RESEARCH ARTICLE

Characteristics of various fibrolytic isozyme activities in the rumen microbial communities of Japanese Black and Holstein Friesian cattle under different conditions

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Abstract

Rumen microorganisms produce various fibrolytic enzymes and degrade lignocellulosic materials into nutrient sources for ruminants; therefore, the characterization of fibrolytic enzymes contributing to the polysaccharide degradation in the rumen microbiota is important for efficient animal production. This study characterized the fibrolytic isozyme activities of a rumen microbiota from four groups of housed cattle (1, breeding Japanese Black; 2, feedlot Japanese Black; 3, lactating Holstein Friesian; 4, dry Holstein Friesian). Rumen fluids in all cattle groups showed similar concentrations of total volatile fatty acids and reducing sugars, whereas acetic acid contents and pH were different among them. Predominant genera were commonly detected in all cattle, although the bacterial compositions were different among cattle groups. Zymograms of whole proteins in rumen fluids showed endoglucanase activities at 55 and 57 kDa and xylanase activity at 44 kDa in all cattle. Meanwhile, several fibrolytic isozyme activities differed among cattle groups and individuals. *Treponema*, *Succinivibrio*, *Anaeroplasma*, *Succiniclasticum*, *Ruminococcus*, and *Butyrivibrio* showed positive correlations with fibrolytic isozyme activities. Further, endoglucanase activity at 68 kDa was positively correlated with pH. This study suggests the characteristics of fibrolytic isozyme activities and their correlations with the rumen microbiota.

KEYWORDS
amplicon sequencing, endoglucanase, rumen microbial community, xylanase, zymogram

1 | INTRODUCTION

Ruminants are an important source of essential animal products, such as dairy products, meat, and leather and have played a key role in sustaining human cultures. Ruminants do not produce fibrolytic enzymes to degrade ingested feed; thus, they depend on symbiotic microorganisms in the rumen. The rumen is an evolved forestomach that serves as one of the four stomachs of ruminants. The rumen fluid contains a complex microbial community consisting of bacteria (up to 10^{11} cells/ml), fungi (10^3 to 10^5 zoospores/ml), and protozoa (10^4 to 10^6 cells/ml) (Kamra, 2005). The rumen microbial community produces various fibrolytic enzymes, such as endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), beta-glucosidases (EC 3.2.1.21), xylanases (EC 3.2.1.8), and lignin peroxidase (EC 1.11.1.14). These fibrolytic enzymes efficiently degrade unavailable plant materials into fermentable carbohydrates (i.e., oligosaccharides and monosaccharides). Finally, carbohydrate-utilizing microorganisms convert fermentable carbohydrates into volatile fatty acids (VFAs), which are major nutrient sources for ruminants (Seshadri et al., 2018). In general, *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* are considered the major fibrolytic bacteria, whereas anaerobic fungi colonize plant tissue and appear to degrade lignified tissue that is not degraded by other microorganisms (Krause et al., 2003). Ruminants can convert human-indigestible lignocellulose into readily accessible animal products because of the highly active fibrolytic enzymes of the symbiotic rumen microbial community.

Understanding the mechanisms of lignocellulose digestion in the rumen provides not only knowledge to elucidate the ecological features of a complex rumen microbial community but also strategies for efficient and stable animal production. Recent investigations using next-generation sequencing technology have identified several factors to determine rumen fibrolytic microbial communities. For example, a survey of the rumen and camelid foregut microbial communities from 32 animal species and 35 countries revealed that the bacterial community structure varies with diet and host, whereas geography only had a minor contribution to the observed variation (Henderson et al., 2015). Exploring the overall bovine ruminal bacterial populations of five age groups, from 1-day-old calves to 2-year-old cows characterized that the rumen bacterial communities were not only influenced by diet, but also by the age of the animal (Jami et al., 2013). Assessment of rumen microbiota from a cohort of 709 beef cattle suggested that some rumen microbial features are heritable and could be influenced by host genetics (Li, Li, et al., 2019). The rumen bacterial community structure of sheep changes significantly with different forage-to-concentrate ratios; however, the numbers of methanogens, protozoa, and anaerobic fungi do not change significantly (Li, Teng, et al., 2019). These studies indicated that the host species, genetics, age, and diet are the main determinants of rumen microbial composition. Several studies also reported the relationship between fibrolytic enzyme activity and rumen microbial community. Sheep fed the diet containing 70% of either alfalfa hay or grass hay and 30% concentrate exhibited no correlations between endoglucanase and xylanase activities and the relative abundance of

F. succinogenes, *R. albus*, and *R. flavefaciens* in rumen fluid (Saro et al., 2014). Supplementation of an extract of *Sapindus rarak* saponins in short-term trial decreased the RNA concentrations of *R. albus* and *R. flavefaciens* and xylanase activity, whereas no correlation between cell wall degrading microorganisms and the fibrolytic activity was observed (Wina et al., 2006). From these previous studies, it was hypothesized that the characteristics of fibrolytic isozyme activities and rumen microbial communities are different among cattle under different conditions. However, the characteristics of fibrolytic isozyme activities in rumen microbial communities in various cattle are unknown. In addition, the relationship between the fibrolytic isozyme activities in rumen microbiota and rumen microbial compositions is still obscure, although fibrolytic isozymes play critical roles in the degradation of polysaccharides comprising lignocellulosic biomass. In this study, we focused on the fibrolytic isozyme activities and investigated the relationship between fibrolytic isozyme activities and rumen microbial community in housed Japanese Black and Holstein Friesian cattle for providing an important knowledge on the characteristics of fibrolytic isozymes and microorganisms that contribute to the degradation of plant materials.

