https://doi.org/10.17221/86/2024-CJAS

The effect of different forages on rumen microbiota and milk production performance in Holstein dairy cows

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Citation: Wang S.H., Liao H.H, Lee C.X., Chen H.M., Chen L.Y., Chuang S.T., Hsu J.T. (2024): The effect of different forages on rumen microbiota and milk production performance in Holstein dairy cows. Czech J. Anim. Sci., 69: 356–366.

Abstract: Optimising the feed composition, especially the forage choice, and reducing costs are essential for improving dairy production efficiency. Different forage sources and proportions were used to formulate rations containing equal energy and protein, and their effects on rumen microbiota and milk production performance of dairy cows were evaluated in two experiments. In experiment 1, thirty lactating cows (235 ±13 d postpartum; milk production 29.1 ± 1.0 kg/day) were divided into Pangola and Bermuda groups. In experiment 2, twenty lactating cows (79.9 ± 8.1 d postpartum; milk production 34.7 ± 0.6 kg/day) were divided into Pangola and oat groups. In experiment 1, the Simpson index for rumen microbiota of the Pangola group was significantly higher than in the Bermuda group ($P < 0.05$). Analysis of the weighted unique fraction (UniFrac) distances indicated significant differences in the beta diversity of the community composition of rumen microbiota between Pangola, Bermuda and oat groups in both experiments ($P < 0.001$). The relative abundance of *Prevotella brevis* was significantly higher in the Pangola group than in the oat group in experiment 2 ($P < 0.05$). The somatic cell counts (SCCs), C18:0, and C18:1 in milk were significantly higher in the Bermuda group than in the Pangola group ($P < 0.05$) in experiment 1. On the other hand, milk crude protein (CP) and solids-not-fat (SNF) were significantly higher in the Oat group than in the Pangola group ($P < 0.05$) in experiment 2; however, milk urea nitrogen (MUN) was significantly higher in the Pangola group ($P < 0.05$). In conclusion, a switch of forage (Pangola *vs* Bermuda) at a lower proportion of the diet under the high forage level condition (experiment 1) caused only minor changes in rumen microbiota diversity (Simpson index, beta diversity) and milk production performance (milk SCCs, C18:0 and C18:1). On the other hand, a switch of forage (Pangola *vs* oat) at a higher proportion of the diet under the low forage level condition (experiment 2) resulted in greater changes in rumen microbiota diversity (beta diversity, relative abundances of bacterial taxa, *P. brevis* relative abundance) and milk production performance (milk CP, SNF, and MUN).

Keywords: bovine; forage proportion; microbiota diversity; milk composition; native Pangola hay

Although using local forage can benefit cows in the dairy industry (Lee et al. 1999), the amount of produced domestic forage can supply only 51% of the total forage required for ruminant animals (Hsu 2006). When supply and storage capacity for domestic forage are inadequate due to climate

Supported by the Ministry of Agriculture, Taiwan, Republic of China (Grant No.: 112AS-2.3.1-LI-L1).

https://doi.org/10.17221/86/2024-CJAS

restrictions, hay must be imported to compensate for the shortage of high-quality local forage. Taiwan's main forage grass imports are alfalfa, Bermuda hay, and Oat hay. Recently, imported forage prices have surged dramatically, making dairy farmers more concerned about optimising domestic forage use.

Rumen microorganisms can decompose plant fibre in the feedstuff, producing volatile fatty acids (VFAs) which are the primary energy source for dairy cows. The rumen microbiota refers to the high-density, diverse, and complex microbial community, including bacteria, archaea, protozoa, and fungi. Bacteria account for 50–70% of the rumen microbiota, principally composed of several species of *Ruminococcus*, *Butyrivibrio*, *Prevotella*, *Fibrobacter, Coprococcus, and Porphyromonas* (Matthews et al. 2019). An alternative feed source may change the total number of bacteria in the rumen and the relative abundance of each bacterial group. For example, the proportion of *Lactobacillus* increases when the feed contains more concentrate. Rumen microorganisms can degrade plant cellulose and hemicellulose (Koike and Kobayashi 2009), but this is affected by various factors, including the type of forage, crop maturity, and the members of the cellulolytic bacterial communities (Castillo-Gonzalez et al. 2014).

