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Author	Ji-Yeong Ku1†, Mi-Jin Lee2†, Youngwoo Jung1, Kwang-Man Park1, Jonghun Baek1, Youngjun Kim1, Ji-Seon Yoon1, Kyoung-Seong Choi3, Hak-Jong Choi4*, Jinho Park1*
Affiliation	1Department of Veterinary Internal Medicine, College of Veterinary Medicine, Jeonbuk University, Iksan 54596, Republic of Korea 2Department of Veterinary Nursing, College of Health Science, Wonkwang University, Iksan 54538, Republic of Korea 3Department of Animal Science and Biotechnology, College of Ecology and Environmental Science, Kyungpook National University, Sangju 37224, Republic of Korea 4Technology Innovation Research Division, World Institute of Kimchi, Gwangju 61755, Republic of Korea
ORCID (for more information, please visit https://orcid.org)	Ji-Yeong Ku (https://orcid.org/0000-0002-7819-7830) Mi-Jin Lee (https://orcid.org/0000-0001-9439-2284) Youngwoo Jung (https://orcid.org/0009-0003-0297-2356) Kwang-Man Park (https://orcid.org/0000-0002-3809-0633) Jonghun Baek (http://orcid.org/0009-0000-2428-5643) Youngjun Kim (http://orcid.org/0000-0002-8997-1086) Ji-Seon Yoon (https://orcid.org/0000-0002-7354-3255) Kyoung-Seong Choi (https://orcid.org/0000-0002-2271-5360) Hak-Jong Choi (https://orcid.org/0000-0003-1185-0919) Jinho Park (https://orcid.org/0000-0001-5235-5717)
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CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	**Fill in information in each box below**
First name, middle initial, last name	Jinho Park
Email address – this is where your proofs will be sent	jpark@jbnu.ac.kr
Secondary Email address	
Address	79, Gobong-ro, Iksan-si, Jeonbuk-do, Republic of Korea
Cell phone number	+82-10-2670-3046
Office phone number	+82-63-850-0949
Fax number	+82-63-850-0910

Abstract

The gut microbiome of cattle suppresses pathogens and aids host immunity. However, the gut microbiome of newborn calves is still developing; therefore, diarrhea caused by pathogen infection is common. Rapid changes in the gut microbiome due to diarrhea have a significant impact on the health and growth of calves. Until recently, there have been few studies on the changes in the gut microbiome following infection with major digestive pathogens that cause diarrhea in Hanwoo (Korean indigenous cattle) calves. Therefore, this study was conducted to identify viral digestive pathogens that cause severe diarrhea in Hanwoo calves. Seven normal calves without diarrhea and eight calves with diarrhea were selected, and their feces were collected to analyze pathogens and the gut microbiome. Bovine rotavirus (BRV) and bovine coronavirus (BCoV) were detected in the feces of the calves with diarrhea. There was no significant difference in the alpha diversity of the microbiome between normal calves and calves infected with viruses; however, a significant decrease in NPShannon and Shannon indices and a significant increase in Simpson index were observed in calves infected with BRV compared to calves infected with BCoV. In addition, beta diversity of the microbiome differed distinctly between normal calves and calves infected with BRV or BCoV. At the class level, BRV infection increased *Gammaproteobacteria* and *Actinobacteria*, whereas BCoV infection increased *Clostridia* and decreased *Bacilli*. In addition, the abundance of *Lactobacillus* was significantly reduced upon infection with BRV and BCoV. In this study, we confirmed the differences in the gut microbiome based on viral pathogens causing diarrhea in Hanwoo calves. The results of pathogen-targeting research are expected to be helpful in preventing common pathogens in calves.

Keywords: gut microbiome, Hanwoo calf, bovine rotavirus, bovine coronavirus, diarrhea

Introduction

Many microorganisms living in the intestine are known to play important roles in maintaining homeostasis and host health as commensal microorganisms by producing metabolites that cannot be synthesized in the body or inhibiting the growth of pathogens [1, 2]. Among commensal bacteria, opportunistic pathogenic bacteria (pathobionts) exhibit pathogenicity depending on the environmental conditions [3, 4]. Changes in the gut microbiome occur owing to various factors, such as the environment, feed, and pathogen infection. Among these, diarrhea caused by pathogen infection causes rapid changes in the gut microbiome [5-7]. Compared to healthy calves, the calves with diarrhea have reduced gut microbiome diversity and a microflora dominated by harmful bacteria [8]. In particular, newborn calves are easily infected with pathogens because their gut microbiome is not fully developed, and diarrhea caused by pathogen infection in the early stages of growth can have significant impacts on subsequent growth and can even lead to death [9, 10].

McGuirk [11] defined diarrhea as feces with a fecal score of 2 (loose but consistent enough to remain on bedding) or 3 (watery feces that shift through bedding material). Diarrhea is a serious disease that affects newborn calves under 30 days of age and causes significant economic losses to farms [12-14]. Diarrhea in Hanwoo (Korean indigenous cattle) calves is caused by viruses (such as bovine viral diarrhea virus [BVDV], bovine rotavirus [BRV], bovine coronavirus [BCoV], etc.), protozoa (such as *Giardia* spp., *Eimeria* spp., *Cryptosporidium* spp.), and bacteria (such as *Escherichia coli* K99) [15-18]. Additionally, cases of simultaneous infection with two or more pathogens have been frequently confirmed [19]. In general, when diarrhea occurs in calves, clinical symptoms such as loss of appetite, dehydration, electrolyte imbalance, and metabolic acidosis appear. However, the infection mechanism differs depending on the type of pathogen, resulting in differences in clinical symptoms [17].

To date, many studies have been conducted on various pathogens causing diarrhea in Hanwoo calves and the resulting clinical symptoms; however, few studies have been conducted on the changes in the gut microbiome of Hanwoo calves following infection with each pathogen [20-22]. To prevent diarrheal symptoms caused by pathogen infection, vaccines, farm management, and colostrum intake are necessary. Early diagnosis of each pathogen using predictive indicators and accordingly treating and managing the calves are necessary. Confirming the relationship between pathogens and the gut microbiome of calves will help understand the effect of each pathogen on calves and will likely help prevent pathogenic infection. Therefore, the gut microbiomes of normal Hanwoo calves and those infected with BRV or BCoV with diarrheal symptoms were compared to analyze the effects of each pathogen on the gut microbiome.

Materials and Methods

Animals

Fecal samples from eight Hanwoo calves under 30 days of age with diarrhea and seven of those without diarrhea were collected from several farms in Gyeongsangnam-do, Republic of Korea. The fecal consistency of diarrhea samples is loose or watery, while normal samples without diarrhea are solid and semi-solid. The collected feces were conducted pathogen tests and gut microbiomes were compared.

All fecal samples were collected directly from the calves' anus by massaging the rectal wall with a finger to induce defecation. The collected feces were transported to the laboratory in a refrigerated state and stored at -20 °C until analysis.

Pathogen Detection

For BRV and BCoV testing in the collected fecal samples, 1 g of the fecal sample was placed in a 15 mL sterile tube (Conical Tube, SPL Life Sciences, Pocheon, Korea), mixed with 10 mL of PBS, and centrifuged at 3000 × g for 10 min. DNA/RNA was extracted from the supernatant using an automated extraction kit (AutoXT PGS DNA/RNA Kit; iNtRON). The extracted RNA was used to detect BVDV, BRV, and BCoV by real-time reverse transcription polymerase chain reaction (Real-time RT-PCR) using a commercialized bovine diarrhea virus triple test kit (PowerChek™ Bovine Disease Virus Triplex Real time PCR Kit, Kogene Biotech, Seoul, Korea). The reaction solution was prepared by adding 5 μL of the extracted RNA to 15 μL of the Real-time RT-PCR Premix. Afterwards, the reaction was performed at 50 °C for 30 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min each [23].