2 | MATERIALS AND METHODS

2.1 | Cattle and diets

All research in this study was conducted in accordance with the regulations of the Institutional Animal Care and Use Committee of Tohoku University (approval numbers: 2019AgA-033 and 2019AgA-001). Cattle with different ages, body weights, and ratios of concentrate to roughage was used in this study to investigate the effects of these parameters on the characteristics of fibrolytic isozyme activities in various housed cattle. Six Japanese Black cattle were selected, three of which were breeding cattle, whereas the other three were feedlot cattle. In addition, six Holstein Friesian cows were selected, three of which were lactating cows, whereas the other three were dry cows. The characteristics of the cattle used in this study are shown in Table 1. Twelve cattle were housed at Tohoku University (Miyagi, Japan) and fed the corresponding diets over 1 month for the adaptation of rumen microbial communities before sampling rumen fluids (Table 2). Cattle were allowed ad libitum access to water, and the regulation of drinking water was not carried out before sampling. Five hundred milliliters of rumen fluid was collected using a stomach tube 2 h after morning feeding at 9:00 a.m. Collected rumen fluid was immediately transferred to the laboratory and strained with a 1×1 mm mesh to remove coarse solids within 30 min. Strained rumen fluids were used for chemical analysis and DNA and protein extraction.

2.2 | Chemical analysis

Chemical characteristics of rumen fluids (pH, VFAs, and reducing sugars) were determined as described in previous studies (Takizawa,

TABLE 1 Cattle groups and characteristics of cattle in this study

Cattle	No.	Age (month)	Body weight (kg)	Concentrate[a] (kg/day)	Roughage[a] (kg/day)	Concentrate (%)
Breeding Japanese Black	1	109	534	0	Ad libitum	0
	2	38	495	0	Ad libitum	0
	3	100	678	0	Ad libitum	0
Feedlot Japanese Black	1	29	812	9	2	82
	2	26	714	9	2	82
	3	22	570	9	2	82
Lactating Holstein Friesian	1	92	873	12	21	36
	2	56	673	12	21	36
	3	35	594	10	21	32
Dry Holstein Friesian	1	36	620	3	21	11
	2	21	529	3	21	11
	3	21	529	3	21	11

[a]The amount of feeding was represented as the fresh matter.

TABLE 2 Nutritional compositions of the diet used in this study

Item (% fresh matter)	Breeding Japanese Black Orchardgrass silage	Feedlot Japanese Black		Lactating and dry Holstein Friesian		
		Concentrate	Rice straw	Concentrate	Beet pulp	Orchardgrass silage
DM	73.5	88.1	89.9	87.6	88.3	77.3
NDF	44.2	23.5	59.4	17.6	36.6	50.8
ADF	28.3	8.6	37.1	8.5	20.6	30.0
ADL	2.6	1.1	2.6	1.8	1.0	3.3
NFC	12.6	46.1	11.1	48.0	42.1	15.0
Crude protein	8.5	13.1	2.6	16.4	7.1	8.2
Crude fat	2.4	2.8	0.4	3.0	0.0	1.1
Crude ash	9.6	4.8	17.7	5.7	8.6	5.4
Ca	0.4	0.3	0.2	0.9	1.1	0.1
P	0.3	0.5	0.1	0.5	0.1	0.2
Mg	0.2	0.2	0.1	0.3	0.3	0.1
K	3.5	0.6	0.3	0.6	0.3	1.8

Abbreviations: ADF, acid detergent fiber; ADL, acid detergent lignin; DM, dry matter; NDF, neutral detergent fiber; NFC, nonfiber carbohydrate.

Abe, et al., 2020; Takizawa et al., 2018) with some modifications. Briefly, the pH of strained rumen fluid was immediately measured without storage using a pH meter (HORIBA, Kyoto, Japan). Two milliliters of strained rumen fluid was additionally filtered through a cellulose acetate membrane filter (0.45-μm pore diameter; ADVANTEC, Tokyo, Japan). The filtered liquids were stored at −20°C until chemical analysis. The concentration of VFAs was measured by high-performance liquid chromatography (JASCO, Tokyo, Japan) using an ion-exchange column (RSpak KC-811; Shodex, Tokyo, Japan) and an ultraviolet detector (870-UV; JASCO). The eluent was a mixture of 3 mmol/L $HClO_4$ and acetonitrile (9:1) The temperature of the oven was set to 60°C, and the flow rate was 1.0 ml/min. Reducing sugars were detected colorimetrically using Somogyi–Nelson reagents

(Nelson, 1944; Somogyi, 1945) using an ultraviolet–visible (UV-vis) spectrophotometer (Shimadzu, Kyoto, Japan).

2.3 | Quantitative analysis of bacterial abundance by real-time PCR

Total DNA was extracted from 1.5 ml of strained rumen fluid using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA), according to the manufacturer's instructions. The concentration and quality of DNA extracts was determined using a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). DNA extract was 100-fold diluted (approximately 2.6 ng/μl) and used as a template.

