Studies on the effects of feeding by-products and calcium salts of long-chain fatty acids on rumen fermentation characteristics and microbiome

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ABSTRACT

The utilization of by-products and feed additives is a feasible strategy for sustainable livestock production. In chapter 1, the effect of supplementary desalted mother liquor (DML) as replacement of salt in concentrate on Thai native cattle was evaluated. Salt could be replaced by DML up to 2% as NaCl in concentrate without adverse effects on nitrogen balance, rumen conditions, blood metabolites and methane (CH_4) emission of Thai native cattle. In chapter 2, the usability of wine lees as feed for ruminants in fattening conditions was investigated. The wine lees substituted for the fattening ration up to 20% DM had no adverse effects on apparent digestibility, ruminal fermentation, and nitrogen balance and decreased an oxidative stress marker in plasma. The results indicated that wine lees have a potential to be an important alternative as a partial substitute for antioxidant products. In chapter 3, the effects of supplementary calcium salts of long-chain fatty acids (CSFA) on the rumen microbiome and CH_4 production were evaluated via *in vitro* method. The inclusion of CSFA significantly changed the rumen microbiome, leading to the acceleration of propionate production and the reduction of CH_4 production. Therefore, CSFA may be a promising candidate for reduction of CH_4 emission from ruminants. Finally, in chapter 5, the difference of the rumen microbiome between Japanese Black steers and Japanese Black sires × Holstein dams crossbred (F1) steers was evaluated as a pre-experiment for the *in vivo* study to elucidate the effect of CSFA on rumen fermentation and microbiome. The Japanese

Black rumen microbiome showed higher relative abundances of fibrolytic bacteria and, consequently, relatively more enzymes associated with cellulose and hemicellulose degradation.

Key words: calcium salts of long-chain fatty acids, desalted mother liquor, methane, rumen fermentation, rumen microbiome, wine lees

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Chapter 2, 3, 4, and 5 are the peer reviewed version of the following articles, respectively:

Sato, Y., Angthong, W., Butcha, P., Takeda, M., Oishi, K., Hirooka, H., & Kumagai, H. (2018). Effects of supplementary desalted mother liquor as replacement of commercial salt in diet for Thai native cattle on digestibility, energy and nitrogen balance, and rumen conditions. *Animal Science Journal*, *89*(8). https://doi.org/10.1111/asj.13028.

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Yoshiaki Sato

CHAPTER 1

General introduction

1.1. Benefit and disadvantage of ruminant production

Demand for animal protein is increasing with the growing world population (Eisler et al., 2014). Livestock provides readily digestible protein and essential nutrients and can make critical contributions to ending hunger and improving food security and nutrition (FAO, 2018). Recently, however, livestock production has been criticized due to feed-food competition. About 36 percent of world consumption of cereals, mainly coarse grains, goes to feed (Alexandratos and Bruinsma, 2012). Unlike pigs and poultry, ruminants have an ability to convert human inedible foodstuffs to human edible protein such as milk and meat; however, in fact, large amount of human edible feeds is offered to ruminants to increase productivity. It is important to exploit feeds not suitable for human consumption.

In addition, livestock is commonly considered to be unsustainable because livestock production globally contributes to anthropogenic greenhouse gas (GHG) emissions. Global livestock sector accounts for approximately 14.5% of the global anthropogenic GHG emissions (Gerber et al., 2013). In particular, methane (CH_4) released from enteric fermentation of ruminants contributes to 39% in livestock sector (Gerber et al., 2013). Furthermore, CH_4 produced by enteric ruminal fermentation not only increases environmental negative impacts but also leads to energy loss amounting 2–12% of the gross energy intake (Johnson and Johnson, 1995). Therefore, increasing efficacy of production with decreasing CH_4 production is indispensable for sustainable livestock production in the future.

1.2. Feeding by-products and feed additives for ruminants

Use of by-products such as food waste and crop residue in animal nutrition has attracted a lot of attention as a strategy to reduce feed-food competition and environmental impacts of livestock production as well as decreasing their disposal costs. Gustavsson et al. (2011) estimated

that roughly one-third of food produced for human consumption is lost or wasted globally, which amounts to about 1.3 billion tons per year. Many by-products are enriched in nutrients such as protein, fiber, and fat, which are essential for animal growth (Table 1-1). Some researchers studied on the utilization of by-products as feeds for ruminants: e.g., palm oil by-products (Abubakr et al., 2015), pineapple fruit residue (Gowda et al., 2015), cassava chip (Chanjula et al., 2007), and tamarind kernel powder extract residue (Wang et al., 2016; Wang et al., 2017).

Table 1-1. Chemical compositions of by-products (%DM)

By-products	DM (%)	OM	CP	EE	NDF	References
Dried brewers grains	89.8	95.1	18.7	4.9	67.5	Faccenda et al. (2019)
White grape pomace	30.5	93.3	9.3	4.8	30.6	Baumgärtel et al. (2007)
Red grape pomace	27.3	94.3	15.5	7.0	50.7	Baumgärtel et al. (2007)
Apple pomace	14.9	97.4	4.5	3.9	38.4	Kara et al. (2018)
Dried tomato pomace	92.0	93.0	22.2	15.0	49.2	Aghajanzadeh-Golshani et al. (2010)
Tamarind kernel powder extract residue	88.5	91.6	42.4	15.0	-	Wang et al. (2017)
Rice gluten meal	-	95.0	46.4	3.4	28.6	Kumar et al. (2016)
Distillers dried grains with solubles	-	96.1	32.0	11.5	58.9	Belyea et al. (2010)

DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber

The utilization of feed additives for ruminants is also a feasible strategy for sustainable livestock production. Feed additives have been used for increasing productivity and decreasing CH_4 production from ruminants. For example, monensin (Guan et al., 2006; Odongo et al., 2007),

which is one of the most common antibiotics in livestock production, linseed oil (Martin et al., 2008), and red seaweeds such as *Asparagopsis armata* and *A. taxiformis* (Roque et al., 2019; Kinley et al., 2020; Roque et al., 2021) have reduction effect on CH_4 production from rumen.

However, some by-products and feed additives also have a negative effect on ruminal digestion. For example, Ikwuegbu and Sutton (1982) reported that feeding linseed oil reduced digestion of organic matter, particularly fiber, and the number of protozoa in rumen. It is important to clarify the appropriate amount to feed and the effect on rumen characteristics and microbiome for the utilization of by-products and feed additives as animal feeds.

1.3. Rumen microbiome

Ruminants have a four chambered stomach (rumen, reticulum, omasum, and abomasum). Among them, rumen is an evolved forestomach that allows microbial digestion of feeds. Ruminants themselves do not produce the enzymes required to degrade most complex plant polysaccharides, while the rumen provides an environment for a rich and dense consortium of anaerobic microbes that fulfil this metabolic role (Henderson et al., 2015). The complex rumen microbes digest and ferment ingested feeds (Terry et al., 2019), producing volatile fatty acids (VFAs) which are major energy sources for ruminants. Therefore, the rumen microbes significantly contribute to convert human inedible feeds to high-quality protein such as milk and meat.

The rumen microbiome is significantly influenced by various factors such as host species and diet, and diet is the most influenced factor (Henderson et al., 2015). Recently, many researchers have investigated the effect of feeds on the rumen microbiome by next-generation sequencing (NGS). Lyons et al. (2017) reported that the relative abundance of *Succinivibrionaceae* and *Veillonellaceae* which are succinate and propionate producers, respectively, were higher and that of *Ruminococcaceae*, which is a family linked with high CH_4

emitters, were lower in the lambs fed linseed oil than in the control group. Shen et al. (2017) suggested that supplementary nisin, a bacteriocin, changed the abundance of some fibrolytic bacteria and modulated rumen fermentation, resulting in the reduction of CH_4 production without adversely affecting feed digestion. Thus, identifying diet-microbe interaction can help us better understand how ingested feeds affect ruminal fermentation and CH_4 emission. It is essential for a deeper understanding the nutritive value of feeds for ruminants to investigate the effect on rumen microbiome.

The effects of feeds on performance, rumen characteristics, or digestibility are different between breeds. For example, starch digestibility was higher in Angus bulls than Nellore when the animals were fed diets containing whole shelled corn or ground corn with silage (Carvalho et al., 2019). Olijhoek et al. (2018) suggested that digestibility of nutrients (dry matter, organic matter, neutral detergent fiber, and fat) was higher for Jersey than Holstein cows. Furthermore, they reported that the molar proportion of acetate and propionate also differed between the two breeds. Differences of the effect of feeds are expected to be due to the difference of rumen microbiome between the breeds of cattle. Latham et al. (2018) reported that unweighted UniFrac distance of rumen bacterial community and the relative abundance of some taxa (e.g., *Prevotellaceae* and *Ruminococcaceae*) clearly differed between Angus and Brahman steers fed low-quality rice straws added by some levels of protein. Therefore, the effects of cattle breeds on the rumen microbiome should be clarified before feeding experiments.

1.4. Objectives of studies

The objective of the thesis was to evaluate the effect of feeding by-products and calcium salts of long-chain fatty acids (CSFA) on rumen fermentation characteristics and microbiome. In Chapter 2, *in vivo* experiment was performed to evaluate the effect of supplementary desalted mother liquor (DML) as replacement of NaCl in diets for fattening Thai native cattle on

digestibility, energy, nitrogen balance and ruminal condition. In Chapter 3, the effects of dietary wine lees for ruminants were investigated using *in vitro* and *in vivo* studies. In Chapter 4, the effects of supplementary CSFA on the rumen microbiome and CH_4 production were evaluated via *in vitro* method. Finally, in Chapter 5, the difference of the rumen microbiome between Japanese Black steers and Japanese Black sires × Holstein dams crossbred (F1) steers was evaluated as a pre-experiment for the *in vivo* study to elucidate the effect of CSFA on rumen fermentation and microbiome.

CHAPTER 2

Effects of supplementary desalted mother liquor as replacement of commercial salt in diet for Thai native cattle on digestibility, energy and nitrogen balance, and rumen conditions

2.1. Introduction

In recent years, the shortage of conventional feeds has become a substantial constraint against animal production. Concentrate feeds such as soybean and its by-products are considerably expensive, resulting in increasing feeding costs. Nowadays, utilizing industrial by-products as animal feed is receiving considerable attention to overcome the problem of feeding costs.

Desalted mother liquor (DML) is by-product of nucleic acid-related compounds (sodium inosinate and sodium guanylate) for food additives produced by a seasoning company in Thailand. The liquor contains nucleic acid-related compounds such as inosine (HxR), guanosine (GR), inosine monophosphate (IMP) and guanosine monophosphate (GMP) because a small portion remains in the liquor after crystallization. Although DML is mainly mixed with monosodium glutamate by-product and utilized as liquid fertilizer, DML can possibly be fed to cattle as a source of nitrogen (N) because nucleic acid-related compounds are rich in N; for example, IMP and GMP contain about 16% and 19% of N, respectively. Kimura et al. (2010) demonstrated that the supplementary GR and HxR activated *in vitro* ruminal fermentation, particularly in roughage substrate conditions. Kanyinji et al. (2009) observed that nutrient intake, digestibility, ruminal fermentation pattern and N balance in goats fed high amounts of concentrate supplemented with HxR did not differ from those that received urea.

Desalted mother liquor also contains high levels of sodium chloride (NaCl); approximately 50% on a dry matter (DM) basis. Some researchers showed that digestibility of nutrients was not affected by high salt levels. Cardon (1953) reported that Hereford cows fed 6.8 kg alfalfa hay and 0.82 kg salt per head daily did not decrease cellulose digestibility compared with animals fed the same diets without salt. Chicco et al. (1971) showed apparent digestibility of DM, cellulose or protein in steers grazing tropical pasture given free access to concentrate with 30% salt were not different from those in steers without salt. However, high salt levels might have

negative effects on N balance. Leibholz et al. (1980) reported that the N accumulation of calves offered the diet containing 6.50% NaCl on a fresh matter (FM) basis was less than that of the calves fed on diets containing 0.50%, 2.54% or 4.58% NaCl on a FM basis. Chicco et al. (1971) also reported that less N was retained in the animals consuming high salt rations. Therefore, the amount of the upper limit of DML supplementation should be considered, although DML is rich in N.

Our previous study indicated that DML supplementation enhanced *in vitro* DM and neutral detergent fiber expressed exclusive of residual ash (NDFom) digestibility compared with NaCl supplementation in roughage substrate conditions (unpublished data). Sakai et al. (2017) demonstrated that N retention and energy loss into methane (CH_4) emission decreased, although there was no effect on nutrient digestibility when 3% NaCl was added to concentrate by supplementing DML. However, the effect of DML on nutrient digestibility and CH_4 emissions are not clear because ingredient compositions of concentrate in their experimental diets were diversified to adjust accurately crude protein (CP) and total digestible nutrients (TDN) contents. Therefore, the objective of this study was to clarify the effects of DML as replacement of 1% or 2% NaCl in concentrate for fattening beef cattle on *in vivo* digestibility, rumen conditions, blood metabolites, CH_4 emissions, N and energy balance of Thai native cattle.

2.2. Materials and Methods

This experiment was conducted at the Ruminants Feeding Standard Research and Development Center, Department of Livestock Development, Khon Kaen, Thailand (16.341° N, 102.821° E), from September 2014 to December 2014. The animals used in the experiment were managed according to the guidelines of Kyoto University and Khon Kaen Animal Nutrition Research and Development Center Animal Ethics Committee.

The DML used in the experiment was prepared by a seasoning factory in Kamphaeng

Phet Province, Thailand. This is the liquor separated from nucleic acid in the fermenting process.

2.2.1. Animals, diets and experimental design

Four Thai native cattle, about 3 years old with initial body weight (BW) of 245 ± 4.6 kg (mean ± SD), were assigned in a 4 × 4 Latin square design for digestibility and respiration trials following a preadaptation period for 5 days. Each experimental period lasted 15 days, including 9 days of adaptation and 6 days of sampling.

The following four concentrate feeds were prepared: C1, 1% NaCl was added as commercial salt; C2, 2% NaCl was added as commercial salt; D1, 1% NaCl in C1 was replaced by DML; D2, 2% NaCl in C2 was replaced by DML, on a DM basis, respectively. (Table 2-1). Urea was added to the concentrate of C1 and C2 in order to be isonitrogenous to the concentrate of DML treatments D1 and D2. The concentrate feeds were prepared before the experiments started, except for commercial salt and DML. Commercial salt and DML were mixed into each concentrate feed before every morning feeding in order to supply equal amounts of NaCl.

2.2.2. Animal management and feeding

The cattle were housed individually in holding pens from days 1 to 4 and subsequently transferred to individual pens with head hoods from days 5 to 15 in each experimental period. Rice straw was chopped into 10 cm lengths on average, and the concentrate feeds and rice straw were fed separately at a ratio of 60:40 on a DM basis. The concentrate-to-roughage ratio was determined according to the feeding method of fattening cattle in the center and the experiment by Sakai et al. (2017). Tap water was offered *ad libitum*. The amount of total diets provided was 1.9% of BW on a DM basis in two equal portions daily, at 09:30 and 17:00 hours. No refusal was found throughout all experimental periods.

Table 2-1. Ingredients and chemical composition of experimental diets

	Concentrate				Rice Straw	Desalted mother liquor
	C1	C2	D1	D2		
Ingredient (%DM)						
Rice bran	9.1	9.1	9.1	9.1		
Soybean meal	11.6	11.6	11.6	11.6		
Cassava chip	25.5	25.5	25.5	25.5		
Palm kernel oil cake	26.8	25.8	26.1	24.2		
Ground corn meal	23.8	23.8	23.8	23.8		
Desalted mother liquor	—	—	1.9	3.8		
Commercial Salt	1.0	2.0	—	—		
Vitamin and mineral mix	2.0	2.0	2.0	2.0		
Urea	0.2	0.2	—	—		
Chemical composition (%)						
Dry matter	87.5	86.8	84.4	82.6	87.0	38.9
Crude protein[a]	14.3	14.0	14.0	14.2	2.7	27.0
Ether extract[a]	3.1	3.1	3.0	2.9	0.9	ND
Non-fibrous carbohydrate[a]	46.3	46.2	45.6	44.7	9.5	ND
aNDFom[a]	29.9	29.5	30.2	30.4	76.4	ND
ADFom[a]	17.7	16.6	18.1	16.8	50.5	ND
Acid detergent lignin[a]	4.7	4.6	4.7	4.8	5.1	ND
Crude ash[a]	6.5	7.2	7.2	7.7	10.5	ND
NaCl[a]	1.1	2.1	1.1	2.2	ND	53.0
Gross energy (MJ/kg DM)	18.5	17.8	18.0	18.1	16.3	ND

ND, not determined; aNDFom, neutral detergent fiber assayed with a heat-stable amylase and expressed exclusive of residual ash; ADFom, acid detergent fiber exclusive of residual ash; C1, 1% NaCl on a dry matter (DM) basis was added by commercial salt; C2, 2% NaCl on a DM basis was added by commercial salt; D1, 1% NaCl on a DM basis was replaced by desalted mother liquor; D2, 2% NaCl on a DM basis was replaced by desalted mother liquor.

[a]On a dry matter basis

2.2.3. Total fecal and urinary collection

Total fecal and urinary collection was conducted during 6 consecutive days, from day 10 to day 15 in each experimental period, after the adaptation. All feces excreted by each cattle were collected into plastic cases and the weight recorded and then about 1 kg was sampled daily. At the final day of each period, all collected feces from each animal were mixed and divided into two portions; one portion was stored at -20°C for analysis of N content, and the other portion was dried at 60°C for 72 h, then ground through a 1 mm screen and stored for analyses of chemical composition and gross energy (GE) contents. Urine was collected into plastic buckets with enough H_2SO_4 (20% v/v, 100–200 ml per day) to keep pH below 3.0 and the volume was recorded in each period. All collected urine from each animal was mixed and about 500 ml of the total was taken. The samples were stored at -20°C until analysis of N and GE contents. Body weight of each animal was weighed at the final day of each period before feeding in the morning.

2.2.4. Rumen fluid and blood sampling

On day 15 of each period, rumen fluid was collected via orogastric tubing from each animal at 0 and 4 hr after feeding in the morning. The rumen fluid samples were filtered through four layers of cheesecloth and pH was determined using a pH meter (pH 700, Eutech Instruments, Singapore, Singapore), then centrifuged at 1,000 × *g* for 10 min and the supernatants were stored at -20°C until further analysis.

Blood samples were also collected from jugular veins into heparinized tubes before morning feeding at the final day of each period. The samples were centrifuged at 3,000 × *g* for 10 min and the plasma parts were transferred into tubes (5 ml/tube) and stored at -20°C until further analyses.

2.2.5. Determination of methane emission

Ruminal CH_4 emission was determined according to the procedure described by Sakai et

al. (2017). The CH_4 emission was measured by using a ventilated head hood calorimetry system as described by Suzuki et al. (2008). The system consisted of a head hood, flow meter (thermal flow cell FHW-N-S; Japan Flow Cell, Ltd., Mintoku, Japan), oxygen (O_2) analyzer (Xentra 4100; Servomex, Ltd., Crowborough, UK), carbon dioxide (CO_2) and CH_4 analyzer (infra-red gas analyzer VIA300; Horiba, Kyoto, Japan). Flow rate was set at 240 L/min. Under usual circumstances, the CH_4 emission is calculated using the following equations:

$$F_{in} = \frac{100 - (O_{2out} + CO_{2out} + CH_{4out})}{100 - (O_{2in} + CO_{2in} + CH_{4in})} \times F_{out}$$

$$CH_4 = CH_{4out} \times F_{out} - CH_{4in} \times F_{in}$$

where F_{in} = flow rate of inlet gas (L/min), F_{out} = flow rate of outlet gas (L/min), O_{2in} = the O_2 concentration of the inlet gas (%), O_{2out} = the O_2 concentration of the outlet gas (%), CO_{2in} = the CO_2 concentration of the inlet gas (%), CO_{2out} = the CO_2 concentration of the outlet gas (%), CH_{4in} = the CH_4 concentration of the inlet gas (%), CH_{4out} = the CH_4 concentration of the outlet gas (%), CH_4 = the emission of CH_4 (L/min). However, unfortunately, the O_2 concentration could not be determined due to trouble with the O_2 analyzer and we could not calculate F_{in}. Therefore, we assumed the difference between F_{in} and F_{out} was very small and CH_4 production was calculated according to the following equation:

$$CH_4 = CH_{4out} \times F_{out} - CH_{4in} \times F_{out}$$

Before the experiment, gas recovery tests were conducted by comparing the measurement of CO_2 with the amount of pure CO_2 injected into each head hood. Average CO_2 recovery of four head hoods was 99.5 ± 6.24% (mean ± SD). The gas analyzers were calibrated using pure N_2 gas and span gas (CH_4, 1990 ppm; CO_2, 1.926%) prior to each measurement. Methane measurement

was conducted for 23.5 hr per day from 09:30 to 09:00 hours of the next day for three consecutive days. Volume of CH_4 (L/day) was converted to energy by using a conversion factor of 39.54 kJ/L (Brouwer, 1965).

2.2.6. Chemical analysis

Feed and fecal samples were analyzed for DM, CP, ether extract (EE), and crude ash contents according to the standards of the Association of Official Analytical Chemists (AOAC 2000): 930.15, 976.05, 920.39 and 942.05, respectively. The organic matter (OM) content was calculated as weight loss through ashing. Neutral detergent fiber assayed with a heat-stable amylase and expressed exclusive of residual ash (aNDFom), ADFom and acid detergent lignin (ADL) were analyzed according to the procedure described by Van Soest et al. (1991). The content of nonfibrous carbohydrate (NFC) was calculated by the equation: NFC = 100 - (CP + EE + NDF + crude ash). Apparent digestibility of DM, OM, CP, NFC, aNDFom, ADFom and EE were estimated by subtracting nutrients contained in the feces from nutrients contained in the dietary intake. The concentration of NaCl was determined by Mohr's titration method (Fischer and Peter, 1968). Gross energy (GE) contents of diets, feces and urine were determined using a bomb calorimeter (6400; Parr, Moline, IL, USA). Digestible energy intake (DEI) and metabolizable energy intake (MEI) were calculated by the equations: DEI = GE intake - energy losses in feces; MEI = DEI - energy losses in urine and CH_4 production. Nitrogen content of urine was determined by a Kjeldahl method (AOAC 2000). Ammonia (NH_3) concentration of rumen fluid was measured by distillation (AOAC 2000). Briefly, the supernatants were distilled by steam distillation and titrated by hydrochloric acid. Ruminal volatile fatty acid (VFA) concentrations were measured by gas chromatography (GC2010; Shimadzu, Kyoto, Japan) using a 25 m × 0.53 mm capillary column (BP21 0.5 P/N 054474; SGE Analytical Science Pty Ltd., Melbourne, Victoria, Australia) following Chuntrakort et al. (2014). The flow rate of carrier gas (N_2) was 30 ml/min. The temperature of injection, column and detector were 180, 130 and 250 °C, respectively. The plasma

samples were analyzed for glucose, blood urea nitrogen (BUN), creatinine, cholesterol, aspartate aminotransferase (AST), alanine aminotransferase (ALT) by the automatic analyzer (LX20PRO; Beckman Coulter, Brea, CA, USA), and for protein, albumin and potassium by the automatic analyzer (Cobas IT 5000; Roche Diagnostics, Mannheim, Germany).

2.2.7. Statistical analyses

The data were analyzed as a 4 × 4 Latin square design using the GLM procedure of Statistical Analysis System (SAS) (1998). The mathematical model was $Y_{ijkl} = \mu+T_i+P_j+A_k+e_{ijkl}$, where μ = the overall means, T_i = the effect of the treatment (i = 1,2,3,4), P_j = the effect of the period (j = 1,2,3,4), A_k = the effect of animal (k = 1,2,3,4), and e_{ijkl} = residual error. Least square means were compared using the Tukey–Kramer method. Contrast statements were used to compare commercial salt versus DML and 1% NaCl versus 2% NaCl contents in concentrate. Differences among means were considered statistically significant at $P < 0.05$ and differences at $0.05 \leqq P < 0.10$ were accepted as showing tendencies toward significance.

2.3. Results

2.3.1. Feed intake and apparent digestibility

Feed intake of animals fed experimental concentrates is presented in Table 2-2. The intake of DM was not significantly different among the treatments. The CP intake of cattle fed C1 was higher than those of C2 and D1 ($P < 0.05$). The intakes of OM, CP, EE and NFC in DML treatments were lower than those of commercial salt treatments ($P < 0.05$). The intake of ADFom, EE and NFC in 1% NaCl treatment were higher than those in 2% NaCl treatment ($P < 0.05$). Table 2-3 shows apparent digestibility of the experimental diets. ADFom digestibility in the D2 treatment was higher than that in C2 and D1 treatments ($P < 0.05$) and was superior in DML treatments relative to commercial salt treatments ($P < 0.05$). Meanwhile, EE digestibility of DML

treatments was lower than salt treatments ($P < 0.05$). Digestibility of DM, OM, CP, NFC and aNDFom were not affected by treatments.

Table 2-2. Body weight and nutrient intake in Thai native cattle fed experimental diets

	Treatment[1]					P-value	
	C1	C2	D1	D2	SEM	1% vs 2%	C vs D
Body weight (kg)	271.6	271.3	273.0	272.8	1.24	NS	NS
Nutrient intake (g/day/kg $BW^{0.75}$)							
Dry matter	70.3	70.0	69.6	69.8	0.23	NS	†
Organic matter	64.7	64.1	63.7	63.7	0.21	NS	*
Crude protein	6.91^{a}	6.74^{bc}	6.68^{c}	6.82^{ab}	0.023	NS	*
aNDFom	33.6	33.4	33.5	33.7	0.11	NS	NS
ADFom	21.3^{a}	20.8^{b}	21.3^{a}	20.8^{b}	0.07	*	NS
Ether extract	1.58^{a}	1.56^{ab}	1.54^{b}	1.50^{c}	0.005	*	*
Non-fibrous carbohydrate	22.6^{a}	22.4^{a}	22.0^{b}	21.7^{b}	0.07	*	*

aNDFom, neutral detergent fiber assayed with a heat-stable amylase and expressed exclusive of residual ash; ADFom, acid detergent fiber exclusive of residual ash; BW, body weight; NS, not significant; 1% vs 2%, adding 1% NaCl vs 2% NaCl; C vs D, commercial salt vs desalted mother liquor; SEM, standard error of mean; C1, 1% NaCl on a dry matter (DM) basis was added by commercial salt; C2, 2% NaCl on a DM basis was added by commercial salt; D1, 1% NaCl on a DM basis was replaced by desalted mother liquor; D2, 2% NaCl on a DM basis was replaced by desalted mother liquor.

[abc]Means in a row with different superscripts significantly differ ($P < 0.05$).

*$P < 0.05$; †$P < 0.10$.

[1]The roughage-concentrate ratio was 40:60.

Table 2-3. Apparent digestibility (%) of the experimental diets

	Treatment[1]					P-value	
	C1	C2	D1	D2	SEM	1% vs 2%	C vs D
Dry matter	65.6	65.1	65.1	65.3	0.45	NS	NS
Organic matter	69.0	68.7	68.5	68.8	0.44	NS	NS
Crude protein	59.6	54.7	55.3	58.7	1.57	NS	NS
Non-fibrous carbohydrate	84.7	87.0	85.5	84.1	1.20	NS	NS
aNDFom	59.6	58.6	59.3	60.4	0.84	NS	NS
ADFom	58.5^{ab}	57.6^{b}	58.1^{b}	60.8^{a}	0.50	NS	*
Ether extract	84.6	84.3	83.3	82.1	0.68	NS	*

aNDFom, neutral detergent fiber assayed with a heat-stable amylase and expressed exclusive of residual ash; ADFom, acid detergent fiber exclusive of residual ash; NS, not significant; 1% vs 2%, adding 1% NaCl vs 2% NaCl; C vs D, commercial salt vs desalted mother liquor; SEM, standard error of mean; C1, 1% NaCl on a dry matter (DM) basis was added by commercial salt; C2, 2% NaCl on a DM basis was added by commercial salt; D1, 1% NaCl on a DM basis was replaced by desalted mother liquor; D2, 2% NaCl on a DM basis was replaced by desalted mother liquor.

^{ab}Means in a row with different superscripts significantly differ ($P < 0.05$).

*$P < 0.05$.

[1]The roughage-concentrate ratio was 40:60.

2.3.2. Nitrogen balance

Nitrogen balance in animals fed the experimental concentrates is shown in Table 2-4. The intake N of animals in the D2 treatment was higher than that in the D1 treatment ($P < 0.05$) and similar to those of C1 and C2 treatments, and DML treatments had lower N intake than commercial salt treatments ($P < 0.05$). Fecal N, urinary N, N retention or proportion of N retention to intake N did not significantly differ among the treatments.