Gene extraction and gene amplification

The total genomic DNA of the microorganisms present in the 15 collected fecal samples was extracted using a DNA extraction kit (FastDNA SPIN Kit for Soil, MP BIO). The V3/V4 region of the 16S rRNA gene was amplified by PCR using the primers 341F and 805R. The metagenome of microorganisms extracted from each fecal sample was used as a template (PTC-200 Peltier thermal cycler, MJ Research, Waltham, MA, USA) (Table 1). The PCR conditions were pre-denaturation at 94 °C for 3 min, followed by 28 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 40 s, and elongation at 72 °C for 1 min, followed by final extension at 72 °C for 5 min. Afterwards, secondary amplification for attachment of the Illumina NexTera barcode was performed using the i5 forward primer and i7

reverse primer (Table 1). The PCR conditions were pre-denaturation at 94 °C for 3 min, followed by 8 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 40 s, and elongation at 72 °C for 1 min, followed by final extension at 72 °C for 5 min.

The amplified PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA) and subjected to electrophoresis to select DNA with a sequence length of 300 bp or longer. DNA fragment lengths were confirmed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). A library was constructed from the amplified products and sequenced using MiSeq (Illumina).

Gut Microbiome Analysis

The base sequence data obtained through MiSeq results were classified by sample using the Mothur program (https://www.mothur.org). The paired-end reads for each sample were then made into a single contig, and sequence filtering was performed to meet the criteria through quality control [24]. The filtered reads were subjected to alpha diversity analysis (operational taxonomic unit [OTU], rarefaction curve, Shannon-Weaver, Chao1, etc.), and the microbial community structure and relationships at the phylum, class, order, family, genus, and species levels were identified using the EzBioCloud server (www. ezbiocloud. net/) and the CL community (ChunLab Inc., Seoul, Republic of Korea). Clustering was confirmed using the unweighted pair group method with arithmetic average (UPGMA), and beta diversity was measured using unweighted unique fraction metric (UNIFRAC) analysis [25-27]. Microbial changes were analyzed using principal coordinate analysis (PCoA) plots [28]. Linear discriminant analysis effect size (LEfSe) was used to identify bacterial taxa at $p < 0.05$, and a linear discriminant analysis (LDA) score >2.0, using the Galaxy workflow framework (https://huttenhower.sph.harvard.edu/galaxy/).

Statistical analysis

The relative abundances of major phyla, classes, orders, families (median relative abundance > 0.1%), and major genera (median relative abundance > 0.01%) were calculated, and comparisons between the groups were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). Normality was analyzed using the Shapiro-Wilk test, and comparisons between groups were performed using the Kruskal-Wallis test. In all statistical analyses, significance was set at $p < 0.05$.

Results

Pathogen Detection

Of the eight calves with diarrhea, BRV was detected in four and BCoV was detected in four, all of which were confirmed to be single infections rather than multiple infections. No pathogens were detected in the normal calves without diarrhea.

Sequence reads

To compare the differences in the gut microbiome based on the cause of infection in Hanwoo calves, 864,232 sequence reads (an average of 57,6158 reads per calf) were obtained from the feces of four calves with BRV, four with BCoV, and seven normal calves, securing a sufficient number of OTUs required for analysis.

Alpha diversity

Alpha diversity analysis was performed on richness and evenness indices to determine the species diversity and distribution of the gut microbiome. There were no significant differences in the richness and evenness indices among the normal, BRV-infected, and BCoV-infected calves. However, there were significant differences in the NPShannon, Shannon, and Simpson evenness indices between calves with BRV and those with BCoV (Fig. 1).

Community Membership and Structure

Clustering and beta diversity analyses were performed to compare and analyze the species diversity of the gut microbiome and confirm the similarity relationship. Clustering showed that the microbiome were clearly differentiated between normal and BRV-infected calves, and between normal and BCoV-infected calves, and this was consistent with the results of PCoA analysis (Fig. 2).

Taxonomic composition

A comparison of the gut microbiomes of normal calves, those infected with BRV, and those with BCoV at the phylum level showed that calves infected with BRV showed a decrease in *Firmicutes* and an increase in *Proteobacteria*, which were significantly different from those of normal calves and those infected with BCoV (Fig. 3-A). Compared with normal calves, those infected with BRV showed a significant increase in *Gammaproteobacteria*

and *Actinobacteria* at the class level, and those infected with BCoV showed a significant increase in *Clostridia* and a decrease in *Bacilli* (Fig. 3-B).

At the order level, calves infected with BRV showed a significant increase in *Enterobacteriales*, whereas those infected with BCoV showed a significant increase in *Clostridiales* and a significant decrease in *Lactobacillales* (Fig. 4-A). At the family level, calves infected with BRV showed a significant increase in *Enterobacteriaceae* (Fig. 4-B). At the genus level, *Bifidobacterium* was increased in rotavirus-infected calves, although not significantly (Fig. 4-C). In addition, the relative frequencies of *Lactobacillaceae* and *Lactobacillus* significant decreased in both BRV- and BCoV-infected calves at the family and the genus levels, respectively (Fig. 4-B, C).

Linear Discriminant Analysis Effect Size (LEfSe) Analysis

The gut microbiota associated with normal, BRV-infected, and BCoV-infected calves were identified through LEfSe analysis. As a result, 2 phyla, 4 classes, 4 orders, 4 families, 4 genera and 16 species were identified (Fig. 5-A). The results of the cladogram based on LEfSe analysis showed that normal calves and those infected with BRV showed differences in the distribution of *Firmicutes* and *Proteobacteria* at the phylum level, whereas normal calves and those infected with BCoV showed differences in *Clostridia* and *Bacilli* at the class level (Fig. 5-B, C).

The relative abundance of seven species of *Lactobacillus* with LDA values higher than 2.0 in normal calves were analyzed, and the *Lactobacillus gasseri*, *L. amylovorus*, *L. panis*, *L. helveticus* groups; *L. rodentium*; and *L. vaginalis* tended to decrease with BRV or BCoV infection. Specifically, *L. rodentium* group were significantly decrease in BRV or BCoV infection calves. In addition, *L. gasseri*, *L. amylovorus*, *L. panis* and *L. helveticus* groups showed a significant decrease in BCoV infection calves compared to normal calves. However, *L. faecis* increased only in the calves infected with BCoV (Fig. 6-A, Supplementary 1). Although there was no significant difference between the groups, the relative abundance of *L. reuteri* was more than 10 % (Fig. 6-B).

Discussion

To investigate the differences in the gut microbiome of Hanwoo calves based on the pathogen causing diarrhea, calves with diarrhea were selected and infection with BRV or BCoV was confirmed. Comparison of the gut microbiomes of these calves revealed distinct differences between normal calves and those infected with BRV and BCoV. Changes in the taxonomic composition were also observed.

Diarrhea reduces gut microbiome diversity [29]. In this study, the number and diversity of gut microorganisms were reduced in BRV-infected calves compared to normal calves, but there was no difference in BCoV-infected calves. However, a previous study reported that the number of gut microbial species increased in calves infected with BRV, and that both BRV and BCoV infections reduced gut microbiome diversity [30]. In addition, this study showed that the microbiome was distinctly different among the normal calves and those infected with BRV and BCoV. These differences in results might have occurred because various factors, such as the environment, feed, and age of the calf, as well as the time elapsed after pathogen infection, affect the gut microbiome. In addition, because the number of calves tested was limited, more research is required with larger sample sizes.

At the phylum level, the gut microbiome of calves is dominated by *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* [7, 31]. In this study, the relative frequencies of *Firmicutes* and *Bacteroidetes* decreased, whereas those of *Proteobacteria* increased in the gut microbiome of calves infected with BRV compared to normal calves. This change was confirmed to be similar to that of the gut microbiome of BRV-infected calves reported previously [30, 32]. However, the gut microbiome of calves infected with BCoV was not significantly different from that of normal calves. It is known that in calves with diarrhea, *Firmicutes* and *Proteobacteria* increase, while *Bacteroidetes* decreases, resulting in an increase in the *Firmicutes*/*Bacteroidetes* ratio [33]. Therefore, a decrease in *Firmicutes* in the gut microbiome of calves is thought to be a characteristic of BRV infection.