There is a strong correlation between milk fat yield and the ratio of the phyla Firmicutes to Bacteroidota. A lower Bacteroidota/Firmicutes ratio leads to an increased milk fat percentage (Jami et al. 2014). Bainbridge et al. (2016) showed moderate correlations between bacterial communities and milk yield, protein percentage, and fat yield. Since the 16S rRNA gene sequencing was first used to study rumen microbial ecosystems, even low-abundance species can be detected. Bacterial community structure is affected by dietary changes. In particular, higher dietary complexity favours microbiome diversity (Henderson et al. 2015). Xue et al. (2018) revealed a significant correlation between rumen bacteria, short-chain fatty acids in the rumen, and dairy cow lactation performance. They suggested that the rumen pan bacteriome and the core bacteriome potentially contribute to variations in milk production traits.

In order to achieve a reasonable number of cows in each treatment group and to test different proportions of forage in the total mixed ration (TMR), we use two experiments to compare native Pangola hay and the two most common imported forage sources (Bermuda hay and oat hay) on an equal dietary energy and protein basis to evaluate how varying forage sources affect dairy cow physiology and milk production. The knowledge of the rumen core microbiomes and milk production performance provides novel insights into future strategies for diversifying the use of domestic forage.

MATERIAL AND METHODS

Ethics statement

All experimental procedures were conducted according to the guidelines of the Institutional Animal Care and Use Committee of the Northern Region Branch of the Livestock Research Institute (NRBLRI), Ministry of Agriculture (MOA), Taiwan, Republic of China (IACUC No.: TLRI HCB IACUC 112-4).

Animals and design

Experiment 1. Thirty lactating Holstein cows were used at NRBLRI. They were 235 ± 13 d postpartum (mean ± SEM; range = 83–373) with daily milk production of 29.1 ± 1.0 kg (range 20–40). Cows were housed in a free-stall house with free access to fresh drinking water. They were fed twice (at 05:30 a.m. and 2:00 p.m.) and milked twice (at 05:00 a.m. and 4:00 p.m.) per day. The cows were randomly assigned to two treatment groups (Pangola and Bermuda groups), matching them for parity, lactation days, and milk yield. The major feedstuffs of TMR included Pangola or Bermuda hay in addition to alfalfa hay, soybean hulls, corn silage, soybean meal, and ground rice/corn/soybean meal-based concentrate. The proportion of Pangola or Bermuda hay in the TMR was 12.72% and 12.96%, and the amount of feed (as dry matter) was 25 kg/cow/day.

Experiment 2. Twenty lactating Holstein cows (79.9 ± 8.1 d postpartum, range = 30–167) with daily milk production of 34.7 ± 0.6 kg (range = 19.4–56.6) were used at NRBLRI. All cows were treated in the same way as in experiment 1 except for the experimental diets. The proportion of Pangola or Bermuda hay in the TMR was 20.01% and 19.73%, and the amount of feed (as dry matter; DM) was 22.7 kg/cow/day.

The C4-plants Pangola hay (strain A254) used in both experiments was harvested between

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June and August 2022 from the pasture area of the NRBLRI. Bermuda (C4-plants) hay and oat (C3-plants) hay were imported from the United States. Table 1 shows the nutritional composition of Pangola hay, Bermuda hay, and oat hay. The neutral detergent fibre (NDF) and acid detergent fibre (ADF) of Pangola hay were higher than in Bermuda hay and oat hay. Table 2 presents the TMR ingredients and nutrient composition for experiments 1 and 2. The TMR was formulated according to the Nutrient Requirements of Dairy Cattle (NRC 2001). During the experimental period, cows were pre-fed for 18 days. Afterwards, milk samples were collected over three days, and rumen fluid samples were collected at the end of the experimental period.

Collection of rumen fluid samples

The cow's rumen contents (approximately 250 ml) were collected at 09:00 by a veterinarian using an oral stomach tube with a vacuum sampler and then filtered through gauze to remove solids. Rumen fluid samples were immediately frozen in a –80 °C freezer.

DNA extraction and next-generation sequencing

Extraction of genomic DNA. Total genomic DNA from rumen fluid samples was extracted using the column-based method (e.g. QIAamp PowerFecal DNA Kit; Qiagen, Hilden, Germany). DNA concentration was determined using a Qubit 4.0 Fluorometer (Thermo Scientific, Waltham, USA) and it was adjusted to 1 ng/ul for the following process.