Quantitative analysis of bacterial abundance was performed as described previously (Takizawa, Abe, et al., 2020). Real-time PCR was conducted using primers for general bacteria (forward, 5′-CGGC AACGAGCGCAACCC-3′; reverse, 5′-CCATTGTAGCACGTGTGTA GCC-3′) (Denman & McSweeney, 2006). Samples were assayed in triplicate in a 25-μl reaction mixture comprising 12.5 μl of MightyAmp for Real Time (TB Green Plus; TaKaRa Bio, Shiga, Japan), 0.5 μl of 10-μM forward primer, 0.5 μl of 10-μM reverse primer, 1-μl template, and 10.5-μl sterile water. The PCR condition for general bacteria was as follows: 98°C for 2 min, followed by 40 cycles of 98°C for 30 s, 60°C for 15 s, and 68°C for 30 s. All real-time PCR amplifications and detections were performed in triplicate using a Thermal Cycler Dice real-time system (TaKaRa Bio). Copy number of bacterial 16S rRNA genes were calculated with a standard curve that was previously constructed by using plasmid DNA containing the inserts of bacterial 16S rRNA genes (Takizawa, Asano, et al., 2020). Target genes were amplified using aforementioned primers, and PCR products were purified using FastGeneTM Gel/PCR Extraction kit (NIPPON Genetics Co., Ltd, Tokyo, Japan) according to the manufacturer's instructions. Purified PCR products were ligated to T-Vector pMD19 (Simple) (TaKaRa Bio). Constructed plasmids were transformed into High Efficiency DH5α Competent Cells (GMbiolab Co., Ltd, Taichung, Taiwan) and then incubated on Luria-Bertani agar plate containing 50 mg/L⁻ ampicillin. The colonies were picked up and cultivated in the Luria–Bertani liquid medium containing 50 mg/L ampicillin. After the incubation, the plasmid DNA was extracted using FastGene™ Plasmid Mini Kit (NIPPON Genetics Co., Ltd). The concentration of the plasmid DNA was determined using a NanoDrop ND-1000 (Thermo Fisher Scientific). The copy number of each plasmid was calculated using the molecular weight of nucleic acid and the length of the plasmid DNA.

2.4 | Analysis of rumen bacterial community

DNA extracts were purified using Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA) and then quantified using the Synergy H1 (Omega Bio-Tek, Norcross, GA, USA) and the QuantiFluor dsDNA System (Promega, Madison, WI, USA). DNA libraries were constructed based on two-step tailed PCR for the 16S rRNA V3-V4 region using primers with a barcode (341f, 5′-CCTACGGGNGGCWGCAG-3′; 805r, 5′-GACTACHVGGGTATCTAATCC-3′). PCR amplifications were performed using ExTaq HS (TaKaRa Bio, Shiga, Japan), and the PCR conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 5 min, and a final extension at 72°C for 5 min. After purification using Agencourt AMPure XP, the concentrations were measured as aforementioned, and the quality was confirmed using a Fragment Analyzer and dsDNA 915 Reagent Kit (Advanced Analytical Technologies, Ankeny, IA, USA). The constructed libraries were subjected to 300-bp paired-end sequencing on an Illumina MiSeq with MiSeq Reagent Kit v3 (600 cycles). Raw sequencing reads, which were divided into forward and reverse, were assembled using the Initial Process in the Ribosomal Database Project (RDP)

pyrosequencing pipeline (http://pyro.cme.msu.edu/). The primer sequences were removed from the sequencing reads. Reads of 150 bp or less or those containing a sequence with a quality value of 20 or less were removed. Chimeric sequences were removed using the Fungene chimera check (http://fungene.cme.msu.edu/). Chimera-filtered sequences were classified phylogenetically using the RDP Classifier with a cutoff value of 0.8. All sequence data were deposited in the DDBJ sequence read archive under the accession number DRA012323 (DRX295195–DRX295206).

2.5 | SDS-PAGE and zymograms

SDS-PAGE and zymograms were performed according to a previous study (Takizawa, Abe, et al., 2020) with some modifications. In brief, strained rumen fluids were mixed with equal volumes of 2× sample buffer comprising 20% (v/v) glycerol, 4% (w/v) SDS, 0.01% (w/v) bromophenol blue, 0.125 mol/L tris-HCl (pH 7.4), 10% (v/v) 2-mercaptoethanol (pH 6.8), 2 mmol/L phenylmethylsulfonyl fluoride, and 1× proteinase inhibitor. Proteins were extracted using Lysing Matrix E tubes (MP Biomedicals), followed by centrifugation at $12,000 \times g$ for 10 min. The supernatant was heated at 70°C for 20 min and immediately cooled on ice. In this study, the volume of loaded protein extracts was standardized for comparing not only the banding patterns of fibrolytic isozymes but also the fibrolytic isozyme activity per a volume of rumen fluid among samples. Eight microliters of protein extract was loaded on 8% polyacrylamide gels containing 0.15% (w/v) carboxymethyl cellulose sodium salt or 1.0% (w/v) xylan from beechwood for endoglucanase and xylanase zymograms, respectively. SDS-PAGE was performed at 200 V for 60 min. Separated proteins were refolded in phosphate–citrate buffer (20 mmol/L, pH 6.5) containing 1.5% (w/v) β-cyclodextrin for 30 min. The gel was soaked in sodium acetate buffer (50 mmol/L, pH 6.5) for 15 min. The endoglucanase zymogram was conducted at 37°C for 90 min in sodium acetate buffer (30 mmol/L, pH 7.3), and the xylanase zymogram was performed at 37°C for 6 h. To visualize the endoglucanase and xylanase activities, the gels were stained with 0.1% (w/v) Congo Red for 20 min and de-stained with 1 mol/L NaCl. To enhance the visualization of active bands, the gels were soaked in 0.3% (v/v) acetic acid. Band densities were analyzed using ImageJ software (Schindelin et al., 2012).