There are an increasing number of reports on changes in the gut microbiome in response to pathogens that cause diarrhea. *Cryptosporidium* spp., *Eimeria* spp., and *Giardia* spp., which are frequently detected in Hanwoo calves with diarrhea, have also been found to cause imbalances in the gut microbiome during infection [34-36]. However, studies targeting pathogens in calves are limited, and only a few have been conducted on Hanwoo calves. In addition, in this study, the results of the cladogram based on LEfSe analysis showed that normal calves and those infected with BRV showed differences in the distribution of *Firmicutes* and *Proteobacteria* at the phylum level, whereas those infected with BCoV showed differences in *Clostridia* and *Bacilli* at the class level. This is similar to previous studies that showed significant differences in the gut microbiome depending on the pathogen causing diarrhea in calves, and it is

193 necessary to select specific microorganisms with a correlation [22, 30, 37]. In the present study, protozoan parasites
194 (*Cryptosporidium* spp., *Eimeria* spp., and *Giardia* spp.) were not detected in the feces of the selected calves (data not
195 shown), and only BRV and BCoV were identified. However, because infections with other potential pathogens may
196 exist and influence the results, further studies targeting various pathogens are expected to elucidate the association
197 between BRV and BCoV and the gut microbiome, which will help predict and diagnose diarrhea in calves.

198 *Bifidobacterium* is the dominant species in the intestine of newborn calves and is found in high relative abundance
199 in healthy calves [38]. *Bifidobacterium* produces lactic acid and short-chain fatty acids (SCFAs) and can inhibit the
200 colonization of pathogenic bacteria in the intestines, so it is important for the health of calves, and *Bifidobacterium*
201 strains are used as probiotics [39, 40, 41]. However, *Bifidobacterium* was hardly present in the fecal of most calves in
202 this study. Although the relative abundance was high in some BRV-infected calves, the difference between individuals
203 was large and there was no significant difference compared to other groups. Although the cause is still unknown, it
204 has been reported that *Bifidobacterium* in the gut microbiome of calves are at their highest on the 7th day after birth
205 and then decrease with growth [42, 43, 44]. In addition, the calves used in this study were at a stage where they
206 consume a mixture of milk and concentrate feed, which is thought to have affected the decrease in *Bifidobacterium*
207 [45]. In addition, calves infected with BRV and BCoV showed a decrease in *Lactobacillus* compared to normal calves.
208 In calves infected with BRV, where *Lactobacillus* was significantly reduced, *Bifidobacterium* was significantly
209 increased, whereas in calves infected with BCoV, *Lactobacillus* was significantly reduced and *Clostridium_g21* was
210 increased. This suggests that the composition of the intestinal microbiota changes depending on the pathogen.
211 However, previous studies have reported an increase in *Lactobacillus* in calves with diarrhea [8, 32, 46, 47]. When
212 calves show diarrhea symptoms, D-lactate and L-lactate levels increase, which increases lactic acid-producing bacteria
213 such as *Lactobacillus*, and the decrease in intestinal pH aids the growth of acid-stable *Lactobacillus*. However, a
214 previous study reported that *Lactobacillus* abundance decreases 24 h before the clinical manifestation of diarrhea [48].
215 Therefore, in this study, it is possible that the time elapsed between the onset of diarrhea and sampling and the clinical
216 condition of the calves caused the difference in the *Lactobacillus* ratio. In addition, because *Lactobacillus* is expected
217 to be related to the health status of Hanwoo calves, *Lactobacillus* can be utilized as a useful microorganism for
218 maintaining the intestinal environment of healthy Hanwoo calves. In particular, unlike calves infected with BRV, the
219 calves infected with BCoV showed increase in *L. faecis*. Hence, *L. faecis* can be used as an indicator for the prevention
220 and diagnosis of BCoV in calves through gut microbiome analysis. In addition, *Clostridium*, *Enterococcus*, and
221 *Escherichia*, which are significantly associated with diarrhea in calves, are the main microorganisms that play a

pathogenic role, and it is thought that these microorganisms have a growth advantage in the changed intestinal environment caused by diarrhea [49]. Therefore, diarrhea caused by BRV and BCoV infections can make calves more vulnerable to pathogenic microorganisms, worsen the imbalance of the gut microbiome, and decrease their immune function.

BRV and BCoV, the main pathogens causing diarrhea in calves, showed significant differences in the gut microbiome compared to that in healthy calves that did not show diarrhea when infected. In addition, significant differences in the gut microbiome were confirmed, depending on the pathogen. Analysis of the gut microbiome targeting each pathogen, which has rarely been studied so far, has revealed microorganisms associated with each pathogen, and utilizing these microorganisms as indicators can help improve the early detection of diseases and treatment efficiency through standardized physiological indicators.

Competing Interests

No potential conflict of interest relevant to this article was reported

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Author's Contributions

Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee of the National Institute of Animal Science, Republic of Korea (JBNU IACUC No. NON2023-123).

References

1. Kamada N, Chen GY, Inohara N, Núñez G. Control of pathogens and pathobionts by the gut microbiota. Nat Immunol. 2013;14:685-90. https://doi.org/10.1038/ni.2608

2. Ivanov II, Honda K. Intestinal commensal microbes as immune modulators. Cell Host Microbe. 2012;12:496-508. https://doi.org/10.1016/j.chom.2012.09.009

3. Hornef M. Pathogens, commensal symbionts, and pathobionts: discovery and functional effects on the host. ILAR J. 2015;56:159-62. https://doi.org/10.1093/ilar/ilv007

4. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol. 2009;9:313-23. https://doi.org/10.1038/nri2515

5. Amin N, Seifert J. Dynamic progression of the calf's microbiome and its influence on host health. Comput Struct Biotechnol J. 2021;19:989-1001. https://doi.org/10.1016/j.csbj.2021.01.035

6. The HC, Le S-NH. Dynamic of the human gut microbiome under infectious diarrhea. Curr Opin Microbiol. 2022;66:79-85. https://doi.org/10.1016/j.mib.2022.01.006

7. Kim E-T, Lee S-J, Kim T-Y, Lee H-G, Atikur RM, Gu B-H, et al. Dynamic changes in fecal microbial communities of neonatal dairy calves by aging and diarrhea. Animals. 2021;11:1113. https://doi.org/10.3390/ani11041113

8. Slanzon GS, Ridenhour BJ, Moore DA, Sischo WM, Parrish LM, Trombetta SC, et al. Fecal microbiome profiles of neonatal dairy calves with varying severities of gastrointestinal disease. PLoS One. 2022;17:e0262317. https://doi.org/10.1371/journal.pone.0262317v

9. Kerr CA, Grice DM, Tran CD, Bauer DC, Li D, Hendry P, et al. Early life events influence whole-of-life metabolic health via gut microflora and gut permeability. Crit Rev Microbiol. 2015;41:326-40. https://doi.org/10.3109/1040841X.2013.837863

10. Stecher B. The roles of inflammation, nutrient availability and the commensal microbiota in enteric pathogen infection. In: Conway T, Cohen P, editors. Metabolism and bacterial pathogenesis. Washington D.C.: ASM Press; 2015. p. 297-320.