Table 2-4. Nitrogen balance in Thai native cattle fed experimental diets

	Treatment[1]					P-value	
	C1	C2	D1	D2	SEM	1% vs 2%	C vs D
N balance (g/day/kg $BW^{0.75}$)							
Intake N	1.11^{c}	1.08^{ab}	1.07^{a}	1.09^{bc}	0.004	NS	*
Fecal N	0.46	0.48	0.47	0.45	0.023	NS	NS
Urinary N	0.26	0.27	0.27	0.27	0.013	NS	NS
Retention N	0.39	0.32	0.33	0.38	0.027	NS	NS
N retention / N intake (%)	35.2	30.0	30.4	34.3	2.45	NS	NS

[abc]Means in a row with different superscripts significantly differ ($P < 0.05$).

NS, not significant; 1% vs 2%, adding 1% NaCl vs 2% NaCl; C vs D, commercial salt vs desalted mother liquor; BW, body weight; N, nitrogen; SEM, standard error of mean; C1, 1% NaCl on a dry matter (DM) basis was added by commercial salt; C2, 2% NaCl on a DM basis was added by commercial salt; D1, 1% NaCl on a DM basis was replaced by desalted mother liquor; D2, 2% NaCl on a DM basis was replaced by desalted mother liquor.

*$P < 0.05$.

[1]The roughage-concentrate ratio was 40:60.

2.3.3. Ruminal fermentation and blood metabolites

There were no significant differences among the treatments in pH and NH_3-N of ruminal fluid at 0 and 4 hr after feeding (Table 2-5). At 0 hr post-feeding, no significant differences were observed in total VFA content or molar percentages of individual VFA among the treatments. However, at 4 hr post-feeding, the molar percentage of acetic acid tended to be higher in DML treatments than commercial salt treatments ($P = 0.08$). Iso-valeric acid proportions in commercial salt treatments were higher than that in DML treatments and butyric acid proportion in the 1% NaCl treatment was higher than that in the 2% NaCl treatment ($P < 0.05$). Blood metabolite contents of each treatment are shown in Table 2-6. The contents of glucose, BUN, creatinine, cholesterol, AST, ALT, protein, albumin and potassium did not show significant differences among the treatments.

Table 2-5. Ruminal conditions in Thai native cattle fed experimental diets

	Time after feeding (hr)	Treatment[1]				SEM	P-value	
		C1	C2	D1	D2		1% vs 2%	C vs D
pH	0	7.1	7.1	7.0	7.1	0.02	NS	NS
	4	6.7	6.7	6.8	6.7	0.03	NS	NS
NH_3-N (mgN/100mL)	0	10.3	9.6	10.9	10.1	1.19	NS	NS
	4	5.1	5.2	5.3	5.7	0.58	NS	NS
Volatile fatty acid								
Total(mmol/L)	0	167.1	242.3	216.1	250.7	29.52	NS	NS
	4	225.6	260.7	284.4	270.4	29.70	NS	NS
Acetic acid (%)	0	68.0	70.0	70.8	71.7	1.44	NS	NS
	4	71.1	71.9	72.0	73.2	0.54	NS	†
Propionic acid (%)	0	21.6	20.2	19.6	19.0	1.11	NS	NS
	4	19.0	18.6	18.6	17.9	0.50	NS	NS
Butyric acid (%)	0	8.3	8.1	8.0	7.7	0.22	NS	NS
	4	8.0	7.8	7.8	7.5	0.10	*	†
Iso-butyric acid (%)	0	0.6	0.5	0.4	0.5	0.08	NS	NS
	4	0.4	0.4	0.4	0.3	0.03	NS	†
Valeric acid (%)	0	0.9	0.6	0.7	0.6	0.12	NS	NS
	4	0.7	0.7	0.7	0.5	0.10	NS	NS
Iso-valeric acid (%)	0	0.7	0.7	0.6	0.5	0.05	NS	†
	4	0.7	0.6	0.6	0.5	0.10	NS	*
Acetate: propionate	0	3.7	3.8	3.7	3.9	0.18	NS	NS
	4	4.1	4.1	4.0	4.5	0.20	NS	NS

NH_3-N, ruminal ammonia nitrogen.

NS, not significant; 1% vs 2%, adding 1% NaCl vs 2% NaCl; C vs D, commercial salt vs desalted mother liquor; SEM, standard error of mean; C1, 1% NaCl on a dry matter (DM) basis was added by commercial salt; C2, 2% NaCl on a DM basis was added by commercial salt; D1, 1% NaCl on a DM basis was replaced by desalted mother liquor; D2, 2% NaCl on a DM basis was replaced by desalted mother liquor.

*$P < 0.05$; †$P < 0.10$.

[1]The roughage-concentrate ratio was 40:60.

Table 2-6. Plasma metabolites of Thai native cattle fed experimental diets

	Treatment[1]					P-value	
	C1	C2	D1	D2	SEM	1% vs 2%	C vs D
Glucose (mg/100mL)	63.8	64.8	69.3	64.0	2.74	NS	NS
BUN (mg/100mL)	7.3	8.0	8.0	7.5	0.64	NS	NS
Creatinine (mg/100mL)	1.8	1.9	1.9	1.7	0.09	NS	NS
Cholesterol (mg/100mL)	130.3	131.8	129.3	124.8	7.59	NS	NS
AST (U/L)	43.0	49.0	45.3	43.8	2.97	NS	NS
ALT (U/L)	20.5	21.5	21.3	21.0	0.99	NS	NS
Protein (g/100mL)	7.5	7.5	7.5	7.6	0.08	NS	NS
Albumin (g/100mL)	3.3	3.4	3.4	3.4	0.04	NS	†
Potassium (mmol/L)	4.1	4.1	4.0	4.1	0.18	NS	NS

BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NS, not significant; 1% vs 2%, adding 1% NaCl vs 2% NaCl; C vs D, commercial salt vs desalted mother liquor; SEM, standard error of mean; C1, 1% NaCl on a dry matter (DM) basis was added by commercial salt; C2, 2% NaCl on a DM basis was added by commercial salt; D1, 1% NaCl on a DM basis was replaced by desalted mother liquor; D2, 2% NaCl on a DM basis was replaced by desalted mother liquor.

†$P < 0.01$.

[1]The roughage-concentrate ratio was 40:60.

2.3.4. Methane emissions and energy partition

Methane emission, energy partition and energy efficiency of animals in each treatment are presented in Table 2-7. There were no significant differences between the treatments in CH_4 emissions expressed as any unit. Gross energy intake (GEI) of C1 treatment was higher than the other treatments ($P < 0.05$). The energy losses in feces and urine, or CH_4 emission did not differ significantly among the treatments. The energy efficiency (i.e., DEI/GEI, MEI/GEI and MEI/DEI) also did not show significant differences among the treatments.

Table 2-7. Methane emission, energy partition and energy efficiency of Thai native cattle fed experimental diets

	Treatment[1]					P-value	
	C1	C2	D1	D2	SEM	1% vs 2%	C vs D
Methane emission							
kJ/kg $BW^{0.75}$	95.5	96.2	95.9	96.2	1.31	NS	NS
MJ/ kg DMI	1.4	1.4	1.4	1.4	0.02	NS	NS
MJ/day	6.4	6.4	6.4	6.5	0.08	NS	NS
Energy partition							
GEI (kJ / kg $BW^{0.75}$)	1241.5[a]	1206.5[b]	1208.8[b]	1213.9[b]	4.04	*	*
Feces (%GEI)	32.0	33.1	33.5	32.6	0.51	NS	NS
Urine (%GEI)	2.2	2.3	2.3	1.9	0.12	NS	NS
Methane (%GEI)	7.7	8.0	7.9	7.9	0.10	NS	NS
Energy efficiency							
DEI/GEI	0.7	0.7	0.7	0.7	0.01	NS	NS
MEI/GEI	0.6	0.6	0.6	0.6	0.01	NS	NS
MEI/DEI	0.9	0.9	0.9	0.9	0.00	NS	NS

BW, body weight; DMI, dry matter intake; GEI, gross energy intake; DEI, digestible energy intake; MEI, metabolic energy intake; NS, not significant; 1% vs 2%, adding 1% NaCl vs 2% NaCl; C vs D, commercial salt vs desalted mother liquor; SEM, standard error of mean; C1, 1% NaCl on a dry matter (DM) basis was added by commercial salt; C2, 2% NaCl on a DM basis was added by commercial salt; D1, 1% NaCl on a DM basis was replaced by desalted mother liquor; D2, 2% NaCl on a DM basis was replaced by desalted mother liquor.

[ab]Means in a row with different superscripts significantly differ ($P < 0.05$).

*$P < 0.05$.

[1]The roughage-concentrate ratio was 40:60.

2.4. Discussion

2.4.1. Feed intake and apparent digestibility

The animals completely ingested the offered concentrate. The result implies that the concentrate, in which 1% and 2% NaCl were replaced by DML, was palatable. The differences

of intake of OM, CP (i.e., N intake), ADFom, EE and NFC among the treatments (Table 2-2) have been observed due to the differences in chemical composition (Table 2-1).

Digestibility of DM, OM, CP, NFC and aNDFom were not affected by the treatments. However, EE digestibility of DML treatments was lower than commercial salt treatments ($P < 0.05$), although the reason was unclear from this experiment.

The ADFom digestibility of D2 was higher than C2 and D1 ($P < 0.05$) and was superior in DML treatments relative to commercial salt treatments ($P < 0.05$). The components of aNDFom are cellulose, hemicellulose and lignin, and ADFom is constituted of cellulose and lignin. Therefore, our result indicated that DML supplementation might have enhanced fiber digestibility, especially cellulose digestibility. Cellulose, lignin and hemicellulose are bound by physical bonds (Harmsen et al., 2010; Zhang et al., 2015). In addition, chemical bonding exists between cellulose and lignin (Harmsen et al., 2010; Kabir et al., 2012; Li et al., 2007) and between cellulose and hemicellulose (Harmsen et al., 2010). Therefore, it is likely that nucleic acid-related compounds in DML enhanced activity of ruminal microorganisms and improved the isolation of the bonds and promoted cellulose degradation. Kimura et al. (2010) showed that the supplementary GR and HxR to roughage as substrate increased *in vitro* NDFom digestibility. Thus, nucleic acid-related compounds in DML affect fiber digestibility. Moreover, we found that *in vitro* NDFom digestibility in roughage substrate conditions of DML supplementation treatments (16.4%) was significantly higher than that of salt supplementation treatments (12.1%) in our previous study (Sato et al., 2013, unpublished data). In many developing countries, and in Asia in particular, ruminants are fed on straw from cereal crops, mainly rice and wheat (Preston and Leng, 1987). As population pressure increases and the area devoted to food-crop production is extended, the use of straw for animal feeding will increase. Therefore, DML is the effective feed additive in improving fiber digestibility, in particular in the tropics.

2.4.2. Nitrogen balance and ruminal fermentation

Sakai et al. (2017) demonstrated that N retention in animals fed the concentrate replacing 1% NaCl on a DM basis by DML was higher than in animals fed the concentrate with 1% NaCl on a DM basis and 2% NaCl replaced by DML on a DM basis. In the present study, the fecal N, urinary N, N retention and retention rate (N retention/N intake) were similar in treatments, indicating that DML supplementation up to 2% NaCl on a DM basis has no negative effect on N balance. The result was inconsistent with Sakai et al. (2017), but the reason was unclear in our experiment.

Slyter et al. (1979), in an *in vivo* study, found that the critical concentration of NH_3-N for maintaining maximum rumen microbial growth was about 2 mg NH_3-N/100 ml when the steers were fed the diet consisted of 70% concentrate and 30% roughage. On the other hand, excessively high levels of NH_3-N, up to 80 mg NH_3-N/100 ml did not inhibit microbial growth (Satter and Slyter, 1974). In our study, the rumen NH_3-N at 0 hr and 4 hr post-feeding had no significant difference among the treatments and the values were 9.60–10.91 and 5.06–5.72 mg NH_3-N/ 100 ml, respectively (Table 2-5), which were within the normal range.

Molar percent acetate at 4 hr after feeding tended to be higher in DML treatment than commercial salt treatment ($P = 0.08$; Table 2-5). The tendency of increasing proportions of acetic acid when adding DML to the concentrates might have been consistent with the higher digestibility of ADFom in DML treatments (Table 2-3). Some studies demonstrated adding NaCl had no effect on ruminal molar percent normal butyric acid (Croom et al., 1982; Leibholz et al., 1980; Rogers et al., 1979). However, in the present study, normal butyric acid at 4 hr post-feeding was significantly lower in animals fed 2% NaCl concentrate as compared with 1% NaCl concentrate ($P < 0.05$). The reason for this inconsistency in the result from the present study with those from the previous studies is obscure.

2.4.3. Blood metabolites

There were no significant differences of the blood metabolite concentrations among the treatments, in glucose, BUN, creatinine, cholesterol, AST, ALT, protein and albumin. Plasma potassium concentrations increased when high dose of NaCl was offered to bulls (Blanco et al., 2014). This is related to reducing secretion of aldosterone. Aldosterone increases sodium reabsorption and potassium excretion (Finco, 1997). When excess salt is consumed, plasma osmolality is increased which has a negative feedback on aldosterone, decreasing concentrations to promote sodium excretion (Digby et al., 2011). As a result, blood potassium is increased. In our study, the plasma concentration of potassium (4.00–4.10 mmol/L) were within the normal range of values (3.9–5.8 mmol/L; Kaneko et al., 2008) and did not show significant difference between 1% NaCl treatment and 2% NaCl treatment, which is likely due to the low supplementation of NaCl.

2.4.4. Methane emissions and energy partition

Methane emission is influenced by fiber fractions digestibility (Santoso et al., 2007). However, there was no difference in CH_4 emission (MJ/day) between DML and NaCl treatment (Table 2-7; $P > 0.05$) although ADFom digestibility of DML treatments was higher than that of NaCl treatments (Table 2-3; $P < 0.05$). The result was consistent with the data reported by Sakai et al. (2017). In their study, the CH_4 emissions from cattle on the basis of metabolic body size (kJ/kg $BW^{0.75}$) in the animals given the concentrate 2% DM NaCl replaced by DML was similar to those in the animals fed the other concentrates; 1% NaCl on a DM basis was added as commercial salt and 1% NaCl on a DM basis was replaced by DML. The present results suggest that adding DML could improve ADFom digestibility and commercial salt could be replaced by DML up to 2% without adverse effects on N balance, rumen conditions, blood metabolites and CH_4 emission. Therefore, DML is a valuable alternative ingredient. The development of animal feeding systems using DML, site-specific by-products from food processing industries, could

improve the local nutrient cycle. Further studies are needed to evaluate the effect of DML on growth and carcass traits.

CHAPTER 3

In vitro and *in vivo* evaluations of wine lees as feeds for ruminants: Effects on ruminal fermentation characteristics, nutrient digestibility, blood metabolites and antioxidant status

3.1. Introduction

Wine production plays a major role in worldwide fruit production, and in 2018, had reached 292 million hectoliters in the world (Office Internationale de la Vigne et du Vin (OIV), 2019). During wine production, a huge amount of by-products is produced leading to a high cost associated with their management and disposal. Effective utilization of the by-products as animal feeds is a feasible strategy to overcome these issues and further aids in reducing feeding cost. In particular, wine lees are by-products of wine manufacturing and defined by EEC regulation No. 337/79 as the residue formed at the bottom of wine containers, after fermentation, during storage or after treatments, as well as the residue obtained after the filtration or centrifugation of this product. Wine lees mostly used for the recovery of tartaric acid, bioactive polyphenols (Kontogiannopoulos et al., 2017) or as fermentation nutrient supplement (Dimou et al., 2015). However, there have been few studies on the usability of wine lees as animal feed. A study by Molina-Alcaide et al. (2008) has reported that wine lees had high crude protein (CP) content, ranging from 14.1% to 20.3% dry matter (DM), and showed high *in vitro* digestibility in ruminants. Moote et al. (2014) suggested that inclusion of approximately 7% of wine lees, on a DM basis, in the feed for fattening steers did not affect the growth performance and meat quality except for the meat color. Moreover, their antioxidative effects on animals are expected since the wine by-products are rich in polyphenols that are well known for natural antioxidants (Kanner et al., 1994; López-Vélez et al., 2003).

Oxidation is a chemical reaction that involves the loss of electrons, and can produce free radicals. Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and the scavenging systems (Textor and Wilcox, 2001). Hydroxyradicals and peroxynitrite induced by ROS cause damage to lipid membrane, enzyme and deoxyribonucleic acid that results in immunodeficiency and growth and breeding disorders, and thereby decreases the productivity of animals (Miller et al., 1993; Kankofer, 2002; Urban-Chmiel

et al., 2009). Various mechanisms exist in most animals for detoxification of ROS; however, occasionally detoxification is not sufficient (Chauhan et al., 2014).

Many researchers have reported the usability of antioxidants as animal feeds. Due to the antioxidant property of wine by-products, dietary grape pomace has been reported to improve the antioxidant capacity in lambs (Kafantaris et al., 2017; Zhao et al., 2018). In contrast, few studies have reported on the antioxidant capacity of wine lees for utilization as animal feed. Ishida et al. (2015) have reported that the inclusion of wine lees in diets for wethers decreased the excretion of 8-hydroxy-2′-deoxyguanosine (8-OHdG) that is a biomarker of oxidative stress.

A high amount of saturated fatty acids (SFA) was included in ruminant products (meat and milk) due to the ruminal biohydrogenation of unsaturated fatty acids (UFA) (Lourenço et al., 2010). Ingesting these products increases the potential risk of consumer's health. Therefore, the biohydrogenation process needs to be controlled for composing better fatty acid profiles in the products from ruminants. Dietary manipulation of fatty acid profiles may be an effective and practical strategy. Various studies have described that dietary antioxidants, such as vitamin E and polyphenols could protect polyunsaturated fatty acids (PUFA) from ruminal biohydrogenation (Gobert et al., 2008; Cabiddu et al., 2009; Lee et al., 2014). However, no studies have been conducted to explore the protective effect of wine lees for PUFA from ruminal biohydrogenation.

The present study was aimed to investigate the effect of dietary wine lees on *in vitro* digestibility, ruminal fermentation, fatty acid profiles, ruminal phenolic contents, and antioxidant capacity. The effect of graded level of wine lees supplementation in the fattening ration on nutrients digestibility, nitrogen balance, ruminal fermentation, blood metabolites, and plasma oxidative stress marker was also investigated in the present study.

3.2. Materials and methods

3.2.1. Materials

Wine lees were obtained from red wine that was brewed through bentonite fining at a local winery located in Nantan district, Kyoto, Japan. The wine lees were filtered through four layers of gauze to reduce their moisture contents and were kept in a freezer at -20°C prior to the experiments. During the trials, the samples were preserved in a refrigerator at 4°C. In the present study, both *in vitro* and *in vivo* experiments were carried out to evaluate thoroughly the dietary effects of wine lees on ruminants. The present study consisted of three experiments that were conducted at Kyoto University, Japan. These experiments were approved by the Kyoto University Animal Ethics Committee (26-33).

3.2.2. *In vitro* experiments

In vitro experiments were conducted twice to investigate the effect of wine lees on the characteristics of ruminal fermentation, (*in vitro* I), fatty acid profiles, ruminal phenolic contents, and antioxidant capacity (*in vitro* II).

3.2.2.1. Experimental design and incubation procedure

For *in vitro* experiments, the rolled barley and the wine lees were used as substrates (Table 3-1), and the four treatments were prepared as follows: 100% DM rolled barley was used as a control (WL0), and three treatments were prepared through the replacement of 7.5% DM, 15.0% DM and 22.5% DM with wine lees (WL7.5, WL15, WL22.5, respectively). Incubation was provided *in vitro* according to the procedure described by Tilley and Terry (1963). The rumen fluid was collected from two Japanese Black steers (body weight (BW); 618 ± 36 kg) at 26 months of age in the late fattening period through orogastric tubing before morning feeding. Rumen fluid was collected into a flask, transported to the laboratory, and filtered through four layers of gauze,

and centrifuged at 500 × *g* for 5 min. The supernatants of rumen filtrates from the two rumen fluid samples were mixed equally and buffered (1:4, v/v) by adding an artificial saliva solution (McDougall, 1948). Each substrate of the treatments (0.5 g DM) was incubated at 39°C using 40 mL of the mixture in the test tube for 48 h. Each treatment had six replicates. Samples of inoculum without any substrate (blank) were also prepared.

In *in vitro* I, total gas production was measured every 12 h during the incubation. After incubation, the aliquots were centrifuged at 500 × *g* for 5 min. The supernatant and residue were stored at -20°C until further use. Three residue samples out of six replicates were used to determine CP digestibility, and remaining three residue samples were used for neutral detergent fiber expressed exclusive of residual ash (NDFom) digestibility. The DM digestibility of all residues was measured. In supernatants of all samples, ammonia nitrogen (NH_3-N), pH, and volatile fatty acids (VFA) were analyzed. In *in vitro* II, after an incubation of 48 h, the test tubes were centrifuged at 500 × *g* for 5 min, and the supernatant and residue samples were stored at -20°C until further analysis. Three samples of six residues and supernatants were used to determine fatty acids profiles, and remaining three samples were used for determination of ruminal phenolic contents and antioxidant capacity.

Table 3-1. Chemical compositions, fatty acids compositions and antioxidant capacity of the substrates used in *in vitro* experiments

Item[1]	Wine lees[2]	Rolled barley
Chemical compositions (%DM)		
Dry matter (%)	47.5	92.4
Organic matter	67.8	97.7
Crude protein	9.3	12.7
Ether extract	0.4	1.9
NDFom	20.9	26.3
ADFom	9.6	7.3
Crude ash	32.2	2.3
Non-fibrous carbohydrate	37.3	56.9
Calcium	3.20	0.06[3]
Phosphorous	0.27	0.37[3]
% of fatty acids		
Palmic acid (16:0)	20.4	21.9
Margaric acid (17:0)	N.D.	0.12
Stearic acid (18:0)	8.7	2.4
Behenic acid (22:0)	2.40	0.22
Lignoceric acid (24.0)	N.D.	0.14
Palmitoleic acid (16:1)	1.27	0.16
Oleic acid (18:1 cis)	19.5	16.9
Eicosenoic acid (20:1)	N.D.	0.93
Erucic acid (22:1)	N.D.	0.26
Nervonic acid (24:1)	N.D.	0.66
Linoleic acid (18:2 n-6 trans)	38.7	51.1
α-Linolenic acid (18:3 n-3)	9.1	5.1
Eicosadienoic acid (20:2)	N.D.	0.17
ΣSFA	31.5	24.7
ΣMUFA	20.7	18.9
ΣPUFA	47.8	56.4
Antioxidants capacity		
Polyphenols (mg CAE/gDM)	3.5	1.0
DPPH radical scavenging activity (μmol TE/gDM)	13.2	4.2

[1] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DM, dry matter; NDFom, neutral detergent fiberexpressed exclusive of residual ash; ADFom, acid detergent fiber expressed exclusive of residual ash; CAE, caffeic acid euivalent; TE,trolox equivalent; DPPH, 1,1-diphenyl-2-picrylhydrazyl

[2] N.D., not detected

[3] The value was determined by National Agriculture and Food Research Organization (2009)

3.2.2.2. Chemical analysis

The substrates used in the experiments were analyzed for DM, CP, ether extract (EE), and crude ash (CA) (methods 930.15, 976.05, 920.39 and 942.05, respectively) according to the standards of the Association of Official Analytical Chemists (AOAC 2000). Organic matter (OM) was calculated as weight loss through ashing. The NDFom and acid detergent fiber that was expressed exclusive of residual ash (ADFom) contents were determined according to Van Soest et al. (1991). The content of nonfibrous carbohydrate (NFC) was calculated using the equation: NFC = 100 - (CP + EE + NDFom + CA). Phosphorus and calcium content in the samples were determined following the colorimetric method (Gomori, 1942), and the atomic absorption spectrophotometer (AA-660F; Shimadzu, Kyoto, Japan), respectively. The *in vitro* digestibility of DM, CP, and NDFom was calculated as the disappearance per unit of their original contents. Residual DM, CP, and NDFom were corrected for the blanks.

The total polyphenol content of corresponding substrates was determined, and incubation residues and supernatants were isolated from methanol extracts. Wet dietary samples and residues were crushed and homogenized using methanol/water (80/20, v/v), and then centrifuged at 8000 × *g* for 15 min. The supernatants were diluted to 250 mL using methanol/water (80/20, v/v) and utilized in the analysis. The incubation supernatants (1 mL) were also diluted to 250 mL using methanol/ water (80/20, v/v). The contents of total polyphenols were determined by the colorimetric method employing the Folin-Denis assay (Swain and Hills, 1959). The antioxidant capacity of the extracts was evaluated spectrophotometrically using the free radicals 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to the method of Brand-Williams et al. (1995). The obtained values of polyphenol contents and DPPH radical scavenging ability in the fermentation supernatant and residue were corrected by subtracting the blank value as ruminal fluid also possessed the antioxidant capacity.

After incubation, the ruminal NH_3-N content in liquid was determined following the microdiffusion method (Conway, 1947). The VFA concentrations of the supernatants after

incubation were measured using the gas chromatography system (GC-14B, Shimadzu Co., Ltd., Kyoto, Japan) equipped with an flame-ionization detector (FID). The chromatography was carried out using a packed glass column (Thermon 3000-3% Shimalite TPA 60/80 3.2 nmφ × 2.1 m, Shimadzu Co., Ltd., Kyoto, Japan), using nitrogen as a carrier gas. The temperatures of the column, detector and injection were 110, 250 and 250 °C, respectively.

For determining the compositions of fatty acids in substrates, incubation residues and supernatants, total lipids were extracted according to the method of Folch et al. (1957). The extracted lipids were dried under nitrogen gas and fatty acid methyl ester (FAME) was prepared by methylation of the fatty acids using BF_3-methanol (Metcalfe and Schmitz, 1961). The prepared FAME was analyzed using gas chromatography system (GC-2014, Shimadzu Co., Ltd., Kyoto, Japan) equipped with a capillary column (TC-2560 0.25 mmφ × 100 m × 0.2 μm, GL Science Co., Ltd, Tokyo, Japan) and an FID. The initial column oven temperature was set at 140°C with a hold for 2 min, and thereafter, the temperature was increased to 240 °C at a rate of 4 °C/min with a hold for 20 min. Injector and detector temperatures were set at 250 °C and 260 °C, respectively. Helium gas was used as the carrier gas. Fatty acids were identified by matching their retention times with a standard fatty acids mixture (F.A.M.E. Mix C4-C24; SUPELCO., Pennsylvania, USA).

3.2.2.3. Statistical analysis

The data of the experiments were analyzed using the GLM procedure of the Statistical Analysis System (SAS 1998). The model was $Y_{ij} = \mu + T_i + e_{ij}$, where μ = the overall mean, T_i = the fixed effect of treatment, e_{ij} = residual error. Least square means of each treatment were compared using Tukey-Kramer test. Significance was declared at $P < 0.05$ and $0.05 \leqq P < 0.1$ was considered as a trend. Contrast test was performed to determine the effect of wine lees inclusion on gas production, digestibility, fermentation parameter, antioxidant capacity, and fatty acids profiles.

3.2.3. *In vivo* experiment

3.2.3.1. Animals, feed and experimental design

Four cannulated wethers having an initial BW of 53.3 ± 4.1 kg (mean ± SD) were used in a 4 × 4 Latin square design experiment. The wethers were housed individually in metabolic cages. An adaptation period of nine days was followed by a sample collection period of five days. Each wether was assigned to the following four treatments: C, basal concentrate without wine lees; L, 6.7% DM of concentrate was replaced by wine lees; M, 13.3% DM of concentrate was replaced by wine lees; H, 20.0% DM of concentrate was replaced by wine lees (Table 3-2). The basal concentrate consisted of 14% wheat bran, 9% rice bran, 5% soybean meal, 38% barely grains, 32% flacked corn, 1% calcium carbonate, 1% sodium chloride, and 0.015% vitamin and mineral premix. Wine lees were mixed well with concentrate just before feeding. The wethers were provided with the experimental diets and rice straw at a ratio of 75:25 on a DM basis. The daily amount of total diets was 2.0% of the BW on a DM basis. The diet was supplied daily in two equal portions at 09:00 and 17:00. The wethers had ad libitum access to water throughout the experiment.