2.6 | Statistical analysis

Multiple comparisons were performed according to the Tukey–Kramer method using the R package multcomp, version 1.4-13. Statistical significance was declared at $p < 0.05$, whereas $p < 0.10$ were described as tendencies. Principal coordinate analysis (PCoA) was conducted based on Bray–Curtis dissimilarity using the R package vegan, versions 2.05.0. Spearman's rank correlation coefficients and significant levels were evaluated using the R package corrplot, version 0.84, to determine the relationships between the relative abundance of

rumen bacteria and fibrolytic isozyme activity. Correlations with p values lower than 0.05 were considered statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | Chemical characteristics of rumen fluid

Four cattle groups showed different chemical characteristics of rumen fluid (Figure 1). Reducing sugars were not significantly different between each cattle group. As with reducing sugars, differences in total VFAs were not significant. The dominant VFA was acetic acid, followed by propionic acid, butyric acid, and iso-valeric acid in all cattle groups (Figure S1). Feedlot Japanese Black cattle showed a significantly lower acetic acid percentage ($p < 0.05$); however, the percentage of propionic acid was not significantly different among cattle groups. The ratio of acetic acid to propionic acid in dry Holstein Friesian cows was significantly higher than that in feedlot Japanese Black cattle ($p = 0.04$). Feedlot Japanese Black cattle showed a significantly lower pH than dry Holstein Friesian cows ($p = 0.02$). The trends of these results were consistent with those of previous studies that investigated the effects of forage type and forage level on ruminal fermentation (Carvalho et al., 2020; Jiang et al., 2017).

3.2 | Rumen microbial abundance and structure of rumen bacterial community

Sufficient concentration (263.5 ± 58.9 ng/μl) and quality (260/280: 1.83 ± 0.02) of DNA were extracted from all samples and successfully used to amplify microbial 16S rRNA gene for the following quantitative analysis and amplicon sequencing. Rumen bacterial abundances were quantified by real-time PCR (Figure 2) because rumen bacteria make the greatest contribution to the breakdown and fermentation of lignocellulosic biomass (Gharechahi & Salekdeh, 2018; Koike & Kobayashi, 2009). Bacterial abundance in lactating Holstein cows was significantly higher than that in feedlot Japanese Black cattle ($p = 0.03$).

Next, bacterial communities of the four cattle groups were compared by 16S rRNA gene amplicon sequencing. After sequence processing and the removal of chimeras, 668,320 reads were obtained (average 55,693; minimum 50,540; maximum 64,392) from 12 samples (Table 3). Diversity indices (Chao1, ACE, Shannon index, and Simpson index) were not significantly different between the cattle groups. PCoA illustrated that the structure of the bacterial community was clustered by cattle group (Figure 3). Previous 16S rRNA gene sequencing studies also reported that the ruminal bacterial structure is clustered by sheep group with different forage-to-concentrate
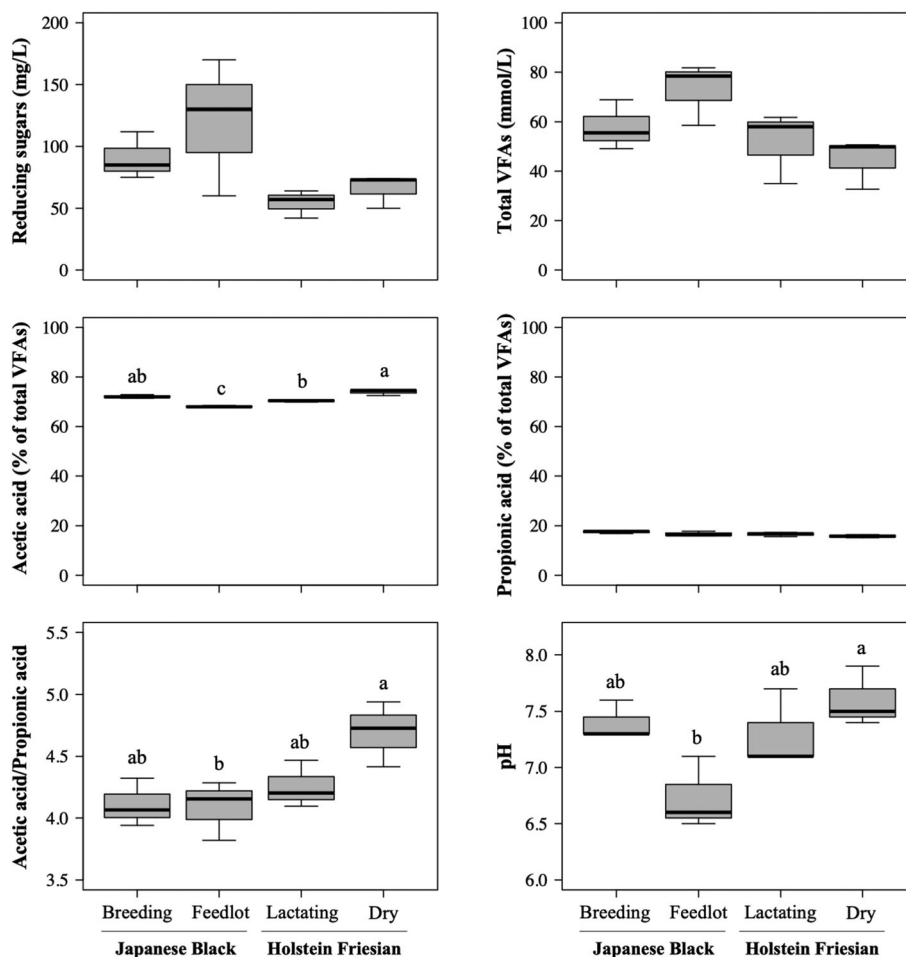