11. McGuirk SM. Disease management of dairy calves and heifers. Vet Clin North Am Food Anim Pract. 2008;24:139-53. https://doi.org/10.1016/j.cvfa.2007.10.003

12. Uetake K. Newborn calf welfare: A review focusing on mortality rates. Anim Sci J. 2013;84:101-5. https://doi.org/10.1111/asj.12019

13. Hur T-Y, Jung Y-H, Choe C-Y, Cho Y-I, Kang S-J, Lee H-J, et al. The dairy calf mortality: the causes of calf death during ten years at a large dairy farm in Korea. Korean J Vet Res. 2013;53:103-8. https://doi.org/10.14405/kjvr.2013.53.2.103

14. Kim U-H, Jung Y-H, Choe C, Kang S-J, Chang S-S, Cho S-R, et al. Korean native calf mortality: the causes of calf death in a large breeding farm over a 10-year period. Korean J Vet Res. 2015;55:75-80. https://doi.org/10.14405/kjvr.2015.55.2.75

15. Chae J-B, Kim H-C, Kang J-G, Choi K-S, Chae J-S, Yu D-H, et al. The prevalence of causative agents of calf diarrhea in Korean native calves. JAST. 2021;63:864. https://doi.org/10.5187/jast.2021.e63

16. Park T, Cho GJ, Yang YJ, Ryu I-S. Analysis of prevalence and risk factors of calf diarrhea using rapid diagnosis kit. Korean J Vet Serv. 2023;46:47-58. https://doi.org/10.7853/kjvs.2023.46.1.47

17. Choe C, Jung Y-H, Do Y-J, Cho A, Kim S-B, Kang H-S, et al. Use of rapid diagnostic kit for the diagnosis of Korean native calf diarrhea. Korean J Vet Serv. 2017;40:61-6. https://doi.org/10.7853/kjvs.2017.40.1.61

18. Lee S-H, Kim H-Y, Choi EW, Kim D. Causative agents and epidemiology of diarrhea in Korean native calves. J Vet Sci. 2019;20:e64. https://doi.org/10.4142/jvs.2019.20.e64

19. Cho Y-I, Han J-I, Wang C, Cooper V, Schwartz K, Engelken T, et al. Case–control study of microbiological etiology associated with calf diarrhea. Vet Microbiol. 2013;166:375-85. https://doi.org/10.1016/j.vetmic.2013.07.001

20. Jang J-Y, Kim S, Kwon M-S, Lee J, Yu D-H, Song R-H, et al. Rotavirus-mediated alteration of gut microbiota and its correlation with physiological characteristics in neonatal calves. J Microbiol. 2019;57:113-21. https://doi.org/10.1007/s12275-019-8549-1

21. Kwon M-S, Jo HE, Lee J, Choi K-S, Yu D, Oh Y-s, et al. Alteration of the gut microbiota in post-weaned calves following recovery from bovine coronavirus-mediated diarrhea. JAST. 2021;63:125–36. https://doi.org/10.5187/jast.2021.e20

22. Kim S-H, Choi Y, Miguel MA, Lee S-J, Lee S-S, Lee S-S. Analysis of fecal microbial changes in young calves following bovine rotavirus infection. Vet Sci. 2023;10:496. https://doi.org/10.3390/vetsci10080496

23. Kim KH, Lee J-Y, Kwak G-H, Cho HU. Prevalence of bovine diarrhea disease from Korean native calves in Jeonbuk western area. Korean J Vet Serv. 2021;44:1-9. https://doi.org/10.7853/kjvs.2021.44.1.1

24. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009;75:7537-41. https://doi.org/10.1128/AEM.01541-09

25. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R. UniFrac: an effective distance metric for microbial community comparison. ISME J. 2011;5:169-72. https://doi.org/10.1038/ismej.2010.133

26. Clarke KR. Non-parametric multivariate analyses of changes in community structure. Austral Ecology. 1993;18:117-43. https://doi.org/10.1111/j.1442-9993.1993.tb00438.x

27. Gower JC. A general coefficient of similarity and some of its properties. Biometrics. 1971;27:857-71. https://doi.org/10.2307/2528823

28. Gower JC. Some distance properties of latent root and vector methods used in multivariate analysis. Biometrika. 1966;53:325-38. https://doi.org/10.1093/biomet/53.3-4.325

29. Zeineldin M, Aldridge B, Lowe J. Dysbiosis of the fecal microbiota in feedlot cattle with hemorrhagic diarrhea. Microb Pathog. 2018;115:123-30. https://doi.org/10.1016/j.micpath.2017.12.059

30. Cui S, Guo S, Zhao Q, Li Y, Ma Y, Yu Y. Alterations of microbiota and metabolites in the feces of calves with diarrhea associated with rotavirus and coronavirus infections. Front microbiol. 2023;14:1159637. https://doi.org/10.3389/fmicb.2023.1159637

31. Oikonomou G, Teixeira AGV, Foditsch C, Bicalho ML, Machado VS, Bicalho RC. Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of Faecalibacterium species with health and growth. PLoS One. 2013;8:e63157. https://doi.org/10.1371/journal.pone.0063157

32. Jessop E, Li L, Renaud DL, Verbrugghe A, Macnicol J, Gamsjäger L, et al. Neonatal calf diarrhea and gastrointestinal microbiota: etiologic agents and microbiota manipulation for treatment and prevention of diarrhea. Vet Sci. 2024;11:108. https://doi.org/10.3390/vetsci11030108

33. Li W, Yi X, Wu B, Li X, Ye B, Deng Z, et al. Neonatal calf diarrhea is associated with decreased bacterial diversity and altered gut microbiome profiles. Fermentation. 2023;9:827. https://doi.org/10.3390/fermentation9090827

34. Fu Y, Zhang K, Yang M, Li X, Chen Y, Li J, et al. Metagenomic analysis reveals the relationship between intestinal protozoan parasites and the intestinal microecological balance in calves. Parasit Vectors. 2023;16:257. https://doi.org/10.1186/s13071-023-05877-z

35. Hares MF, Griffiths BE, Johnson F, Nelson C, Haldenby S, Stewart CJ, et al. Specific pathway abundances in the neonatal calf faecal microbiome are associated with susceptibility to Cryptosporidium parvum infection: a metagenomic analysis. Anim Microbiome. 2023;5:43. https://doi.org/10.1186/s42523-023-00265-5

36. Bessegatto JA, Lisbôa JAN, Martins FDC, Freire RL, Facury Filho EJ, Alfieri AA, et al. Development of the intestinal microbiota of dairy calves and changes associated with Cryptosporidium spp. infection in Brazil. Microorganisms. 2024;12:1744. https://doi.org/10.3390/microorganisms12091744

37. Ichikawa-Seki M, Motooka D, Kinami A, Murakoshi F, Takahashi Y, Aita J, et al. Specific increase of Fusobacterium in the faecal microbiota of neonatal calves infected with Cryptosporidium parvum. Sci Rep. 2019;9:12517. https://doi.org/10.1038/s41598-019-48969-6

38. Gomez DE, Arroyo LG, Costa MC, Viel L, Weese JS. Characterization of the fecal bacterial microbiota of healthy and diarrheic dairy calves. J Vet Intern Med. 2017;31:928-39. https://doi.org/10.1111/jvim.14695

39. Abe F, Norio I, and Seiichi S. Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. J Dairy Sci. 1995;78:2838-2846. https://doi.org/10.3168/jds.S0022-0302(95)76914-4

40. Veiga P, Pons N, Agrawal A, Oozeer R, Guyonnet D, Brazeilles R, et al. Changes of the human gut microbiome induced by a fermented milk product. Sci Rep 2014;4:6328. https://doi.org/10.1038/srep06328

41. Wu Y, Nie C, Luo R, Qi F, Bai X, Chen H, et al. Effects of multispecies probiotic on intestinal microbiota and mucosal barrier function of neonatal calves infected with E. Coli k99. Front microbiol. 2022;12:813245. https://doi.org/10.3389/fmicb.2021.813245

42. Uyeno Y, Sekiguchi Y, and Kamagata Y. rRNA-based analysis to monitor succession of faecal bacterial communities in Holstein calves. Lett Appl Microbiol. 2010;51:570-77. https://doi.org/10.1111/j.1472-765X.2010.02937.x

43. Vlková E, Trojanová I, and Rada V. Distribution of bifidobacteria in the gastrointestinal tract of calves. Folia Microbiol. 2006;51:325-28. https://doi.org/10.1007/BF02931825

44. Song Y, Malmuthuge N, Steele MA, Guan LL. Shift of hindgut microbiota and microbial short chain fatty acids profiles in dairy calves from birth to pre-weaning. FEMS Microbiol Ecol. 2018;94: fix179. https://doi.org/10.1093/femsec/fix179

