Analysis of gut microbiota with cryptosporidiosis based on fecal condition in neonatal dairy calves on a farm in Japan

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Abstract: Cryptosporidiosis is a major cause of diarrhea and is associated with high morbidity in calves. Changes in the gut microbiota exacerbate diarrhea caused by Cryptosporidium parvum (C. parvum) infection in neonatal and weaned calves. However, information on the gut microbiota of neonatal calves with C. parvum infection is scarce, and research into the microbiome of calves is essential for developing preventive and therapeutic interventions. This study aimed to elucidate the gut microbiota of neonatal calves with cryptosporidiosis. We collected 31 fecal samples from 31 neonatal calves on a dairy farm with or without C. parvum antigen [CP(+) or CP(-)] using a kit and analyzed the differences in the microbiota between diarrheal (D) and normal (N) fecal samples with C. parvum infection based on the fecal score. The analyses revealed the α diversity indexes of fecal microbiota in CP(+)N samples were higher than that in CP(+)D samples. Megasophaera spp. and other rumen microbes were identified, and significantly associated with CP(+)N samples compared with CP(+)D samples by LEfSe analysis. We conclude that the specific gut microbiota could characterize fecal microbiota in calves with neonatal cryptosporidiosis without clinical symptoms.

Cryptosporidiosis, a major cause of neonatal diarrhea, has high morbidity in calves (Wyatt et al., 2010) and generally infects pre-weaned calves less than 6 weeks of age (Thomson et al., 2019). Cryptosporidiosis is caused by Cryptosporidium spp., and C. parvum is one of the main gastroenteric pathogens. Calves are mainly infected with C. parvum via the fecal-oral route (McDonald, 2000). Infections have a significant impact on the productivity and economy of farms in terms of milk and meat production, as they reduce growth rates and can cause death (Brook et al., 2008). Prevalence of C. parvum in fecal samples of cattle herds was reported to range from 13% to 100% in Europe (Imre and Dărăbuș, 2011) and was also high at 1.7–100% in Japan (El-Alfy and Nishikawa, 2020). A previous study indicated a correlation between parasite burden and diarrhea severity (Operario et al., 2015); however, another study indicated that infected calves show no or mild symptoms (Shaw et al., 2020). This inconsistency could be a result of the host intestinal microbiota and immune system (El-Alfy and Nishikawa, 2020; El-Deeb et al., 2022).

An association exists between the presence of Cryptosporidium spp. and intestinal microbiota, including a significant increase in Fusobacterium spp. in the feces of calves with cryptosporidiosis (Dorbek-Kolin et al., 2022). The onset of diarrhea in calves may be associated with the gut microbiota. Chen et al. (Chen et al., 2022) suggested that interactions among some bacteria could influence calf diarrhea and that some species of Prevotella spp. may be the core microbiota in calves. These results could indicate that changes in the gut microbiota are also one of the exacterating factors in diarrhea caused by Cryptosporidium spp. infection.

However, information on the gut microbiota in neonatal calves with Cryptosporidium spp. infection without diarrheal symptoms is scarce. Information from Cryptosporidium spp. infection-positive with non-diarrheal fecal samples is needed to develop preventive and therapeutic interventions. Therefore, this study aimed to elucidate the characteristics of the gut microbiota of neonatal calves with cryptosporidiosis based on clinical symptoms, on a dairy farm.

The study was conducted from June to November 2022 on a dairy farm (300 lactating cows) in Japan (42°45′36″N 143°03′00″E). Thirty-one dairy calves (Holstein-Friesian, Jersey, and Japanese Black cattle (by embryo transfer) until 1–2 weeks old) were randomly selected and assigned in this study (Table 1). The calves were housed in wooden pens individually and received the same feeding management after dam-calf separation. Dam-calf separation was performed approximately 12 h after birth with calves receiving Colostrum of the dam with feeding bottles, estimated to contain sufficient concentrations of immunoglobulin G using digital Brix refractometer instrument referring to Bielmann et al. (Bielmann et al., 2010). If the immunoglobulin G levels in the colostrum of the dam were low, the calf was fed powdered colostrum (225 g, HeadStart, Saskatoon Colostrum Company Ltd., Saskatoon, SK, Canada) twice on the day of birth. Fecal samples were collected from the calves during their scheduled health examination the week after birth (1–2 weeks of age). Fecal Cryptosporidium parvum (CP) antigen was detected using a kit (DipFit Cryptosporidium parvum, BIO K387, Bio-X Diagnostics S.A., Rochefort, Belgium) and the presence of the Cryptosporidium spp. antigen was recorded.
The bacterial 16S rRNA sequence of the V3–V4 region was amplified using 2 sets of primers; first-round: V3-V4f_MIX (5′-ACACTCTTTCCTACACGAGTCTTCCGATCT-NNNNN-GACTACHVGGGTATCTAATCC-3ʹ) and V3-V4r_MIX (5′-GAGTGTACGGGAGGCAGCAGTCTTCCGCTTCTCTTCTGCT-NNNNN-ACGCAGACGGCTCAAA-3ʹ), and second-round: 2ndF (5′-AATGATACGGCGACCACCGAGATCTACAC-NNNNN-GACTACHVGGGTATCTAATCC-3ʹ), and second-round: 2ndR (5′-CAAGCAGAAGACGGCATACGAGAT-Index1-GTGACTG-GAGTGTACGGGAGGCAGCAGTCTTCCGCTTCTCTTCTGCT-NNNNN-ACGCAGACGGCTCAAA-3ʹ). Sequencing was performed using the MiSeq system with MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA) at 2 × 300 bp. The Fastq barcode_splitter from the Fastx toolkit (ver. 0.0.14) was employed to selectively extract sequences that exactly matched the primers. Sequences with a low-quality score (<20) were removed, resulting in a final base pair length of 130 or less. Pre-processed sequences were then analyzed using Quantitative Insights into Microbiology Ecology (QIIME) 2 (v2022.8) and clustered into Operational Taxonomic Units (OTUs) based on the Greengenes database (https://greengenes.lbl.gov/Download/), with a 97% similarity threshold.