Table 3-2. Feed and chemical compositions of the experimental diets used in *in vivo* experiment

Item[1]	Concentrate[2]				Rice straw
	C	L	M	H	
Feed composition (%DM)					
Fattening ration[3]	100.0	92.7	85.3	78.0	
Soybean meal	0.0	0.67	1.3	2.0	
Wine lees	0.0	6.7	13.3	20.0	
Chemical composition (%DM)					
Dry matter (%)	87.4	84.8	82.1	79.5	87.3
Organic matter	92.4	90.7	89.1	87.4	88.4
Crude protein	12.7	12.7	12.7	12.6	4.3
Ether extract	6.6	6.2	5.7	5.3	1.7
NDFom	40.1	40.1	40.0	39.9	69.4
ADFom	12.0	12.5	13.0	13.5	44.5
Non-fibrous carbohydrate	33.0	31.7	30.7	29.6	13.0
Calcium	0.41	0.60	0.78	0.97	0.31
Phosphorous	0.38	0.37	0.37	0.36	0.38
Antioxidants capacity					
Polyphenols (mg CAE/gDM)	1.2	1.3	1.5	1.7	1.3
DPPH radicals scavenging activity (μmol TE/gDM)	3.2	3.9	4.5	5.2	2.9

[1] DM, dry matter; NDFom, neutral detergent fiberexpressed exclusive of residual ash; ADFom,acid detergent fiber expressed exclusive of residual ash; CAE, caffeic acid euivalent; TE,trolox equivalent; DPPH, 1,1-diphenyl-2-picrylhydrazyl

[2] C, basal concentrate without wine lees; L, 6.7%DM of concentrate was replaced by wine lees; M, 13.3%DM of concentrate was replaced by wine lees; H, 20.0%DM of concentrate was replaced by wine lees.

[3] Fattening ration was constituted with 14% wheat bran, 9% rice bran, 5% soybean meal, 38% barely grains, 32% flacked corn, 1% calcium carbonate, 1% sodium chloride, and 0.015% vitamin and mineral premix on a fresh matter basis.

3.2.3.2. Sampling

During the sample collection period, residues and feces were obtained and weighted regularly prior to morning feed. These samples were accumulated separately throughout the period for each wether. All samples of experimental feeds, residues and feces were dried at 60°C for 48 h, and grinded using a Wiley mill to pass through a 1 mm screen for chemical analysis. Urine was collected into vessels containing 20% sulfuric acid to prevent the loss of nitrogen. The volume of urine was measured daily, and 50 mL representative samples were collected. The urine samples were mixed per wether per treatment according to the original excretion quantities, and stored at -20°C until further analysis. On the last day (day 14) of each collection period, ruminal fluid (approximately 100 mL) was collected through the rumen cannulae of each wether at 0 and 4 h after morning feed. The ruminal liquid was filtered through four layers of gauze and immediately analyzed for ruminal pH using a glass electrode pH meter (Horiba Ltd., Kyoto, Japan). Thereafter, ruminal fluid was centrifuged at 500 × *g* for 5 min and the supernatants were stored at -20°C until VFA and NH_3-N analysis. At 0 h after feeding on the final day of the collection period (day 14), blood samples were collected from each wether through jugular vein puncture into vacuum tubes containing heparin sodium. The collected blood was centrifuged at 1,460 × *g* for 10 min and the plasma was stored at -20°C until the analysis of blood metabolites.

3.2.3.3. Chemical analysis

The contents of DM, CP, EE, CA, OM, NFC, NDFom, ADFom, total polyphenols and the free radicals DPPH in experimental diets were analyzed as described in the *in vitro* experiments. Apparent digestibility of DM, OM, CP, EE, NFC, NDFom, and ADFom was estimated by subtracting nutrients that were present in the feces from the dietary intake nutrients. Total digestible nutrients (TDN) intake was calculated using the following equation: TDN content = 5.81 + 0.869 × digestible DM, reported by Heaney and Pigden (1963). Urine samples were analyzed for urinary nitrogen using the Kjeldahl procedure described by AOAC (2000). The NH_3-

N content and the VFA concentrations of the ruminal fluid were measured as mentioned in the *in vitro* experiments.

The concentrations of metabolites in the blood plasma samples were analyzed. Concentrations of glucose, non-esterified fatty acid (NEFA), albumin, blood urea nitrogen (BUN), total cholesterol, phospholipids, calcium, and inorganic phosphorus (IP) were analyzed using diagnostic kits (Glucose-HRII, NEFA-HR Albumin-HRII, L type Wako UN, L type Wako CHO.H, L type Wako Phospholipids and Inorganic phosphorus- HRII; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Total protein was determined using a refractometer (SPR-Ne; Atago Co., Ltd., Tokyo, Japan). Glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), and γ-glutamyl transpeptidase (γ-GTP) activities were analyzed according to the standard methods established by the Japan Society of Clinical Chemistry (Kotani et al., 1994). The malondialdehyde (MDA) concentration in plasma was measured using a commercially available kit (ZeptoMetrix Co., New York, America).

3.2.3.4. Statistical analysis

The data in the experiment were analyzed using Statistical Analysis System (SAS 1998). The carry-over effects from the previous periods were tested using the GLIMMIX procedure.

Subsequently, the data were analyzed using the MIXED procedure. The model was $Y_{ijkl} = \mu+T_i+P_j+A_k+e_{ijkl}$, where μ = the overall mean, T_i = the fixed effect of treatment, P_j = the fixed effect of period, A_k = the random effect of animal and e_{ijk} = residual error. Significance was declared at $P < 0.05$ and $0.05 \leqq P < 0.1$ was considered as a trend as described above. The Contrast test was performed to determine the effect of wine lees inclusion. The Tukey-Kramer test was used to detect differences between the least square means.

3.3. Results

3.3.1. Chemical compositions of wine lees

Wine lees used in the experiments were observed to have low OM, CP, EE, NFC, and phosphorus contents and high calcium content and antioxidant capacity as compared to rolled barley (Table 3-1). Wine lees contained high stearic acid, α-linolenic acid, and total SFA as compared to rolled barley. However, linoleic acid and total PUFA were observed to be low in wine lees.

3.3.2. *In vitro* experiment

3.3.2.1. *In vitro* gas production and digestibility

In vitro gas production and digestibility of different substrates are shown in Table 3-3. Gas production at 24 h was quadratically increased with the inclusion of wine lees ($P < 0.01$), and a significantly higher value was observed in WL7.5, WL15, and WL22.5 as compared to WL0 ($P < 0.05$). On the contrary, gas production at 48 h was linearly decreased with wine lees inclusion ($P < 0.01$). At 36 h after incubation, WL0 and WL7.5 showed high gas production as compared to WL22.5 ($P < 0.05$). The digestibility of DM and CP was linearly decreased with wine lees inclusion ($P < 0.01$), while NDFom digestibility was linearly increased ($P < 0.05$).

Table 3-3. *In vitro* gas production after 12, 24, 36 and 48 h incubation and digestabilities of experimental substrates

	Treatment[2]					P-value	
Item[1]	WL0	WL7.5	WL15	WL22.5	SEM[3]	Linear	Quadratic
Gas production (mL/0.5gDM)							
12 h	41.0	40.2	41.2	38.7	1.30	0.32	0.53
24 h	73.0^{b}	86.2^{a}	83.3^{a}	83.2^{a}	2.39	0.0054	0.011
36 h	103.0^{a}	104.3^{a}	99.5^{ab}	97.5^{b}	1.37	0.071	0.24
48 h	104.5^{a}	105.5^{a}	101.3^{ab}	99.0^{b}	1.25	0.039	0.20
Digestibility (%)							
Dry matter	78.1^{a}	76.3^{b}	73.8^{c}	71.5^{d}	0.35	< 0.001	0.47
Crude protein	57.1^{a}	49.4^{b}	47.9^{b}	49.1^{b}	1.32	0.0056	0.010
NDFom	53.6	61.1	60.2	61.1	1.74	0.017	0.093

[abcd] LSMeans with different superscripts within same row significantly differed ($P < 0.05$).

[1] DM, dry matter; NDFom, neutral detergent fiber expressed exclusive of residual ash

[2] 100% DM rolled barley as a control (WL0), and replaced 7.5%DM, 15.0%DM and 22.5%DM of wine lees (WL7.5, WL15, WL22.5, respectively).

[3] SEM, standard error of means

3.3.2.2. Volatile fatty acids and ammonia nitrogen

The ruminal fermentation characteristics of the experimental substrates are shown in Table 3-4. Ruminal NH_3-N contents of WL7.5, WL15, and WL22.5 were lower than that of WL0 ($P < 0.05$), and significant linear and quadratic effects ($P < 0.01$) were observed. Total VFA concentrations and the proportion of each VFA were not significantly different among the treatments.

Table 3-4. VFA and ruminal ammonia nitrogen concentrations after 48 h incubation in *in vitro* experiment

Item[1]	Treatment[2]				SEM[3]	P-value	
	WL0	WL7.5	WL15	WL22.5		Linear	Quadratic
pH	6.4	6.4	6.4	6.4	0.02	0.097	0.35
Ruminal ammonia nitrogen (mgN/dL)	7.1[a]	5.8[b]	5.2[b]	5.2[b]	0.20	0.0002	0.013
Total VFA(mmol/L)	82.9	76.0	87.1	85.7	5.01	0.41	0.60
VFA composition							
Acetic acid (%)	46.8	48.8	47.5	48.2	1.44	0.68	0.69
Propionic acid (%)	45.8	45.7	43.1	43.5	1.07	0.083	0.82
Butryic acid (%)	7.3	5.5	9.4	8.3	1.11	0.21	0.76
Acetic acid : Propionic acid	1.0	1.1	1.1	1.1	0.05	0.24	0.76

[ab] LSMeans with different superscripts within same row significantly differed ($P < 0.05$).

[1] VFA, volatile fatty acid

[2] 100% dry matter (DM) rolled barley as a control (WL0), and replaced 7.5%DM, 15.0%DM and 22.5%DM of wine lees (WL7.5, WL15, WL22.5, respectively).

[3] SEM, standard error of means

3.3.2.3. Polyphenols and antioxidants capacity

Polyphenol contents and antioxidant capacity of the experimental substrates, and incubation residues and supernatants are shown in Table 3-5. The polyphenol contents of the fermented residue were linearly increased with wine lees inclusion ($P < 0.01$) and the values of WL15 and WL22.5 were higher than that of WL0 ($P < 0.05$), while no significant difference was found in the supernatants despite a numerical increase. There was a tendency of linear increase for total polyphenol contents after incubation with increasing wine lees levels ($P = 0.09$). For DPPH radicals scavenging ability of the residue after incubation, there was a linear effect of wine lees inclusion ($P < 0.05$). Total DPPH radical scavenging ability after fermentation was linearly increased with the inclusion of wine lees ($P < 0.01$).

Table 3-5. Polyphenols contents and DPPH radicals scavenging ability of the experimental substrates, incubation residues and supernatants after 48 h incubation in *in vitro* experiment

	Treatment[2]					P-value	
Item[1]	WL0	WL7.5	WL15	WL22.5	SEM[3]	Linear	Quadratic
Polyphenols contents (mgCAE/0.5gDM)							
Substrate[4]	0.52	0.61	0.71	0.80	-	-	-
Residue	0.38^{c}	0.46^{bc}	0.76^{a}	0.62^{ab}	0.054	0.0027	0.090
Supernatant	0.27	0.24	0.43	0.46	0.176	0.36	0.90
Total after incubation	0.65	0.70	1.19	1.08	0.211	0.093	0.90
DPPH radicals scavenging ability (μmolTE/0.5gDM)							
Substrate[4]	2.11	2.45	2.78	3.12	-	-	-
Residue	1.14	1.25	1.71	1.49	0.142	0.046	0.28
Supernatant	1.43	1.74	2.03	2.24	0.268	0.053	0.86
Total after incubation	2.57	3.00	3.73	3.73	0.278	0.0094	0.46

[abc] LSMeans with different superscripts within same row significantly differed ($P < 0.05$).

[1] DM, dry matter; CAE, caffeic acid eqivalent; TE,trolox equivalent; DPPH, 1,1-diphenyl-2-picrylhydrazyl

[2] 100%DM rolled barley as a control (WL0), and replaced 7.5%DM, 15.0%DM and 22.5%DM of wine lees (WL7.5, WL15, WL22.5, respectively).

[3] SEM, standard error of means

[4] Calculated by Table 3-1.

3.3.2.4. Fatty acid compositions

Fatty acid profiles of the fermented residues and the supernatants after incubation are shown in Table 3-6. In the residues after incubation, the proportions of elaidic acid and α-linolenic acid were linearly decreased and increased, respectively, with wine lees inclusion ($P < 0.01$). Furthermore, there was a linear effect on linoleic acid and eicosenoic acid proportions ($P < 0.05$). Higher linoleic acid and α-linolenic acid proportions in WL22.5 as compared to WL0 made a significant difference in PUFA contents between WL0 and WL22.5 ($P < 0.05$). Similar to SFA, no significant difference was observed among the treatments. In the supernatants, elaidic acid was a dominant fatty acid. The proportions of linoleic acid and total PUFA were linearly increased due to wine lees inclusion ($P < 0.05$).

Table 3-6. Fatty acids profiles (%) of the incubation residues and supernatants after 48 h incubation in *in vitro* experiment

	Treatment[2]					P-value	
Item[1]	WL0	WL7.5	WL15	WL22.5	SEM[3]	Linear	Quadratic
Residue							
Palmic acid (16:0)	23.5	23.3	22.3	23.0	0.43	0.23	0.32
Margaric acid (17:0)	0.13	0.25	0.05	0.07	0.067	0.23	0.54
Stearic acid (18:0)	6.2	6.1	5.6	5.7	0.27	0.14	0.76
Behenic acid (22:0)	0.19	0.28	0.23	0.30	0.059	0.34	0.76
Lignoceric acid (24.0)	0.13	0.21	0.18	0.24	0.049	0.21	0.87
Palmitoleic acid (16:1)	0.11	0.16	0.22	0.17	0.077	0.50	0.56
Elaidic acid (18:1 trans)	25.8^{a}	25.7^{a}	24.1^{ab}	20.6^{b}	1.01	0.0051	0.13
Oleic acid (18:1 cis)	13.2	13.0	13.3	14.0	0.28	0.055	0.16
Eicosenoic acid (20:1)	0.92	0.90	1.00	1.01	0.035	0.044	0.65
Erucic acid (22:1)	0.20	0.10	0.06	0.17	0.076	0.72	0.23
Nervonic acid (24:1)	0.22	0.20	0.25	0.32	0.077	0.33	0.54
Linoleic acid (18:2 n-6 trans)	26.3	26.3	28.7	29.9	0.91	0.011	0.53
α-Linolenic acid (18:3 n-3)	2.8^{c}	3.2^{bc}	3.7^{ab}	4.1^{a}	0.18	0.0007	0.84
Eicosadienoic acid (20:2)	0.40	0.29	0.25	0.40	0.063	0.91	0.075
ΣSFA	30.2	30.3	28.5	29.5	0.72	0.25	0.57
ΣMUFA	40.5^{a}	40.1^{ab}	39.0^{ab}	36.3^{b}	0.92	0.011	0.24
ΣPUFA	29.0^{b}	29.5^{ab}	32.5^{ab}	34.0^{a}	1.03	0.0046	0.60
Supernatant[4]							
Palmic acid (16:0)	28.8	29.1	26.8	30.5	0.98	0.54	0.12
Stearic acid (18:0)	18.5	20.5	20.8	19.2	1.75	0.77	0.34
Palmitoleic acid (16:1)	1.3	1.0	0.9	0.6	0.46	0.31	0.93
Elaidic acid (18:1 trans)	45.2	44.0	44.0	41.2	1.62	0.14	0.65
Oleic acid (18:1 cis)	4.4	3.7	4.8	4.7	0.45	0.34	0.45
Linoleic acid (18:2 n-6 trans)	1.7	1.8	2.7	3.8	0.68	0.049	0.51
ΣSFA	47.3	49.6	47.6	49.7	1.34	0.41	0.94
ΣMUFA	51.0	48.6	49.6	46.6	1.82	0.17	0.85
ΣPUFA	1.7	1.8	2.7	3.8	0.68	0.049	0.51

[abc]LSMeans with different superscripts within same row significantly differed ($P < 0.05$).

[1] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids

[2] 100% dry matter (DM) rolled barley as a control (WL0), and replaced 7.5%DM, 15.0%DM and 22.5%DM of wine lees (WL7.5, WL15, WL22.5, respectively).

[3] SEM, standard error of means

[4] Other fatty acids detected in residue were not detected

3.3.3. *In vivo* experiment

3.3.3.1. Carry-over effect

Significant carry-over effects were detected only in three variables: digestibility of NFC, fecal N, and NEFA in blood ($P < 0.05$).

3.3.3.2. Feed intake and digestibility

The nutrient intake, apparent digestibility, and TDN of each experimental diet are shown in Table 3-7. No refusal was observed during all experimental periods and mixing wine lees with concentrate did not affect the DM intake. The NFC and EE intake were linearly decreased with wine lees inclusion ($P < 0.01$) due to a linear decrease in nutritional contents in the experimental diets with an increase in the proportion of wine lees. On the contrary, ADFom intake was increased with increasing wine lees levels ($P < 0.01$) and the value of H was higher than that of C and L ($P < 0.05$). There was no significant difference for apparent digestibility values among the treatments.

3.3.3.3. Nitrogen balance

No significant difference was observed for intake N, fecal N, urinary N, retention N, and proportion of retention N in order to intake N among the treatments (Table 3-8).

Table 3-7. Nutritional intake, apparent digestibilities and TDN of the wethers fed experimental diets

Item[1]	Treatment[2]				SEM[3]	P-value	
	C	L	M	H		Linear	Quadratic
Intake (g/BW$^{0.75}$/day)							
Dry matter	52.1	52.1	52.9	53.4	0.77	0.095	0.71
Organic matter	47.6	46.8	46.5	46.0	0.66	0.054	0.73
Crude protein	5.6	5.6	5.6	5.7	0.08	0.14	0.88
Non-fibrous carbohydrate	14.7^{a}	14.3^{ab}	14.0^{b}	13.7^{b}	0.19	0.0015	0.89
Ether extract	2.9^{a}	2.7^{b}	2.5^{c}	2.4^{d}	0.03	< 0.0001	0.99
NDFom	24.5	24.4	24.4	25.1	0.39	0.15	0.61
ADFom	10.3^{b}	10.4^{b}	10.9^{ab}	11.2^{a}	0.20	0.0028	0.51
Digestibility (%)							
Dry matter	72.6	72.7	70.2	70.4	1.26	0.11	1.00
Organic matter	74.6	74.9	73.3	73.7	1.52	0.41	0.98
Crude protein	70.3	72.4	71.1	70.7	1.33	1.00	0.28
Non-fibrous carbohydrate	87.3	87.7	84.7	85.9	1.45	0.17	0.75
Ether extract	86.2	90.4	89.7	88.5	1.96	0.41	0.14
NDFom	65.1	65.3	64.9	65.9	2.05	0.76	0.79
ADFom	43.1	47.8	44.8	41.9	4.58	0.58	0.29
TDN (%)	68.9	69.0	66.9	67.0	1.31	0.11	1.00

[abcd]LSMeans with different superscripts within same row significantly differed ($P < 0.05$).

[1] BW, body weight; NDFom, neutral detergent fiber expressed exclusive of residual ash; ADFom, acid detergent fiber expressed exclusive of residual ash; TDN, total digestible nutrition

[2] C, basal concentrate without wine lees; L, 6.7% dry matter (DM) of concentrate was replaced by wine lees; M, 13.3%DM of concentrate was replaced by wine lees; H, 20.0%DM of concentrate was replaced by wine lees.

[3] SEM, standard error of means

Table 3-8. Nitrogen balance of the wethers fed experimental diets in *in vivo* experiment

Item[1]	Treatment[2]				SEM[3]	P-value	
	C	L	M	H		Linear	Quadratic
N balance (g/day/$BW^{0.75}$)							
Intake N	0.89	0.90	0.90	0.91	0.012	0.14	0.88
Fecal N	0.26	0.25	0.26	0.27	0.015	0.66	0.31
Urinary N	0.30	0.36	0.30	0.31	0.034	0.85	0.36
Retention N	0.33	0.29	0.35	0.33	0.042	0.63	0.66
Proportion of retention N to intake N (%)	36.6	31.9	38.4	36.3	4.83	0.73	0.72

[1] BW, body weight; N, nitrogen

[2] C, basal concentrate without wine lees; L, 6.7% dry matter (DM) of concentrate was replaced by wine lees; M, 13.3%DM of concentrate was replaced by wine lees; H, 20.0%DM of concentrate was replaced by wine lees.

[3] SEM, standard error of means

3.3.3.4. Ruminal pH, volatile fatty acid production, and ammonia nitrogen concentration

The ruminal pH, VFA concentrations and NH_3-N contents at 0 and 4 h after morning feed are shown in Table 3-9. There was no significant difference in ruminal pH and NH_3-N contents at 0 and 4 h after morning feed among the treatments. There were linear and quadratic effects on total ruminal VFA concentration at 4 h after feeding with the inclusion of wine lees ($P < 0.05$). On the contrary, there was a linear decrease in propionic acid proportion at 4 h after morning feed ($P < 0.05$) with an increase in wine lees levels. Accordingly, the ratio of acetic acid to propionic acid was increased with wine lees inclusion ($P < 0.05$).

Table 3-9. Ruminal fermentation characteristics of the wethers fed each experimental diet at 0 and 4 h after feeding

Item[1]	Time after feeding	Treatment[2]				SEM[3]	P-value	
		C	L	M	H		Linear	Quadratic
pH	0 h	6.6	7.0	6.7	6.9	0.22	0.52	0.44
	4 h	6.0	5.8	5.8	6.0	0.11	0.94	0.09
Ruminal ammonia nitrogen (mgN/dL)	0 h	19.7	15.5	19.0	17.5	3.22	0.81	0.63
	4 h	6.1	9.5	10.3	6.9	2.77	0.72	0.13
Total VFA (mmol/L)	0 h	76.6	68.1	86.1	75.8	12.45	0.70	0.92
	4 h	93.7^{b}	112.8^{ab}	125.4^{a}	114.3^{ab}	6.62	0.012	0.018
VFA composition								
Acetic acid (%)	0 h	58.8	61.4	61.4	62.8	2.70	0.26	0.79
	4 h	56.2	54.9	58.4	61.0	2.56	0.089	0.36
Propionic acid (%)	0 h	25.0	20.2	17.9	18.2	3.15	0.11	0.38
	4 h	29.9	27.7	23.8	21.5	2.91	0.020	0.99
iso-Butyric acid (%)	0 h	2.3	2.3	2.8	2.8	0.83	0.064	0.064
	4 h	0.75	0.42	0.79	0.88	0.509	0.12	0.14
Butyric acid (%)	0 h	13.8	16.1	18.0	16.2	1.24	0.50	0.98
	4 h	13.2	17.0	17.0	16.6	1.75	0.66	0.57
Acetic acid : Propionic acid	0 h	2.5	3.3	3.5	3.5	0.50	0.14	0.37
	4 h	2.0	2.1	2.7	3.0	0.44	0.043	0.81

[ab]LSMeans with different superscripts within same row significantly differed ($P < 0.05$).

[1] VFA, volatile fatty acids

[2] C, basal concentrate without wine lees; L, 6.7% dry matter (DM) of concentrate was replaced by wine lees; M, 13.3%DM of concentrate was replaced by wine lees; H, 20.0%DM of concentrate was replaced by wine lees.

[3] SEM, standard error of means

3.3.3.5. Blood metabolites and oxidative stress marker

Table 3-10 shows the concentrations of blood metabolites and the MDA levels in wethers fed each experimental diet. There was a linear effect of wine lees inclusion on the GOT concentration ($P < 0.05$) and the value in M and H tended to be lower than that in C ($P = 0.09$ and 0.06, respectively). The IP concentration was linearly increased with wine lees inclusion ($P < 0.05$). The MDA level tended to be lower for H than C ($P = 0.07$) and there was a linear effect of wine lees inclusion on the MDA level ($P < 0.05$).

Table 3-10. Plasma metabolites concentrations and MDA level of the wethers fed experimental diets

	Treatment[2]					P-value[4]	
Item[1]	C	L	M	H	SEM[3]	Linear	Quadratic
Total protein (g/dL)	7.3	7.3	7.1	7.2	0.15	0.33	0.82
Albumin (g/dL)	3.7	3.6	3.6	3.7	0.09	0.86	0.28
Phospholipid (mg/dL)	85.3	84.0	94.3	98.0	10.58	0.20	0.75
Total Cholesterol (mg/dL)	124.8	126.3	136.0	140.8	13.90	0.24	0.87
NEFA (mEq/L)	0.25	0.28	0.27	0.23	0.07	0.74	0.56
GOT (U/L)	60.0	52.8	51.3	50.3	2.91	0.016	0.18
GPT (U/L)	12.3	9.8	12.0	12.5	1.24	0.47	0.14
γ-GTP (U/L)	55.3	59.0	60.8	58.0	2.93	0.32	0.17
BUN (mg/dL)	17.6	16.7	17.4	19.9	1.94	0.27	0.26
Ca (mg/dL)	8.9	9.7	9.3	9.6	0.33	0.15	0.28
IP (mg/dL)	7.8	7.1	9.6	10.6	1.22	0.031	0.35
Glucose (mg/dL)	77.0	73.5	74.8	71.3	4.99	0.35	1.00
MDA (nmol/mL)	5.1	3.8	3.6	3.4	0.51	0.020	0.18

[1] NEFA, nonesterified fatty acid; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; γ-GTP, γ-glutamic transpeptidase; BUN, blood urea nitrogen; IP, inorganic phosphorus; MDA, malondialdehyde

[2] C, basal concentrate without wine lees; L, 6.7% dry matter (DM) of concentrate was replaced by wine lees; M, 13.3%DM of concentrate was replaced by wine lees; H, 20.0%DM of concentrate was replaced by wine lees.

[3] SEM, standard error of means

3.4. Discussion

3.4.1. Chemical compositions of wine lees

Wine lees include various kinds of polyphenols, such as condensed tannin, anthocyanins, and flavonols (Molina-Alcaide et al., 2008; Pérez-Serradilla and Castro, 2011; Barcia et al., 2014). The wine lees used in the experiments were observed to have higher polyphenolic contents and DPPH radical scavenging ability than those of rolled barley. Alonso et al. (2002) have reported that the antioxidant ability was related to the total polyphenolic contents; however, the relationship of antioxidant ability was not observed to particular compounds. Some individual polyphenolic compounds had more contribution to the total antioxidant ability in comparison to others. Malenčić (2008) has also suggested a positive relationship between polyphenolic contents and DPPH radical scavenging ability, consistent with the present study. Wine lees used in the present study included high proportion of crude ash (32.2% DM). Sancho-Galán et al. (2020) recently reported that red wine lees have high ash concentration of 33.3% DM, which is consistent with our result. The high proportion of ash in wine lees in the present study might be strongly influenced by the bentonite used for fining.

3.4.2. *In vitro* experiments

The *in vitro* gas production is an index of nutrient utilization. In the present study, a linear decrease was observed in gas production at 48 h after incubation with an increase in the percentage of wine lees in the substrate. Wine lees had lower OM and NFC contents, and higher polyphenolic contents than those of rolled barley. Moreover, the ability of polyphenols to bind with proteins firmly is the most crucial aspect of their nutritional and toxicological effects on ruminal microbes (Hagerman and Butler, 1981). Sinz et al. (2019) have shown that phenolic extracts from grape seed and green tea decreased the *in vitro* gas production. Kamalak et al. (2005) have also suggested a negative correlation between gas production and tannin content, especially condensed

tannin. On the contrary, the gas production after 24 h incubation was linearly increased with wine lees inclusion. Although the reason behind this was not apparent, dietary wine lees might activate ruminal microbial activity, promoting ruminal fermentation at only the initial stage of incubation.

Stürm et al. (2007) have demonstrated that tannin contents had a negative effect on ruminal CP degradation. In the present study, *in vitro* CP digestibility was decreased in the wine lees treatments. Furthermore, wine lees inclusion decreased NH_3-N concentration that was attributed to the lower CP digestibility. On the contrary, *in vitro* NDFom digestibility was increased upon wine lees inclusion. These results might have two possible explanations. Firstly, NDFom in wine lees might be easier to be fermented by ruminal microorganisms than that in rolled barley. Secondly, polyphenols in wine lees might have a positive effect on rumen fibrolytic bacteria, thus increasing the fiber digestibility. Patra et al. (2011) reported that supplementary *Terminalia chebula* (harad) and *Allium sativum* (garlic) that is rich in polyphenol increased NDF and ADF digestibility.