45. Vlkova E, Rada V, Trojanova I, Killer J, Šmehilová M, Molatova Z. Occurrence of bifidobacteria in faeces of calves fed milk or a combined diet. Arch Anim Nutr. 2008;62:359-65. https://doi.org/10.1080/17450390802327662

46. Gomez DE, Li L, Goetz H, MacNicol J, Gamsjaeger L, Renaud DL. Calf diarrhea is associated with a shift from obligated to facultative anaerobes and expansion of lactate-producing bacteria. Front Vet Sci. 2022;9:846383. https://doi.org/10.3389/fvets.2022.846383

47. Li L, Renaud DL, Goetz HM, Jessop E, Costa MC, Gamsjäger L, et al. Effect of time of sample collection after onset of diarrhea on fecal microbiota composition of calves. J Vet Intern Med. 2023;37:1588-93. https://doi.org/10.1111/jvim.16801

48. Schwaiger K, Storch J, Bauer C, Bauer J. Abundance of selected bacterial groups in healthy calves and calves developing diarrhea during the first week of life: Are there differences before the manifestation of clinical symptoms? Front microbiol. 2022;13:958080. https://doi.org/10.3389/fmicb.2022.958080

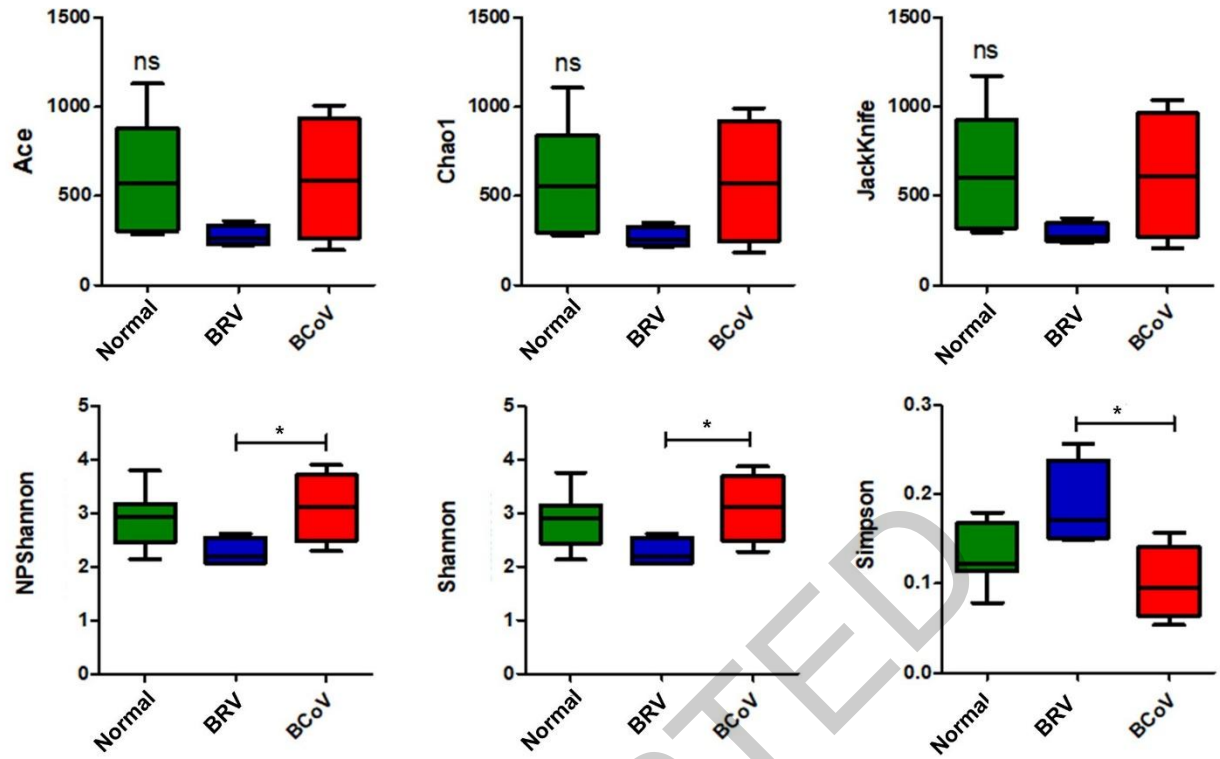
49. Cho Y-i, Yoon K-J. An overview of calf diarrhea-infectious etiology, diagnosis, and intervention. J Vet Sci. 2014;15:1-17. https://doi.org/10.4142/jvs.2014.15.1.1

Tables and Figures

Table legends

Table 1. Primers and gene sequences of polymerase chain reaction (PCR)

Primer	Sequence (5′ → 3′)
341F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCAG
805R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGT ATCTAATCC
Illumina index i5 S502	ATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAG CGTC
i7index i7 N701	CAAGCAGAAGACGGCATACGAGATTCGCCTTGTCTCGTGGGCTCGG



383

384 Fig. 1. Alpha diversity analysis. Comparison of gut microbiome of normal calves, calves infected with bovine

385 rotavirus (BRV), and calves infected with bovine coronavirus (BCoV). *; $p < 0.05$.

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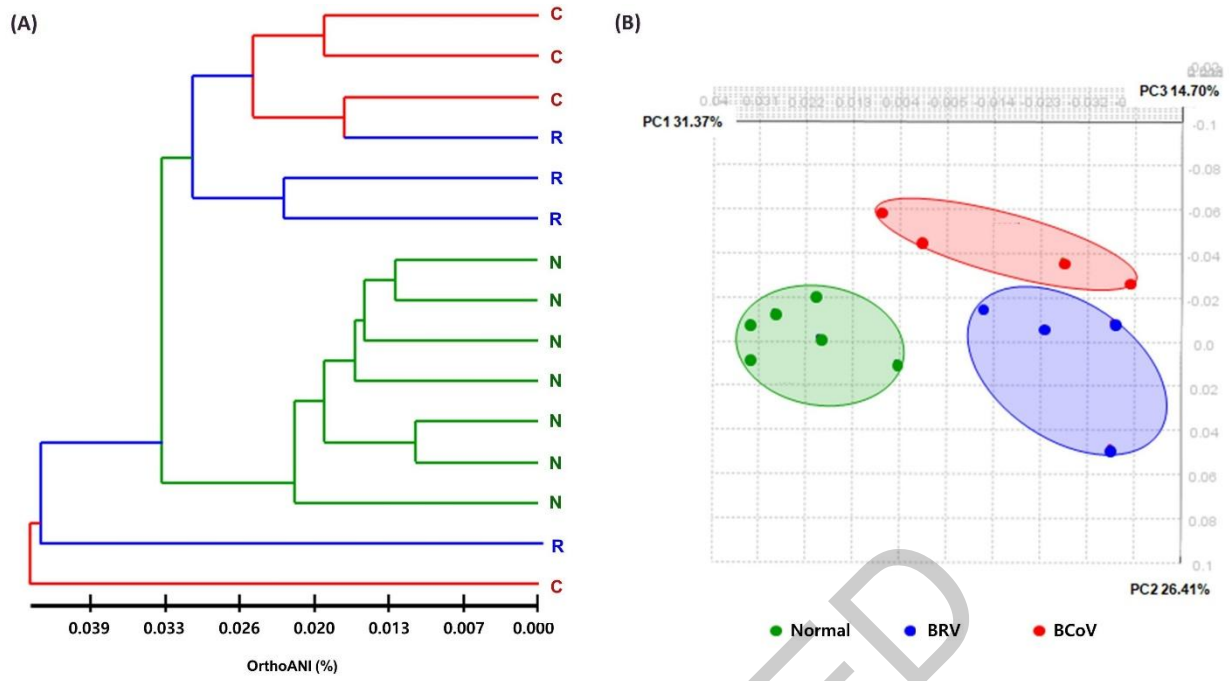

388

389 Fig. 2. Comparison of the gut microbiome of normal calves, calves infected with bovine rotavirus (BRV), and those

390 infected with bovine coronavirus (BCoV). Hierarchical clustering (A) and principal coordinate analysis (B) of the

391 gut microbiome of calves.