Table 1. The nutrient composition of Pangola, Bermuda and oat hay used in the experimental diets

Items (%)*	Pangola hay	Bermuda hay	Oat hay
DM	91	94	90
CP*	4.10	12.88	4.06
EE	1.17	1.89	1.03
NDF	61.37	58.10	45.52
ADF	34.19	31.04	25.95

*Dry matter basis (%)

ADF = acid detergent fibre; CP = crude protein; DM = dry matter; EE = ether extract; NDF = neutral detergent fibre

PCR amplification and purification. The full-length 16S genes (V1–V9 regions) were amplified with barcoded 16S gene-specific primers. According to the Amplification of Full-Length 16S Gene with Barcoded Primers for Multiplexed SMRTbell Library Preparation and Sequencing Procedure (Pacific Biosciences, Menlo Park, USA), samples with a bright main strip around 1 500 bp were chosen and purified using the AMPure PB beads for the subsequent library preparation.

SMRTbell library construction and sequencing. In brief, an equal volume of each barcoded PCR product was pooled, and 500–1 000 ng of pooled amplicon sample was used for DNA damage repair, followed by end-repair/A-tailing and ligation steps to introduce the universal hairpin adapters into double-stranded DNA fragments. After purification with AMPure PB beads to remove the adapter dimer, the SMRTbell library was incubated with sequencing primer v4 and Sequel II Binding Kit 2.1 (Pacific Biosciences, Menlo Park, USA) for the primer annealing and polymerase binding. Finally, sequencing was performed in the circular consensus sequence (CCS) mode on a PacBio Sequel IIe instrument to generate the High Fidelity HiFi reads with a predicted accuracy (Phred Scale) of 30.

Milk and hay composition analysis

Milk fat, crude protein (CP), lactose, solids-not-fat (SNF), milk urea nitrogen (MUN), citric acid, total saturated fatty acids (TSFA), total unsaturated fatty acids (TUSFA), *de novo* fatty acids (FA), mixed FA, preformed FA, myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), and oleic acid (C18:1) concentrations were determined at the NRBLRI milk-testing laboratory using the Fourier transform mid-infrared spectroscopy (FT-MIR) devices MilkoScan™ FT$^+$ (FOSS, Hillerød, Denmark). Somatic cell counts (SCCs) were determined using Fossomatic™ FC (FOSS, Hillerød, Denmark). FA analysis was performed using the Foss FA Origin package (Schwarz et al. 2018), which divides the milk FAs into three groups: *de novo*, mixed, and preformed. The *de novo* FA group includes butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), C14:0, and myristoleic acid (C14:1). The mixed FA group includes C16:0 and palmitoleic acid (C16:1). Finally, the

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Table 2. The ingredients and nutrient composition of the total mixed ration used in experiments 1 and 2 (DM values)

Items	Experiment 1		Experiment 2	
	Pangola	Bermuda	Pangola	Oat
Ingredient composition (%)				
Corn silage	22.96	22.90	26.39	26.48
Concentrate[1]	29.31	29.23	28.48	28.57
Alfalfa hay	14.38	14.34	6.15	6.17
Bermuda hay	–	12.96	–	–
Pangola hay	12.72	–	20.01	–
Oat hay	–	–	–	19.73
Soybean hull	12.72	12.68	2.0	2.0
Steam–flaked corn	–	–	1.96	1.96
Soybean meal, 44% CP	5.33	5.32	11.74	11.78
Lipid[2]	1.58	1.58	1.96	1.97
Sodium bicarbonate	0.80	0.80	1.32	1.32
Premix[3]	0.20	0.20	0.02	0.02
Total	100	100	100	100
Nutrient composition[4]				
DM(%)	44	44	44	44
CP (%)	16.4	17.2	16.7	16.9
EE (%)	3.5	3.5	3.8	3.8
NDF (%)	36.4	34.5	34.3	28.8
ADF (%)	21.9	20.4	18.4	15.1
NEL (MJ/kg)	6.36	6.40	6.78	6.95
Forage to concentrate ratio	40:60	40:60	40:60	40:60

[1]Concentrate included ground rice (29.4%), ground corn (29.4%), soybean meal (28.5%), fish meal (3%), molasses (5%), salt (1.2%), limestone (1%), dicalcium potassium (0.8%), sodium bicarbonate (0.8%), magnesium oxide (0.4%), vitamin premix (0.03%), mineral premix (0.02%) (as fed basis); [2]lipid = energy booster 100 × dry fat supplement contains 98% total fatty acids; [3]each kg of premix contains Vitamin A (10 000 000 IU), Vitamin D3 (1 600 000 IU), Vitamin E (70 000 IU), Fe (50 g), C (10 g), Zn (40 g), I (0.5 g), Se (0.1 g), Co (0.1 g); [4]the nutrient composition value is calculated according to NRC (2001)
ADF = acid detergent fibre; CP = crude protein; DM = dry matter; EE = ether extract; IU = international unit; NDF = neutral detergent fibre; NEL = net energy for lactation

preformed FA group includes pentadecylic acid (C15:0), margaric acid (C17:0), C18:0, C18:1, linoleic acid (C18:2), linolenic acid (C18:3), arachidic acid (C20:0), eicosadienoic acid (C20:2), behenic acid (C22:0), and lignoceric acid (C24:0) (Schwarz 2018).