After fermentation, rumen digesta flows to the lower gastrointestinal tract *in vivo*, and phenolic compounds in diets are absorbed or degraded in the gastrointestinal tract (Perez-Maldonado and Norton, 1996a), and transferred to blood plasma or organ. In the present study, polyphenolic contents and DPPH radical scavenging ability increased with an increase in the levels of wine lees due to the higher contents of polyphenols in the wine lees than in the rolled barely. This indicates that wine lees inclusion may enhance the antioxidant capacity in plasma or organs as with other wine by-products (Kafantaris et al., 2017; Zhao et al., 2018). Furthermore, total DPPH radical scavenging ability after fermentation also tended to increase linearly with the inclusion of wine lees, reflecting an increase in the total polyphenol contents ($P = 0.09$). Although it is unknown whether the status of antioxidants in rumen contributes to those in host animal tissue, the higher antioxidant status in the rumen was expected to protect dietary fatty acids from ruminal biohydrogenation (Lee et al., 2014). Dietary antioxidants in rumen might break the chain reaction of lipoperoxidation by trapping lipophilic peroxyl radicals or reducing the quantity of oxygenated

radicals (Gobert et al., 2009).

The wine lees used in the present study contained sufficient stearic acid (8.7%) and linoleic acid (38.7%). Furthermore, the major components of fatty acids in wine lees were C16 (C16:0 and C16:1) and C18 (C18:0, C18:1, C18:2 and C18:3). The results were consistent with that of a study by the Gómez et al. (2004) in which the lipid composition of sherry wine lees was determined. To the best of our knowledge, the present study investigates, for the first time, the effect of wine lees on fatty acid profiles of rumen residues and liquids after incubation. During ruminal fermentation, rumen microorganisms can cause biohydrogenation of dietary lipids, which is a detoxication mechanism for microbes from PUFA (Vasta et al., 2010), leading to the conversion of PUFA to SFA. Consequently, ruminal products, such as meat are rich in SFA that increase the risk of cardiovascular disease. Polyphenols are known to inhibit the ruminal biohydrogenation (Vasta et al., 2010; Jafari et al., 2017). Therefore, dietary polyphenols have the potential to increase PUFA in ruminant products, such as meat or milk (Lourenço et al., 2008; Cabiddu et al., 2009; Doreau et al., 2011). In the present study, wine lees inclusion linearly increased the PUFA contents both in the fermented residues and supernatants. Higher polyphenolic contents and antioxidant capacity with wine lees inclusion might have contributed to the protection of PUFA from biohydrogenation. This protective effect indicates the potential to improve the fatty acid profiles in ruminant products by feeding wine lees. In previous studies on wine polyphenols, Rivas-Cañedo et al. (2013) have reported that supplementation of red wine extract rich in polyphenols delayed oxidation in sheep meat.

3.4.3. *In vivo* experiment

Various studies have reported that feeds, which include tannin at a high level, reduced DM intake (Barry and Duncan, 1984; McSweeney et al., 1988; Silanikove et al., 1996; Frutos et al., 2004), while dietary polyphenols, such as resveratrol (Ma et al., 2015) and quercetin (Benavides et al., 2013) had no effects on DM intake. Therefore, the observed difference in the effect of

polyphenol on DM intake is likely attributed to the different amounts of supplementation, polyphenolic components, and the associated effects of the other dietary components. Moreover, polyphenols have been known to form a complex with protein, fiber, and carbohydrates, and decrease the digestibility. Condensed tannins significantly reduced the apparent digestibility of dietary protein and consequently increased fecal N excretion (Waghorn, 1996). In the present study, wine lees had no effects on DM intake, the digestibility of DM, CP, NFC, and NDFom, and nitrogen balance in the in vivo experiment. This indicates that the concentrate diets could replace up to 20% DM by wine lees. Interestingly, difference was observed in the effect of dietary wine lees on CP digestibility between in vivo and *in vitro* experiments. Polyphenols, especially tannins, generally form protein complexes that protect protein from digestion in the rumen and inhibit the growth and activity of proteolytic bacteria (Patra and Saxena, 2011). However, some polyphenol-protein complexes are resolved in the lower gastrointestinal tract (Perez-Maldonado and Norton, 1996b), allowing the digestion and absorption there. Thus, the difference of the reaction of polyphenols and protein between in rumen and post-ruminal gastrointestinal tract may cause the difference of the result of CP digestibility between *in vivo* and *in vitro* experiments.

Wine lees inclusion increased total VFA production at 4 h after the feeding. The result was consistent with that of the study by Foiklang et al. (2016) that reported higher total VFA concentrations in dairy steers fed diets supplemented with grape pomace powder in comparison to those fed a control diet. In the present study, propionic acid proportion was decreased with wine lees inclusion, while the acetic acid proportion was tended to increase with wine lees ($P = 0.09$). Polyphenol concentration in experimental feeds might be related to the acetate and propionate production. Jayanegara et al. (2012) reported that acetate proportion tended to increase ($P = 0.08$) while propionate proportion tended to decrease ($P = 0.08$) with increasing levels of dietary tannins, which might be consistent with our results.

Total protein and albumin in blood are used as indices of protein metabolism. In the present study, wine lees inclusion did not affect these parameters *in vivo*. However, the CP digestibility

was decreased *in vitro*, thus indicating that polyphenols in wine lees bound to protein in rumen and subsequently, the protein complex was dissociated in hindgut. BUN represents kidney functions as BUN is transported through the kidney. In the present study, wine lees had no effects on kidney functions because BUN of the experimental animals was within the normal range (8-20 mg/L, Kaneko et al., 2008). This observation was in agreement with that of the previous study in dairy ewes fed grape seed (Nudda et al., 2015). Calcium and IP concentrations in plasma were determined as indicators of mineral metabolism. Plasma calcium concentration was not affected upon wine lees supplementation. However, plasma IP concentration was linearly increased with wine lees inclusion. This trend was observed in the wethers fed dietary middle and high amount of wine lees was tended to be higher than that in the wethers fed no wine lees diet ($P = 0.09$ and 0.06, respectively). This result indicate that dietary wine lees improved the phosphorous absorption by the animals; however, the mechanism was obscure in our experiment. Further study is needed to investigate the relationship of dietary polyphenol to phosphorous utilization.

Beef cattle are fed high quantities of grains to increase weight gain and shorten their fattening period. However, grains have been found to increase oxidative stress in animals (Mercier et al., 2004). Antioxidant status in plasma is a useful index to investigate the antioxidant ability of feeds served to the animal. We expected that wine lees inclusion enhanced the antioxidant ability since the polyphenolic contents and DPPH radical scavenging ability in the fermented residues were increased *in vitro*. In the present study, it is anticipated that wine lees inclusion decreased the plasma MDA that is a representative index of lipid peroxidation (Janero, 1990). This was consistent with Saito et al. (2016) who reported that peanut by-products, especially its skin, had the potential to reduce the MDA level of goats.

One of the limitations of the present study was that there were significant carry-over effects in digestibility of NFC, fecal N, and NEFA in blood due to the short length of the adaptation period. Moreover, in the present study, beef cattle and wethers were used for *in vitro* and *in vivo* experiments, respectively. The results in wethers were not completely compatible with those in

beef cattle because there might be some functional differences such as NDF and nitrogen digestibility between sheep and cattle (Amaning-Kwarteng et al., 1986). Considering these limitations of the present study, further studies with an increase in sample size will be needed to correctly evaluate the effects of dietary wine lees.

3.5. Conclusions

Conclusively, the wine lees, one of the by-products of wine-manufacturing, were replaced with the fattening ration of up to 20% DM. The replacement had no adverse effects on apparent digestibility, ruminal fermentation, and nitrogen balance. Furthermore, a decrease was observed in an oxidative stress marker *in vivo*. Although the gas production and digestibility of DM and CP was decreased with an increase in the level of wine lees *in vitro*, wine lees inclusion protected PUFA during ruminal fermentation.

In the present study, beef cattle and wethers were used for *in vitro* and *in vivo* experiments, respectively. The results in wethers were not completely compatible with those in beef cattle because there might be some functional differences such as NDF and nitrogen digestibility between sheep and cattle (Amaning-Kwarteng et al., 1986). In order to evaluate the effects of wine lees on performance of beef cattle, further studies will be needed.

CHAPTER 4

Calcium salts of long-chain fatty acids from linseed oil decrease methane production by altering the rumen microbiome *in vitro*

4.1. Introduction

Methane (CH_4) is an important global greenhouse gas because it has a global warming potential 28 times as strong as that of carbon dioxide (CO_2) over a 100 years timeframe (IPCC, 2014). Livestock are the largest emitter of anthropogenic CH_4 and the global emission of CH_4 from livestock production was estimated as 195 Tg/year in 2003–2012 (Saunois et al., 2016); CH_4 released from enteric fermentation of ruminants accounts for 39% of CH_4 from livestock sector (Gerber et al., 2013). Methane is the end product of anaerobic fermentation in the digestive process of ruminants, contributing an energetic loss of 2–12% of the gross energy (Johnson and Johnson, 1995). Therefore, mitigating enteric CH_4 emission from ruminants is required not only for reducing the environmental load but for improving the efficiency of animal production.

Dietary supplementation of lipids or independent fatty acids (FA) is one of the feasible feeding strategies to mitigate enteric CH_4 emission from ruminants (Beauchemin et al., 2008; Eugène et al., 2008; Martin et al., 2010). Beauchemin et al. (2008), through a meta-analysis, demonstrated that CH_4 production from ruminants was decreased by 5.6% with each 1% addition of supplemental fat. Among fats, polyunsaturated fatty acids (PUFA) are especially able to depress ruminal methanogenesis. Martin et al. (2008) demonstrated that a 5.7% supply of linseed oil that includes a high proportion of PUFA significantly reduced CH_4 emitted from dairy cows by 64% *in vivo*. The reduction of enteric CH_4 production from ruminants in response to dietary fats or FA is due to their toxic effects against a wide variety of rumen microorganisms, including bacteria, protozoa, archaea, and fungi (Machmüller et al., 1998; Dohme et al., 2001; Yang et al., 2009; Abubakr et al., 2014; Maia et al., 2007). However, dietary lipids or FA also cause the reduction of other traits such as dry matter (DM) intake and nutrient digestibility (Eugène et al., 2008; Martin et al., 2008; Dohme et al., 2001), as well as CH_4 production.

Calcium salts of long-chain fatty acids (CSFA) have been widely used in dairy and beef production as a rumen-protected fat in practical farm conditions (Jenkins et al., 2007; Bain et al.,

2016). Although dietary unprotected lipids significantly inhibit rumen microorganism activity, CSFA prevents problems related to rumen microbial fermentation and digestion (Jenkins et al., 2007). As a result, dietary CSFA have generally no or little adverse effect on nutrient digestibility in ruminants (Bain et al., 2016; Grummer et al., 1988; Reddy et al., 2003; Manso et al., 2006; Weiss et al., 2004; Purushothaman et al., 2008). Furthermore, CSFA partially escapes biohydrogeneration (BH) of fatty acids by rumen microbes. Wu et al. (1991) reported that net BH of total unsaturated C18 in diets with added CSFA and animal-vegetable blend fat were 57.3% and 87.2%, respectively, in dairy cows. Therefore, dietary CSFA can effectively increase unsaturated fatty acids contents in cow's lower digestive tract, increasing meat quality such as linoleic acid concentration (Bain et al., 2016), and milk yield and quality (Weiss et al., 2004; Purushothaman et al., 2008; McNamara et al., 2003).

Recently, the effect of CSFA on CH_4 production emitted from ruminants has attracted considerable interest. For example, Kliem et al. (2019) reported that diets with the addition of 2.2 g oil/kg DM as CSFA from palm and linseed oil decreased CH_4 production in dairy cows. This is probably because unsaturated fatty acids in CSFA were not completely protected from dissociation (Fotouhi et al., 1992), and were slowly released as free fatty acids in the rumen, influencing rumen microorganisms involved in CH_4 production. Nevertheless, the effects of CSFA on rumen microbiome have been little reported, and the impact of graded level of dietary CSFA on rumen CH_4 production is unclear. Therefore, the objective of the present study was to evaluate the effects of supplementary CSFA on *in vitro* ruminal fermentation, digestibility, CH_4 production and ruminal microbiome by comparing with those of fumarate and monensin that are major inhibitors of enteric CH_4 emission from ruminants (Asanuma et al., 1999; Odongo et al., 2007; Ungerfeld et al., 2007; Eckard et al., 2010). In the present study, we hypothesize that the FA may be gradually released from CSFA in the rumen and alter the microbiome, inhibiting CH_4 production with little negative effect on rumen fermentation.

4.2. Materials and methods

The experiment was approved by the Kyoto University Animal Ethics Committee (Permit Number: 31–33) and performed at the Graduate school of Agriculture, Kyoto University from July to August 2019. The CSFA used in the present study was received from Taiyo Yushi Corp., a Japanese commercial chemical manufacturer. The product contained 56.7% linseed oil and 27.6% silica gel as the fatty acids absorbent. The molar ratio of FA to calcium in CSFA was adjusted to 2.8. The FA were constituted with 5.5% palmitic acid (C16:0), 0.1% palmitoleic acid (C16:1), 3.3% stearic acid (C18:0), 18.2% oleic acid (C18:1), 15.6% linoleic acid (C18:2), 56.8% α-linolenic acid (C18:3) and 0.5% other fatty acids. Rolled barley was used as a substrate in the study. The substrate was ground in a Wiley mill to pass a 1 mm screen before use.

4.2.1. Experimental design

The following five treatments (FAL, FAH, FUM, MOM, and CON) were used in the experiments. CSFA was supplemented at 2.25% DM and 4.50% DM of the substrate—namely FAL and FAH, respectively. Based on the linseed oil concentration of the CSFA used in this study, the linseed oil concentration in FAL and FAH were 1.5% and 3.0%, respectively. Fumarate was added to a final concentration of 15 mM (FUM). One treatment received monensin at 20 mg/kg DM of the substrate (MOM). The doses of fumarate and monensin were determined based on Shirohi et al. (2012) and Joyner et al. (1979). The control treatment (CON) contained only substrate. Monensin was dissolved in ethanol before adding to test tubes in MON. Therefore, an equal volume of ethanol, 14.9 μL, was added into the other test tubes.

4.2.2. Animals, diets, and feeding

Two ruminal-cannulated Corridale wethers with initial body weight (BW) of 58.6 ± 6.2 kg (mean ± SD) were used. The animals fed on ryegrass straw and concentrate at a ratio of 30:70

on a DM basis for 23 days. The amount of total diets provided was 2% of BW on a fresh matter (FM) basis in two equal portions daily, at 08:30 and 17:00. The ingredient compositions of the concentrate were as follows: 35.2% rice bran, 54.0% rolled barley, 6.9% alfalfa meal, 3.4% soybean meal, and 0.6% vitamin-mineral premix on a DM basis calculated using Standard Tables of Feed Composition in Japan (NARO, 2009). Mineral blocks and water were offered *ad libitum*.

4.2.3. Procedure of *in vitro* experiment

On day 24, about 200 mL ruminal fluid was collected through the rumen cannula from each wether before morning feeding and was transferred to the laboratory within 30 min. The sample was filtered through four layers of cheesecloth. Subsequently, the two strained liquids were mixed equally. The filtered sample were mixed with artificial saliva (McDougall, 1948) in a ratio of 1:4 under anaerobic condition. The artificial saliva was sterilized by autoclaving and made anaerobic by a CO_2 flushing before mixing. A 40 mL mixture was transferred to each test tube containing 0.5 g DM of rolled barley and respective feed additives. The test tube was closed with a silicone rubber stopper fitted with a plastic syringe (Suzuki et al., 1995) to collect fermentation gas and incubated at 39˚C for 48 h. Each treatment was set up in three replicates.

During incubation, the total cumulative gas production at 0, 3, 6, 9, 12, 18, 24, 30, 36, 42, and 48 h, and CH_4 and CO_2 production at 12 and 24 h were measured. After incubation, test tubes were placed in ice-cold water to stop fermentation and immediately analyzed for pH using a pH meter (Horiba Ltd., Kyoto, Japan). Next, 1.5 mL of culture was subsampled for microbiome analysis and stored at -80˚C until further use. A 0.5 mL of the culture was mixed with 4.5 mL methyl green formalin sodium chlorate (MFS) solution for protozoa count (Ogimoto and Imai, 1981). All of the remaining culture was then centrifuged at 500 × *g* for 5 min to separate the residue and supernatant. The supernatant was mixed with 25% (w/v) meta-phosphoric acid at a 5:1 ratio and stored at -20˚C until the analyses of volatile fatty acids (VFA) and ammonia nitrogen (NH_3-N) concentrations. The residue was transferred to a nylon bag to determine the digestibility

of DM and neutral detergent fiber expressed exclusive of residual ash (NDFom).

4.2.4. Chemical analyses

DM, crude protein (CP), ether extract (EE), and crude ash contents of the feeds and substrate were analyzed according to the standards of the Association of Official Analytical Chemists (AOAC 2000; 930.15, 976.05, 920.39, and 942.05, respectively). The NDFom and acid detergent fiber expressed exclusive of residual ash (ADFom) contents were determined according to Van Soest et al. (1991). The content of non-fibrous carbohydrate (NFC) was calculated using the following formula; NFC = 100 - (CP + EE + NDFom + crude ash). Chemical compositions of the feeds and substrate are shown in Table 4-1. The DM and NDFom digestibility were determined by the procedure described by Sato et al. (2019). The total CH_4 and CO_2 production were analyzed by gas chromatography (INORGA, LC Science, Nara, Japan) equipped with a thermal conductivity detector (TCD). For the analysis of VFA concentrations, collected samples were centrifuged at 15,000 × *g* at 4˚C for 15 min. The concentrations of VFA in the supernatants were determined by gas chromatography (GC14-B, Shimadzu, Kyoto, Japan) equipped with a FID using a packed glass column (Thermon 3000–2% Shimalite TPA 60/80 3.2 mmφ × 2.1, Shimadzu Co., Ltd., Kyoto, Japan). The temperature of injection, column, and detector were 250, 115, and 250˚C, respectively. The NH_3-N concentration was determined by the steam distillation in a micro-Kjeldahl system (Kjeltec 2300, Foss Japan Ltd., Tokyo, Japan). Briefly, 3 mL of the supernatant after incubation was distilled with NaOH and the NH_3-N was trapped in a boric acid solution. Then, the solution was titrated with 0.1 N H_2SO_4 to determine NH_3-N concentration.

Table 4-1. Chemical compositions of feeds and substrate (% DM)

Item[1]	Concentrate	Ryegrass straw	Rolled barley
Dry matter (%)	87.9	87.6	87.8
Organic matter	97.0	95.7	97.5
Crude protein	14.5	7.0	13.5
Ether extract	3.4	2.4	2.5
NDFom	33.4	64.5	32.1
ADFom	9.0	39.6	10.3
Non-fibrous carbohydrate	45.7	21.8	49.4
Crude ash	3.0	4.3	2.5

[1] NDFom, neutral detergent fiber expressed exclusive of residual ash; ADFom, acid detergent fiber expressed exclusive of residual ash

4.2.5. Microbial DNA extraction, 16S rRNA gene amplicon preparation, and sequencing

Frozen culture samples were thawed on ice and centrifuged at 12,000 × *g* for 15 min. The supernatant was removed, and the pellet was used for DNA extraction by the method reported by Frias-Lopez et al. (2008). Extracted DNA was stored at −20˚C until further analysis. For each sample, the V3-V4 hypervariable region of the 16S rRNA gene was amplified using the primer set reported by Takahashi et al. (2014) with added the Illumina overhang adapter sequences (forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG, reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG), according to the 16S sample preparation guide (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). The amplicons were then sequenced on Illumina MiSeq platform (Illumina, San Diego, CA, USA), which generated paired 300-bp reads.

4.2.6. Sequence read processing and analysis

QIIME 2 (2019.4) package (http://qiime2.org) was used for sequence data analysis

(Bolyen et al., 2019). The adapter of the sequences was first trimmed using the cutadapt plugin (Martin, 2011). The pair-end reads were then merged, quality filtered (Q20), and dereplicated using vsearch (Rognes et al., 2016) and quality-filter plugin (Bokulich et al., 2013). Subsequently, chimeras were identified and removed, and operational taxonomic units (OTUs) clustering using a similarity threshold of 97% were performed with the vsearch plugin (Rognes et al., 2016). Multiple sequence alignment of the sequences was performed using Multiple Alignment using Fast Fourier Transform (MAFFT) program (Katoh and Standley, 2013) and masked (Lane, 1991) to remove highly variable regions using the qiime alignment command. A phylogenetic tree was then constructed with FastTree2 using the qiime phylogeny plugin (Price et al., 2010). The taxonomy of the sequence variants was assigned using the q2-feature-classifier plugin (Bokulich et al., 2018) against the Silva 132 OTUs sequences (Quast et al., 2012). The OTUs were rarefied to a depth of 3,966, which was the lowest sample depth, for alpha and beta diversity analysis. For analysis of alpha diversity, richness (observed-OTUs and Chao1 (Chao, 1984)) and diversity (Shannon diversity index (Shannon, 1948)) were estimated using the q2-diversity plugin. Non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis dissimilarities of OTUs was performed using R package 'vegan' (Oksanen et al., 2019) and visualized in R using 'ggplots2' (Wickham, 2016). Ward linkage hierarchical clustering using Spearman distance of OTUs was performed using the R function "hclust." In order to identify differentially abundant microbial taxa at the phylum and genus levels, we normalized the count matrices of taxa with a negative binomial distribution using DESeq2 (Love et al., 2014). Relative abundance was calculated using the normalized data, and the minor phylum and genus (average relative abundance < 1% for all treatments) were excluded from statistical analysis.

4.2.7. Statistical analyses

Data, except for Bray–Curtis dissimilarities of OTUs and abundant bacterial taxa, were analyzed using GLM procedure of Statistical Analysis System (SAS, 2008). The mathematical

model was:

$$Y_{ij} = \mu + T_i + e_{ij}$$

Where μ = the overall means, T_i = the effect of treatment, and e_{ij} = residual error. Multiple comparisons among the least square means were performed using the Tukey-Kramer method. In order to evaluate differences Bray–Curtis dissimilarities among the treatments, permutational multivariate analysis of variance (PERMANOVA) test was conducted with 9999 permutations using R package 'vegan' (Oksanen et al., 2019). Differentially abundant bacterial taxa were identified using a negative binomial Wald test in DESeq2 (Love et al., 2014). The obtained p-values were corrected according to Benjamini and Hochberg procedure (Benjamini and Hochberg, 1995). Differences were considered statistically significant at $P < 0.05$.

4.3. Results

4.3.1. *In vitro* gas production, methane production, and nutrient digestibility

The effects of the feed additives on *in vitro* gas production, CH_4 production, and digestibility are shown in Table 4-2. Among the treatments, FUM had the highest total gas production ($P < 0.05$) at the time points of incubation investigated. Compared to CON, the total gas production at 12 h after incubation in FAL was higher ($P < 0.05$) and that in FAH was similar ($P > 0.05$) but the total gas productions at 48 h after incubation in FAL and FAH were lower ($P < 0.05$). The total gas production in MON was lower ($P < 0.05$) than that in CON in the time points of incubation investigated. The total CH_4 production after 12 h and 48 h incubation and digestibility-adjusted CH_4 in FAL, FAH, and MON were significantly lower than those in CON ($P < 0.05$), and the lowest CH_4 production was produced in FAH. No significant differences were observed for all parameters related to CH_4 production between CON and FUM ($P > 0.05$). The

DM and NDFom digestibility in FAL and FAH were lower ($P < 0.05$) than those of the other treatments.

Table 4-2. Effects of feed additives on *in vitro* gas production, CH_4 production and digestibility of concentrate as substrate

	Treatment[2]					
Item[1]	CON	FAL	FAH	FUM	MON	SEM[3]
Gas production (mL/0.5gDM)						
12 h	88.9^{c}	97.0^{b}	92.7^{bc}	103.8^{a}	82.0^{d}	1.23
24 h	124.1^{b}	117.4^{c}	106.9^{d}	140.6^{a}	112.8^{d}	1.21
48 h	134.9^{b}	124.1^{c}	111.3^{d}	154.5^{a}	120.9^{c}	1.47
CH_4 production						
Total CH_4 after 12 h incubation (mL/0.5gDM)	6.3^{a}	4.7^{bc}	3.0^{d}	5.3^{ab}	4.1^{c}	0.23
Total CH_4 after 48 h incubation (mL/0.5gDM)	13.3^{a}	7.9^{b}	4.4^{c}	12.4^{a}	8.6^{b}	0.46
Adjusted CH_4 after 48h incubation (mL/g IVDMD)	32.4^{a}	20.0^{b}	12.0^{c}	30.3^{a}	21.1^{b}	1.15
Adjusted CH_4 after 48h incubation (mL/g IVNDFD)	123.7^{a}	80.6^{b}	46.6^{c}	115.5^{a}	80.7^{b}	5.10
CO_2 production after 48h incubation (mL/0.5gDM)	90.4^{b}	87.2^{bc}	80.7^{bc}	107.5^{a}	77.5^{c}	2.49
Digestibility (%)						
IVDMD	82.3^{a}	79.1^{b}	74.2^{c}	81.8^{a}	81.9^{a}	0.42
IVNDFD	67.3^{a}	61.4^{b}	59.7^{b}	67.0^{a}	66.9^{a}	0.97

^{abcd} LSMeans in a row with different superscripts significantly differ ($P < 0.05$)

[1] DM, dry matter; IVDMD, *in vitro* dry matter digestibility; IVNDFD, *in vitro* neutral detergent fiber digestibility

[2] CON, non-supplementation; FAL, 2.25% DM calcium salt of long-chain fatty acid supplementation; FAH, 4.50% DM calcium salt of long-chain fatty acid supplementation; FUM, fumarate supplementation; MON, monensin supplementation

[3] SEM, standard error of means

4.3.2. Characteristics of rumen fermentation and protozoa population

The results of rumen fermentation and the protozoa population are presented in Table 4-3. The pH in FAL and FAH were similar to those in CON and MON ($P > 0.05$), and higher than that in FUM ($P < 0.05$). No differences were observed among the treatments for total VFA concentration, the proportion of iso-butyrate, n-butyrate, and iso-valerate. The percentages of acetate in FAL, FAH, and MON were lower ($P < 0.05$) than that in CON. In contrast, higher proportions of propionate were observed in FAL, FAH, FUM, and MON than in CON ($P < 0.05$). Lower ratios of acetate to propionate were observed in all additive treatments, compared to CON. NH_3-N concentration in FAL and FAH were lower than those in CON, FUM, and MON ($P < 0.05$). Compared with CON and FUM, smaller number of protozoa was observed in FAL ($P < 0.05$) and even fewer in FAH and MON ($P < 0.05$).

4.3.3. Diversity and structure of rumen microbiome

The number of observed OTUs and Chao1 index in FAH and MON were lower ($P < 0.05$) than those in CON, while no differences were observed among CON, FAL, and FUM (Fig 4-1). For the Shannon diversity index, CON has the highest, followed by FAL and FUM, and FAH and MON showed the lowest values ($P < 0.05$) (Fig 4-1). PERMANOVA analysis confirmed that there were significant differences of rumen microbial communities among the treatments ($P < 0.001$), and NMDS using the Bray-Curtis dissimilarity metric (Fig 4-2) and hierarchical clustering of the microbiota community (Fig 4-3) revealed distinct clustering patterns that separated the microbiota in FAL, FAH and MON from that in CON and FUM.

