similarities; bp, base pairs; FDR, false discovery rate; LEfSe, Linear discriminant analysis effect size; OUT, operational taxonomic unit; PcOA, principal coordinate analysis; RDP, ribosomal database project

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gastrointestinal disease (Rodriguez et al., 2015). Current knowledge on *C. difficile* strains in humans in Switzerland is limited, and there are no data for horses.

Clostridium perfringens is a well-characterized cause of intestinal and extraintestinal disease in various species, including horses. Yet, it can be found in 0–8% in feces of healthy adult horses other than broodmares, where the prevalence can approach 35% (Bacciarini et al., 2003; Waggett et al., 2010). It is more commonly (6–41 %) found in horses with gastrointestinal disease (Herholz et al., 1999) although cause versus effect is difficult to differentiate, since *C. perfringens* is able to readily grow when changes in the microbiota have occurred. Enterocolitis in foals has been associated with *C. perfringens* Type A and Type C (East et al., 1998). The role of *C. perfringens* as an etiologic agent of colitis in adults is less clear. There is some evidence to support that enterotoxigenic *C. perfringens* (CPE producing strains) or beta-2 toxin is associated with enteric disease in horses (Herholz et al., 1999), however this is still unclear. Recently a newly detected toxin, termed *netF,* has been identified in foals with severe neonatal enterocolitis. The toxin was shown to be cytotoxic, and *netF* positive isolates belonged to a clonal population; (Gohari et al., 2015) however, it could not be detected in a subsequent study surveying diarrheic neonatal foals (Gohari et al., 2014). The role this toxin plays in the pathophysiology of enterocolitis is therefore unclear.

The objectives of this study were to investigate the prevalence of *C. difficile* and *C. perfringens* in the Swiss horse population based on health status (healthy horses, horses with diarrhea and horses with colic) and to investigate whether repetitive sampling on different days would increase the cumulative prevalence compared to one-time sampling. The second objective was to assess differences in microbiota composition between horses with colic that were shedding or not shedding *C. difficile*.

2. Methods

The study was performed in accordance with the Swiss Animal Legislation.

2.1. Animals

Healthy group: In total 103 fecal samples from healthy horses in Switzerland were collected between October 2014 and November 2015. Eight boarding stables were contacted in various areas of Switzerland and individual horse owners were asked to participate. Inclusion criteria for horses on these farms were more than one year of age, willingness to participate and availability of a fecal sample on the day of sampling. Exclusion criteria were treatment with antibiotics or non-steroidal anti-inflammatories, or a history of colic within the preceding six months. A single fecal sample was collected from these horses.

Colic group: Horses presented to the Veterinary Teaching hospital in Berne and Zurich between February and May 2015 for colic were included.

Diarrhea group: Horses presented for diarrhea or horses that developed diarrhea while they were hospitalized at the Veterinary Teaching hospital in Zurich between January 2013 and April 2016. Inclusion criteria were horses with ≥2 of the following signs: loose feces > 2 consecutive times, fever > 38.2C and leucopenia (< 4700 cells/μL). Loose feces were defined as feces that was amorphous when placed in a fecal container. Antimicrobial administration was recorded for every horse. Fecal samples were collected of the ground using gloved hands and care was taken to avoid contamination with bedding material. Fecal samples were stored at 4 °C for up to 48 h if not processed immediately.

2.2. Culture and molecular techniques

All fecal samples from healthy horses and horses with colic were analyzed using culture and molecular typing. Of the horses with diarrhea, only samples positive for *C. difficile* antigen or toxins and/or *C. perfringens* using were chosen for molecular typing. Presence of *C. difficile* in diarrhea horses was assessed using the TECHLAB® C. DIFF QUIK CHEK COMPLETE® ((Abbott, Baar, Switzerland)) according to the manufacturer`s instructions. For detection of *C. perfringens* in diarrhea horses a 10 μl loopful of feces was inoculated onto Columbia-Blood Agar (ThermoFisher Scientific, Pratteln, Switzerland) and incubated anaerobically at 37 °C for 24 h. *C. perfringens* colonies were identified by colony morphology and double-zone hemolysis. Christie-Atkins-Munch–Peterson (CAMP) testing was performed on suspicious colonies.

Fecal samples from healthy horses, horses with colic and selected diarrhea horses were further analyzed using culture and molecular typing. Culture was performed as previously described (Schoster et al., 2012). DNA was extracted from confirmed *C. difficile* and *C. perfringens* isolates from pure 24 h culture on blood agar with a commercial Chelex resin-based DNA extraction kit (InstaGene Matrix, Bio-Rad, California), using the manufacturer's instructions. The resulting DNA was used as template for subsequent PCR tests. For *C. difficile*, PCR detection of genes encoding toxins A (*tcdA*) and B (*tcdB*) and the binary toxin (*cdtA/cdtB*) was tested by a multiplex amplification targeting the above genes plus a segment of the 16S rDNA gene as an internal positive control (Persson et al., 2008). A previously described capillary ribotyping method and the Webribo server (https://webribo.ages.at) were used to analyze all positive *C. difficile* isolates (Fawley et al., 2015). When the ribotype was a recognized international ribotype previously identified by the PHLS Anaerobic Reference Unit (Cardiff, UK), the appropriate numerical designation (i.e. 078) was used. Otherwise, nomenclature was assigned by the Webribo server and entered into the databank if not yet existing. Typing of *C. perfringens* was based on genes encoding six toxin genes (*cpa, cpb, etx, iap, cpe* and *cpb2*), as described previously (Baums et al., 2004). Presence of *netF* was assessed as described previously (Gohari et al., 2015).