All hay samples were dried in an oven at 65 °C for 48 h, then ground and preserved for chemical analysis. Hay samples were sent to the feed analysis centre (LRI, Tainan, Taiwan). DM, CP and ether extract (EE) in feed were determined according to the method described by Association of Official Analytical Chemists International (methods 934.01, 990.13 and 920.85), and the NDF and ADF were determined following Van Soest et al. (1991).

Statistical analysis

The data were analysed using SAS software (v.9.4; SAS Institute Inc., Cary, USA). All data were compared between treatments using an independent samples *t*-test implemented in SAS. *P*-values of less than 0.05 were considered statistically significant, while those between 0.05 and 0.1 were considered a trend. To analyse the sequence similarities among different amplicon sequence variants (ASVs), multiple sequence alignment was conducted using the QIIME2 (v.2024.2)alignment MAFFT (v.7) (Katoh and Standley 2013) against the NCBI database. ASVs abundance information was rarefied to the minimum sequence depth to normalise

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the variations in sequence depth across samples. Subsequent analysis of alpha and beta diversities was performed using the normalised data. Alpha diversity was indicative of the species complexity within individual samples based on different criteria output, including observed species and Shannon and Simpson indices (Whittaker 1972). Observed species is the number of different species represented in the microbial community. The Shannon index is an information statistical index that assumes all species are represented in a sample and that they are randomly sampled. The Simpson index is a dominance index because it gives more weight to common or dominant species. Beta diversity analysis was used to evaluate the differences between samples regarding species complexity. Beta diversity parameters and the weighted UniFrac (Lozupone and Knight 2005) were calculated using the QIIME2 pipeline.

RESULTS

Rumen microbiota

Alpha diversity analysis results of the two experiments are listed in Table 3. In experiment 1, the Simpson index of the Pangola group was significantly higher than that of the Bermuda group ($P < 0.05$). However, there were no significant differences in observed ASVs and the Shannon index between the groups. There were no significant differences in observed ASVs, Shannon and Simpson indexing in experiment 2. The ASV's beta diversity was determined using the weighted UniFrac method; an analysis of the weighted UniFrac distances indicated significant differences in the beta diversity of community composition between the groups ($P < 0.001$) in both experiments (Figures 1 and 2). The five most abundant phyla, class, order, family, genus and species are presented in Tables 4 and 5. In experiment 1, the most abundant phylum was Bacteroidota, its relative abundance in the Pangola and Bermuda groups being 66.6% and 63.7%, respectively. The second and the third relatively abundant phyla were Firmicutes and Proteobacteria. The

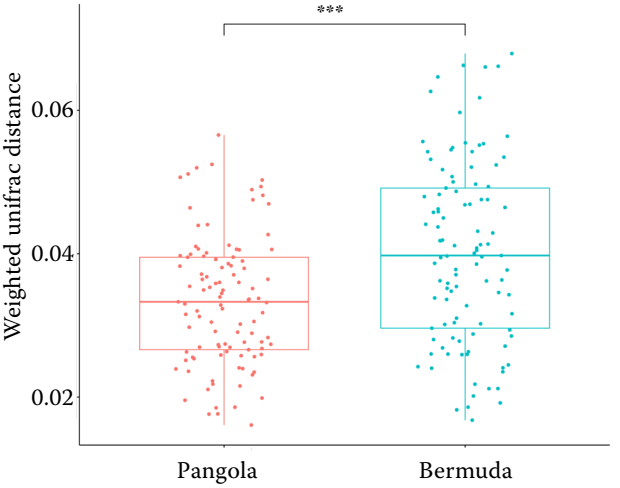

Figure 1. Boxplots of weighted UniFrac beta diversity between the Pangola and Bermuda groups (experiment 1)
***Significant at $P < 0.001$