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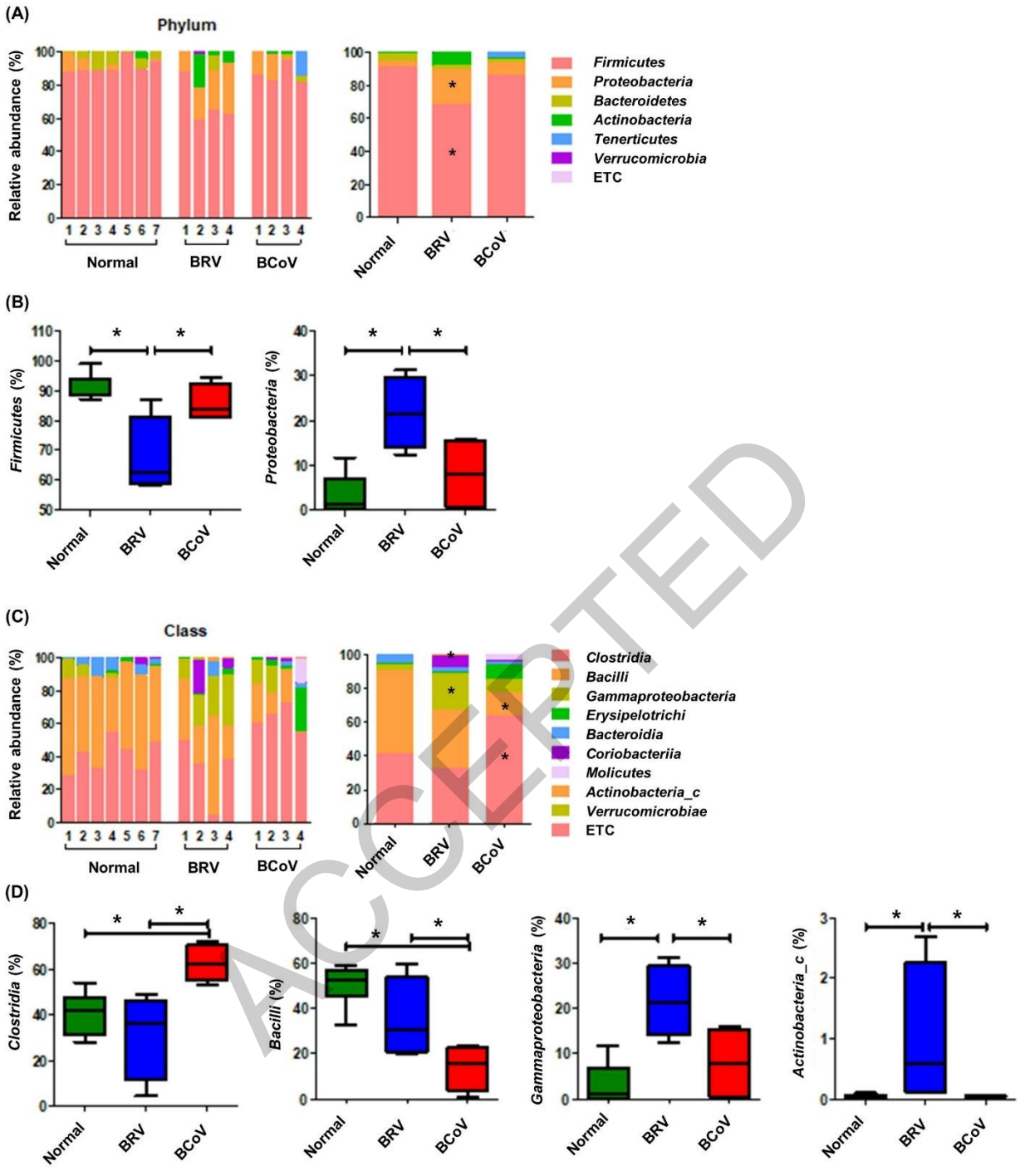


Fig 3. Comparison of the gut microbiome between normal calves, bovine rotavirus (BRV)-infected calves, and bovine coronavirus (BCoV)-infected calves. Individual and average taxonomic compositions and major microorganisms at the phylum (A and B) and class (C and D) levels. * in A and C indicates microorganisms that are significantly different from normal calves. *; $p < 0.05$.

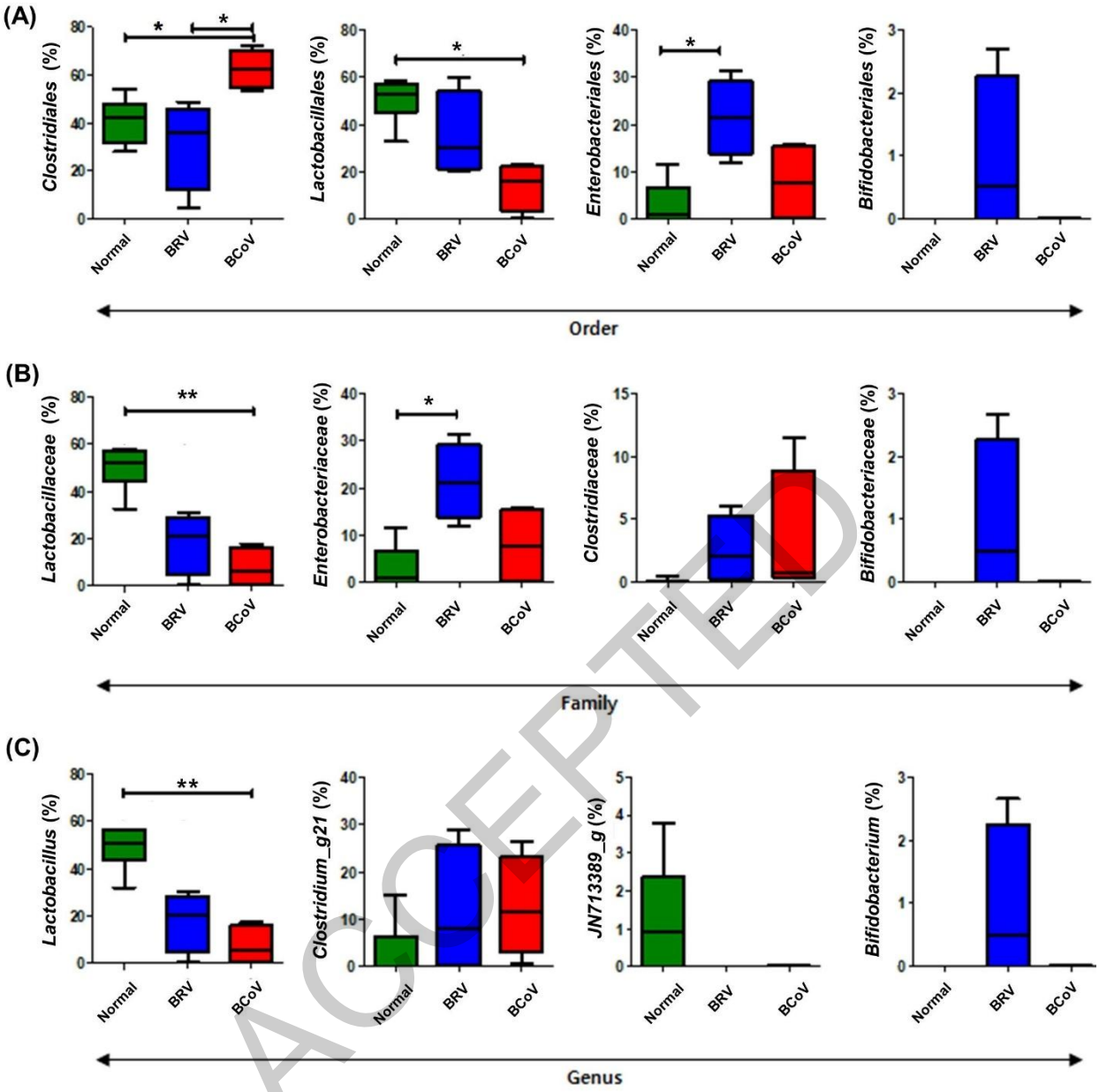


Fig 4. Taxonomic composition of major gut microbiome of normal calves, bovine rotavirus (BRV)-infected calves, and bovine coronavirus (BCoV)-infected calves at the order (A), family (B), and genus (C) levels. JN713389_g; unknown genus of Oscillospiraceae. *; $p < 0.05$.