2.3. DNA extraction, 16S rRNA gene amplification, purification and sequencing

An aliquot of two hundred milligrams of feces was extracted using a commercial kit ([b] E.Z.N.A. Stool DNA Kit, Omega Bio-Tek Inc, GA, USA) according to manufacturer's recommendations. Adequate DNA quality and quantity were assessed by spectrophotometry (NanoDrop, Roche, ON, Canada. Amplification of the V4 region of the 16S rRNA gene, purification and sequencing were performed as previously described (Schoster et al., 2015). Briefly, primers targeting the V4 region of the 16S rRNA gene were designed with overhanging adapters for annealing to the Illumina index primers in the second PCR step. PCR products were purified and Illumina index primers were attached during the second PCR step. PCR products were purified and evaluated by gel-electrophoresis in 1.5% agarose gel. The samples were sequenced at the University of Guelph's Agriculture & Food Labusing an Illumina MiSeq (Illumina RTA v1.17.28; MCS v2.2).

2.4. Bioinformatics and statistics

The open-source software package, MOTHUR (v1.33.0) was used to process the sequences (Schloss et al., 2009). Paired end reads were aligned and then sequences were aligned with the SILVA 16S rRNA gene reference database (www.arb-silva.de) (Quast et al., 2013). Sequences with ambiguous base calls, inappropriate length (> 244 base pairs (bp) or < 239 bp), runs of homopolymers of > 8 bp, and sequences corresponding to chloroplasts, mitochondria, Archaea and Eukaryotes were removed. Chimeras were identified using uchime (Edgar et al., 2011) and removed. The remaining sequences were classified using the ribosomal database project (RDP) classifier (http://rdp.cme.msu.edu/index.jsp). Subsampling was performed to normalize sequence numbers for further comparison. This consisted of random

selection of a number of sequences from each sample that corresponded to the lowest sequence abundance of all samples. Completeness of sampling effort was assessed visually using rarefaction curves.

Alpha diversity was described using the Chao richness, Shannon's evenness and inverse Simpson's index. Only bacterial taxa accounting for more than one percent of the total were used for statistical analysis. Data were determined to be non-parametric based on examination of quantile plots and Shapiro-Wilk testing. Relative abundances and alpha diversity indices were compared between horses shedding *C. difficile* and non-shedders using the Wilcoxon test. False discovery rate (FDR) adjustments using the Benjamin Hochberg procedure were performed for comparisons of relative abundance of taxa between age groups and between foals with and without diarrhea.

Dendrograms based on community overlap (classical Jaccard index) and structure (Yue&Clayton index of dissimilarity) were created and visualized by FigTree v1.4.0 (http://tree.bio.ed.ac.uk/). Community overlap and structure were compared between groups by parsimony test, analysis of molecular variance (AMOVA), and analysis of similarities (ANOSIM) applied to the Jaccard and Yue&Clayton data, respectively. Dissimilarity was also visualized using principal coordinate analysis (PCoA). Linear discriminant analysis effect size (LEfSe) was performed to identify differentially abundant operational taxonomic units (OTUs) with 97% sequence similarity between groups (Segata et al., 2011). The Chi square test (or Fisher's exact test if less than 5 horses were present in a group) was used to compare the prevalence of *C. difficile* and *C. perfringens* between healthy horses and horses with colic and between cumulative prevalence and single day prevalence as well as inter-day prevalence in horses with colic. The Chi square test was also used to analyze the association of survival, antimicrobial use and diagnosis with clostridial shedding. A p-value of < 0.05 was considered significant for all comparisons. A commercial program was used for all statistical analyses (JMP Statistical discoveries, Version 11).

3. Results

3.1. Demographic data of animals

Fecal samples from a total 352 horses (103 healthy horses, 98 horses with colic and 151 horses with diarrhea) were evaluated for presence of *C. difficile* and *C. perfringens*. Prevalence based on health status is shown in Fig. 1. More horses with colic shed *C. perfringens* and *C. difficile* than healthy horses, but significance depended on which day the horses sampling was performed (Day 1 $p = 0.07$ and $p = 0.23$ for *C. perfringens* and *C. difficile*, respectively, Day 2 $p = 0.09$ and $p < 0.001$ and Day3/10 $p = 0.5$ and $p = 0.0008$).

3.2. Cumulative sampling

Horses with colic were sampled on three days. The single day prevalence of *C. difficile* and *C. perfringens* ranged from 1 to 10% and 3–8%, respectively (Fig. 1). The cumulative prevalence (positive on any day) was 19/98 (19%) for *C. difficile* and 16/98 (16%) for *C. perfringens*. The

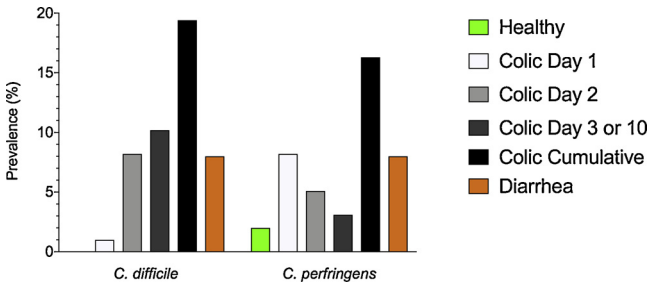

Fig. 1. Prevalence of *C. difficile* and *C. perfringens* in healthy Swiss horses (n = 103), horses with colic (n = 98) and horses with diarrhea (n = 151).