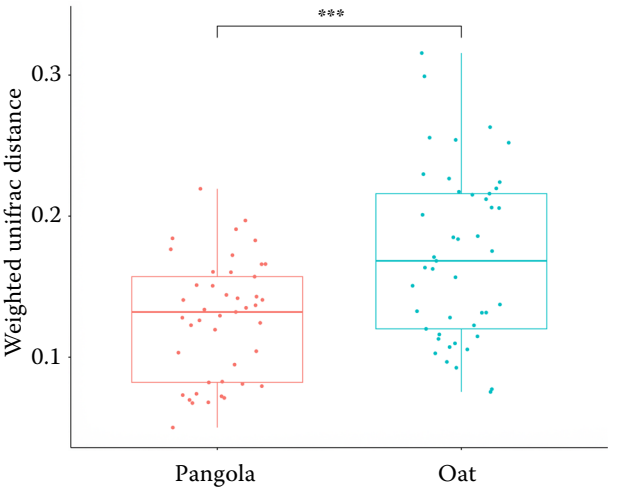

Figure 2. Boxplots of weighted UniFrac beta diversity between the Pangola and oat groups (experiment 2)
***Significant at $P < 0.001$

Table 3. Effect of TMR with different forage sources on the alpha diversity index of the rumen microbiota

Measurement	Experiment 1				Experiment 2			
	Pangola	Bermuda	SEM	*P*-value	Pangola	oat	SEM	*P*-value
Observed ASVs	1 067	1 063	76.79	0.972	914.9	912.0	73.40	0.443
Shannon index	9.418	9.374	0.123	0.544	9.231	8.945	0.219	0.098
Simpson index	0.998	0.997	0.001	0.029	0.998	0.994	0.003	0.058

SEM = standard error of the mean; TMR = total mixed ration

https://doi.org/10.17221/86/2024-CJAS

Table 4. The five most abundant bacterial taxa (%) in the rumen in experiment 1

Relative abundance (%)	Pangola	Bermuda	SEM	*P*-value
Phylum				
Bacteroidota	66.6	63.7	2.11	0.174
Firmicutes	24.2	26.5	1.86	0.236
Proteobacteria	2.39	2.51	0.59	0.848
Candidatus Melainabacteria	1.73	1.86	0.34	0.715
Tenericutes	1.66	1.69	0.22	0.904
Firmicutes/Bacteroidota	38.8	42.7	4.10	0.162
Class				
Bacteroidia	66.5	63.5	2.09	0.169
Clostridia	18.9	20.4	1.56	0.329
Negativicutes	3.92	4.62	0.49	0.160
Gammaproteobacteria	2.18	2.20	0.59	0.965
Candidatus Melainabacteria	1.73	1.86	0.34	0.715
Order				
Bacteroidales	66.5	63.5	2.09	0.171
Eubacteriales	18.9	20.4	1.55	0.329
Acidaminococcales	3.35	3.56	0.37	0.569
Aeromonadales	2.18	2.20	0.59	0.966
Vampirovibrionales	1.73[b]	1.86[a]	0.34	0.716
Family				
Prevotellaceae	49.3	48.1	2.74	0.660
Oscillospiraceae	9.32	9.45	0.86	0.885
Lachnospiraceae	5.97	7.15	0.68	0.093
Tannerellaceae	5.19	4.58	0.91	0.503
Acidaminococcaceae	3.35	3.56	0.37	0.569
Genus				
Prevotella	47.6	46.4	2.73	0.659
Parabacteroides	5.17	4.56	0.90	0.501
Succiniclasticum	3.35	3.56	0.37	0.572
Paludibacter	2.43	1.86	0.64	0.380
Ruminococcus	2.15	2.93	0.49	0.137
Species				
Prevotella ruminicola	25.7	24.4	1.82	0.464
Prevotella brevis	12.2	10.9	1.08	0.229
Succiniclasticum ruminis	3.35	3.56	0.37	0.572
Parabacteroides distasonis ATCC 8503	2.43	2.30	0.38	0.721
Parabacteroides merdae	2.59	2.13	0.69	0.606

[a,b]Indicats significant difference between two groups (*P*-value < 0.05)