FIGURE 1　Chemical characteristics of rumen fluids in breeding Japanese Black cattle, feedlot Japanese Black cattle, lactating Holstein cattle, and dry Holstein cattle. Different letters indicate a statistically significant difference ($p < 0.05$, $n = 3$)

ratios (Li, Teng, et al., 2019). Based on the phylum-level taxonomic composition, 26 phyla were identified in total, 18 of which were detected in all cattle (Table 3). Breeding Japanese Black cattle had five unique phyla, whereas feedlot Japanese Black cattle and lactating Holstein cow harbored one unique phylum each. Another single phylum was detected in breeding Japanese Black cattle and lactating Holstein cow. Bacteroidetes was the most dominant phylum, followed by Firmicutes, unclassified Bacteria, and Fibrobaceteres (Figure 4a). Feedlot Japanese Black cattle showed a lower abundance of Firmicutes than lactating and dry Holstein Friesian cows ($p < 0.05$); however, the abundance of Proteobacteria was higher than that of other cattle groups ($p < 0.05$). In the genus-level taxonomic
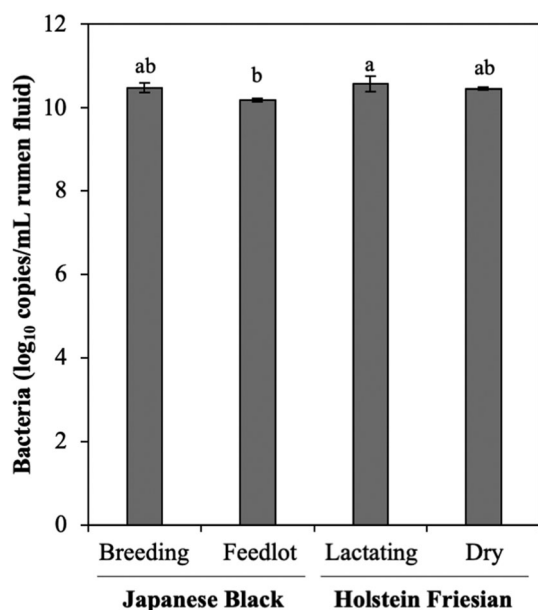
composition, the xylanolytic genus *Prevotella* (Miyazaki et al., 1997) was the most dominant genus, followed by unclassified Bacteroidetes, unclassified *Bacteroidales*, unclassified Bacteria, and the cellulolytic genus *Fibrobacter* (Béra-Maillet et al., 2004) in all cattle (Figure 4b). The relative abundance of unclassified *Clostridiales* in feedlot Japanese Black cattle was significantly higher than that in breeding Japanese Black cattle and dry Holstein Friesian cows ($p < 0.05$). Similarly, the relative abundance of unclassified *Lachnospiraceae*, *Ruminococcus*, and *Anaeroplasma* in feedlot Japanese Black cattle was significantly lower than that in other cattle groups ($p < 0.05$). Meanwhile, the relative abundance of *Succinivibrio* in feedlot Japanese Black cattle was significantly higher than that in the other cattle groups ($p < 0.05$).

Some cellulolytic species of the genus *Ruminococcus* efficiently degrades cellulose into acetate and reducing sugars; thus, it is one of the important degraders of plant cell wall polysaccharides in the cattle rumen (Baba et al., 2017; Dai et al., 2015). During the fattening period, Japanese Black cattle are generally fed a high-energy diet one to three times daily, and the diet comprises 80%–90% of concentrate (8–10 kg/day) and 10%–20% of roughage (1–2 kg/day). In this study, the feedlot Japanese Black cattle were also fed much less roughage than the other cattle groups, which suggests that the relative abundance of *Ruminococcus* decreased in feedlot Japanese Black cattle because the polysaccharides included in the diet were not sufficient for the growth of *Ruminococcus*. Meanwhile, *Anaeroplasma abactoclasticum* 6-1, *A. bactoclasticum* JR, *A. intermedium* 7LA, and *A. varium* RM10 grow on maltose, glucose, and starch and produce acetate, formate, and lactate (Joblin & Naylor, 2002). Liu et al. (2016) reported that *Anaeroplasma* is more abundant in alfalfa with high amounts of crude protein and ether extract than in rice straw with low amounts of neutral detergent fiber, acid detergent fiber, and ash (Liu et al., 2016), which was inconsistent with the results of this study. Our results indicated that decreases in *Ruminococcus* and *Anaeroplasma*, which produce acetic acid, partly resulted in a low ratio of acetic acid to total VFAs and acetic acid to propionic acid (Figure 1). However, further investigations are needed to clarify why the relative abundance of *Anaeroplasma*, which utilizes soluble sugars and starch,

FIGURE 2 Bacterial abundances in breeding Japanese Black cattle, feedlot Japanese Black cattle, lactating Holstein cows, and dry Holstein cows. Error bars represent the standard deviations. Different letters indicate a statistically significant difference ($p < 0.05$, $n = 3$)

TABLE 3 Number of reads and alpha diversity of rumen bacterial community

	Breeding Japanese Black	Feedlot Japanese Black	Lactating Holstein Friesian	Dry Holstein Friesian
Read	56,435 ($\pm$ 1927)	52,303 ($\pm$ 1478)	57,096 ($\pm$ 5307)	56,939 ($\pm$ 2299)
Bacterial phylum[a]	21 ($\pm$ 0)	18 ($\pm$ 1)	18 ($\pm$ 0)	18 ($\pm$ 0)
Bacterial genus	165 ($\pm$ 3)	130 ($\pm$ 14)	127 ($\pm$ 3)	135 ($\pm$ 2)
Chao1	208.93 ($\pm$ 0.25)	169.68 ($\pm$ 24.96)	177.11 ($\pm$ 18.55)	167.33 ($\pm$ 4.50)
ACE	198.60 ($\pm$ 5.51)	163.35 ($\pm$ 18.96)	163.51 ($\pm$ 9.07)	164.43 ($\pm$ 5.53)
Shannon index	2.71 ($\pm$ 0.00)	2.62 ($\pm$ 0.08)	2.66 ($\pm$ 0.05)	2.61 ($\pm$ 0.03)
Simpson index	0.89 ($\pm$ 0.00)	0.87 ($\pm$ 0.02)	0.88 ($\pm$ 0.01)	0.88 ($\pm$ 0.00)

Note: The values represent the mean $\pm$ standard deviations ($n = 3$).