Table 1
Ribotypes and toxinprofiles of *C. difficile* isolated from horses with colic (n = 19) and horses with diarrhea (n = 10).

Ribotype	Toxin profile	Overall Prevalence x/29 (%)	Colic (n = 19)	Diarrhea (n = 10)
PR17515	A + B+CDT-	11 (38%)	11	0
014/0	A + B+CDT-	5 (17.4%)	4	1
078	A + B+CDT+	5 (17.4%)	1	4
PR22353	A + B+CDT-	1 (3.4%)	0	1
AI-78	A + B+CDT-	1 (3.4%)	0	1
PR21821	A + B+CDT-	1 (3.4%)	0	1
046	A + B+CDT-	1 (3.4%)	0	1
005	A + B+CDT-	1 (3.4%)	0	1
PR21823	A + B+CDT-	1 (3.4%)	1	0
PR21819	A + B+CDT-	1 (3.4%)	1	0
PR19783	A + B+CDT-	1 (3.4%)	1	0

Table 2
Types and toxins of *C. perfringens* and their prevalence in healthy horses (n = 2), horses with colic (n = 16) and horses with diarrhea (n = 6).

Type	Toxin gene	Overall n = 24 (%)	Healthy (n = 2)	Colic (n = 16)	Diarrhea (n = 6)
A	cpa	13 (54)	1	8	4
A	cpa, cpb2	4 (16)	1	3	0
A	cpa, cpe	3 (13)	0	2	1
A	cpa, cpe, netF	1 (4)	0	0	1
C	cpa, cpb	3 (13)	0	3	0

cumulative prevalence of *C. perfringens* shedding way significantly higher compared to single day shedding ($p > 0.0001$, $p < 0.0001$ and $p = 0.003$ for Day 1, Day 2 and Day3/10 samples). The cumulative prevalence of *C. difficile* shedding way significantly higher compared to single day shedding on most days ($p = 0.18$, $p < 0.0001$ and $p < 0.0001$ for Day 1, Day 2 and Day3/10 samples). Inter-day prevalence was not significantly different between Day 1, 2 and 3/10 for *C. perfringens* (all $p = 1$). Horses shed significantly more *C. difficile* on Day 2 compared to Day 1 ($p = 0.03$), but this was not significantly different between Day 1 and Day 3/10 ($p = 1$) and Day 2 and Day 3/10 ($p = 0.29$).

3.3. Association of diagnosis, antimicrobial treatment and survival with clostridial shedding

Of the 97 horses with colic, 26 (26%) were treated with antimicrobials at the time of sample collection, 15 (15%) underwent surgery and 94 (96%) survived. Horses that were euthanized were euthanized due to their primary disease, not due to clostridial shedding. Data for one horse were not available. Diagnosis for the horses with colic included equine gastric ulcer syndrome (n = 9), primary gastric impaction (n = 2), inflammatory lesions (inflammatory bowel disease, hepatitis, peritonitis, n = 7), large intestinal non-strangulating lesions (meteorism, large colon displacement and large colon impaction, n = 61), small intestinal strangulating lesions (n = 8), and unknown (n = 10). Horses with colic that had surgery were significantly more likely to shed *C. difficile* ($p = 0.01$, OR 4.7 95%CI 1.44–15.34), but not *C. perfringens* ($p = 0.74$, OR 1.2, 95%CI 0.36–4.51). There was no significant correlation between the diagnosis and shedding of *C. difficile* and *C. perfringens* ($p = 0.83$ and $p = 0.11$ respectively). Horses with colic that were treated with antimicrobials were significantly more likely to shed *C. difficile* ($p = 0.02$, OR 3.64, 95CI 1.25–10.61) but not *C. perfringens* ($p = 0.12$, OR 253 95%CI 0.83–7.72). Analysis of shedding and survival was not performed as only three horses with colic did not survive, and they were euthanized due to their primary lesion not due to clostridial shedding.