SEM = standard error of the mean

Table 5. The five most abundant bacterial taxa (%) in the rumen in experiment 2

Relative abundance (%)	Pangola	Oat	SEM	*P*-value
Bacteroidota	69.0	65.5	2.60	0.187
Firmicutes	25.9	30.4	2.29	0.066
Spirochaetes	1.24	0.78	0.20	< 0.05
Proteobacteria	0.89	0.29	0.36	0.117
Tenericutes	0.84	1.01	0.25	0.502
Firmicutes/ Bacteroidota	37.9	47.9	5.51	0.094
Class				
Bacteroidia	68.5	65.2	2.62	0.225
Clostridia	18.9	22.6	2.32	0.125
Negativicutes	5.79	6.66	0.70	0.192
Spirochaetia	1.24	0.78	0.20	< 0.05
Erysipelotrichia	0.93	0.72	0.14	0.161
Order				
Bacteroidales	68.5	65.2	2.62	0.225
Eubacteriales	18.9	22.6	2.32	0.125
Acidaminococcales	4.89	5.73	0.62	0.192
Spirochaetales	1.24	0.78	0.20	< 0.05
Erysipelotrichales	0.93	0.72	0.14	0.161
Family				
Prevotellaceae	50.4	48.8	4.29	0.711
Oscillospiraceae	9.48	10.8	1.65	0.426
Muribaculaceae	5.78	5.41	0.89	0.682
Lachnospiraceae	5.40	7.12	1.40	0.236
Tannerellaceae	5.18	4.24	0.85	0.289
Genus				
Prevotella	48.8	47.5	4.36	0.776
Parabacteroides	5.16	4.23	0.84	0.285
Succiniclasticum	4.89	5.73	0.62	0.190
Sodaliphilus	4.17	4.02	0.81	0.852
Marseillibacter	2.13	1.79	0.34	0.331
Species				
Prevotella ruminicola	26.8	26.0	3.99	0.84
Prevotella brevis	11.8	8.10	1.08	< 0.05
Succiniclasticum ruminis	4.89	5.73	0.62	0.19
Sodaliphilus pleomorphus	4.17	4.02	0.81	0.85
Parabacteroides distasonis ATCC 8503	2.83	1.80	0.76	0.20
Parabacteroides merdae	2.59	2.13	0.69	0.606

P-value < 0.05 indicates significant difference between two groups

SEM = standard error of the mean

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Firmicutes to Bacteroidota ratio in the Pangola and Bermuda groups was 38.8% and 42.7%, respectively. The mean relative abundances of bacterial taxa in the rumen did not show any significant differences at the phylum, class, order, family, genus, and species levels between the Pangola and Bermuda groups.

In experiment 2, the most abundant phylum was Bacteroidota, with relative abundances of 69.0% and 65.5% in the Pangola and oat groups, respectively. The second and the third relatively abundant phyla were Firmicutes and Spirochaetes. The Firmicutes to Bacteroidota ratio was 37.9% and 47.9% in the Pangola and oat groups, respectively. The mean relative abundances of bacterial taxa in the rumen were significantly different ($P < 0.05$) between the Pangola and oat groups at the phylum, class, order and species levels. At the species level, the *Prevotella brevis* relative abundance was significantly higher ($P < 0.05$) in the Pangola group than in the oat group.

Milk production performance

Table 6 shows the effects of TMR with different forage sources on milk yield and composition. In experiment 1, the milk somatic cell counts (SCCs) ($\times 10^4$ cells/ml) were significantly higher in the Bermuda group than in the Pangola group ($P < 0.05$). In contrast, milk production (kg/day), CP (%), lactose (%), SNF (%), MUN (mmol/l), and citric acid (mmol/l) were not significantly different between treatments. In experiment 2, milk CP and SNF were significantly higher in the Oat group than in the Pangola group ($P < 0.05$). However, MUN was

Table 6. Effect of TMR with different forage sources on milk yield and composition

Measurement	Experiment 1				Experiment 2			
	Pangola	Bermuda	SEM	*P*-value	Pangola	oat	SEM	*P*-value
Milk yield (kg/d)	27.5	28.9	1.60	0.39	36.4	33.9	2.25	0.31
Fat (%)	3.93	4.35	0.25	0.26	4.71	5.16	0.35	0.21
Crude protein (%)	3.31	3.38	0.06	0.24	3.15	3.48	0.09	< 0.05
Lactose (%)	4.84	4.81	0.05	0.67	4.92	4.90	0.05	0.76
Solids-not-fat (%)	8.79	8.86	0.06	0.26	8.72	9.02	0.10	< 0.05
SCC (10^4 cells/ml)	13.5	33.0	8.45	< 0.05	22.8	34.0	9.80	0.26
Urea nitrogen (mmol/l)	2.59	2.63	0.12	0.68	2.67	2.27	0.15	< 0.05
Citric acid (mmol/l)	9.18	8.60	0.33	0.08	8.33	8.31	0.30	0.96