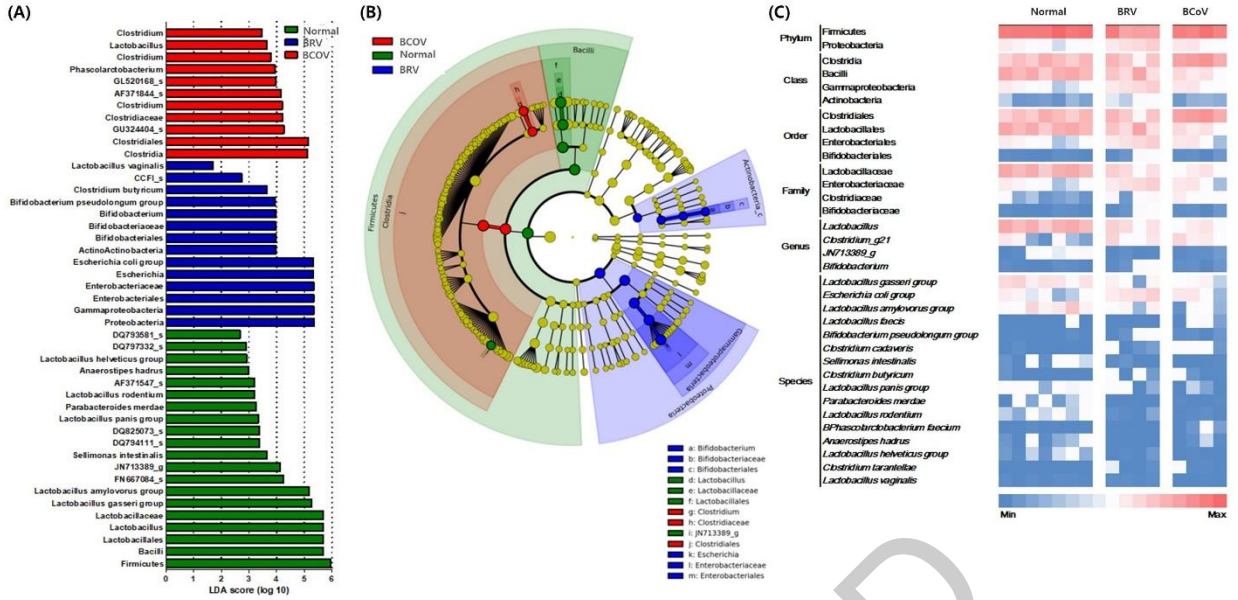


Fig 5. Analysis of the gut microbiome of normal calves, bovine rotavirus (BRV)-infected calves, and bovine coronavirus (BCoV)-infected calves at the species level. LDA score analysis (A), cladogram analysis (B), and relative abundance analysis of individual gut microbiome (C). GL520168_s; unknown species of Clostridium_g36, AF371844_s; Clostridium, GU324404_s; unknown species of Sporobacter, CCFI_s; Clostridium hominis, DQ793581_s; unknown species of Oscillospiraceae, DQ797332_s; Uncultured bacterium clone RL248_aai98a04, AF371547_s; unknown species of Blautia, DQ825073; Uncultured bacterium clone RL185_aan85a07, DQ794111; unknown species of Ruminococcus_g2, JN713389_s; unknown species of Oscillospiraceae, FN667084_s; unknown species of Lactobacillus.

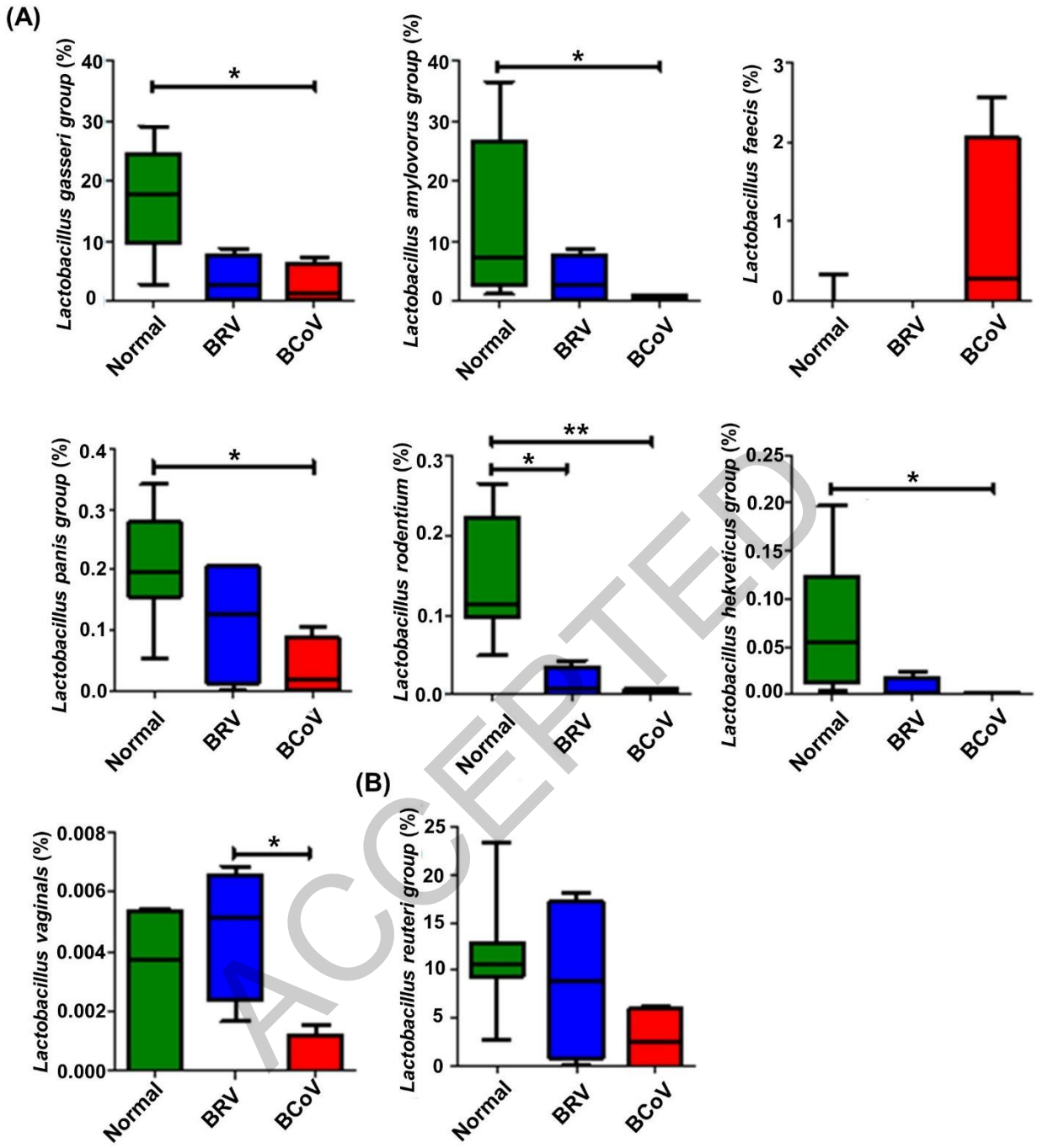
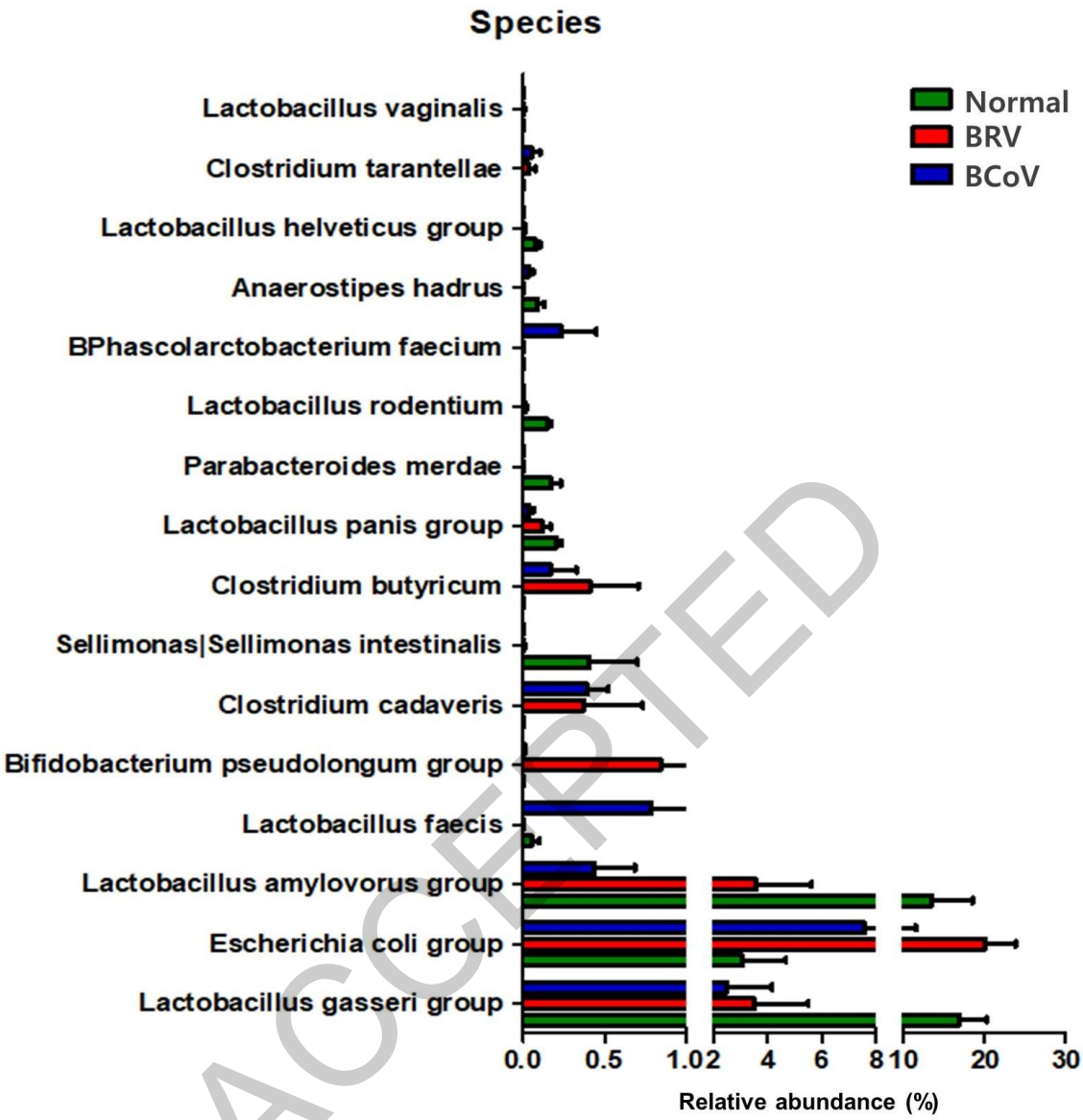