Of the 151 horses with diarrhea, 74 (49%) were treated with

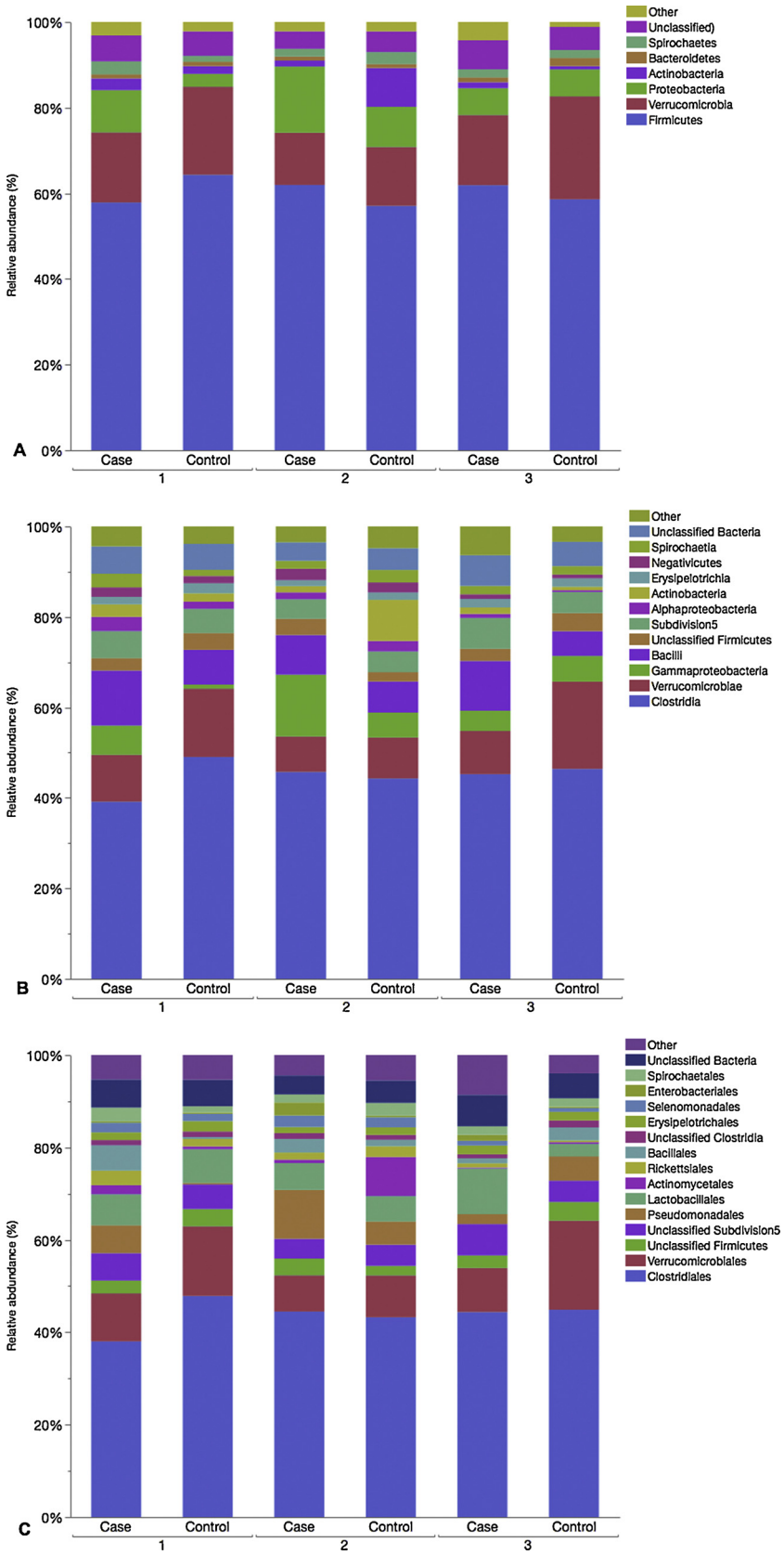

Fig. 2. Relative abundance of phyla (A), classes (B) and orders (C) in the fecal bacterial microbiota of horses with colic shedding *C. difficile* (n = 7) and non-shedders (n = 7) on 3 consecutive days.

Table 3
Relative abundance of significantly different taxa (before multiple comparison adjustment) in the fecal bacterial microbiota of horses with colic shedding *C. difficile* (n = 7) and non-shedders (n = 7) on 3 consecutive days.

	Day	Median relative abundance % (range %)		p-value	FDR adjusted p-value
		Case	Control		
Phylum					
Spirochaetes	1	2.1 (1.5-8.2)	1.2 (0.5-2.4)	0.018	0.108
Bacteriodetes	3	0.6 (0.2-3.6)	1.8 (1.5-2.4)	0.048	0.286
Class					
Clostridia	1	38.8 (21.7-55.3)	48.8 (43.1-56.8)	0.047	0.286
Spirochaetia	1	2.1 (1.6-8.2)	1.2 (0.6-2.4)	0.02	0.217
Order					
Clostridiales	1	38.3 (21.6-53.9)	47.6 (42.2-54.4)	0.253	0.189
Spirochaetales	1	2.1 (1.6-8.2)	1.3 (0.6-2.4)	0.018	0.189
Lactobacillales	3	9.1 (2.1-24.6)	2.6 (0.06-6.9)	0.035	0.525
Family					
Carnobacteria	3	5.1 (0.6-14.1)	0.3 (0.02-3.3)	0.009	0.097

FDR: False discovery rate adjusted (Benjamin Hochberg procedure), Case: Shedding *C. difficile*, Control: Not shedding *C. difficile.*

antimicrobials before sampling. Horses with diarrhea were significantly more likely to shed *C. difficile* (p = 0.009, OR 6.4, 95% CI 1.39–30.6) and *C. perfringens* (p = 0.016, OR 5.9, 95%CI 1.23–27.7) when treated with antimicrobials.

3.4. Molecular characterization

Molecular characterization was not possible in all isolates from horses with diarrhea as repeat culture in the laboratory were molecular typing was performed failed in one case of *C. difficile* and four cases of *C. perfringens*. The most common isolated *C. difficile* ribotype was PR17515 (11/29; 38%), followed by 014/0 and 078 (both 5/29; 17%). Other ribotypes detected were PR22353, 005, AI-78, PR21821, 046, PR21823, PR21819 and PR19783 (Table 1). Only one horse shed *C. difficile* on multiple days (day 1 and day 2), two different ribotypes were detected (014/0 and PR17515). None of the horses shed *C. perfringens* on more than one day. Of the horses with colic and those with diarrhea 1 and 2 respectively, shed both *C. difficile* and *C. perfringens* at the same time.