Table 4-3. Effects of feed additives on pH, NH_3-N, protozoa population and VFA after 48 h incubation

	Treatment[2]					
Item[1]	CON	FAL	FAH	FUM	MON	SEM[3]
pH	6.48[a]	6.49[a]	6.46[a]	6.32[b]	6.40[ab]	0.02
NH_3-N (mgN/dL)	26.7[a]	20.8[c]	19.4[c]	26.5[a]	24.6[b]	0.35
Protozoa ($\times 10^5$/mL)	4.3[a]	3.1[b]	2.0[c]	4.4[a]	1.9[c]	0.19
VFA						
Total VFA (mmol/L)	130.6	127.1	124.8	132.1	121.8	4.88
Acetate (%)	49.6[a]	44.0[b]	42.4[b]	45.6[ab]	44.9[b]	0.91
Propionate (%)	35.2[c]	40.7[ab]	43.4[a]	39.1[b]	41.8[ab]	0.63
iso-Butyrate (%)	0.22	0.00	0.00	0.11	0.00	0.06
n-Butyrate (%)	10.1	9.4	7.6	10.0	8.1	1.09
iso-Valerate (%)	2.6	2.9	2.1	2.9	2.2	0.24
n-Valerate (%)	2.3[c]	3.1[b]	4.5[a]	2.3[c]	3.0[b]	0.14
Acetate:Propionate	1.4[a]	1.1[bc]	1.0[c]	1.2[b]	1.1[bc]	0.03

[abcd] LSMeans in a row with different superscripts significantly differ ($P < 0.05$)

[1] NH_3-N, ammonia nitrogen; VFA, volatile fatty acids

[2] CON, non-supplementation; FAL, 2.25% DM calcium salt of long-chain fatty acid supplementation; FAH, 4.50% DM calcium salt of long-chain fatty acid supplementation; FUM, fumarate supplementation; MON, monensin supplementation

[3] SEM, standard error of means

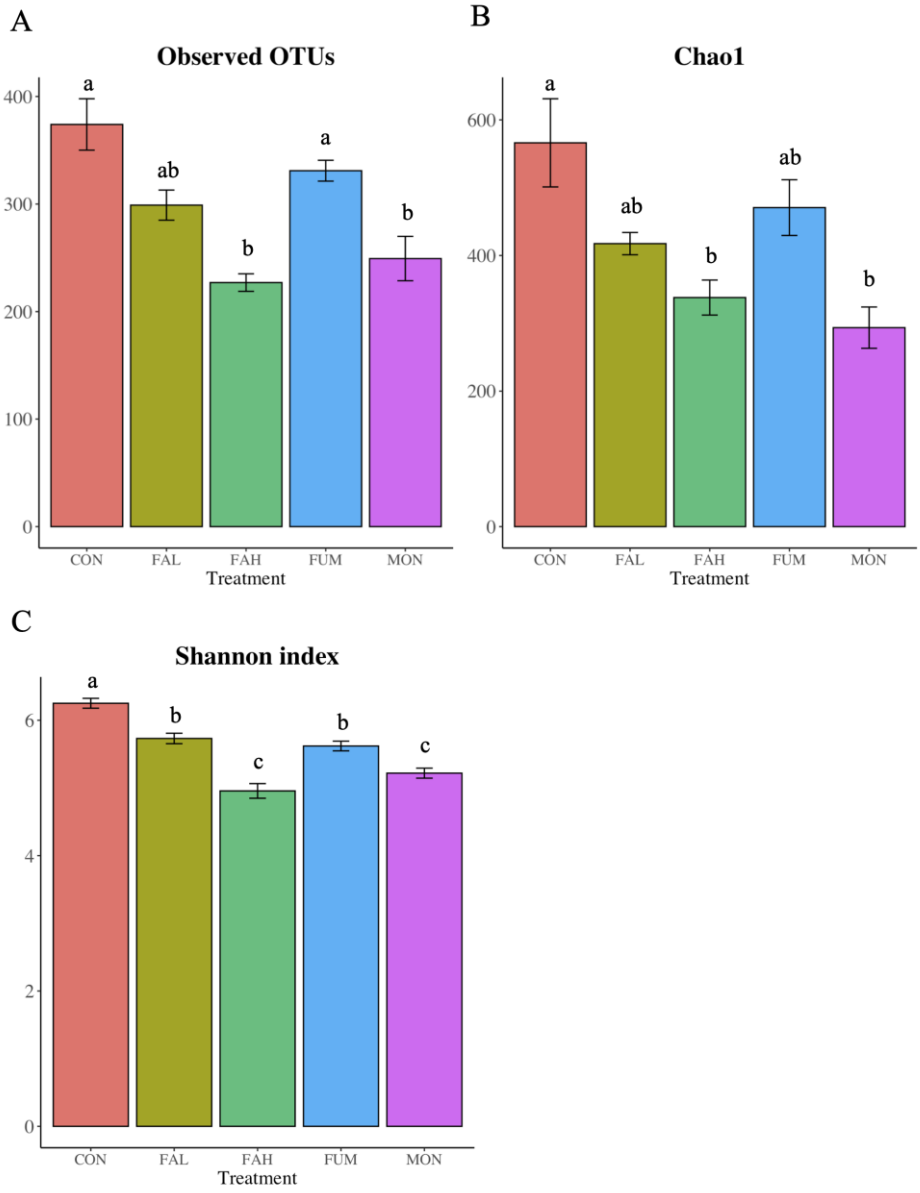

Fig 4-1. Effects of feed additives on alpha diversity. Data are presented as mean ± SE (n = 3 per treatment). (A) Observed OTUs, (B) Chao1, and (C) Shannon index in microbiomes after incubation. Different superscripts (abc) indicate significant differences ($P < 0.05$). CON = non-supplementation; FAL = 2.25% DM calcium salt of long-chain fatty acid supplementation; FAH = 4.50% DM calcium salt of long-chain fatty acid supplementation; FUM = fumarate supplementation; MON = monensin supplementation.

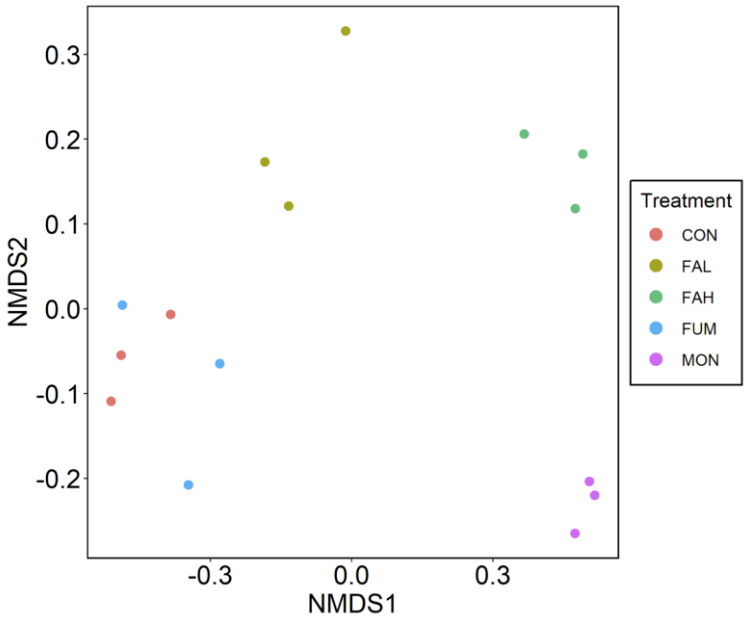

Fig 4-2. Non-metric multidimensional scaling (NMDS) plots of the Bray-Curtis dissimilarities of microbiota. CON = non-supplementation; FAL = 2.25% DM calcium salt of long-chain fatty acid supplementation; FAH = 4.50% DM calcium salt of long-chain fatty acid supplementation; FUM = fumarate supplementation; MON = monensin supplementation.

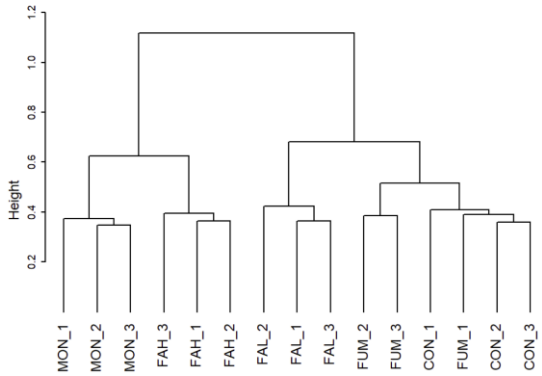

Fig 4-3. Ward linkage hierarchical clustering of microbiota based on Spearman distance. CON = non-supplementation; FAL = 2.25% DM calcium salt of long-chain fatty acid supplementation; FAH = 4.50% DM calcium salt of long-chain fatty acid supplementation; FUM = fumarate supplementation; MON = monensin supplementation; 1-3, sample number.

4.3.4. Bacterial abundance

Fig 4-4 shows the relative abundance of microbiota at the phylum level, and different abundant taxa is presented in Fig 4-5. At the phylum level, the microbiota in all treatments was dominated by Firmicutes, Bacteroidetes, and Proteobacteria. The abundant of the phyla Bacteroidetes in MON was lower than that in CON ($P < 0.05$). The abundant of the phyla Firmicutes in FAH, FUM and MON was increased compared with CON and FAL ($P < 0.05$), and that of Proteobacteria in FAL, FAH and MON was higher than that in CON ($P < 0.05$).

At the genus level, *Methanobrevibacter*, which accounted for over 99% of the phylum Euryarchaeota, in FAH was lower than that in CON ($P < 0.05$). Among the phylum Bacteroidetes, *Bacteroidales* BS11 gut group and *Rikenellaceae* RC9 gut group was higher in CON than FAL, FAH and MON ($P < 0.05$). There was significant difference of unclassified Bacteroidales between CON and the other treatments ($P < 0.05$). Regarding the phylum Firmicutes, many genera (*Succiniclasticum Anaerovibrio*, *Megasphaera*, *Schwartzia*, *Selenomonas.1*, *Veillonellaceae* UCG.001, uncultured *Veillonellaceae*, and unclassified *Veillonellaceae*) were higher in MON than CON ($P < 0.05$). Similarly, adding CSFA at high level (treatment FAH) increased *Succiniclasticum*, *Selenomonas.1* and *Megasphaera* compared to those of CON ($P < 0.05$). Additionally, *Streptococcus* was higher in FAH than in the other treatments ($P < 0.05$) and *Schwartzia* was increased in FUM ($P < 0.05$). *Ruminococcus*.2 in all additive treatments, especially FAH and MON, were significantly decreased compared with that in CON ($P < 0.05$). Among the phylum *Proteobacteria*, *Ruminobacter* was lower in FUM than other treatments ($P < 0.05$), but higher in FAH and MON than in CON ($P < 0.05$). *Succinivibrio* was increased in FAL and FAH ($P < 0.05$) compared with that in CON and MON. *Pyramidobacter* (the phylum Synergistetes) was higher in MON than that in other treatments ($P < 0.05$).

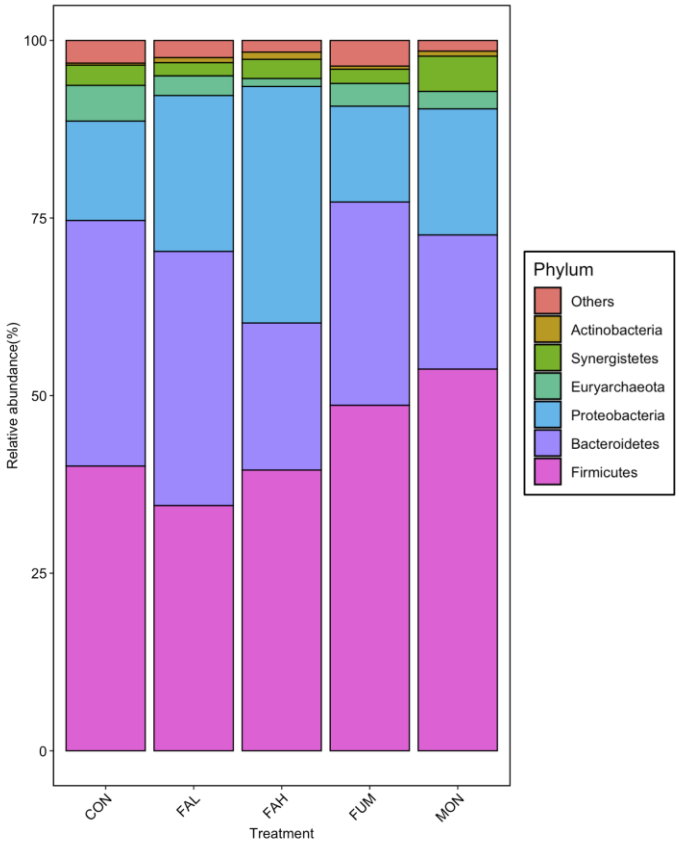

Fig 4-4. Relative abundance (%) of rumen microbiome at phylum level. All phyla comprising less than 1% of the total abundance in all treatments were combined into the "Others" category. CON = non-supplementation; FAL = 2.25% DM calcium salt of long-chain fatty acid supplementation; FAH = 4.50% DM calcium salt of long-chain fatty acid supplementation; FUM = fumarate supplementation; MON = monensin supplementation.

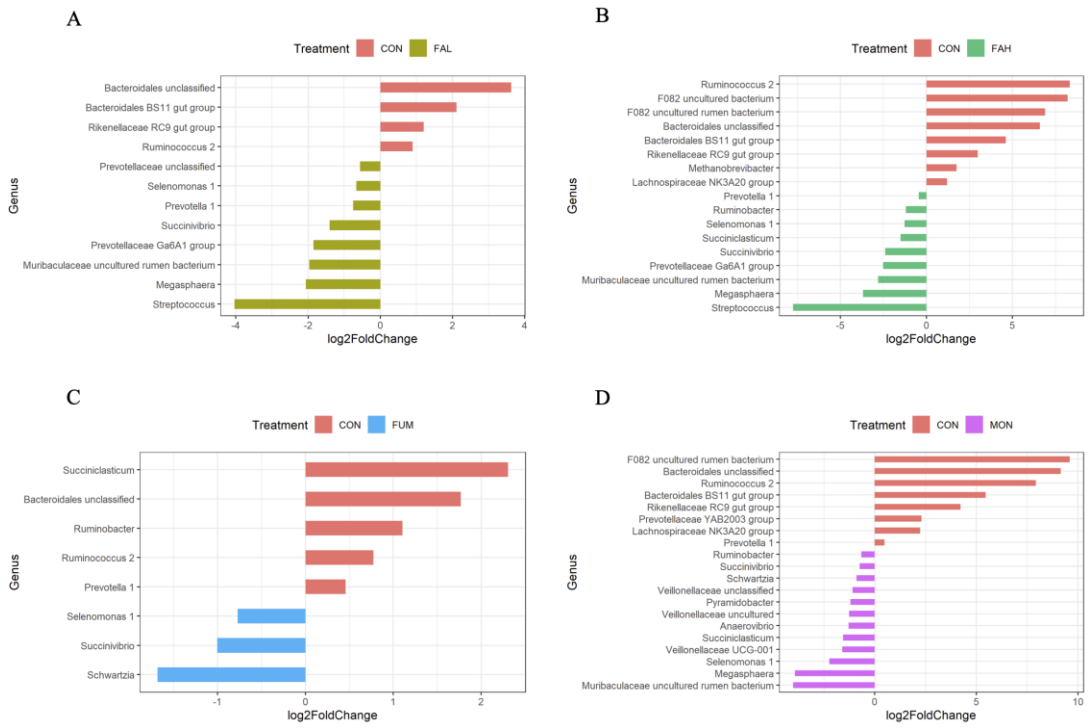

Fig 4-5. Significantly differentially abundant microbial taxa at the genus level. Genera with significant differences (adjusted $P < 0.05$) identified using DESeq2 between (A) CON and FAL, (B) CON and FAH, (C) CON and FUM, and (D) CON and MON. Only taxa $\geq 1\%$ relative abundance for at least one treatment are shown. CON = non-supplementation; FAL = 2.25% DM calcium salt of long-chain fatty acid supplementation; FAH = 4.50% DM calcium salt of long-chain fatty acid supplementation; FUM = fumarate supplementation; MON = monensin supplementation.

4.4. Discussion

We evaluated the effect of CSFA on *in vitro* rumen fermentation, CH_4 production, digestibility, and rumen microbiota. Many studies showed that supplementary linseed decreases ruminal CH_4. A meta-analysis by Martin et al. (2010) demonstrated that for each 1% addition of supplemental linseed, CH_4 production decreased by 4.8%. In the present study, compared with control (no additive), low and high amounts of CSFA supplementation (FAL and FAH) reduced CH_4 production (mL/g IVDMD) by 38.2% and 63.0%, respectively. We found that addition of CSFA led to 21.0–25.5% decreases per 1% of linseed oil addition. Thus, in this study, the percentage of CH_4 reduction due to CSFA supplementation was higher than that reported by Szumacher-Strabel et al. (10.1% reduction per 1% of linseed oil addition in *in vitro*) (2004), indicating that the CSFA used in the present study has a substantially high reduction effect on CH_4 production. We presumed that silica might be a key factor to increasing the CH_4 reduction effect of CSFA. Shinkai et al. (2012) reported that cashew nut shell liquid pellet with 40% silica powder has a larger reduction effect on CH_4 production than that with 11.3% silica powder and several ingredients. They hypothesized that the cashew nut shell liquid pellet with 40% silica powder easily diffuses in the rumen, leading to a remarkable decrease in CH_4 production (2012). Similarly, unsaturated fatty acids might diffuse from CSFA with 27.6% silica gel, and efficiently suppressed microbial activity related to CH_4 production.

Furthermore, adding CSFA at a high dose level more clearly reduced CH_4 production when compared with adding supplementary fumarate and monensin. Monensin and fumarate are feed additives that can reduce CH_4 production from ruminants. Odongo et al. (2007) reported that monensin reduced CH_4 production from dairy cows without the negative effect on DM intake and milk yield. Asanuma et al. (1999) demonstrated that the use of fumarate as a feed additive could reduce methanogenesis and increase propionate production in the rumen, leading to the reduction of CH_4 production. Therefore, the results in the present study indicate that CSFA is one of the

potent inhibitors of methanogenesis. In the present study, supplementary fumarate did not reduce CH_4 production probably because rolled barley was used as the substrate. García-Martínez et al. (2005) reported that adding fumarate to batch culture under a low-forage substrate condition have less CH_4 reduction effect compared with a high-forage substrate condition.

Methane production in the rumen is due to methanogenesis of methanogens, and rumen methanogens use mainly H_2 to reduce CO_2 to CH_4 (Leahy et al., 2010). Protozoa, which produce H_2 in the hydrogenosomes (Müller, 1993), are also involved in methanogenesis because some of the methanogens attach to the cell surface of protozoa (Vogels et al., 1980). Guyader et al. (2014) demonstrated by a meta-analysis that there was a positive linear correlation between protozoal numbers and CH_4 emissions. Fatty acids, especially PUFA, have adverse effects on methanogens and protozoa (Ikwuegbu and Sutton, 1982; Sutton et al., 1983). In the present study, the genus *Methanobrevibacter*, which is the dominant methanogen in the rumen (Whitford et al., 2001; Janssen and Kirs, 2008; Henderson et al., 2015), and the count of protozoa were decreased with the levels of CSFA, suggesting that FA released from CSFA might influence these microorganisms.

Increasing propionate production decreases available H_2 for methanogenesis since propionate formation is a competing alternative to H_2 formation (Janssen, 2010). Therefore, the increase of propionate in the rumen is associated with reduction in CH_4 production. In the present study, the percentage of propionate was increased by CSFA supplementation, corresponding with the result of fumarate and monensin inclusion. These results are consistent with previous studies related to the supplementation of linseed oil in dairy cows and steers diets (Ueda et al., 2003; Van Gastelen et al., 2017; Li et al., 2015). In the rumen, there are two pathways for propionate production; succinate pathway (the main pathway) and acrylate pathway (Jeyanathan et al., 2014). In the succinate pathway, fumarate is reduced to succinate, and succinate is converted to propionate by some bacteria. The genera *Ruminobacter* (Wang et al., 2020) and *Succinivibrio* (Russell and Rychlik, 2001) are involved in succinate production, while *Succiniclasticum* (Van Gylswyk, 1995), *Selenomonas* (Van Gylswyk et al., 1997), and Schwartzia (Van Gylswyk et al.,

1997) ferment succinate and produce propionate via the succinate pathway in rumen. In the present study, the genera *Ruminobacter*, *Succinivibrio*, and *Selenomonas.*1, *Succiniclasticum* were increased by supplementation of CSFA at a high level or monensin compared to control, indicating that *Ruminobacter* and *Succinivibrio* might produce succinate used by *Selenomonas* and *Succiniclasticum* for propionate production in CSFA or monensin supplementation. However, the inclusion of fumarate increased the genus *Schwartzia*. Thus, the main bacteria related to propionate production via succinate pathway were different between the treatments (FAH and MON) and fumarate although all feed additives increased the proportion of propionate. Furthermore, the genera *Streptococcus* and *Megasphaera* were also increased by the inclusion of CSFA at a high level. *Streptococcus bovis* produces lactate (Russell et al., 1981; Maroune et al., 1987), while *Megasphaera elsdenii* is a utilizer of lactate for the production of butyrate and propionate (Russell et al., 1981; Maroune et al., 1987; Hino et al., 1993). Thus, our results indicate that supplementary CSFA may also promote propionate production via acrylate pathway as well as succinate pathway.

Rumen protected fats such as CSFA prevent ruminal fermentation and digestion problems caused by fat feeding (Jenkins and Bridges, 2007). Therefore, we expected that no or little negative effects of supplementary CSFA on rumen fermentation and digestibility would be observed as with the results of other studies (Bain et al., 2016; Grummer, 1988; Reddy et al., 2003; Manso et al., 2006; Weiss and Wyatt, 2004; Purushothaman et al., 2008). In the present study, however, supplementary CSFA decreased DM and NDFom digestibility, resulting in the inhibition of total gas production after 48 h incubation. Decreased ruminal ammonia was also observed with CSFA inclusion. The results indicate that FA released from CSFA might be sufficiently detrimental to the activity of ruminal microorganisms. Yang et al. (2009) reported that dietary soybean oil and linseed oil to dairy cows decreased the counts of cellulolytic bacteria. We observed a strong decrease in the genus *Ruminococcus*.2 (the family *Ruminococcaceae*), *Rikenellaceae* RC9 gut group (the family *Rikenellaceae*), unclassified Bacteroidales (the order

Bacteroidales), and Bacteroidales BS11 gut group (the family *Bacteroidaceae*) with the addition of CSFA. *Ruminococcus* is one of the main cellulolytic bacteria in the rumen, accounting for about 10^6 cells/mL of rumen content (Koike and Kobayashi, 2001). Dai et al. (2015) demonstrated that *Ruminococcus* primarily synthesized putative cellulases and hemicellulases. It is well known that long chain fatty acids inhibit the growth of gram-positive bacteria (Maczulak et al., 1981), and supplementary linseed oil reduces *Ruminococcaceae* (Yang et al., 2009; Popova et al., 2019), which agrees with the results in the present study. *Rikenellaceae* may be associated with either primary or secondary degradation of structural carbohydrates (Pitta et al., 2010). Various studies have reported that supplementary oil such as sunflower oil (Asma et al., 2013), linseed oil (Janssen, 2010), and tucumã oil (Ramos et al., 2018) reduced the relative abundance of *Rikenellaceae* RC9, consistent with our findings. Some *Bacteroidales* are associated with fiber degradation. *Bacteroidales* BS11 is specialized in fermenting many different hemicellulosic monomers, producing acetate and butyrate for the host (Solden et al., 2017). Hence, the reduced abundance of these bacterial taxa might be a reason for the decreased digestibility observed after CSFA supplementation. The inclusion of monensin also decreased these taxa because monensin preferentially inhibits gram-positive bacteria (Russell and Strobel, 1989) as linseed oil. In contrast to CSFA, no reduction in fiber digestibility by monensin was observed. This may be probably due to higher abundance of some taxa which belong to the phylum Firmicutes in MON than in FAH. Bensoussan et al. (2017) found that cellulosome components, which are an extracellular multi-enzyme complex considered to be one of the most efficient plant cell wall-degrading strategies, were prevalent in Firmicutes. Among the phylum Firmicutes, *Selenomonas.1*, which was significantly increased with monensin compared to FAH, might have enhanced fiber digestion in the present study. *Selenomonas ruminantium* improves fiber digestion by cooperating with other cellulolytic bacteria (Sawanon and Kobayashi, 2006; Sawanon et al., 2011).

Interestingly, cumulative gas production after supplementing CSFA at a low level was higher than in control at 12 h after incubation in spite of decreased CH_4 production. These results

indicate that CSFA supplementation inhibits the activity of rumen microbes related to CH_4 production in the initial stage of ruminal fermentation without toxic effect on other rumen bacteria. The characteristic of CSFA may be worthy of *in vivo* investigation, since rumen contents and liquid flow out of the rumen *in vivo*. Hartnell and Satter (1979) reported that ruminal turnover rates of liquid, grain, and hay were 8.1, 4.4, and 3.9% per hour, respectively, in dairy cows. Considering these turnover rates and our results, dietary CSFA may be able to decrease CH_4 production with little or no negative effect on rumen fermentation and digestibility *in vivo*.

One of the limitations of the present study was that the low sample size (n = 3 per treatment) with only one *in vitro* trial. Moreover, we evaluated the effect of CSFA using only one substrate although the effect of fat on rumen fermentation can be influenced by the concentrate and roughage ratio of feeds (Bayat et al., 2017). Therefore, further studies with an increase in sample size and substrates will be needed to increase the reliability of the effect of CSFA on CH_4 production.

In conclusion, although *in vitro* digestibility was reduced with increasing concentration of CSFA, addition of CSFA significantly changed rumen microbiome, resulting in the acceleration of propionate production, and the reduction of CH_4 production. These findings present CSFA as a promising candidate for reduction of CH_4 emission from ruminants. However, some differences of the observation were reported between *in vivo* and *vitro* (Sato et al., 2020). Therefore, future studies are needed to confirm the *in vivo* effect of dietary CSFA on CH_4 production, productivity, rumen microbiome, and digestibility, and to determine the optimal amount of CSFA in a diet for ruminants.

CHAPTER 5

Taxonomic and functional characterization of the rumen microbiome of Japanese Black cattle revealed by 16S rRNA gene amplicon and metagenome shotgun sequencing

5.1. Introduction

Ruminants can convert indigestible plant biomass to high-quality edible animal proteins, including milk and meat, for human consumption. This process occurs via microbial fermentation in the rumen, which is the first of four stomach chambers in ruminants. Rumen microbes comprise mainly bacteria, ciliate protozoa, fungi, archaea, and viruses (bacteriophages) at concentrations of $\leq 10^{11}$, 10^6, 10^6, 10^9, and 10^{10} cells or particles/mL, respectively (Morgavi et al., 2013). Ruminants do not directly produce the enzymes required to degrade plant fiber. Rather, the complex microbiome in the rumen digests and ferments this material (Terry et al., 2019) and supplies volatile fatty acids (VFAs) for host ruminant growth.

Research on the rumen microbiome has recently been accelerated by the development of next-generation sequencing technologies. Several studies have suggested that the cattle rumen microbiome contributes to host traits such as feed efficiency (Shabat et al., 2016; Delgado et al., 2019), methane yield (Roehe et al., 2016; Danielsson et al., 2017), developmental stage (Malmuthuge et al., 2019), breed (De Mulder et al., 2018; Li et al., 2019), and milk production (Indugu et al., 2017, Xue et al., 2020) as well as environmental factors such as diet (Bohra et al., 2019; Wang et al., 2019). Therefore, determining the rumen microbiome community structure and function is important for improving livestock production.

Japanese Black (JB) cattle is a Wagyu breed and major beef breed in Japan (Gotoh et al., 2014; Gotoh et al., 2018). The breed accounts for 97% of the nationwide distribution among Wagyu breeds (Hirooka, 2014). Japanese Black cattle can deposit very large quantities of intramuscular fat (23.3% in the longissimus dorsi muscle by age 24 months) and undergo substantial marbling (Gotoh et al., 2009). In Japan, the beef is evaluated using a 12-point beef marbling standard (BMS) score, which is used for evaluating the marbling of beef in Japan (a higher score indicates more abundant marbling). The BMS score of JB is considerably higher than that of other Wagyu breeds (e.g., Japanese Brown): 6.9 for JB; (Inoue et al., 2021); 3.4 for

Japanese Brown steer (Sasaki et al., 2006). In addition, the BMS score of JB is high compared with that of JB × Holstein crossbred (approximately 3.25; Mukai et al., 2004). Kim et al. (2020) and Krause et al. (2020) reported that the rumen microbial population affected the marbling score of Honwoo Korean beef cattle and Angus steers, respectively. This indicates that the rumen microbiome of JB cattle may be correlated with their ability to produce superior marbled beef, and JB cattle may possess a unique rumen microbiome that markedly differs from that of other beef cattle breeds.

Only one study has comprehensively investigated the rumen microbiome of JB cattle via the next-generation sequencing technology (Ogata et al., 2019). The authors used 16S ribosomal ribonucleic acid (rRNA) gene amplicon sequencing to assess the effects of a long-term high-grain diet on the rumen microbiota of JB cattle. They reported that unclassified *Ruminococcaceae* and unclassified *Lachnospiraceae* were the most abundant bacterial genera (Ogata et al., 2019). In contrast, no study has examined the functions, particularly carbohydrate-active enzymes (CAZyme), encoded by the JB rumen microbiome. These key enzymes break down complex carbohydrates and glycoconjugates (Cantarel et al., 2009).