[a]Twenty-six phyla were identified in total, 18 of which were detected in all cattle. Breeding Japanese Black cattle had five unique phyla, whereas feedlot Japanese Black cattle and lactating Holstein cow harbored one unique phylum each. Another single phylum was detected in breeding Japanese Black cattle and lactating Holstein cow.
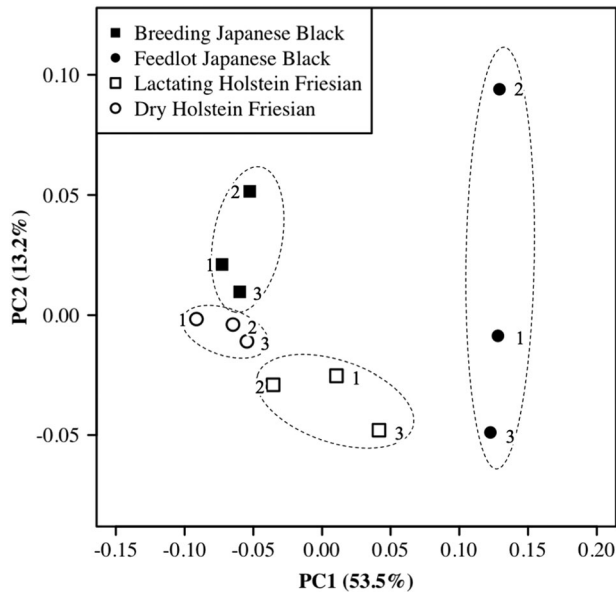
FIGURE 3 Principal coordinates analysis of genus-level bacterial communities in breeding Japanese Black, feedlot Japanese Black, lactating Holstein Friesian, and dry Holstein Friesian cattle, evaluated by 16S rRNA gene amplicon sequencing. The number with each plot represents the cattle number (Table 1)

was lower in feedlot Japanese Black cattle, even though these cattle were fed higher contents of starch and non-fiber carbohydrate. *Succinivibrio* is the predominant genus from the rumen when the diet of the animal is high in starch (Li, Teng, et al., 2019; O'Herrin & Kenealy, 1993); thus, *Succinivibrio* possibly contributes to starch digestion in the rumen. Taken together, our results indicate that the change in the microbial community results in differences in the chemical features of rumen fluids between cattle groups.

3.3 | Fibrolytic isozyme activities of rumen microbial communities

Zymograms for endoglucanase and xylanase activities were produced from SDS-PAGE gels of the protein extracts (Figure 5 and Table S1). Endoglucanase and xylanase activities were visualized and compared among cattle groups and individual cattle. Several similarities in endoglucanase activities were observed in all cattle (Figure 5a), although the characteristics of cattle (i.e., age, ratio of concentrate to roughage, nutritional composition, and cattle type) were different between the individuals (Table 2). All cattle showed a wide range of endoglucanase activities from 37 to 250 kDa. Prominent bands representing the activities of two endoglucanases at 55 and 57 kDa were also detected in all cattle. Endoglucanases with a wide range of molecular masses are secreted by various ruminal bacteria such as *R. albus* (Ohara et al., 2000), *R. flavefaciens* (Rincon et al., 2001), *F. succinogenes* (Bera et al., 1996), and *Prevotella bryantii* (Matsui et al., 2000). Although the origin of the isozymes determined in the present study has yet to

be identified, these predominant cellulolytic bacteria were commonly detected in all cattle groups (Figure 4b). These results suggest that core microbiota produced endoglucanases and played key roles in cellulose digestion in the rumen microbial community. Endoglucanase zymograms showed differences in endoglucanase activity among cattle groups. Endoglucanase activity at 68 kDa was detected in breeding Japanese Black cattle, lactating Holstein Friesian cows, and dry Holstein Friesian cows. However, obvious activity was not detected in feedlot Japanese Black cattle. In this study, the relative abundance of the genus *Ruminococcus* including some cellulolytic species decreased in feedlot Japanese Black cattle (Figure 4b). The nucleotide sequence of the celB endoglucanase gene from *R. flavefaciens* FD-1 revealed an open reading frame of 1943 nucleotides that encodes a polypeptide of 632 amino acids with a molecular weight of 69,414 (Vercoe et al., 1995). Our results suggest that the low abundance of cellulolytic bacteria such as *Ruminococcus* resulted in an absence of the activity of endoglucanase at 68 kDa in size.

As with endoglucanase, all cattle showed a wide range of xylanases from 20 to 250 kDa (Figure 5b). Xylanase with low activity at 44 kDa was commonly detected in all cattle. It has been shown that xylanases with a wide molecular mass are produced by many rumen microorganisms such as *Prevotella ruminicola* (Matsui et al., 2000), *R. albus* (Moon et al., 2011), and *R. flavefaciens* (Saluzzi et al., 2001). Although the origin of the xylanases determined in the present study has yet to be identified, these predominant xylanolytic bacteria were commonly detected in all cattle groups (Figure 4b). These results suggest that a core microbiota secretes xylanases and plays key roles in xylan digestion in the rumen microbial community. Meanwhile, each cattle group had a different pattern of xylanase activity. Breeding Japanese Black cattle and dry Holstein Friesian cows showed xylanase activity at 52 and 53 kDa, respectively, whereas these activities were not clearly observed in feedlot Japanese Black cattle and lactating Holstein Friesian. The expression level of the xylanase gene (*xynA*) in *Clostridium cellulovorans* is lower when cells are grown in di- or monomeric sugars, such as lactose, mannose, glucose, galactose, maltose, sucrose, and locus bean gum (Han et al., 2003). It was speculated that feedlot Japanese Black cattle and lactating Holstein Friesian cows provide large amounts of di- or monomeric sugars produced from starch, which results in a decrease in the transcription levels of xylanases of xylanolytic rumen microorganisms.