Fig 6. Relative frequencies of Lactobacillus in normal calves, calves infected with bovine rotavirus (BRV), and those infected with bovine coronavirus (BCoV). Relative abundance of seven species of Lactobacillus with LDA values ≥ 2.0 in normal calves (A) and relative abundance of L. reuteri (B). *; $p < 0.05$.



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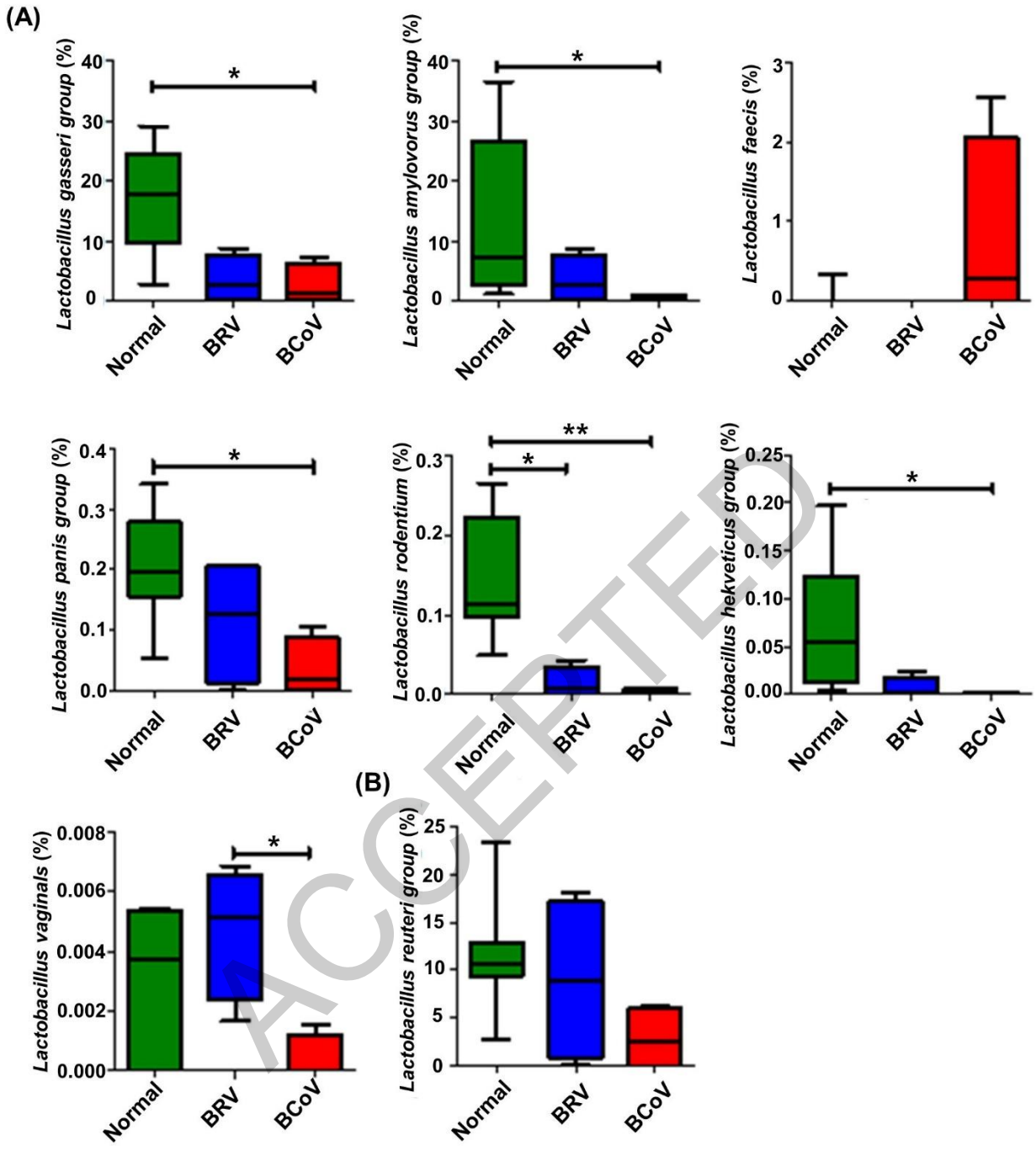

Supplementary 1. Relative abundance of gut microbiome differing between normal calves, bovine rotavirus (BRV)-infected calves, and bovine coronavirus (BCoV)-infected calves at the species level.