The objective of this study was to identify the taxonomic and functional characteristics of the JB rumen microbiome. We performed 16S rRNA gene amplicon and metagenome shotgun sequencing to compare the rumen microbiomes of JB against those of JB sires × Holstein dams crossbred (F1) administered the same diet. Metagenome shotgun sequencing captures the microbial diversity and functional potential of the rumen microbiome. The F1 was used as the control, as the breed is the predominant non-Wagyu breed cattle used for beef production in Japan and presents with less muscular marbling than JB cattle.

5.2. Materials and methods

This study was conducted on a commercial farm in Handa City, Aichi Prefecture, Japan

(34°89′N, 136°94′E). The experimental design and protocol were approved by the Kyoto University Animal Ethics Committee (permit number R2-119).

5.2.1. Animals, diets, and feeding

A total of six JB and six F1 steers were used during the early fattening period. Their average ages were 14.7 ± 1.44 months and 11.1 ± 0.39 months, respectively. There were two animals per pen and all pens were on the same cattle barn. All cattle were fed bermudagrass and concentrate at an 18:82 dry matter ratio (Table 5-1). The same experimental diets were offered to JB and F1 cattle for 45 days and 52 days, respectively, before sampling of the rumen fluid. Information on the animals is shown in Table 5-2.

Table 5-1. Chemical compositions of the feeds used in the experiment.

	Concentrate	Bermudagrass
Chemical compositions (%)		
Dry matter	87.7	89.4
Organic matter[a]	96.1	94.6
Crude protein[a]	16.3	5.4
Ether extract[a]	4.5	1.2
NDFom[a]	31.2	72.7
ADFom[a]	10.0	40.5
Non-fibrous carbohydrate[a]	44.1	15.3

[a]On a dry matter basis

NDFom, neutral detergent fiberexpressed exclusive of residual ash; ADFom, acid detergent fiber expressed exclusive of residual ash

Table 5-2. Information of animals used in the experiment.

Sample ID	Breed	Sire	Birthplace	Pen[1]
JB_34	Japanese Black	Hirashigekatsu	Kamikita, Aomori, Japan	A
JB_030	Japanese Black	Takanokuni	Towada, Aomori, Japan	A
JB_14	Japanese Black	Yurishige	Tahara, Aichi, Japan	B
JB_18	Japanese Black	Yurifukuhisa	Hamamatsu, Shizuoka, Japan	B
JB_53	Japanese Black	Yasufukuhisa	Toyohashi, Aichi, Japan	C
JB_91	Japanese Black	Shigekatsusakae	Sannohe, Aomori, Japan	C
F1_60	F1	Umesakaefuku	Tokachi, Hokkaido, Japan	D
F1_52	F1	Umesakaefuku	Futami, Hokkaido, Japan	D
F1_024	F1	Umesakaefuku	Hiroo, Hokkaido, Japan	E
F1_81	F1	Umesakaefuku	Kato, Hokkaido, Japan	E
F1_58	F1	Umesakaefuku	Hiroo, Hokkaido, Japan	F
F1_424	F1	Umesakaefuku	Notsuke, Hokkaido, Japan	F

[1]Animals with same alphabet were kept in the same pen

5.2.2. Sample collection

Rumen contents were collected with stomach tubing (Fujihira Industry Co., Ltd., Tokyo, Japan) from each animal at 4 h after morning feeding. The initially collected sample (~200 mL) was discarded to avoid contamination with saliva. The subsequent rumen sample (~100 mL) was stored. The rumen samples were passed through four layers of cheesecloth and their pH was measured with a pH meter (Horiba Ltd., Kyoto, Japan). Filtered rumen fluid (10 mL) was mixed with 2 mL of 25% (*w*/*v*) *m*-phosphoric acid to determine VFA content and ammonia nitrogen (NH_3-N) concentrations. The latter is the major protein degradation end-product in the rumen. A second 1.5 mL rumen fluid aliquot was subsampled for microbial analysis. Both aliquots were transported on dry ice to the laboratory and stored at -20°C and -80°C, respectively.

5.2.3. Chemical analyses

The feeds were analyzed for dry matter, crude protein, ether extract, and crude ash

contents according to the methodology of the Association of Official Analytical Chemists (AOAC, 2000). Neutral detergent fiber expressed exclusive of residual ash (NDFom) and acid detergent fiber expressed exclusive of residual ash were measured as described by Van Soest et al. (1991). Non-fibrous carbohydrate was determined as follows:

$$\text{Non} - \text{fibrous carbohydrate} = 100 - (\text{crude protein} + \text{ether extract} + \text{NDFom} + \text{crude ash})$$

Rumen fluid samples were centrifuged at 15,000 × *g* at 4°C for 15 min. VFA concentrations in the supernatants were measured via a gas chromatography system (GC14-B; Shimadzu, Kyoto, Japan) fitted with a flame ionization detector and a packed glass column (FAL-M; 10% Shincarbon-All 80/100; 2.1 m × 3.2 mm i.d.; Shimadzu). NH_3-N concentration was measured with steam distillation in a micro-Kjeldahl system (Kjeltec 2300; Foss Japan Ltd., Tokyo, Japan), according to Sato et al. (2020).

5.2.4. Microbial DNA extraction

Rumen samples were thawed and centrifuged at 12,000 × *g* at 4°C for 15 min. The supernatants were discarded, and the pellets were used for DNA extraction as described by Frias-L**o**pez et al. (2008) with small modification. Briefly, 1 mL of lysis buffer, containing 5 mg/mL lysozyme, was added to the microcentrifuge tubes containing the pellets, and the tubes were incubated at 37°C for 30 min. Sodium dodecyl sulfate (final concentration of 1%) was added, followed by the addition of proteinase K (TaKaRa Bio, Shiga, Japan) at a final concentration of 0.5 mg/mL. The tubes were then incubated at 55°C for 20 min and further at 70°C for 5 min to induce cell lysis. Genomic DNA was recovered from the aqueous phase using a combination of phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1) protocols. DNA was precipitated by adding 3M sodium acetate and isopropanol. The DNA was washed with

70% ethanol, suspended in sterile Milli-Q water, and stored at -20°C until further analysis.

5.2.5. Library preparation and sequencing

5.2.5.1. *16S rRNA gene sequencing*

16S rRNA gene sequencing was conducted on the DNA samples of all animals (n = 12). The V3–V4 hypervariable region of the 16S rRNA genes was amplified via PCR with the Pro341F (5'-CCTACGGGNBGCASCAG -3') and Pro805R (5'-GACTACNVGGGTATCTAATCC -3') primers (Takahashi et al., 2014) and overhang adapters (forward: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG, reverse: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG). Libraries were prepared according to the 16S sample preparation guide (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf). Paired-end (2 × 300-bp) sequencing was conducted on an Illumina MiSeq platform (San Diego, CA, USA).

5.2.5.2. *Shotgun metagenomic sequencing*

The collected samples from all animals were used for shotgun metagenomic sequencing (n = 12). The metagenomic library was prepared using the Nextera XT DNA library preparation kit (Illumina). Library quantity and quality were evaluated with a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, MA) and an Agilent Technology 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), respectively. All libraries were pooled and sequenced in the same lane of an Illumina Hiseq X Ten platform (2 × 150-bp).

5.2.6. Bioinformatic analysis

5.2.6.1. *16S rRNA gene sequencing data*

The DADA2 plugin of QIIME2 v. 2020.8 (Callahan et al., 2016) was used for quality

filtering, denoising, and pair-end merging and to construct a feature table of amplicon sequence variants (ASVs). For the taxonomic analysis, the ASVs were assigned against the SILVA (release 132) reference database (Quast et al., 2012). The ASVs taxonomically assigned to mitochondria, chloroplast, and unassigned kingdom were removed from the feature table. The feature table was further filtered for ASVs detected <10 times in the entire dataset and in only one animal. All sequence data were rarefied to the lowest sample depth of 65,699 sequences per sample for diversity analysis. For alpha diversity analysis, the richness (observed ASVs) and Shannon diversity indices (Shannon, 1948) were estimated using the 'Phyloseq' package of R (Mcmurdie and Holmes, 2013). Beta diversity was assessed via non-metric multidimensional scaling ordination based on ASV Bray-Curtis dissimilarity in the 'vegan' package of R (Oksanen et al., 2019) and visualized with 'ggplots2' in R (Wickham, 2016).

5.2.6.2. *Metagenomic sequence data*

Illumina adapters and low-quality reads were trimmed with Trimmomatic v. 0.39 (Bolger et al., 2014). To remove host DNA contamination, the trimmed reads were mapped with BWA-MEM to the bovine reference genome ARS-UCD1.2/bosTau9 (Li, 2013). The filtered reads were assembled with SPAdes v. 3.13.0 (Bankevich et al., 2012) using the "--meta" option (Nurk et al., 2017). Contigs of >300 bp were retained for further analysis. A summary of the genome assembly statistics is shown in Table 5-3. Open reading frames (ORFs) were predicted with MetaGeneMark v. 3.38 according to the assembled contigs (Zhu et al., 2010). A nonredundant gene catalog with 95% identity and 90% coverage was identified using CD-HIT v. 4.7 (Fu et al., 2012). Clean reads from each sample were mapped with BWA-MEM to the gene catalog (Li, 2013). Gene abundance was calculated as transcripts per million based on the number of reads mapped to the gene.

Predicted nonredundant genes were annotated against the non-redundant (NCBI-nr) database by DIAMOND v. 0.9.23 (Buchfink et al., 2014) using an e-value cutoff of 1×10^{-10}. Outputs were imported into MEGAN v. 6.19.9 to estimate the taxonomic composition using the

lowest common ancestor algorithm with default parameters (Huson et al., 2016). Only taxonomically classified genes at the domain level were used to evaluate the relative abundance of taxa. CAZymes, including glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), auxiliary activities, and carbohydrate-binding modules (CBMs), were identified via unique gene annotation in dbCAN2 (Zhang et al., 2018), which integrates three tools and databases for CAZyme prediction. These include a HMMER search against the dbCAN CAZyme domain hidden Markov model database, DIAMOND search against the CAZy database, and Hotpep search against the conserved CAZyme short peptide database. Only CAZyme domains annotated by ≥2 tools were conserved as recommended by Zhang et al. (2018). Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) were assigned using eggNOG-mapper v. 2.0.1 (Huerta-Cepas et al., 2017) based on eggNOG v.5.0 (Huerta-Cepas et al., 2019).

Table 5-3. Genome assembly statistics of shotgun metagenome data.

Sample ID	Number of contigs (> 300 bp)	Largest contigs (bp)	Total length (bp)	N50 (bp)
JB_34	516,042	177,279	330,322,746	656
JB_030	346,060	204,005	289,813,331	1,101
JB_14	569,793	194,530	357,059,197	643
JB_18	863,231	155,794	542,897,703	635
JB_53	704,003	379,867	488,875,009	755
JB_91	492,186	252,228	342,114,958	748
F1_60	357,813	74,964	227,977,875	658
F1_52	406,160	317,234	267,624,869	682
F1_024	543,000	217,651	433,828,559	1,003
F1_81	404,736	132,986	273,322,510	723
F1_58	548,843	118,765	378,599,811	752
F1_424	566,737	253,503	411,500,377	817

5.2.6.3. *Metagenome-assembled genome (MAG) reconstruction*

Metagenomic bins were constructed with contigs using the MetaBAT2 v.2.15 (Kang et al., 2019) with "--minContigLength 2000 --minContigDepth 2" options. All bins were aggregated and dereplicated with dRep v.2.6.2 (Olm et al., 2017) with the "dRep dereplicate" command. This command first estimates genome quality (completeness and contamination) with CheckM v.1.1.3. (Parks et al., 2015). Genomes with ≥50% completeness and <10% contamination were retained as medium-quality rumen MAGs (Bowers et al., 2017), which were then dereplicated at 99% average nucleotide identity (ANI).

5.2.6.4. *Phylogenetic and functional analyses of MAGs*

The MAGs retained from our data set were classified using GTDB-tk v.1.3.0 (Chaumeil et al., 2020). Genomes were defined as novel species if the ANI and alignment fraction determined via GTDB-tk were <95% and <0.65, respectively. For MAGs, ORFs were determined using Prodigal v.2.6.3 (Hyatt et al., 2010). The ORFs were annotated with KO using eggNOG-mapper v2.0.1 (Huerta-Cepas et al., 2017). We focused on the potential of MAGs to ferment carbohydrates into short-chain fatty acids. For acetate production, acetate kinase (*ackA*; K00925), phosphate acetyltransferase (*pta*; K00625), and acylphosphatase (*acyP*; K01512) were used as markers. Potential of propionate production was evaluated by the presence of genes annotated with methylmalonyl-CoA mutase (MUT; K01847) and methylmalonyl-CoA/ethylmalonyl-CoA epimerase (MCEE; K05606). Phosphate butyryltransferase (*ptb*; K00634) and butyrate kinase (*buk*; K00929) were regarded as potential genes involved in butyrate production. Malate dehydrogenase (*mdh*; K00024), fumarate hydratase (*fum*; K01676, K01677, and K01678), and fumarate reductase (*frd*; K00239, K00240, K00241, K00244, K00245, and K00247) were used as markers of succinate production. L-lactate dehydrogenase (*ldh*; K00016) also used as a marker for lactate production. Subsequently, CAZymes were identified for the ORFs using dbCAN2 (Zhang et al., 2018), as described above. The predicted CAZymes were annotated against the

NCBI-nr database using DIAMOND v. 0.9.23 (Buchfink et al., 2014).

5.2.7. Quantitative real-time PCR analysis

Numbers of total bacteria and cellulolytic bacteria (*Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *Ruminococcus albus*) 16S rRNA gene copies were estimated with quantitative real-time PCR (qPCR). The designed primers are shown in Table 5-4. To derive the DNA standards, PCR products that included the primer sequence for the qPCR assays were generated. All the PCR products were purified using Wizard SV Gel and PCR Clean-up System (Promega, Tokyo, Japan). Each PCR product was analyzed by electrophoresis in 2.5% agarose gel to confirm a single band. DNA sequencing of the purified PCR products was performed to confirm the specificity of the primer set. DNA concentration of the PCR products was quantified with a Qubit 4 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The DNA concentration and amplicon size were used to calculate the number of amplicon copies. Standard curves were created using 10-fold dilution series of the PCR products. The qPCR assay was carried out using TB Green™ Premix Ex Taq™ II (Takara Bio Inc.) on the Thermal Cycler Dice real-time system III (TaKaRa Bio Inc.). The amplification was performed at 95 °C for 30 s, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Data generated by the qPCR assay were $\log_{10}$-transformed before the statistical analysis.

Table 5-4. Primers for qPCR assay.

Target	Primer	Sequence(5′–3′)	Product size (bp)	Reference
General bacteria	Forward	CGGCAACGAGCGCAACCC	130	Denman and McSweeney (2006)
	Reverse	CCATTGTAGCACGTGTGTAGCC		
Fibrobacter succinogenes	Forward	GTTCGGAATTACTGGGCGTAAA	121	Denman and McSweeney (2006)
	Reverse	CGCCTGCCCCTGAACTATC		
Ruminococcus albus	Forward	CCCTAAAAGCAGTCTTAGTTCG	175	Koike and Kobayashi (2001)
	Reverse	CCTCCTTGCGGTTAGAACA		
Ruminococcus flavefaciens	Forward	TCTGGAAACGGATGGTA	295	Koike and Kobayashi (2001)
	Reverse	CCTTTAAGACAGGAGTTTACAA		

5.2.8. Statistical analyses

VFA, NH_3-N and qPCR data were analyzed using the GLM procedure in SAS v. 9.4 (SAS Institute Inc., Cary, NC). The mathematical model was as follows:

$$Y_{ij} = \mu + B_i + e_{ij}$$

where μ = overall means, B_i = breed effect, and e_{ij} = residual error.

For 16S rRNA gene sequencing analysis, a permutational multivariate analysis of variance test was performed with 9,999 permutations in the 'vegan' package of R (Oksanen et al., 2019) to identify significant differences between breeds. Statistical significance of the Shannon index and that of the observed ASVs were identified using the Wilcoxon signed-rank test in R. Differences were considered as statistically significant when $P < 0.05$ and trending when $0.05 \leq P < 0.1$.

For metagenomic analysis, significant differences between breeds in terms of relative

taxon abundance, KEGG pathways, and CAZymes were determined by linear discriminant analysis (LDA) effect size analysis (Segata et al., 2011). An LDA > 2.0 at a $P < 0.05$ was considered to indicate a significant difference. An LDA > 2.0 at $0.05 \leq P < 0.1$ was considered to indicate a trend.

5.2.9. Data availability

All the sequence data were deposited in the DDBJ database (accession number: DRA011676).

5.3. Results

5.3.1. Rumen parameters

The ruminal pH tended to be higher in JB than in F1 (P = 0.08) but the difference between breeds was not significant for the total and individual VFA or NH_3-N concentrations (Table 5-5)

Table 5-5. Rumen fermentation parameters (pH, NH_3-N, and VFA) of Japanese Black (JB) and Japanese Black × Holstein (F1) steers.

Item[1]	Breed		SEM[2]
	JB	F1	
pH	6.76[A]	6.51[B]	0.091
NH_3-N (mgN/dL)	9.65	12.2	1.23
VFA			
Total VFA (mmol/L)	79.7	89.3	5.99
Acetate (mmol/L)	45.6	49.3	2.97
Propionate (mmol/L)	19.6	24.0	3.00
iso-Butyrate (mmol/L)	1.1	1.0	0.19
n-Butyrate (mmol/L)	10.9	12.1	1.25
iso-Valerate (mmol/L)	1.6	1.9	0.18
n-Valerate (mmol/L)	0.9	1.1	0.08
Acetate (%)	57.2	55.6	1.40
Propionate (%)	24.7	26.4	2.18
iso-Butyrate (%)	1.4	1.1	0.22
n-Butyrate (%)	13.6	13.5	0.89
iso-Valerate (%)	2.0	2.2	0.22
n-Valerate (%)	1.1	1.2	0.04
Acetate:Propionate	2.4	2.2	0.21

[AB] LSMeans in a row with different superscripts significantly indicate a trend of statistical significance (P < 0.1)

[1] NH_3-N, ammonia nitrogen; VFA, volatile fatty acids

[2] SEM, standard error of means

5.3.2. Rumen bacterial diversity according to 16S rRNA gene sequencing

An average of 280,994 ± 46,773 reads per sample was obtained by 16S rRNA gene sequencing. A total of 12 rumen samples presented with 3,164 ASVs. In terms of alpha diversity, JB and F1 did not differ significantly in terms of the number of observed ASVs and Shannon diversity indices (Fig. 5-1). In terms of beta diversity, the non-metric multidimensional scaling plot revealed distinct clustering patterns separating the JB and F1 rumen microbiota, although only one JB resembled F1 (Fig. 5-2A). Permutational multivariate analysis of variance revealed a significant difference in the rumen microbial community between JB and F1 ($P < 0.05$). There were 2,496 and 2,022 ASVs in the JB and F1 rumen microbiota, respectively. There were 1,354 ASVs (42.8%) common to JB and F1, but 1,142 (36.1%) and 668 (21.1%) ASVs were uniquely identified in JB and F1, respectively (Fig. 5-2B). In taxonomic analysis according to 16S rRNA gene sequencing, *Prevotella_1* was predominant in the JB and F1 rumen microbiota (Table 5-6).

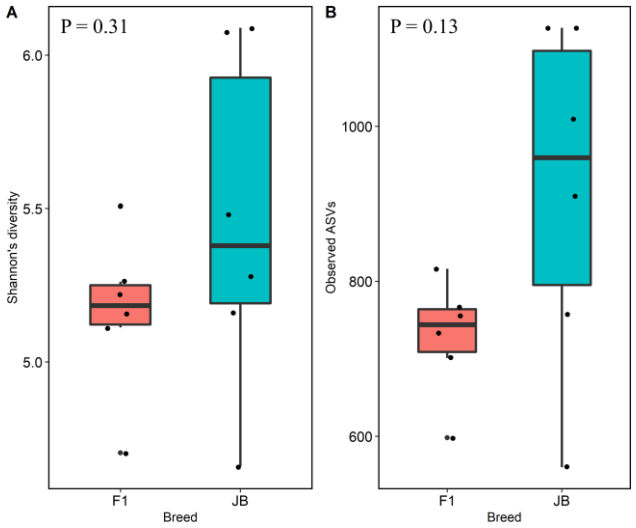

Fig. 5-1 Alpha diversity in the rumen microbiome of Japanese Black (JB) and Japanese Black × Holstein crossbred (F1) steers according to 16S rRNA gene amplicon sequencing. Boxplots shows (A) Shannon diversity indices and (B) observed amplicon sequence variants (ASVs). Statistics were performed by Wilcoxon signed-rank test.

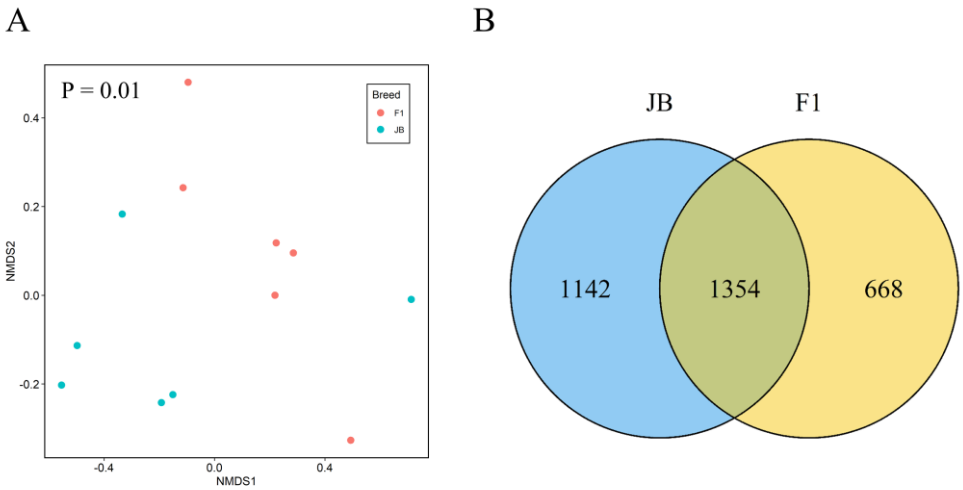

Fig. 5-2 Rumen microbiota diversity in Japanese Black (JB) and Japanese Black × Holstein crossbred (F1) steers using 16S rRNA gene amplicon sequencing. (A) Non-metric multidimensional scaling (NMDS) plots based on Bray-Curtis dissimilarities in ASVs. (B) Venn diagram displaying numbers of ASVs common to or not shared by JB and F1 steers

Table 5-6. Relative abundance (%) of ASVs in the rumen microbiome of JB and F1 steers (mean ± SE) according to 16S rRNA gene sequencing

ASV ID[1]	Relative abundance (%)		Taxonomy					
	JB	F1	Domain	Phylum	Class	Order	Family	Genus
ASV1	1.24±0.407	4.34±1.718	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	NA
ASV2	2.87±2.248	2.65±1.695	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella 1
ASV3	0.40±0.279	3.59±1.367	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	NA
ASV4	3.12±1.871	0.61±0.391	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella 1
ASV5	0.79±0.404	2.38±0.496	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella 1
ASV6	1.12±0.317	1.99±0.387	Bacteria	Firmicutes	Negativicutes	Selenomonadales	Acidaminococcaceae	Succiniclasticum
ASV7	1.33±0.318	1.49±0.284	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	[Eubacterium] coprostanoligenes group
ASV8	0.18±0.104	2.18±0.418	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella 1
ASV9	1.18±0.321	1.13±0.194	Bacteria	Firmicutes	Negativicutes	Selenomonadales	Acidaminococcaceae	Succiniclasticum
ASV10	2.05±2.052	ND	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella 1
ASV11	1.11±0.571	0.90±0.643	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella 1
ASV12	ND	1.83±0.974	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella 1
ASV13	1.67±1.015	0.04±0.012	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella 1
ASV14	0.45±0.158	1.20±0.220	Archaea	Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter
ASV15	0.32±0.110	1.23±0.397	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella 1
ASV16	1.50±0.931	0.00±0.001	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella 1
ASV17	0.25±0.111	1.15±0.349	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella 1
ASV18	1.04±0.973	0.28±0.199	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella 1
ASV19	ND	1.30±1.295	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotellaceae UCG-001
ASV20	0.05±0.039	1.09±1.024	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella 1
ASV21	0.03±0.015	1.05±0.893	Bacteria	Proteobacteria	Gammaproteobacteria	Aeromonadales	Succinivibrionaceae	Succinivibrio

[1] ASVs identified with > 1% at least in more than one breed.
ND, Not detected
NA, Not assign

5.3.3. Metagenome sequencing data analysis

After quality control, 463.09 M reads were generated via metagenome sequencing, with a mean ± SD of 38.59 ± 6.73 M per sample. The ORF predictions revealed 5,641,802 nonredundant genes, of which 887,444 and 448,869 were unique to JB and F1, respectively. There were 2,225,623 (39.4%), 1,320,735 (23.4%), and 38,920 (0.69%) genes mapped to the taxonomy at the domain level, KEGG pathway, and CAZyme, respectively.

5.3.4. Rumen microbiome taxonomic composition according to metagenome sequencing data analysis and qPCR assay

A total of 14 bacterial phyla and one archaea phylum were identified in the rumen samples. The predominant bacterial phyla were Bacteroidetes (JB, 46.7 ± 15.70%; F1, 55.2 ± 15.61%) and Firmicutes (JB, 38.3 ± 12.70%; F1, 28.4 ± 13.10%) (Fig. 5-3; Table 5-7). As with 16S rRNA gene sequencing, bacteria belonging to the genus *Prevotella* were dominant. The dominant bacterial species were *Prevotella ruminicola* (JB, 6.08 ± 0.835%; F1, 7.08 ± 0.515%), followed by *Prevotella* sp. ne3005 (JB, 5.28 ± 0.907%; F1, 4.87 ± 0.763%), *Prevotella* sp. tc2-28 (JB, 5.11 ± 2.422%; F1, 4.75 ± 0.775%), and *Prevotella* sp. tf2-5 (JB, 3.27 ± 0.601%; F1, 5.64 ± 1.163%) (Fig. 5-4). Regarding differential comparison analysis (Fig. 5-5), *Eubacterium ruminantium*, *Fibrobacter succinogenes*, two *Ruminococcus* spp., five unclassified genera of *Ruminococcaceae*, three *Treponema* spp., *Butyrivibrio* sp. AE2032, and *Lachnospiraceae* bacterium NK4A179 were significantly more abundant in the JB rumen than in the F1 rumen (LDA > 2.0 and $P < 0.05$). A total of two *Ruminococcus* spp., and seven unclassified genera of *Lachnospiraceae* tended to be more abundant in the JB than in the F1 rumen (LDA > 2.0 and $P < 0.1$). In contrast, 12 *Prevotella* spp. were more abundant in the F1 than in the JB rumen (LDA > 2.0 and $P < 0.05$). A total of seven *Prevotella* spp. tended to be more abundant in the F1 than in the JB rumen (LDA > 2.0 and $P < 0.1$).

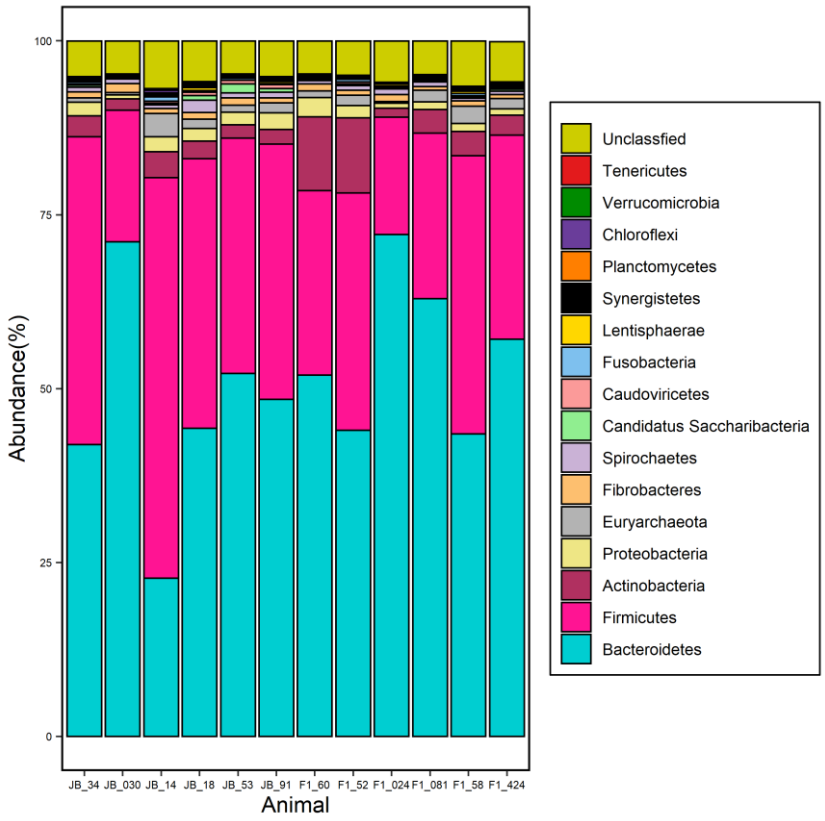

Fig. 5-3 The rumen microbial composition profiles in Japanese Black (JB) and Japanese Black × Holstein crossbred (F1) steers at phylum level using metagenome shotgun sequencing.