Alpha diversity metrics using the Shannon index (diversity, “shannon,” expH’), Simpson index (diversity, “simpson”), and Pielou index (evenness) were calculated to detect differences between CP antigen-positive (+) and antigen-negative (−) groups and between fecal samples from calves with and without diarrhea (N:...
samples with normal fecal condition, D: samples with diarrheal condition, based on the scoring) in the CP (+) groups. Statistical significance among groups in α diversity metrics was analyzed using Welch's t-test. Beta diversity was assessed using principal component analysis (PCA, prcomp) of the robust Aitchison distances (vegdist, "robust.aitchison") in each fecal sample from each group (Martino et al., 2019). To determine the microbiome variation attributable to individual samples, a permutational ANOVA (PERMANOVA, adonis2) was performed with permutations = 100,000. These analyses were conducted using R software version 4.3.2 (http://www.R-project.org/), with the “vegan” and “stats” packages; the results were deemed statistically significant at $P \leq 0.05$.

The linear discriminant analysis effect size (LEfSe) approach was used to identify microbial taxa that were significantly associated with the groups, according to Segata et al. (Segata et al., 2011). Briefly, the LEfSe algorithm contains a Kruskal–Wallis rank sum test to detect differences between classes and linear discriminant analysis (LDA) to detect differences in the relevant features. The parameters were set at $P = 0.05$ and LDA score = 2.0 for computation. LEfSe was performed using the LEfSe Docker container of the biobakery account (biobakery/LEfSe v1.0.0): the data set table was converted into LEfSe format (format_input.py), and LEfSe was executed (run_lefse.py) with the specified settings and no subclass specifications.

At the time of the first fecal examination in the neonatal area of the farm, most calves tested positive for CP antigens (Table 1; 29/31, 93.5%). Five of the 29 CP (+) fecal samples (17.2%) had normal fecal characteristics (CP (+)-N, FCS = 0 and 1), and 24 (82.8%) had runny-to-watery diarrhea (CP (+)-D, FCS = 2 and 3) (Table 1). In the follow-up survey conducted 3 mo after sampling, it was noted that 17 of the 29 CP (+) calves required medical treatments, while 12 calves maintained their vigor and appetite. No significant difference was observed in the frequency of treatments between CP (+)-N and CP (+)-D calves. Figure 1 shows the relative abundance of fecal microbes in CP (+) and CP (-) calves in phylum (A) and family (B) levels. There was no significant difference in the α and β diversity of fecal microbiota between CP (+) and CP (-) calves. Differences in the microbiota of CP (+)-N and CP (+)-D calves are shown in Figure 2. The α-diversity of the fecal microbiota in CP (+)-N samples was higher than that in CP (+)-D samples, as evidenced by the Simpson index (Figure 2B) and Pielou index (Figure 2C). Moreover, we observed a tendency for higher values in the Shannon index calculations (Figure 2A). This study revealed a high diversity of fecal microbiota in CP (+)-N. The β-diversity between CP (+)-N and CP (+)-D showed no significant difference (PERMANOVA; $P = 0.8832$, Figure 2D). In contrast, in the LEfSe analysis, 19 microbes were identified in CP (+)-N (n = 16) and CP (+)-D (n = 3). Specifically, *Megasphaera* spp., *Christensenella* spp., *Mogibacterium* spp., and *Saccharibacteria* (TM7) spp. were specifically identified in CP (+)-N samples, whereas *Lachnospiraceae* and *Actinomycetaceae* were specifically identified CP (+)-D samples (Figure 2E, F).