C. perfringens type A and C were present. Prevalence based on healthy status is shown in Table 2. Detection rates of beta-2 toxin, enterotoxin and netF are shown in Table 2.

3.5. Sequencing quality data

The DNA concentration after extraction ranged from 10 to 100 ng/µL. Final concentration of PCR products before sequencing averaged approximately 30 ng/µL. A total of 4,914,371 V4 16S RNA gene sequences passed all quality control filters. Subsampling was performed based on 58,916 sequences per sample. Sequencing depth was deemed adequate based on rarefaction curves (Additional file 1).

3.6. Microbial community composition of horses shedding C. difficile and non-shedders

Twenty different phyla, 68 classes, and 132 orders were identified.

Table 4
Species significantly enriched in the fecal microbiota of horses presented due to colic and shedding of *C. difficile* (n = 7, cases) or not shedding *C. difficile* (n = 7, control) on 3 consecutive days sampled over time determined by linear discriminant analysis effect size (LEfSe).

OTU	Family	Health	LDA	p-value
Day 1				
Unclassified	Unclassified Bacteria	Case	2.09	0.04
Unclassified	Unclassified Firmicute	Case	2.16	0.01
Unclassified	Unclassified Bacteria	Case	2.38	0.02
Unclassified	Unclassified Bacteria	Case	2.73	0.02
Selenomonas	Veillonellaceae	Control	2.04	0.05
Phascolarctobacterium	Acidaminococcaceae	Control	2.18	0.01
Unclassified	Unclassified Candidatus_Saccharibacteria	Control	2.19	0.05
Unclassified	Unclassified Verrucomicrobia	Control	2.22	0.05
Unclassified	Unclassified Verrucomicrobia	Control	2.39	0.04
Unclassified	Unclassified Lachnospiraceae	Control	2.48	0.05
Unclassified	Unclassified Candidatus_Saccharibacteria	Control	2.56	0.01
Treponema	Spirochetaceae	Control	2.92	0.01
Unclassified	Unclassified Clostridiales	Control	3.04	0.00
Unclassified	Unclassified Clostridiales	Control	3.73	0.03
Day 2				
Treponema	Spirochetaceae	Case	2.00	0.03
Unclassified	Unclassified Proteobacteria	Case	2.05	0.02
Acinetobacter	Moraxellaceae	Case	2.18	0.03
Sporobacter	Ruminococcaceae	Case	2.20	0.03
Akkermansia	Verrucomicrobiaceae	Case	2.24	0.05
Unclassified	Unclassified Bacteria	Case	2.41	0.02
Unclassified	Ruminococcaceae	Case	2.51	0.02
Streptococcus	Streptococcaceae	Case	2.85	0.05
Unclassified	Unclassified Verrucomicrobia	Case	2.95	0.04
Unclassified	Erychopelatrichaceae	Case	2.96	0.03
Sacharofermentans	Ruminococcaceae	Control	2.88	0.03
Day 3				
Unclassified	Unclassified Bacteroidetes	Case	2.01	0.01
Treponema	Spirochetaceae	Case	2.01	0.03
Unclassified	Choriobacteriaceae	Case	2.02	0.03
Unclassified	Unclassified Bacteria	Case	2.09	0.01
Unclassified	Unclassified Bacteria	Case	2.11	0.04
Phascolarctobacterium	Acidaminococcaceae	Case	2.21	0.04
Unclassified	Unclassified Clostridiales	Case	2.24	0.02
Unclassified	Lachnospiraceae	Case	2.25	0.05
Treponema	Spirochetaceae	Case	2.28	0.03
Unclassified	Lachnospiraceae	Case	2.30	0.03
Akkermansia	Verrucomicrobiaceae	Case	2.40	0.03
Unclassified	Unclassified Clostridiales	Case	2.41	0.02
Unclassified	Unclassified Verrucomicrobia	Case	2.43	0.05
Unclassified	Unclassified Firmicutes	Case	2.45	0.01
Unclassified	Unclassified Bacterioidetes	Case	2.46	0.02
Unclassified	Unclassified Bacteria	Case	2.50	0.01
Unclassified	Unclassified Clostridiales	Case	2.50	0.02
Unclassified	Unclassified Clostridiales	Case	2.54	0.02
Sporobacter	Ruminococcaceae	Case	2.68	0.01
Unclassified	Ruminococcaceae	Case	2.74	0.03
Treponema	Spirochetaceae	Case	2.78	0.01
Treponema	Spirochetaceae	Case	2.92	0.05
Unclassified	Veillonellaceae	Case	3.29	0.03
Sacharofermentans	Ruminococcaceae	Case	3.86	0.03
Sacharofermentans	Ruminococcaceae	Case	3.93	0.01
Streptococcus	Streptococcaceae	Control	2.04	0.03
Treponema	Spirochetaceae	Control	2.12	0.03
Unclassified	Unclassified Firmicutes	Control	2.13	0.03
Unclassified	Unclassified Bacteria	Control	2.14	0.03
Unclassified	Unclassified Clostridiales	Control	2.16	0.03
Schwartzia	Veillonellaceae	Control	2.19	0.02
Unclassified	Unclassified Candidatus_Saccharibacteria	Control	2.31	0.01
Unclassified	Unclassified Clostridiales	Control	2.54	0.02
Unclassified	Unclassified Clostridia	Control	2.56	0.04
Unclassified	Ruminococcaceae	Control	2.70	0.05
Unclassified	Unclassified Bacteria	Control	2.81	0.05
Selenomonas	Veillonellaceae	Control	2.82	0.03

Table 5
Alpha diversity indices of fecal bacterial microbiota of horses with colic shedding *C. difficile* (n = 7) and non-shedders (n = 7) on 3 days.