SCC = somatic cell counts; SEM = standard error of the mean; TMR = total mixed ration

Table 7. Effect of TMR with different forage sources on milk fatty acids (FA) composition (g/100 g of milk)

Measurement	Experiment 1				Experiment 2			
	Pangola	Bermuda	SEM	*P*-value	Pangola	oat	SEM	*P*-value
TSFA	2.65	2.93	0.34	0.23	3.38	3.82	0.30	0.15
TUSFA	0.96	1.04	0.05	0.12	0.98	1.04	0.07	0.34
De novo FA[1]	0.77	0.91	0.07	0.40	0.95	1.05	0.09	0.24
Mixed FA[2]	1.47	1.60	0.11	0.24	1.81	2.00	0.13	0.15
Preformed FA[3]	1.33	1.49	0.09	0.54	1.52	1.58	0.11	0.57
C14:0	0.39	0.42	0.04	0.33	0.49	0.57	0.46	0.07
C16:0	1.35	1.54	0.12	0.13	1.72	1.94	0.15	0.13
C18:0	0.35	0.40	0.23	< 0.05	0.45	0.44	0.03	0.81
C18:1	0.78	0.87	0.44	< 0.05	0.86	0.91	0.07	0.47

[1]C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, and C14:1; [2]C16, C16:1; [3]C15:0, C17:0, C18:0, C18:1, C18:2, C18:3, C20:0, C20:2, C 22:0, and C24:0

SEM = standard error of the mean; TMR = total mixed ration; TSFA = total saturated fatty acids; TUSFA = total unsaturated fatty acids

https://doi.org/10.17221/86/2024-CJAS

significantly higher in the Pangola group ($P < 0.05$), and milk production, milk fat, lactose, SCCs, and citric acid were not significantly different between treatments. Table 7 shows the effects of TMR with different forage sources on the composition of milk FAs. In experiment 1, C18:0 and C18:1 concentrations in milk of the Bermuda group were significantly higher than in the Pangola group ($P < 0.05$), but no significant differences in TSFA, TUSFA, *de novo* FA, mixed FA, preformed FA, C14:0, and C16:0 were observed between the groups. In experiment 2, there were no significant differences in milk FA composition.

DISCUSSION

Rumen microbiota diversity and relative abundances of bacterial taxa

Alpha diversity of rumen microbiota was assessed in ASV counts to ensure comparable group results. The Simpson index of the Pangola group was significantly higher than that of the Bermuda group in experiment 1, indicating that dominant species of rumen microbiota varied between the Pangola and Bermuda groups. However, the Shannon index showed no significant differences between groups, which might indicate no diversity difference in the overall species presence between groups. In experiment 2, there were no significant differences in alpha diversity, Shannon index, or Simpson index between the Pangola and oat groups. However, in experiment 2, the alpha diversity index was lower than in experiment 1, which might indicate that the forage proportion in the TMR affects the diversity of rumen microbiota (Table 3). McCann et al. (2014) reviewed that the TMR forage to concentrate ratio was changed to meet lactation requirements, and microbiome composition was also altered. On the other hand, higher-quality forages are significant dietary contributors to maintaining high milk production and impact the rumen microbiome. Feed composition has a determinant effect on shaping the ruminal microbiota. When cows were fed an NDF-rich diet, the diversity of bacteria and fungi in the rumen increased compared with a starch-rich diet (Belanche et al. 2012). Increasing dietary fibre content results in an increase in the abundance and diversity of ruminal bacteria, fungi, and protozoa, while abundance and diversity decrease when dietary forage content is reduced (Sanjorjo et al. 2023). Thus, low forage levels in experiment 2 resulted in no significant difference in the alpha diversity of rumen microbiota.

In this study, we used the weighted UniFrac distance to evaluate sample diversity, and results showed that the rumen microbiota was significantly diverse in experiments 1 and 2 (Figures 1 and 2). A lower diversity of rumen microbiota in experiment 1 may be due to the lower proportion switch of forage sources. Diversity in the Pangola group was smaller than that of the Bermuda (experiment 1) and oat (experiment 2) groups, which we speculate might relate to the difference in the abundance of dominant species because of the change in forage structure and rumen fermentation. Feed particle size is reduced over time through rumination and fermentation (Russell 2002).