Interestingly, zymograms also showed that several cattle had specific endoglucanase activity (Figure 5a). Endoglucanase activities at 37 and 52 kDa were detected only in lactating Holstein Friesian cows (No. 3). Feedlot Japanese Black cattle (No. 2), lactating Holstein Friesian cows (No. 3), and dry Holstein Friesian cows (No. 3) specifically showed endoglucanase activity at 43 kDa. Similarly, xylanase activity was markedly different among individual cattle (Figure 5b). Feedlot Japanese Black cattle (No. 1) showed high xylanase activity at 22 and 56 kDa, whereas these activities were low or undetectable in other feedlot Japanese Black cattle (No. 2 and No. 3). Xylanase activity at 40 and 75 kDa was specifically observed in Feedlot Japanese Black cattle (No. 2) and lactating Holstein Friesian cows (No. 3), respectively. Differences in microbial community composition are predominantly
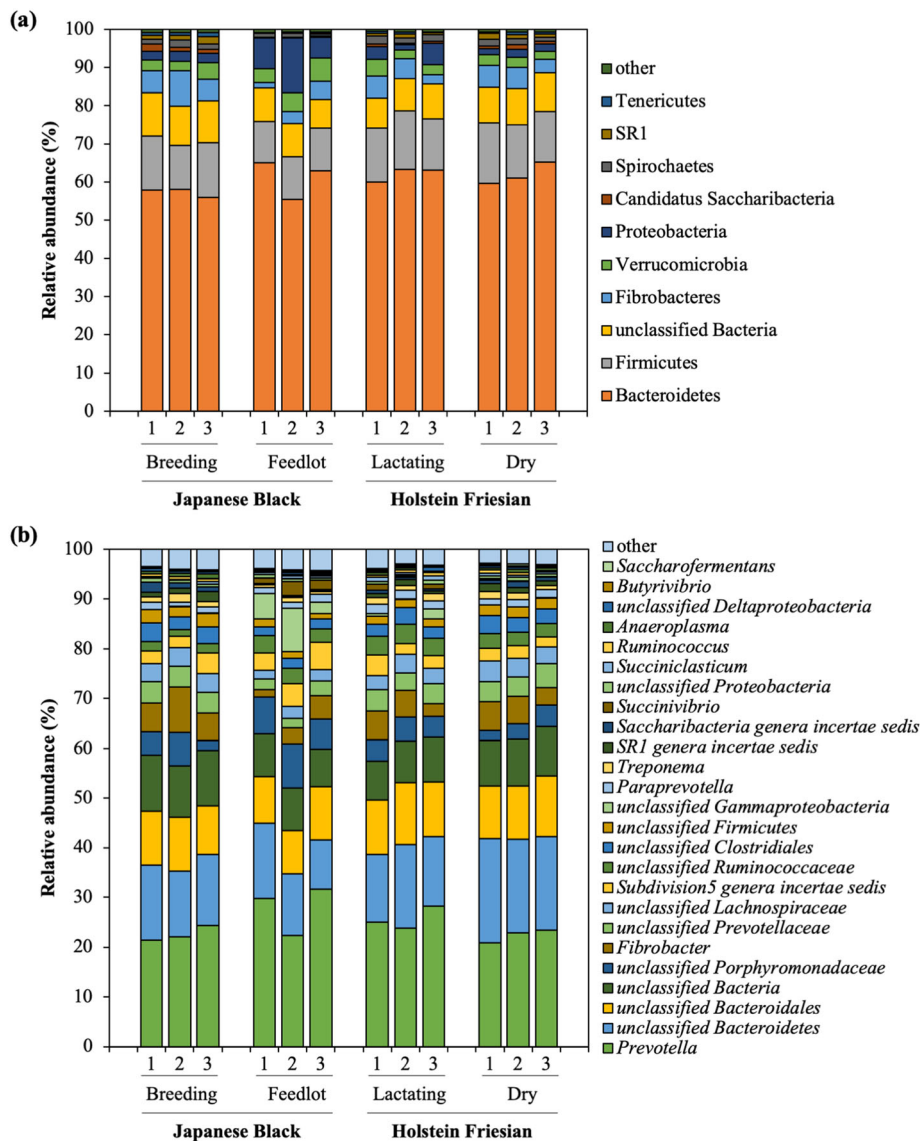
(a)

(b)

attributable to diet (Henderson et al., 2015), whereas host genetics, age, and growth of the host are also the main determinants of the rumen microbial composition (Malmuthuge & Guan, 2017). Additionally, it was reported that major shifts in the composition of microbial communities colonizing switchgrass could be observed within 30 min, and biomass degradation appeared to have occurred between 30 min and 4 h after the switchgrass was placed in the rumen (Piao et al., 2014). In this study, rumen fluids were collected from each cattle 2 h post-feeding. However, the times at which animals finished eating were possibly different among individual cattle, which resulted in differences in rumen microbial communities and fibrolytic isozyme activities. Further investigations using a greater number of cattle are needed to elucidate the mechanisms underlying the different fibrolytic isozyme activities between individual cattle. This study suggests both the similarities and differences in fibrolytic isozyme activities in a rumen microbial community in Japanese Black cattle and Holstein Friesian cows. Our results also suggest that zymograms can show differences in fibrolytic isozyme activity between individual cattle.