	Time point	Median (range)		p-value
		Control	Cases	
Chao Richness index	Day 1	15867.6 (10223.9–19427.3)	14518.4 (10325.4–16136.9)	0.34
	Day 2	12571.7 (10805–154.6)	13340.4 (11324.3–17176.9)	0.48
	Day 3	14943.7 (10946.6–16579)	15784.4 (11007.2–27147.7)	0.48
	All days	12859.9 (10223.9–19427.3)	14518.4 (11032.6–27147.7)	0.75
Simpson Diversity index	Day 1	71.4 (11.5–238.9)	94.9(38.8–294.6)	0.65
	Day 2	44.95 (29.12–124.17)	58.78(19.65–147.58)	0.65
	Day 3	47.52 (18.22–66.70)	92.41(30.48–163.34)	0.08
	All days	56.44 (11.47–238.85)	83.73 (19.65–294.57)	0.24
Shannon evenness index	Day 1	0.73 (0.57–0.78)	0.71(0.62–0.80)	0.95
	Day 2	0.69 (0.61–0.73)	0.70(0.60–0.75)	0.84
	Day 3	0.68 (0.62–0.72)	0.72(0.61–0.76)	0.06
	All days	0.69 (0.57–0.78)	0.71(0.60–0.80)	0.26
Good's coverage	Day 1	0.92 (0.91–0.95)	0.93(0.91–0.95)	0.41
	Day 2	0.94 (0.93–0.95)	0.93(0.92–0.94)	0.65
	Day 3	0.93 (0.92–0.95)	0.93(0.87–0.95)	0.48
	All days	0.93 (0.90–0.95)	0.91(0.86–0.95)	0.85

Case: Shedding *C. difficile*, Control: Not-shedding *C. difficile*.

Six phyla, 12 classes, and 15 orders had a mean relative abundance of more than 1% (Fig. 2). Of 251 families identified, 11 had a mean relative abundance of > 1%. The most abundant families were Lachnospiraceae (10%), Ruminococcaceae (9%), Clostridiaceae (6%), Carnobacteriaceae (3%), Planococcaceae (2%), Eubacteriaceae (2%), Erysipelotrichaceae (2%), Veillonellaceae (2%), Streptococcaceae (1%), and Clostridiales incertae sedis XIII (1%) (Fig. 2).

3.7. Difference of fecal microbial composition of horses with colic shedding *C. difficile* and non-shedders

Six horses shedding *C. difficile* and six non-shedders were included. Inclusion criteria for this part of the study was medical treatment only, no administration of antimicrobials and availability of all three samples. This was done to avoid bias from anesthesia and antimicrobial treatment. The shedders were selected first and then the first non-shedder admitted immediately before or after the shedding horse was selected as control. There were no differences in relative abundance at any taxonomic level (phylum to genus) after FDR adjustment (Table 3). On Day 1, 10 taxa were identified as enriched using LEfSe in the non-shedders. Four taxa were enriched in the *C. difficile* shedding group on Day 1. On Day 2, one taxon was enriched in the non-shedder group whereas ten taxa were enriched in the *C. difficile* shedding group. On Day 3 twelve taxa were enriched in the non-shedder group and twenty-five taxa were enriched in the shedding group. There was no trend for any phylum order or class that predominated in any of the two groups (full list, P-values and LDA values see Table 4). There were no differences in alpha diversity indices observed when separating data by Day or when combining data from all days (Table 5). No clear differences in beta diversity were evident through comparison of Jaccard and Yue& Clayton indices (Fig. 3, Table 6).

4. Discussion

The prevalence of *C. difficile* and *C. perfringens* in healthy horses was comparable to prior studies, where the prevalence ranged between 0 and 10%, 0 and 8% (Tillotson et al., 2002; Waggett et al., 2010; Weese et al., 2001) respectively. Similar to what has been shown for *Salmonella* spp., (Palmer et al., 1985) *C. difficile* and *C. perfringens* cannot be isolated from feces consistently, as shedding is intermittent or numbers are too low for cultural detection. Detection threshold for *C. perfringens* has been shown to be 9 cfu/g feces using this method ;(Schoster et al., 2012) several different methods have been shown to be able to detect 100 spores/g feces of *C. difficile*, including the method used here (Limbago et al., 2012). We could show that multiple sampling increased the prevalence of *Clostridia* spp. from 1 to 10% on a single given day, up to 19% when samples were collected over three days. The cumulative prevalence over the course of one year has also been shown to be higher compared to a single sample in time (Schoster et al., 2012). Therefore, the analysis of more than one fecal sample is recommended in cases were shedding is being evaluated. It needs to be evaluated whether this is also necessary when trying to diagnose horses with clinical disease due to enteric clostridia, as it is reasonable to assume that horses with disease caused by *C. difficile* or *C. perfringens* would shed higher numbers of organisms in feces compared to healthy horses. However, this has not been clearly established.