*Megasphaera* spp. synthesize short-chain fatty acids (Yoshikawa et al., 2018) as lactic acid bacteria, and previous studies have suggested the regulation and maintenance of intestinal homeostasis (Venegas et al., 2019), locally regulating the host intestinal immune response in animals (Binder, 2010; Bachem et al., 2019). Additionally, a decrease in short-chain fatty acid levels is associated with an increase in *Cryptosporidium parvum* infection in mice (Charania et al., 2020). Moreover, *Megasphaera elsdenii* is used as a probiotic in cattle to treat metabolic acidosis and improve pro-

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**Figure 1.** Microbial composition at the phylum (A) and family (B) levels. *Cryptosporidium* infection (-) (n = 2) and (+) (n = 29), and *Cryptosporidium* spp. positive infection (+)/ normal feces (n = 5) and (+)/ diarrhea (n = 24)
ductivity as a commercial product (Lopes et al., 2021) because it can convert lactate to short-chain fatty acids (Sedighi and Alipour, 2019) and maintain ruminal function (Counotte et al., 1981). Additionally, *Megasphaera* spp., was significantly associated with CP (+)-N samples, and could characterize fecal microbiota in calves with neonatal cryptosporidiosis without clinical symptoms. Consequently, *Megasphaera* spp. may serve as a promising candidate for probiotic and therapeutic interventions against cryptosporidiosis.

*Christensenellaceae*, a recently described family in the phylum Firmicutes, is generally detected in mammalian intestine and rumen (Ramayo-Caldas et al., 2020). Previous studies have considered this microbe as an important hydrogen-producing bacterial group utilized for metabolism in the human intestine (Waters and Ley, 2019; Miura et al., 2021) and methane production in the rumen (Andrade et al., 2022). However, their role in the guts of ruminants, particularly neonatal calves, remains unclear. Additionally, *Mogibacterium* and *Saccharibacteria* (TM7) are abundant in rumen fluid. An increased abundance of *Mogibacterium* was associated with high methane production in the rumen (Wallace et al., 2015). Additionally, *Saccharibacteria* may contribute to improved cellulose degradation (Opdahl et al., 2018). Fecal samples were collected from 2-week-old calves without a fully developed rumen. Therefore, these microbes may have been transferred to the calves from their dams or the environment. Microbial transfer from dams to calves is important for constructing the calf microbiota and influencing their subsequent formation (Yeoman et al., 2018). Therefore, calves that did not present with diarrhea may have received sufficient microbiota from their dams in the farm environment.

*Lachnospiraceae* and *Actinomycetaceae* were specifically identified in diarrheal samples with *C. parvum* in the current study by LEfSe analysis. Consistent with our results, Xin et al. (2021) identified *Actinomycetaceae*, *Enterobacteriaceae*, and *Fusobacteriaceae* as specifically detected in diarrheic calves, suggesting that these differences affected short-chain fatty acid production in the intestine. Conversely, an earlier study indicated that *Lachnospiraceae* was more abundant in the fecal sample from diarrheal calves treated with antibiotics (Kim et al., 2021) and can potentially reduce inflammatory response in the intestine (Liu et al., 2022). In the current study, these microbes may be related to the anti-inflammatory response in the intestine.

Here, we could not measure the level of blood immunoglobulins in calves and could only estimate the colostrum intake from the dam using Brix values. Consequently, the evaluation of calves’ microorganisms, and their potential impact on health outcomes, requires further investigation.

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**Figure 2.** Summary of α and β diversity of fecal microbiota with cryptosporidiosis. The blue and orange circles indicate cryptosporidiosis calves showing non-diarrheal (CP(+)-N) and diarrheal (CP(+)-D) symptoms, respectively. Alpha diversity index based on the Shannon (A), Simpson (B), and Pielou (C) indices. Beta diversity was assessed using principal component analysis (PCA) of robust Aitchison distances within each fecal sample from each group based on the bacterial 16S rRNA gene sequence data for fecal samples (D). Permutational multivariate ANOVA (PERMANOVA) clustering and differences in dispersion results are indicated, along with a 95% confidence interval. Linear discriminant analysis (LDA) scores of abundant taxa (E) by LEfSe analysis and cladogram (F) in the *Cryptosporidium* infected but non-diarrhea feces. The red and green highlighted objects indicate specific microbes in the samples of cryptosporidiosis calves showing diarrheal symptoms and cryptosporidiosis calves showing non-diarrheal symptoms, respectively.
immune response to Cryptosporidium spp. in this study is limited by this constraint. A previous study indicated the level of clinical features associated with the immune response of calves in cryptosporidiosis (El-Alfy and Nishikawa, 2020; El-Deeb et al., 2022). Here, all calves could receive sufficient immunoglobulins either from the dam’s colostrum or powdered colostrum, resulting in robust vigor and appetite in all calves before fecal sampling at age 1–2 weeks. We presumed that all experimental calves received sufficient levels of immunoglobulins, and that the composition of the intestinal microbiota is one of the factors in whether calves infected with Cryptosporidium spp. show clinical signs of diarrhea or not.

We focused on non-diarrheal calves infected with Cryptosporidium spp., and this is a novel study suggesting that some bacteria could prevent the clinical burden. Summarily, the microbial composition of non-diarrheal calves with cryptosporidiosis could differ from that of calves with diarrhea. Although further analysis of the gut microbiota of animals with cryptosporidiosis showing no diarrheal symptoms is needed, the microbes specifically identified in fecal samples from non-diarrheal calves with cryptosporidiosis, such as Megasphaera spp. and other rumen microbes, may have the potential to reduce clinical burden associated with cryptosporidiosis in calves.

References


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Notes

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