3.4 | Correlation between fibrolytic isozyme activities and characteristics of cattle or taxonomic compositions of bacterial communities

To identify parameters that potentially influence fibrolytic isozyme activity, correlations between fibrolytic isozyme activities and taxonomic compositions of the bacterial communities were analyzed (Figure 6). The fibrolytic genus *Fibrobacter* was negatively correlated with total endoglucanase activity ($r = -0.68$, $p < 0.01$), endoglucanase of 55 kDa ($r = -0.81$, $p < 0.01$), and total xylanase activity ($r = -0.62$, $p = 0.04$). These results were inconsistent with those of previous studies, which suggested that *F. succinogenes* is the major fibrolytic bacteria (Baba et al., 2017; Dai et al., 2015). Meanwhile, the fibrolytic genus *Butyrivibrio* showed a positive correlation with xylanase activity at 44 kDa ($r = 0.83$, $p < 0.01$). Genome analysis revealed that rumen *Butyrivibrio* bacteria encode a large and diverse spectrum of degradative carbohydrate-active isozymes and utilize a wide variety of substrates, especially xylan and pectin, suggesting that *Butyrivibrio* plays

FIGURE 5 Endoglucanase (a) and xylanase (b) zymograms for breeding Japanese Black cattle, feedlot Japanese Black cattle, lactating Holstein Friesian cows, and dry Holstein Friesian cows. Zymograms were performed at pH 7.3, which was the mean observed pH value among all cattle (see Figure 1). Eight microliters of all protein extracts was respectively loaded on zymogram gels. The original zymogram gels before adjustments of contrasts and exposures are shown in Figure S2

FIGURE 6 Spearman's rank correlations of chemical characteristics of rumen fluid, fibrolytic isozyme activities, and predominant bacterial genera. The color of the square represents each correlation coefficient from −1 (red) to 1 (blue). The size of the square represents each correlation. A larger square indicates a higher correlation, and a smaller square represents a weaker correlation. An asterisk in a square represents a significant correlation ($p < 0.05$). The relative abundances of the top 10 classified genera are shown. A/P, acetic acid/propionic acid

an important role in hemicellulose and pectin degradation in the rumen (Palevich et al., 2020). In this study, it was also suggested that *Butyrivibrio* plays an important role in xylan degradation by producing a xylanase of 44 kDa. Endoglucanase activity at 68 kDa was positively correlated with the soluble sugar-fermenting genus *Treponema* (Stanton & Canale-Parola, 1980) ($r = 0.60$, $p = 0.04$) and the soluble sugar- and starch-utilizing genus *Anaeroplasma* (Joblin & Naylor, 2002) ($r = 0.71$, $p = 0.01$). The amylolysis-associated genus *Succinivibrio* (O'Herrin & Kenealy, 1993) was positively correlated with endoglucanase activity at 57 kDa ($r = 0.60$, $p = 0.04$) and total xylanase activity ($r = 0.69$, $p = 0.02$) but negatively correlated with endoglucanase at 68 kDa ($r = -0.63$, $p = 0.03$). Similarly, the succinate-fermenting genus *Succiniclasticum* (Van Gylswyk, 1995) was positively correlated with xylanase activity at 22 kDa ($r = 0.58$, $p = 0.047$), 44 kDa ($r = 0.64$, $p = 0.03$), and 56 kDa ($r = 0.69$, $p = 0.01$). The fibrolytic genus *Ruminococcus* showed a positive correlation with xylanase activity at 53 kDa ($r = 0.62$, $p = 0.03$) and also tended to be positively correlated with endoglucanase activity at 68 kDa ($r = 0.52$, $p = 0.08$). This result supported our hypothesis, which suggested that a decrease in cellulolytic bacteria such as *Ruminococcus* results in low endoglucanase activity at 68 kDa. Co-culture of cellulolytic *R. flavefaciens* and non-cellulolytic *Selenomonas ruminantium* results in higher digestibility of avicel, orchardgrass hay, rice straw, and alfalfa hay compared to that with *R. flavefaciens* alone (Sawanon & Kobayashi, 2006). Our results suggest that nonfibrolytic bacteria remove succinate and/or lactate from fibrolytic microorganisms for their growth with an increase in the fibrolytic isozyme activities of fibrolytic microorganisms.

This study also revealed correlations between the fibrolytic isozyme activities and taxonomic compositions of the bacterial communities and also indicated key rumen microorganisms related to fibrolytic isozyme activities in Japanese Black cattle and Holstein Friesian cows. In addition, our study offers important insights for improving and sustaining animal production, although further analysis of endoglucanases and xylanases using sufficient numbers of cattle is necessary. Our results suggest that fibrolytic isozymes and microorganisms correlate with fibrolytic isozyme activities and can be used to enhance cellulose digestion in the rumen microbial community in all cattle, which would improve animal production and the bioconversion of lignocellulosic biomass.

In conclusion, this study revealed the structure of the rumen microbial community and the characteristics of fibrolytic isozyme activities in Japanese Black cattle and Holstein Friesian cows fed various ratios of concentrate to roughage. The results showed that the structures of the rumen bacterial community were different among cattle groups. Moreover, we found that endoglucanases of 55 and 57 kDa and xylanase of 44 kDa had activity in all cattle, whereas the differences in several fibrolytic isozyme activities were observed between cattle groups, but also between individual cattle. *Treponema*, *Succinivibrio*, and *Anaeroplasma* showed positive correlations with endoglucanase activities at 57 and 68 kDa. *Treponema*, *Succinivibrio*, *Succiniclasticum*, *Ruminococcus*, and *Butyrivibrio* showed positive correlations with xylanase activities at 22, 44, 53, and 56 kDa. Our study

suggested that these genera play key roles in lignocellulose digestion in Japanese Black and Holstein Friesian cattle; thus, further analysis of the genera related to fibrolytic isozyme activities is important for efficient animal production.

CONFLICT OF INTEREST

The authors declare no competing interests.

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