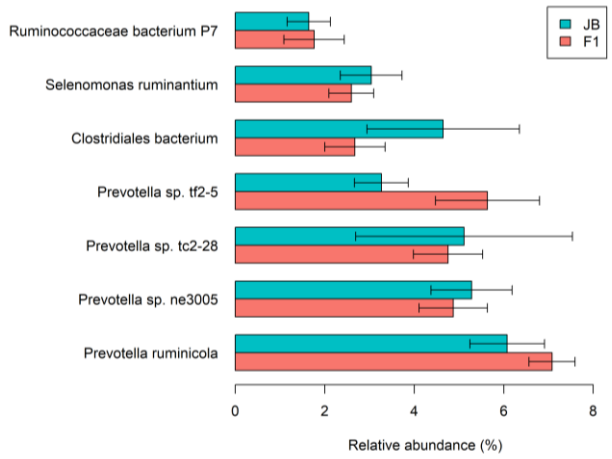

Fig. 5-4 Relative abundance (%) of predominant species in the rumen microbiome of Japanese Black (JB) and Japanese Black × Holstein crossbred (F1) steers according to shotgun metagenome sequencing. Data are presented as mean ± SE (n = 6 per group).

Table 5-7. Relative abundance (%) of each taxon at phylum level in the rumen microbiome of JB and F1 steers (mean ± SE) according to metagenome sequencing data analysis

Phylum	Relative abundance (%)		Breed[1]	LDA score (log10)[2]	P-value[3]
	JB	F1			
Euryarchaeota	1.33 ± 1.058	1.37 ± 1.018	-	N.A.	0.52
Bacteroidetes	46.7 ± 15.70	55.2 ± 15.61	-	N.A.	0.34
Firmicutes	38.3 ± 12.70	28.4 ± 13.10	-	N.A.	0.15
Actinobacteria	2.49 ± 0.765	5.40 ± 3.420	-	N.A.	0.20
Proteobacteria	1.78 ± 0.644	1.40 ± 0.761	-	N.A.	0.26
Fibrobacteres	0.94 ± 0.213	0.76 ± 0.211	-	N.A.	0.20
Spirochaetes	0.85 ± 0.455	0.57 ± 0.479	-	N.A.	0.26
Fusobacteria	0.28 ± 0.166	0.24 ± 0.172	-	N.A.	1.00
Candidatus Saccharibacteria	0.54 ± 0.405	0.20 ± 0.432	JB	3.30	0.02
Synergistetes	0.17 ± 0.082	0.14 ± 0.086	-	N.A.	0.63
Lentisphaerae	0.21 ± 0.124	0.17 ± 0.126	-	N.A.	0.52
Planctomycetes	0.12 ± 0.053	0.06 ± 0.062	JB	3.28	0.08
Verrucomicrobia	0.11 ± 0.050	0.10 ± 0.052	-	N.A.	0.87
Chloroflexi	0.12 ± 0.132	0.07 ± 0.135	-	N.A.	1.00
Tenericutes	0.09 ± 0.056	0.08 ± 0.061	-	N.A.	0.75
Caudoviricetes	0.34 ± 0.186	0.15 ± 0.214	JB	3.08	0.05
Unclassfied	5.41 ± 0.814	5.46 ± 0.858	-	N.A.	1.00

LDA, linear discriminant analysis

N.A., not analyzed because P-value was > 0.1.

[1]Breed with higher abundance (LDA score > 2.0 and P-value < 0.1)

[2]LDA was performed with LEfSe (Segata et al., 2011).

[3]Kruskall-Wallis test

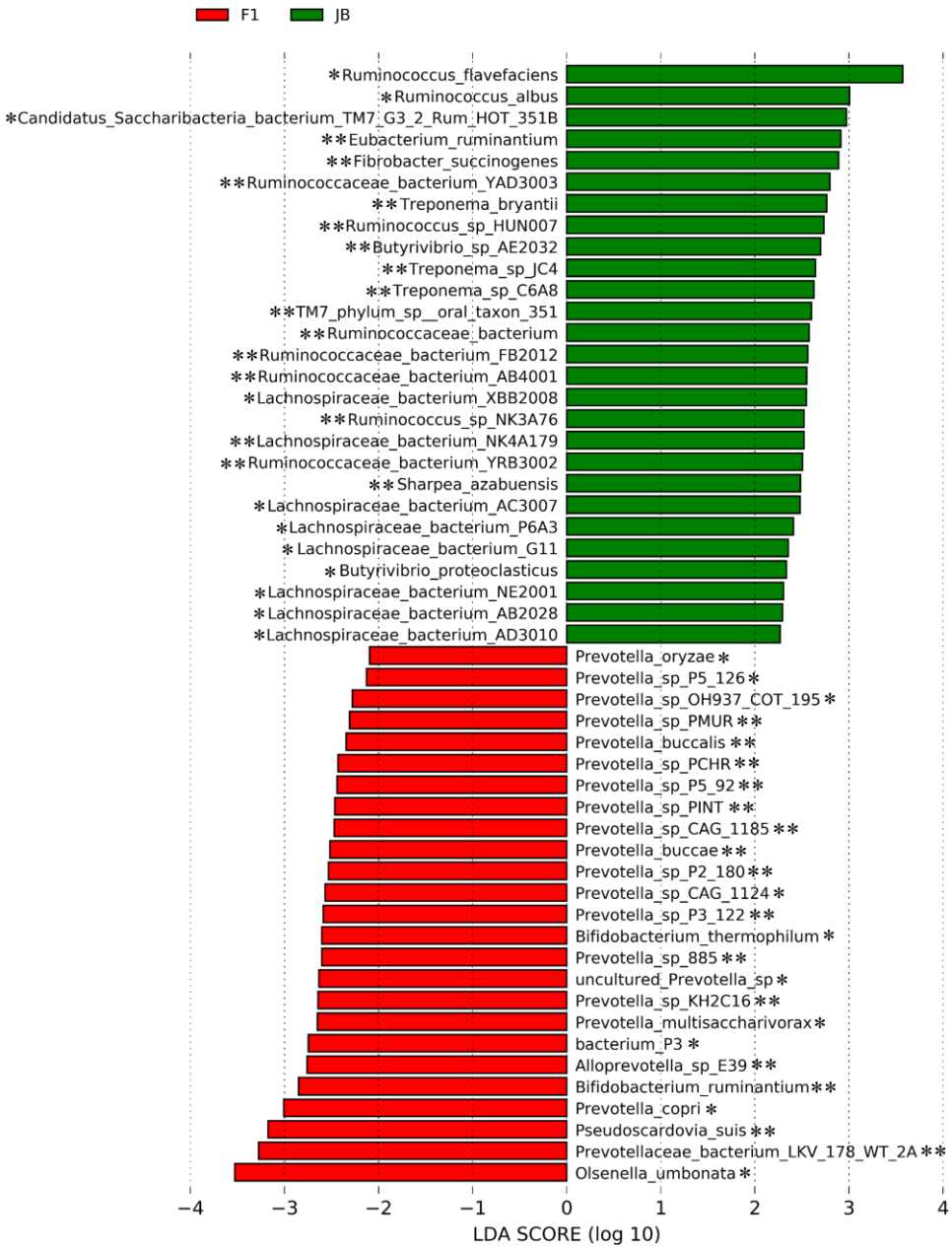

Fig. 5-5 Differences in relative abundances of bacterial species between Japanese Black (JB) and Japanese Black × Holstein crossbred (F1) steers using metagenome shotgun sequencing. Significantly different bacterial species between JB and F1 steers identified by linear discriminant analysis (LDA) effect size (LEfSe) analysis (** LDA > 2.0 and P < 0.05; * LDA > 2.0 and P < 0.1).

The results of qPCR assays are shown in Table 5-8. No significant difference was observed for the 16S rRNA gene copy number of total bacteria between JB and F1 (JB vs. F1, 9.80 vs. 9.81 $\log_{10}$ copies/mL; P = 0.87). The 16S rRNA gene copy number of *F. succinogenes* was significantly higher in the JB rumen than in the F1 rumen (JB vs. F1, 7.47 vs. 6.82 $\log_{10}$ copies/mL; P = 0.02). However, there were no significant differences in 16S rRNA gene copy numbers of *R. albus* (JB vs. F1, 7.61 vs. 7.47 $\log_{10}$ copies/mL; P = 0.49) and *R. flavefaciens* (JB vs. F1, 7.87 vs. 7.54 $\log_{10}$ copies/mL; P = 0.13) between JB and F1 although those in the JB rumen were numerically higher than those in the F1 rumen.

Table 5-8. Copy numbers of bacterial 16S rRNA genes (Log_{10} copies/mL) in the rumen of Japanese Black (JB) and Japanese Black × Holstein (F1) steers.

Target	Breed		SEM[1]	P-value
	JB	F1		
Total bacteria	9.80	9.81	0.053	0.87
Ruminococcus albus	7.61	7.47	0.139	0.49
Ruminococcus flavefaciens	7.87	7.54	0.142	0.13
Fibrobacter succinogenes	7.47[a]	6.82[b]	0.164	0.02

[ab] LSMeans with different superscripts within same row significantly differed (P < 0.05)

[1] SEM, standard error of means

5.3.5. Functional rumen microbiome profiles according to metagenome sequencing data analysis

Although JB and F1 showed differences in the relative abundances of several bacteria, the function profiles (KEGG level 2) were more constant and less diverse compared to the taxonomic composition (Fig. 5-6). According to KEGG pathway analysis (Table 5-9), “purine metabolism” (ko00230) and “pyrimidine metabolism” (ko00240) were more abundant in JB than in F1 (LDA > 2.0 and P < 0.05). Three pathways enriched in JB, including “mismatch repair” (ko03430), “homologous recombination” (ko03440), and “RNA polymerase” (ko03020) were associated with the “genetic information processing” category (LDA > 2.0 and P < 0.05). In contrast, 11 pathways were comparatively enriched in F1. Most of these pathways, including “lysine biosynthesis” (ko00300) “terpenoid backbone biosynthesis” (ko00900), “phenylpropanoid biosynthesis” (ko00940) “biosynthesis of secondary metabolites” (ko01110), were associated with the “metabolism” category (LDA > 2.0 and P < 0.05). “Carbohydrate digestion and absorption” (ko04973) was relatively more abundant in F1 (LDA > 2.0 and P < 0.05).

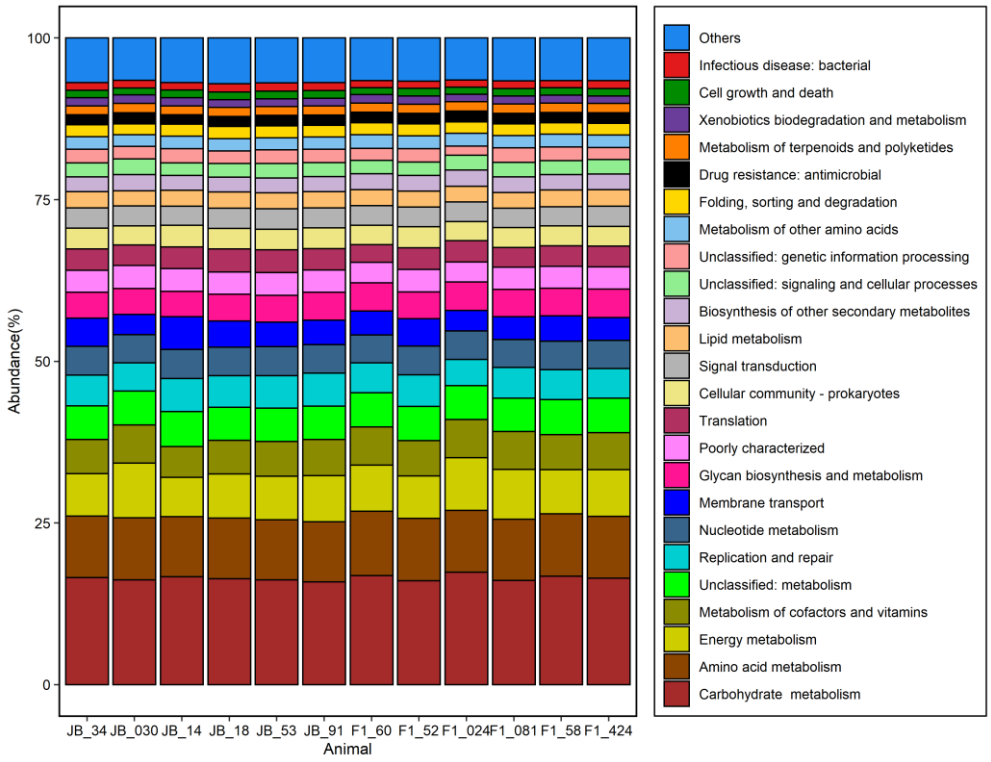

Fig. 5-6 Functional compositions of rumen microbiomes in Japanese Black (JB) and Japanese Black × Holstein crossbred (F1) steers using metagenome shotgun sequencing. Relative KEGG function abundances at level 2.

Table 5-9. Differential KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways between the rumen microbiome of Japanese Black (JB) and Japanese Black × Holstein crossbred (F1) steers.

	Relative abundance (%)[1]			LDA score	
KEGG pathway	JB	F1	Breed[2]	(log10)[3]	P-value[4]
Ribosome	1.37 ± 0.044	1.22 ± 0.063	JB	2.88	0.078
Pyrimidine metabolism	2.18 ± 0.018	2.08 ± 0.023	JB	2.72	0.004
Purine metabolism	2.33 ± 0.035	2.22 ± 0.023	JB	2.71	0.037
Mismatch repair	0.90 ± 0.017	0.84 ± 0.020	JB	2.51	0.037
Homologous recombination	1.05 ± 0.016	0.99 ± 0.027	JB	2.47	0.078
Bacterial secretion system	0.62 ± 0.015	0.59 ± 0.007	JB	2.20	0.078
Bacterial chemotaxis	0.33 ± 0.019	0.30 ± 0.011	JB	2.14	0.055
RNA polymerase	0.24 ± 0.008	0.21 ± 0.005	JB	2.09	0.025
Longevity regulation pathway worm	0.19 ± 0.003	0.16 ± 0.004	JB	2.08	0.004
Cyanoamino acid metabolism	0.30 ± 0.006	0.33 ± 0.007	F1	2.06	0.055
Carbohydrate digestion and absorption	0.12 ± 0.004	0.15 ± 0.007	F1	2.07	0.037
Amino sugar and nucleotide sugar metabolism	1.47 ± 0.008	1.49 ± 0.010	F1	2.10	0.055
Terpenoid backbone biosynthesis	0.38 ± 0.007	0.41 ± 0.004	F1	2.11	0.016
Phenylpropanoid biosynthesis	0.23 ± 0.003	0.25 ± 0.007	F1	2.13	0.010
Lysine biosynthesis	0.52 ± 0.005	0.55 ± 0.009	F1	2.19	0.025
Phenylalanine, tyrosine and tryptophan biosynthesis	0.66 ± 0.028	0.70 ± 0.008	F1	2.26	0.055
Other glycan degradation	0.45 ± 0.008	0.49 ± 0.017	F1	2.30	0.078
Biosynthesis of antibiotics	5.25 ± 0.035	5.35 ± 0.024	F1	2.69	0.025
Biosynthesis of secondary metabolites	6.55 ± 0.047	6.75 ± 0.033	F1	2.97	0.025
Metabolic pathways	16.09 ± 0.109	16.33 ± 0.053	F1	3.03	0.055

LDA, linear discriminant analysis

[1]mean ± SE

[2]Breed with higher pathway abundance

[3]LDA was performed with LEfSe (Segata et al., 2011).

[4]Kruskall-Wallis test

JB and F1 showed differences in several taxa related to carbohydrate degradation in the rumen. Therefore, we analyzed the CAZyme profiles to speculate the enzymes participating in lignocellulose degradation. The CAZyme profiles comprised GHs (JB, 63.2 ± 0.73%; F1, 63.8 ± 1.56%), GTs (JB, 19.9 ± 0.48%; F1, 19.1 ± 1.70%), CEs (JB,8.1 ± 0.35%; F1, 8.0 ± 0.68%), CBMs (JB, 6.7 ± 0.20%; F1, 6.7 ± 0.61%), and PLs (JB, 2.1 ± 0.54%; F1, 2.3 ± 0.52%) (Fig. 5-7A). No genes encoding auxiliary activities were detected in the JB or F1 rumen microbiome. There were 197 CAZymes, including 36 CBMs, 13 CEs, 104 GHs, 27 GTs, and 17 PLs, in the 12 rumen samples. Phylogenetic analysis of the CAZymes revealed that the genus *Prevotella* was the main contributor to all CAZyme categories (Fig. 5-7B), including the GHs (JB, 53.1 ± 6.51%; F1, 53.1 ± 3.41%), GTs (JB, 45.9 ± 6.03%; F1, 47.0 ± 3.22%), CEs (JB, 55.5 ± 5.95%; F1, 60.0 ± 3.06%), PLs (JB, 59.2 ± 3.59%; F1, 65.4 ± 1.80%), and CBMs (JB, 54.0 ± 6.07%; F1, 53.1 ± 4.22%).

A total of seven CAZymes (CBM2, CBM3, CBM22, CE3, GH11, GT0, and PL27) were enriched in JB, whereas three (CBM25, GH76, and PL11) were enriched in F1 (Fig. 5-7C, LDA > 2.0 and $P < 0.05$). In total, two CAZymes (GT32 and CE14) tended to be relatively more abundant in JB and four CAZymes (GH95, GH28, GH51, and PL33) tended to be comparatively richer in F1 (Fig. 5-7C, LDA > 2.0 and $P < 0.1$). *Ruminococcus* spp. contributed most of the GH11, CBM22, CBM3, CBM2, and CE3 CAZymes, whereas *Prevotella* spp. were the main contributors of PL27, GH76, PL33, PL11, GH95, GH28, and GH51 (Fig. 5-7D). *Bifidobacterium* spp. were associated primarily with CBM25.

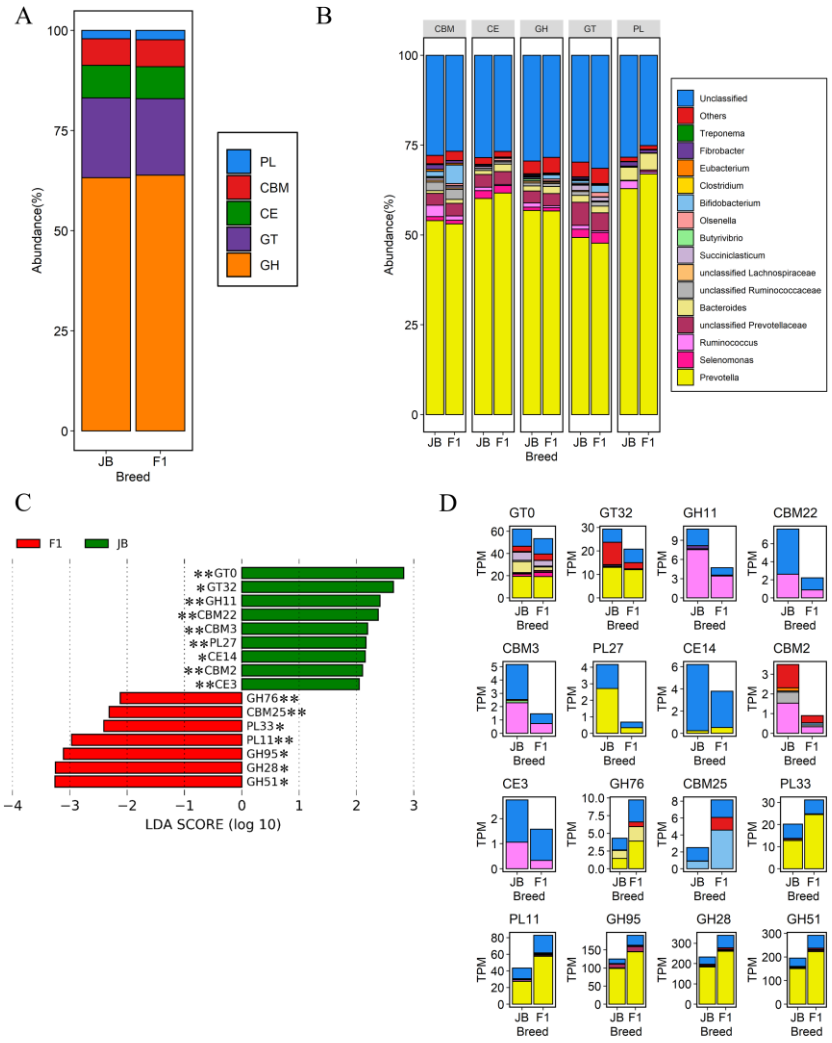

Fig. 5-7 Carbohydrate-active enzyme (CAZyme) compositions of rumen microbiomes in Japanese Black (JB) and Japanese Black × Holstein crossbred (F1) steers using metagenome shotgun sequencing. (A) Relative abundances of CAZymes including glycoside hydrolases (GHs), glycosyltransferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs), and carbohydrate-binding modules (CBMs). (B) Major CAZyme contributors among predominant rumen microbial taxa at genus level. (C) Differences in CAZyme activity between JB and F1 steers tested by linear discriminant analysis (LDA) effect size (LEfSe) analysis (** LDA > 2.0 and P < 0.05; * LDA > 2.0 and P < 0.1). (D) Major contributors to differential CAZyme activity between JB and F1.

5.3.6. Metagenome-assembled genome reconstruction and taxonomic classification

To obtain phylogenetic and functional information on abundant species observed in the JB and F1 rumen, we performed metagenome-assembled genome analysis. In total, 140 MAGs with completeness ≥50% and contamination <10% were generated. Among them, we obtained 114 final dereplicated MAGs (99% ≥ ANI), including 61 and 53 MAGs reconstructed from JB and F1 data, respectively. All 114 MAGs (113 bacterial and one archaeal MAGs) were assigned to the genus level, and only 59 MAGs were classified at the species level using the GTDB-tk (Table 5-10). A total of 54 MAGs (47.4%) were classified as members of the phylum Bacteroidota (Table 5-10). Among them, 27 MAGs (14 JB MAGs and 13 F1 MAGs) were taxonomically annotated to the genus *Prevotella*, which was the predominant taxon according to 16S rRNA gene sequencing.

Table 5-10. Taxonomic classification of Metagenome-Assembled Genomes (MAGs)

Bins ID	GTDB classification						
	Domain	Phylum	Class	Order	Family	Gunus	Specie
F1_MAG1	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Oscillospiraceae	UBA1777	
F1_MAG2	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	UBA932	RC9	
F1_MAG3	Bacteria	Firmicutes_C	Negativicutes	Acidaminococcales	Acidaminococcaceae	Succiniclasticum	Succiniclasticum sp900315925
F1_MAG4	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Acutalibacteraceae	Ruminococcus_E	Ruminococcus_E sp900316555
F1_MAG5	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Acutalibacteraceae	RUG420	RUG420 sp900317085
F1_MAG6	Bacteria	Spirochaetota	Spirochaetia	Sphaerochaetales	Sphaerochaetaceae	UBA9732	
F1_MAG7	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Oscillospiraceae	UBA1777	
F1_MAG8	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	
F1_MAG9	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp900316645
F1_MAG10	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	UBA4372	
F1_MAG11	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Paludibacteraceae	RF16	RF16 sp900317745
F1_MAG12	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp900314915
F1_MAG13	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	UBA4372	
F1_MAG14	Bacteria	Firmicutes_C	Negativicutes	Selenomonadales	Selenomonadaceae	UBA3796	
F1_MAG15	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	
F1_MAG16	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Paludibacteraceae	RF16	RF16 sp900316005
F1_MAG17	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	
F1_MAG18	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp900316265
F1_MAG19	Bacteria	Actinobacteriota	Actinomycetia	Actinomycetales	Bifidobacteriaceae	RUG039	RUG039 sp900314875
F1_MAG20	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	UBA1179	UBA1179 sp900319225
JB_MAG21	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	

JB_MAG22	Bacteria	Firmicutes_A	Clostridia	Saccharofermentanales	Saccharofermentanaceae	Saccharofermentans	
JB_MAG23	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp900316295
JB_MAG24	Bacteria	Firmicutes_C	Negativicutes	Acidaminococcales	Acidaminococcaceae	Succiniclasticum	Succiniclasticum sp900315925
JB_MAG25	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp900320255
JB_MAG26	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	
JB_MAG27	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp002392645
JB_MAG28	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp900316475
JB_MAG29	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Acutalibacteraceae	Ruminococcus_E	Ruminococcus_E sp900314795
JB_MAG30	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Ruminococcaceae	Ruminococcus_D	
JB_MAG31	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Ruminococcaceae	Ruminococcus	
JB_MAG32	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Acutalibacteraceae	Ruminococcus_E	
JB_MAG33	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Oscillospiraceae	UBA1777	UBA1777 sp900320595
JB_MAG34	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Paludibacteraceae	RUG163	RUG163 sp900316995
JB_MAG35	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	UBA932	RC9	
JB_MAG36	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	C941	C941 sp900320245
JB_MAG37	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	UBA4372	UBA4372 sp900313705
JB_MAG38	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	UBA932	RC9	
JB_MAG39	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Oscillospiraceae	UBA2922	
JB_MAG40	Bacteria	Firmicutes_C	Negativicutes	Selenomonadales	Selenomonadaceae	RUG643	RUG643 sp900321035
JB_MAG41	Bacteria	Verrucomicrobiota	Kiritimatiellae	RFP12	UBA3636	UBA3636	UBA3636 sp002448475
JB_MAG42	Bacteria	Firmicutes_C	Negativicutes	Acidaminococcales	Acidaminococcaceae	Succiniclasticum	Succiniclasticum sp900315925
JB_MAG43	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Paludibacteraceae	RF16	RF16 sp900320865
JB_MAG44	Bacteria	Firmicutes	Bacilli	RFN20	CAG-826	UBA733	

JB_MAG45	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	C941	
JB_MAG46	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	
JB_MAG47	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	
JB_MAG48	Bacteria	Firmicutes_C	Negativicutes	Acidaminococcales	Acidaminococcaceae	Succiniclasticum	Succiniclasticum sp900315925
JB_MAG49	Bacteria	Firmicutes_C	Negativicutes	Selenomonadales	Selenomonadaceae	UBA3796	
JB_MAG50	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	C941	C941 sp900320055
JB_MAG51	Bacteria	Spirochaetota	Spirochaetia	Treponematales	Treponemataceae	Treponema_D	
JB_MAG52	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Oscillospiraceae	CAG-103	
F1_MAG53	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp900316645
F1_MAG54	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Acutalibacteraceae	Ruminococcus_E	
F1_MAG55	Archaea	Methanobacteriota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	Methanobrevibacter_A	Methanobrevibacter_A millerae
F1_MAG56	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	P3	UBA1711	UBA1711 sp900316235
F1_MAG57	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Oscillospiraceae	UBA1777	
F1_MAG58	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	UBA932	RC9	RC9 sp900321655
F1_MAG59	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp900315775
F1_MAG60	Bacteria	Firmicutes_A	Clostridia	Lachnospirales	Lachnospiraceae	Stomatobaculum	
F1_MAG61	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp900319305
F1_MAG62	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	UBA4372	
F1_MAG63	Bacteria	Firmicutes_C	Negativicutes	Acidaminococcales	Acidaminococcaceae	Succiniclasticum	Succiniclasticum sp900315925
F1_MAG64	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	UBA932	RC9	RC9 sp002354005
F1_MAG65	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Acutalibacteraceae	Ruminococcus_E	
F1_MAG66	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Acutalibacteraceae	Ruminococcus_E	Ruminococcus_E sp900319655