It is interesting to note that *C. perfringens* prevalence decreased over the course of the hospital stay, while *C. difficile* prevalence increased. A potential explanation could be that *C. perfringens* shedding ceased as horses recovered from colic and their microbiota normalized. The limited microbiota differences seen between *C. difficile* shedders and non-shedders in our study, do not exclude the possibility that more substantial changes occur in association with C. *perfringens* shedding. Larger studies are needed to further explore the microbiota in relation to Clostridial shedding. The increase in *C. difficile* prevalence could be due to slower growth of *C. difficile* compared to C. *perfringens*, resulting in a later rise in prevalence. Another explanation could be potential acquisition of *C. difficile* in the hospital during the stay. This is supported by one predominant ribotype isolated in this study. The high cumulative prevalence of *C. perfringens* (16%) and *C. difficile* (19%) shedding among horses admitted for colic, as well as the potential nosocomial spread of C. *difficile*, further highlights the importance of equine hospital biosecurity. Ideally horses with colic should be admitted to, and housed in a separate ward of the hospital with nursing precautions to minimize the risk of spread of Clostridia spp and other pathogens (e.g. Salmonella, multi drug resistant bacteria etc.) (Ekiri et al., 2009; Kim et al., 2001). Targeted surveillance of high-risk groups, such as horses with gastrointestinal disease is also recommended in equine biosecurity protocols (Weese, 2014) and is further supported by this data.

Antimicrobial treatment significantly increased the odds of shedding Clostridia spp. in both, horses with colic and horses with diarrhea. Antimicrobial treatment leads to quantifiable and sustained alterations in the microbiota, irrespective of the antimicrobial used (Costa et al., 2015). This dysbiosis could explain the why Clostridia spp. are able to overgrow in antibiotic treated animals. We could not corroborate the dysbiosis in horse shedding *C. difficile*, however the power of the study was limited.

4.1. Molecular types

Some strains of *C. difficile* appear to be endemic and cause more severe disease compared to others. In North America one of the endemic strain of *C. difficile* in humans has been identified as BI/NAP1/027/III (REA/PFGE/ribotype/toxinotype) (McDonald et al., 2005) and

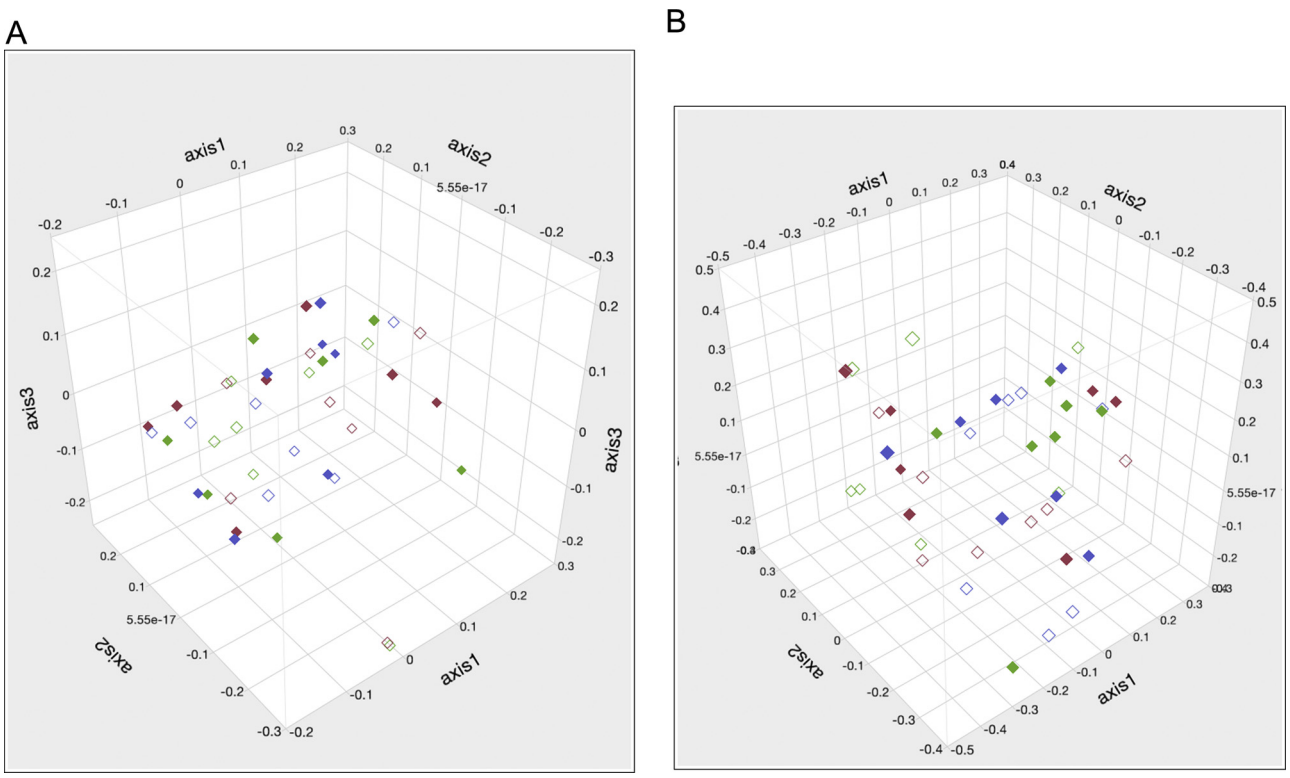

Fig. 3. Principal coordinate analysis based on the Jaccard index (A) and the Yue&Clayton index (B).
Full diamonds: Horses shedding *C. difficile* (n = 7), Empty diamonds: Horses not shedding *C. difficile* (n = 7). blue: Day 1, red Day 2, green Day 3.