F1_MAG67	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Paludibacteraceae	RF16	RF16 sp900319005
F1_MAG68	Bacteria	Actinobacteriota	Actinomycetia	Actinomycetales	Bifidobacteriaceae	Bifidobacterium	Bifidobacterium merycicum
F1_MAG69	Bacteria	Firmicutes_A	Clostridia_A	Christensenellales	CAG-74	UBA4263	UBA4263 sp002395225
F1_MAG70	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp900318625
F1_MAG71	Bacteria	Actinobacteriota	Coriobacteriia	Coriobacteriales	Atopobiaceae	Olsenella	
F1_MAG72	Bacteria	Firmicutes_C	Negativicutes	Acidaminococcales	Acidaminococcaceae	Succiniclasticum	Succiniclasticum sp900315925
F1_MAG73	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp900315525
JB_MAG74	Bacteria	Firmicutes_C	Negativicutes	Selenomonadales	Selenomonadaceae	UBA3796	
JB_MAG75	Bacteria	Patescibacteria	Saccharimonadia	Saccharimonadales	Saccharimonadaceae	UBA2834	
JB_MAG76	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Acutalibacteraceae	Ruminococcus_E	Ruminococcus_E sp900319655
JB_MAG77	Bacteria	Firmicutes_C	Negativicutes	Selenomonadales	Selenomonadaceae	RUG643	
JB_MAG78	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Paludibacteraceae	RF16	
JB_MAG79	Bacteria	Patescibacteria	Saccharimonadia	Saccharimonadales	Saccharimonadaceae	UBA2866	
JB_MAG80	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	C941	
JB_MAG81	Bacteria	Firmicutes_C	Negativicutes	Acidaminococcales	Acidaminococcaceae	Succiniclasticum	Succiniclasticum sp900315925
JB_MAG82	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp002354095
JB_MAG83	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	
JB_MAG84	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Muribaculaceae	C941	
JB_MAG85	Bacteria	Firmicutes_A	Clostridia	Lachnospirales	Lachnospiraceae	UBA2868	
JB_MAG86	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp900316265
JB_MAG87	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	UBA4372	
JB_MAG88	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	

JB_MAG89	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	UBA4334	UBA4334 sp900316505
JB_MAG90	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Acutalibacteraceae	Ruminococcus_E	
JB_MAG91	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Acutalibacteraceae	Ruminococcus_E	
F1_MAG92	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Oscillospiraceae	UBA1777	UBA1777 sp900320595
F1_MAG93	Bacteria	Actinobacteriota	Coriobacteriia	Coriobacteriales	Atopobiaceae	Olsenella	Olsenella sp900313175
F1_MAG94	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Acutalibacteraceae	Ruminococcus_E	
F1_MAG95	Bacteria	Firmicutes_A	Clostridia_A	Christensenellales	CAG-74	GCA-900199385	
F1_MAG96	Bacteria	Firmicutes_C	Negativicutes	Acidaminococcales	Acidaminococcaceae	Succiniclasticum	Succiniclasticum sp900315925
F1_MAG97	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Acutalibacteraceae	Ruminococcus_E	Ruminococcus_E sp900316555
F1_MAG98	Bacteria	Actinobacteriota	Actinomycetia	Actinomycetales	Bifidobacteriaceae	Pseudoscardovia	Pseudoscardovia suis
F1_MAG99	Bacteria	Actinobacteriota	Coriobacteriia	Coriobacteriales	Atopobiaceae	Olegusella	Olegusella sp900314685
F1_MAG100	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	
F1_MAG101	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	
F1_MAG102	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Acutalibacteraceae	Ruminococcus_E	Ruminococcus_E sp900319655
F1_MAG103	Bacteria	Firmicutes_C	Negativicutes	Acidaminococcales	Acidaminococcaceae	Succiniclasticum	Succiniclasticum sp900315925
JB_MAG104	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	UBA932	RC9	
JB_MAG105	Bacteria	Firmicutes_A	Clostridia	Lachnospirales	Lachnospiraceae	UBA2868	UBA2868 sp900320345
JB_MAG106	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	
JB_MAG107	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Paludibacteraceae	RF16	RF16 sp900320865
JB_MAG108	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	UBA932	RC9	RC9 sp002354005
JB_MAG109	Bacteria	Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	Prevotella	Prevotella sp900315035
JB_MAG110	Bacteria	Spirochaetota	Spirochaetia	Treponematales	Treponemataceae	Treponema_D	

JB_MAG111	Bacteria	Firmicutes_A	Clostridia	Oscillospirales	Oscillospiraceae	UBA1777	
JB_MAG112	Bacteria	Firmicutes_C	Negativicutes	Acidaminococcales	Acidaminococcaceae	Succiniclasticum	Succiniclasticum sp900315925
JB_MAG113	Bacteria	Verrucomicrobiota	Kiritimatiellae	RFP12	UBA1067	UBA1067	
JB_MAG114	Bacteria	Firmicutes_C	Negativicutes	Selenomonadales	Selenomonadaceae	RUG643	

5.3.7. Functional analysis of the rumen MAGs

For functional analysis based on KOs found in the rumen MAGs, the rumen MAGs were clearly clustered according to their taxonomy at the phylum level (Fig. 5-8). Of the three genes related to acetate production, *ackA* and *pta* were found in many MAGs, and *acyP* was detected in Actinobacteriota, Firmicutes_A, and Firmicutes_C (Fig. 5-9). Most Bacteroidota and Firmicutes_C harbored genes encoding MUT and MCEE, which are involved in propionate production. Genes associated with butyrate (*ptb* and *buk*) and succinate (*mdh*, *fum* and *frd*) production were detected in most MAGs belonging to Bacteroidota, whereas the lactate production gene *ldh* was not detected in these MAGs.

As we observed that CAZymes differed between the JB and F1 rumen microbiomes, as mentioned above, we investigated the genes encoding CAZymes of MAGs. Of the 7,425 CAZymes detected, only 489 (6.59%) showed a highly similar match (≥95%) with proteins in the NCBI-nr database (Fig. 5-10). The nine CAZymes enriched in the JB microbiome, except for GT0 and GT32, were detected only in JB MAGs (Fig. 5-11). JB_MAG31, which was taxonomically closest to *R. flavefaciens* FD-1 (GCA_000174895.1), contained genes encoding CBM3, CBM22, GH11, and CE3. Carbohydrate-binding module 2 was found in JB_MAG22, JB_MAG30, and JB_MAG105. Highly diverse CAZyme families were detected in MAGs belonging to the genus *Prevotella*. There were differences in some CAZymes in *Prevotella* MAGs between JB and F1. For example, 11 JB *Prevotella* MAGs (78.5%) harbored the genes encoding GH67 (α-glucuronidase), whereas only five F1 *Prevotella* MAGs (38.5%) had these genes.

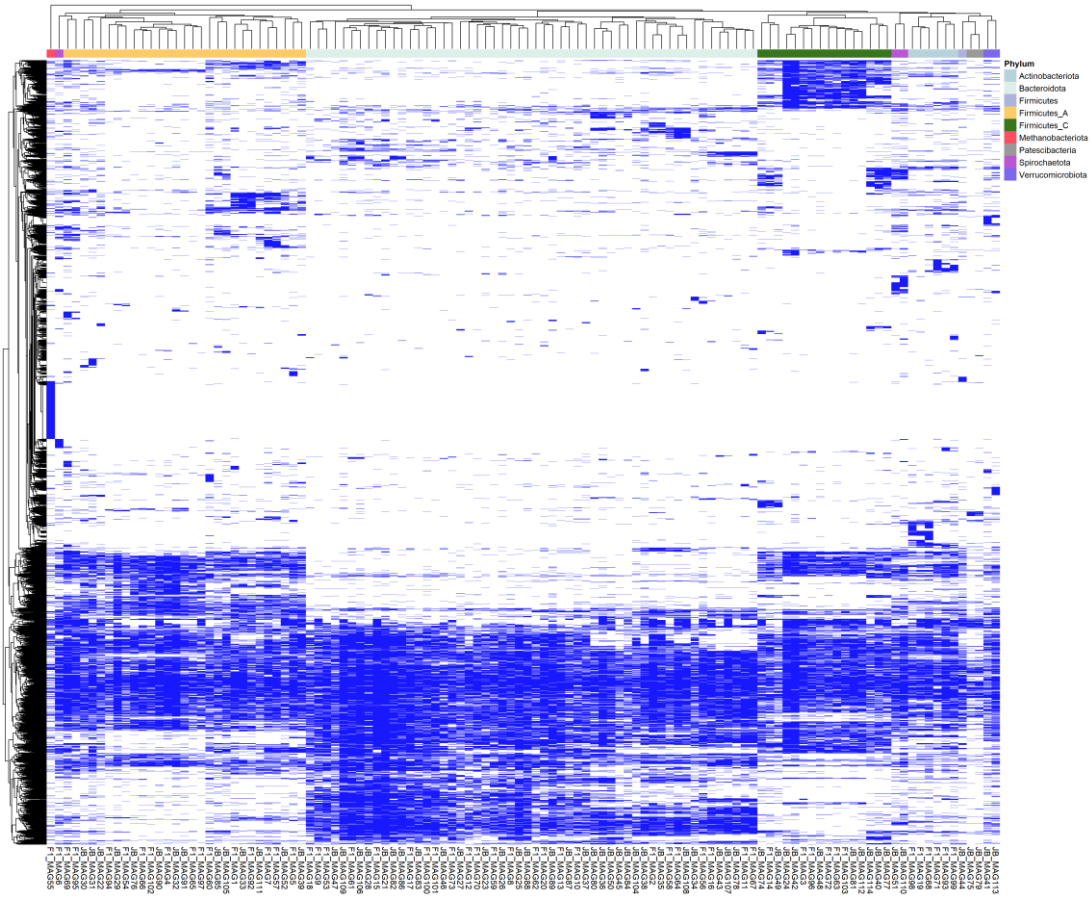

Fig. 5-8 Binary heatmap of KEGG orthology (KO) found in the rumen Metagenomic-Assembled Genomes (MAGs). Each row indicates one KO and each column represents one MAG. Hierarchical clustering was performed on MAGs using Euclidean distance and complete linkage clustering. Presence is depicted in blue, and absence is depicted in white.

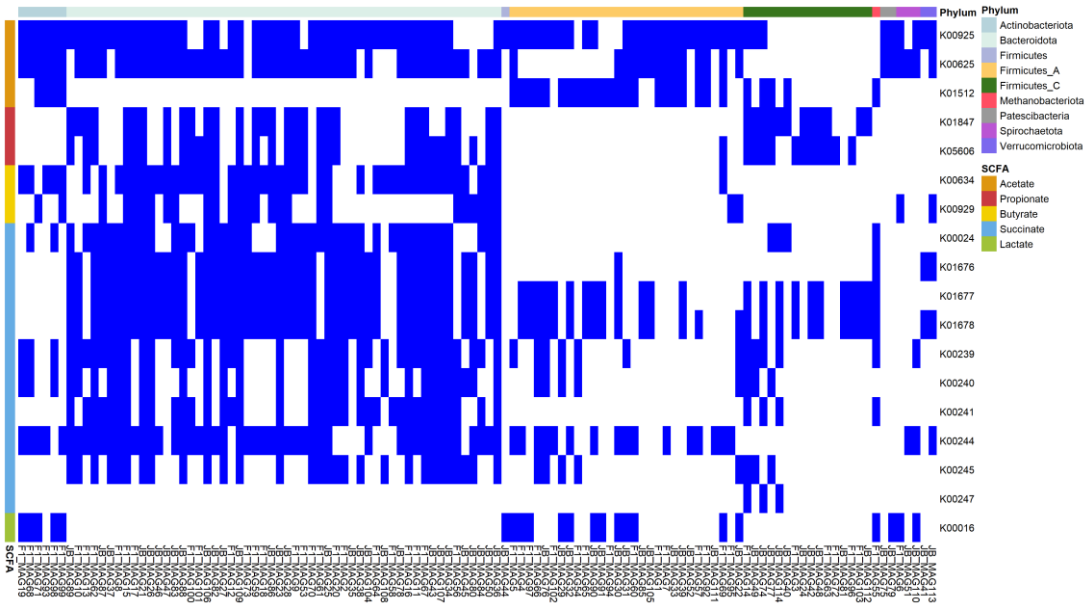

Fig. 5-9 Binary heatmap of KEGG orthology (KO) related to short chain fatty acids (SCFAs) production in the rumen Metagenomic-Assembled Genomes (MAGs). Each row indicates one KO and each column represents one MAG. Presence is depicted in blue, and absence is depicted in white. K00925, acetate kinase; K00625, phosphate acetyltransferase; K01512, acylphosphatase; K01847, methylmalonyl-CoA mutase; K05606, methylmalonyl-CoA/ethylmalonyl-CoA epimerase; K00634, phosphate butyryltransferase; K00929, butyrate kinase; K00024, malate dehydrogenase; K01676, K01677 and K01678, fumarate hydratase; K00239, K00240, K00241, K00244, K00245 and K00247, fumarate reductase; K00016, L-lactate dehydrogenase.

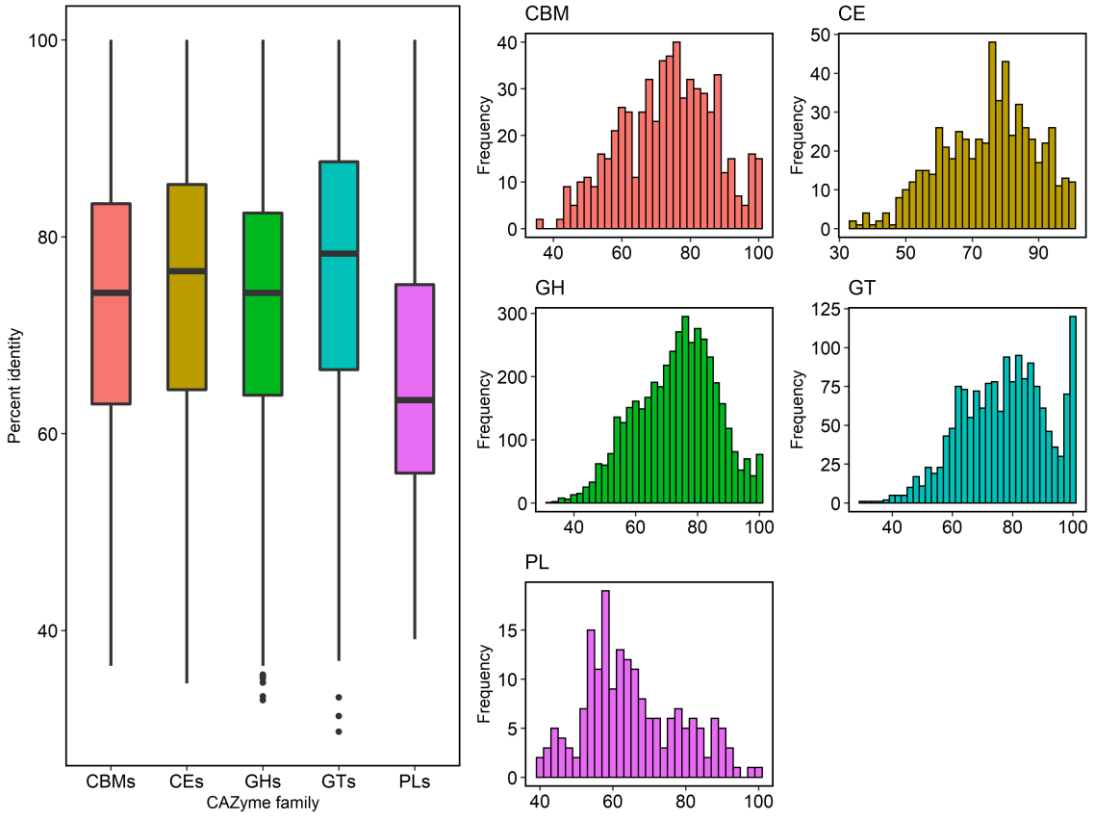

Fig. 5-10 Percentage identity of CAZymes predicted in the rumen Metagenomic-Assembled Genomes (MAGs) against the NCBI-nr database. CBMs, carbohydrate-binding modules; CEs, carbohydrate esterases; GHs, glycoside hydrolases, GTs, glycosyltransferases, PLs, polysaccharide lyases.

Fig. 5-11 Binary heatmap of CAZymes found in the rumen Metagenomic-Assembled Genomes (MAGs). Distributions of CAZymes in JB and F1 MAGs were visualized in heat maps on the left and right side, respectively. CAZymes enriched in JB were surrounded by green lines. Each row indicates one CAZyme and each column represents one MAG. Presence is depicted in blue, and absence is depicted in white.

5.4. Discussion

We performed 16S rRNA gene amplicon and metagenome shotgun sequencing to investigate the differences between JB and F1 steers in terms of their rumen microbiome composition and function. Previous studies demonstrated that the cattle breed influences the rumen microbial communities. Li et al. (2019) reported that the rumen bacterial and archaeal communities in Angus, Charolais, Galloway, Hereford, Holstein, Brown Swiss, and Simmental hybrids are distinct from those in pure Angus and Charolais breeds. These results agree with those of the present study, which revealed differences between JB and F1 steers in terms of the beta diversity of their rumen microbial communities (Fig 5-2A). Only 42.8% of the observed ASVs were common to both JB and F1 (Fig. 5-2B), which indicated that there was a distinct difference between the JB and F1 rumen microbial communities.

As JB and F1 differed in terms of their beta diversity levels according to 16S rRNA gene sequencing, we conducted shotgun sequencing to determine the rumen microbial characteristics of JB. Results showed that the predominant taxa belonged to the genus *Prevotella,* agreeing with the result of 16S rRNA gene sequencing. *Prevotella* spp. degrade and utilize starch and plant cell wall polysaccharides such as xylans and pectins in the rumen (Stewart et al., 1997). Various MAGs of the genus *Prevotella* were recovered and are present in a variety of genes encoding CAZymes associated with acetate, propionate, butyrate, and succinate productions. In addition, most JB *Prevotella* MAGs (78.5%) possessed genes encoding GH67 (α-glucuronidase), which plays an essential role in the hydrolysis of xylan substrate (Lee et al. 2012), whereas only five F1 *Prevotella* MAGs (38.5%) had these genes. This may be because the predominant taxa belonging to the genus *Prevotella* at ASV level differed between JB and F1 (Table 5-6). These finding indicate that *Prevotella* spp. play a key role in degrading plant biomass and producing short-chain fatty acids in the JB rumen via the catalytic activities of enzymes, and that the genes encoding CAZyme of *Prevotella* spp. may differ between the JB and F1 rumen.

Regarding the differences between JB and F1, we found that the relative abundance of several bacterial species, including *F. succinogenes*, *Ruminococcus* spp., *Treponema* spp., and certain members of *Lachnospiraceae* were enriched in the JB rumen microbiome according to metagenome sequencing (Fig. 5-5). Particularly, *F. succinogenes* was quantitatively higher in JB than in F1 according to qPCR assays. Furthermore, we recovered *Ruminococcus* MAG (JB_MAG31), which differs from *Ruminococcus* genomes in the Hungate collection (Closest ANI: 84.0%), from the JB rumen. The GH diversity of JB_MAG31 was high, including that of GH5, GH9, and GH48 (cellulase). Dai et al. (2015) reported that most putative cellulases in the rumen belonged to four GH families (i.e., GH5, GH9, GH45, and GH48) and were primarily synthesized by *Ruminococcus* and *Fibrobacter*, as revealed by metatranscriptomic analyses. Several *Ruminococcus* spp. and *Fibrobacter* spp. are the main cellulolytic bacteria in the rumen (Flint, 1997; Flint et al., 2008). Notably, the gene encoding CBM51, which binds to galactose (Gharechahi and Salekdeh, 2018), was found in JB_MAG31. CBM51 has not been previously detected in *Ruminococcus* genomes from the Hungate collection (Seshadri et al., 2018). *Lachnospiraceae* MAGs from the JB rumen (JB_MAG85 and JB_MAG105) harbored genes encoding chitinase (GH18), amylase (GH13), endoxylanases (GH5), and oligosaccharidases (GH2, GH3, GH36, and GH51), which are related to the degradation of various polysaccharides in the JB rumen. *Treponema* spp. are pectinolytic bacteria (Liu et al., 2014) that interact with cellulolytic bacteria and enhance the digestion of cellulosic materials (Kudo et al., 1987). A total of two *Treponema* MAGs (JB_MAG51 and JB_MAG110) harbored genes encoding the pectin-degrading CAZyme GH53 (endo-β-1,4-galactanase), which can hydrolyze β-1,4 *O*-glycosidic bonds in galactan and arabinogalactan type I (Le Nours et al., 2009).

We analyzed the functional characteristics of the JB and F1 rumen microbiomes and identified the differences in their CAZyme profiles. To the best of our knowledge, this study is the first to report on the CAZyme profile of the rumen microbiome of JB steers. Several CAZymes involved in cellulose and hemicellulose degradation, such as CBM2, CBM3,

CBM22, GH11, and CE3, were relatively more enriched in JB than in F1 (Fig. 5-7C), as the former had a greater relative abundance of *Ruminococcus* spp. than the latter. *Ruminococcus* spp. are major contributors of CBM2, CBM3, CBM22, GH11, and CE3 (Fig. 5-7D). The CAZymes were only found in MAGs reconstructed from the JB rumen microbiome data. Particularly, the *Ruminococcus* MAG (JB_MAG31) harbored those CAZymes, except for CBM2. CBMs typically append to GHs and then target their attached catalytic modules to distinct regions on a substrate (Boraston et al., 2004). CBMs markedly increase the enzyme concentration near the substrate and enhance polysaccharide hydrolysis (Hervé et al., 2010). CBM2 and CBM3 are accompanied by GH family cellulases (Iakiviak et al., 2016, Do et al., 2018). Thus, they may accelerate cellulose degradation by rumen microorganisms in JB. Berg et al. (2009) demonstrated that GH11, CE3, and CBM22, which are associated with hemicellulose degradation, also participate in xylan breakdown in *R. flavefaciens*.

In contrast, JB and F1 showed no significant differences in their cellulolytic enzyme-associated GHs such as GH5 and GH9, which are the predominant cellulase family in the rumen of cattle (Wang et al., 2019; Hess et al., 2011; Jose et al., 2017; Shen et al., 2020). This is likely because the GHs have a variety of substrate specificities in addition to cellulase, and *Prevotella* spp. is the predominant contributor. GH5 are mainly cellulases and include endo-β-1,4-glucanase and β-glucosidase. Nevertheless, there are also non-cellulolytic GH5s such as endo-β-1,4-mannosidases and xylanases (Nguyen et al., 2018).

Several *Prevotella* spp. were enriched in the F1 rumen microbiome according to metagenomic sequencing (Fig. 5-5). *Prevotella* spp. may be related to milk production. Xue et al. (2020) showed that certain *Prevotella* spp. are significantly more abundant in the rumen of Holstein cows with high milk yield and protein content than in the rumen of cows with low milk yield and protein content. CBM25, GH51, GH28, and PL11 CAZymes were enriched in the F1 rumen. These enzymes degraded hemicellulose, pectin, and starch and were produced by the abundant *Prevotella* spp. and *Bifidobacterium* spp. in the F1 rumen (Fig. 5-7D). GH28 and PL11 are associated with pectin degradation (Seshadri et al., 2018), whereas α-*L*-

arabinofuranosidase (GH51) removes the xylan backbone side chains during plant cell wall degradation (Terry et al., 2019). The α-*L*-arabinofuranosidase also uses pectinaceous polysaccharides such as arabinan as substrates (Matsuo et al., 2000). CBM25 are associated mainly with amylolytic enzymes that bind and digest raw starch (Majzlová and Janeček, 2014).

Finally, the birthplaces and parents differed for the animals used in the present study, although they were housed under the same conditions during the experiment period. Therefore, factors other than breed may influence the differences in the rumen microbiome between JB and F1 cattle. To clarify the effect of breed on the JB and F1 rumen microbiome, a further large-scale study is required. Furthermore, this study was performed using DNA-based metagenomic analysis. RNA-based analysis should be needed to distinguish the microbial activity of the JB rumen in the future.

In summary, the present study revealed differences in the rumen microbiome structures and functions of JB and F1 steers. *Prevotella* spp. were predominant both in the JB and F1 rumen, while the genes encoding CAZymes of *Prevotella* spp. may differ between JB and F1. The JB rumen microbiome showed higher relative abundances of fibrolytic bacteria such as *F. succinogenes* and *Ruminococcus* spp. and, consequently, relatively more CAZymes associated with cellulose and hemicellulose degradation. In particular, the absolute abundance of *F. succinogenes* was also higher in JB than in F1. Furthermore, the relative abundance of *Treponema* spp. and *Lachnospiraceae* were higher in the JB rumen than in the F1 rumen. In contrast, several *Prevotella* spp. were enriched in the F1 rumen microbiome and produced comparatively more CAZymes-associated hemicellulose, pectin, and starch degradation. Additionally, there are many novel bacterial species genomes and genes annotated to CAZymes in the JB and F1 rumen. Future studies are needed to validate the association between the rumen microbiome structure and high marbling quality of JB cattle.

CHAPTER 6

General summary

Utilization of by-products and feed additives as animal feeds is feasible strategy for sustainable livestock production. I expected that the use of by-products and feed additives solved the challenges of livestock production such as feed-food competition and environmental negative impact. This thesis highlights the investigation of the nutritional value of dietary by-products (DML and wine lees) and feed additives (CSFA) as feeds for ruminants.

In chapter 2, I evaluated the effect of supplementary DML as replacement of NaCl in diets for fattening beef cattle on digestibility, energy, nitrogen balance and rumen characteristics. Supplementary DML as replacement of NaCl in diets for Thai native cattle improved ADFom digestibility although there was no effect on nitrogen balance, rumen conditions, blood metabolites and CH_4 production. Considering that ruminants in tropics are often fed low-quality roughage such as rice straw, our results indicate that DML is a useful feed additive for ruminants in tropics such as Thailand.

In chapter 3, I investigated the usability of wine lees as feed for ruminants in fattening conditions by *in vitro* and *in vivo* trials. I found that wine lees inclusion protected PUFA from ruminal biohydrogenation during ruminal fermentation from *in vitro* study. In addition, the wine lees substituted for the fattening ration up to 20% DM had no adverse effects on apparent digestibility, ruminal fermentation and nitrogen balance, and decreased an oxidative stress marker (MDA) in plasma *in vivo* although the gas production and DM and CP digestibility were decreased *in vitro*. Thus, wine lees have a potential to be an important alternative as a partial substitute for antioxidant products.

In chapter 4, the effects of supplementary CSFA from linseed oil on ruminal fermentation, digestibility, CH_4 production and rumen microbiome *in vitro*. Although DM and NDFom digestibility decreased with CSFA addition, CH_4 production was drastically reduced. Regarding the rumen microbiome, supplementary CSFA increased the relative abundance of some bacteria related to propionate production such as *Ruminobacter*, *Succinivibrio*, *Succiniclasticum*, *Streptococcus*, *Selenomonas*.1 and *Megasphaera*. Furthermore, the relative abundance of *Methanobrevibacter* and protozoa counts which are associated with CH_4

production was decreased. The results suggested that the inclusion of CSFA significantly changed the rumen microbiome, leading to the acceleration of propionate production and the reduction of CH_4 production. The CSFA may be a promising candidate for reduction of CH_4 emission from ruminants although further *in vivo* study is needed to evaluate the reduction effect on rumen CH_4 production

In chapter 5, I investigated the difference of the taxonomical and functional characteristics of the rumen microbiome between Japanese Black and F1 (Japanese Black sires × Holstein dams) steers. Based on 16S rRNA gene sequencing, the beta diversity revealed differences in microbial community structures between the Japanese Black and F1 rumen. Shotgun sequencing showed that *Fibrobacter succinogenes* and two *Ruminococcus* spp., which are related to cellulose degradation, were relatively more abundant in the Japanese Black steer rumen than in the F1 rumen. Furthermore, the 16S rRNA gene copy number of *F. succinogenes* was significantly higher in the Japanese Black steer rumen than in the F1 rumen according to quantitative real-time polymerase chain reaction analysis. Genes encoding the enzymes that accelerate cellulose degradation and those associated with hemicellulose degradation were enriched in the Japanese Black steer rumen.

In conclusion, by-products, DML and wine lees, can be used as feeds for ruminants without negative effects on digestibility, nitrogen balance and ruminal fermentation. Supplementary CSFA from linseed oil changes the rumen microbiome, leading to significant reduction of CH_4 from rumen. However, CSFA has negative effects on the DM and NDFom digestibility and the effects increase with the addition level. Note that further *in vivo* study is needed to determine the optimal amount of CSFA in a diet for ruminants. Finally, I identified the taxonomical and functional differences between the rumens of Japanese Black and F1 steers. The result indicates that the dietary effect of feeds such as by-products and feed additives on rumen microbiome may be different depend on breeds. Therefore, I should evaluate the feed value of by-products and feed additives considering breed difference of ruminants.

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