Table 6
Difference in bacterial microbial composition (Jaccard index) and structure (Yue&Clayton index) of the fecal bacterial microbiota of colic horses shedding (n = 7) and not shedding (n = 7) enteric *Clostridia* spp.

	Day 1		Day 2		Day 3	
	Jaccard index p-value	Yue& Clayton index p-value	Jaccard index p-value	Yue& Clayton index p-value	Jaccard index p-value	Yue& Clayton index p-value
Parsimony	0.96	0.69	0.68	1	0.68	0.32
AMOVA	0.67	0.87	0.93	0.85	0.046	0.11
ANOSIM	0.76	0.98	0.89	0.75	0.005	0.06
Unifrac	0.99	0.94	0.99	0.89	0.99	0.95

more recently BK/NAP7/078/V in North America and Europe (Goorhuis et al., 2008). This latest strain is of concern due to high-level fluoroquinolone resistance and hypervirulence causing higher morbidity and mortality (Goorhuis et al., 2008). Data on molecular strains in horses are currently limited. Early studies in healthy adult horses have shown marked diversity in ribotypes (Arroyo et al., 2005; Ossiprandi et al., 2010) whereas more recent studies have shown a high prevalence of ribotype 078 and 027, thus potentially linking disease in horses to humans (Arroyo et al., 2007). All isolates obtained were toxigenic *C. difficile*. The most commonly found ribotype of *C. difficile* in this study was PR17515, followed by 014/0 (subtype of 014) and 078. Ribotype 014 was the most common ribotype in a multicenter European human hospital based study, including Switzerland, with a prevalence of 16%; 078 was also detected in that study (Bauer et al., 2011).

Similar to prior studies (Tillotson et al., 2002; Weese et al., 2001), *C. perfringens* type A was most commonly detected here. The role of CPB2 and CPE in equine diarrhea is controversial as they can be isolated from healthy horses and horses with diarrhea, similar to what has been seen in our study (Garmory et al., 2000; Herholz et al., 1999). No association between these toxin genes and diarrhea was identified; however, the sample size was inadequate for proper investigation of this. Recently, an additional toxin named *netF* has been identified in foals with necrotizing enterocolitis and in vitro cell cytotoxicity has been proven (Gohari et al., 2015). *NetF* was detected in only one isolate of *C. perfringens* obtained from one horse with diarrhea in our study. Further studies are needed to investigate the importance of this toxin. It is interesting to note that the *C. perfringens* type C isolates stemmed from horses with colic rather than horses with diarrhea. The small numbers preclude any strong conclusions but the roles of Type C *C. perfringens* and *netF* in enteric disease in adult horses deserve further investigation.

4.2. Microbiota differences between C. difficile shedders and non-shedders

It has been shown that dysbiosis occurs in horses with colic (Stewart et al., 2018; Weese et al., 2014) and other gastrointestinal diseases (Costa et al., 2012; Garrett et al., 2002; Rodriguez et al., 2015) and it has been speculated that isolation rates of enteric clostridia in horses with gastrointestinal disease are higher due to this dysbiosis. There are only limited data available testing this hypothesis. Microbiota differences in horses shedding and not shedding *C. difficile* could not be shown in a study where 10 horses were evaluated. Only three horses shedding *C. difficile* in the above study had gastrointestinal disease, the other two had orthopedic problems (Rodriguez et al., 2015). Similarly, in our study there were only minor differences seen in the microbiota structure and composition between horses shedding *C. difficile* and horses not shedding *C. difficile*. The reason for this is unclear, but the small sample size could influence results as the microbiota has been shown to vary significantly between individuals (Blackmore et al., 2013). It is also possible that the dysbiosis that occurred in horses with colic masked the differences that could be seen due to *C. difficile* shedding. We elected to study the horses with colic as too few healthy horses shed *C. difficile*.

4.3. Limitations

Only horses with colic were sampled on multiple occasions whereas both healthy horses and horses with diarrhea were only sampled once. Samples from horses with diarrhea were initially evaluated with a different method, if positive samples were frozen and re-cultured using the same method as for samples from colic horses and healthy horses. Given that not all samples were positive in the second culture, laboratory technique and repeated freezing and thawing likely had an influence. We elected not to perform any statistics to avoid comparison between differing methods. Only few samples were included in microbiota analysis, however the numbers correspond to other studies in this field (Costa et al., 2012; Rodriguez et al., 2015). Microbiota differences based on *C. perfringens* shedding were not evaluated.

4.4. Conclusion

Pathogenic enteric clostridia could be isolated from horses with and without gastrointestinal disease in Switzerland, and should be considered as etiologic agents of diarrhea. Testing of multiple fecal samples is recommended for detection of shedders. Horses with colic are more likely to shed *C. difficile* compared to healthy horses, but major microbiota differences could not conclusively be identified.

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Declaration of Competing Interest

None.

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