In this study, the NDF and ADF levels in Pangola hay (domestic forage) were higher than those in Bermuda and oat hay, not only because of forage species difference but also because of the climate restrictions on harvest time and hay quality (Lee et al. 1991). Lignin is thought to interfere with microbial degradation of fibre polysaccharides by acting as a physical barrier. Hence, NDF and ADF content or their degradability might cause a difference in bacterial diversity between the groups in both experiments. In experiment 2, the feed intake (as DM) of the oat group (16.98 kg/day) was higher than that of the Pangola group (15.82 kg/day) (data not shown). Wang et al. (2020) mentioned that a short rumen retention time is expected to reduce microbiota diversity, as it selects for only fast-growing taxa. Increasing the feed intake level reduces retention time with a quadratic effect on the alpha diversity of microbial community (Ali et al. 2019). In comparison with starch-rich diets, NDF-rich diets provide less acidic conditions in which microorganisms can grow without restriction (Belanche et al. 2012). Compared to the oat group, the Pangola group should have a longer rumen retention time and higher NDF and ADF contents which might increase the diversity of rumen microbiota. The beta diversity difference between the Pangola and oat groups (Figure 2) was likely due to the fibre content of the forage and its indirect effect on digestibility because the proportion of Pangola and oat hay in the TMR was higher in experiment 2.

As expected, the weighted Unifrac distance value was higher in experiment 2 than in experiment 1,

https://doi.org/10.17221/86/2024-CJAS

indicating that the rumen microbiota diversity might change with the proportion of forage in the low-forage TMR. In experiment 2, the Pangola group was significantly richer at the phylum, class and order levels (i.e. Spirochaetes, Spirochaetia, and Spirochaetales) than the oat group. The Spirochetes contribute significantly to the degradation of plant materials ingested by ruminants (Paster and Canale-Parola 1982).

Rumen bacterial communities are generally dominated by Bacteroidota and Firmicutes (Wallace 2008). Similarly, the most abundant groups observed in our study were Bacteroidota and Firmicutes. However, the Firmicutes/Bacteroidota ratio did not differ between groups in either experiment. Wang et al. (2020) showed that the primary and secondary dominant bacteria in the high-forage (HF) and high-concentrate (HC) treatments were Bacteroidota and Firmicutes. However, in the HC group, the average relative abundance of Bacteroidota (25.36%) was inconsistent with our study. In the present study, C4 plants such as Pangola and Bermuda hay were higher in fibre content than C3 plants (oat hay), and C3 plants store their energy as sugar molecules joining together to form a complex carbohydrate, whereas C4 plants store starch rather than sugar. Different hay structures led to changes in the bacterial community and utilisation efficiency; in particular, the middle lamella and primary wall of thick-walled cells are highly lignified in C4 plants, which might interfere with hay degradation by rumen microbes (Buxton and Redfearn 1997). We observed that the species *Prevotella ruminicola* accounted for a large proportion of the bacterial community, above 24% in the two experiments. In experiment 2, *Prevotella brevis* was significantly more abundant in the Pangola group than in the oat group ($P < 0.05$). *Prevotella* was the dominant genus and was more abundant under high-fibre diets. *Prevotella* species are gram-negative anaerobes and produce various extracellular degradative enzymes, degrading starch and hemicellulose and exhibiting proteolytic activity (Stevenson and Weimer 2007). The high NDF and ADF content in the Pangola group coincided with the observation of more abundant *Prevotella brevis* levels. Therefore, the results indicate that *Prevotella* was affected by the forage source and fibre content between treatments. Similarly, Indugu et al. (2017) also reported differences in rumen bacterial populations due to dietary composition, particularly differences in forage type and proportion in the diets. We measured the volatile fatty acid concentration in rumen fluid in experiment 2, and found significantly higher acetic acid (4.45 *vs* 3.83 mg/ml; $P < 0.05$), isobutyric acid (0.07 *vs*. 0.05 mg/ml; $P < 0.05$) and isovaleric acid (0.14 *vs* 0.09 mg/ml; $P < 0.05$) in Pangola group than in oat group. Higher acetic acid content should be related to higher fibre degradation, and the higher branched-chain fatty acid content should be related to greater amino acid deamination after protein degradation. Higher fibre and protein degradation coincided with the presence of *Prevotella*.

Milk yield, milk fat, and fatty acid composition

In the two experiments, the CP and EE of Pangola hay were lower than in Bermuda hay and oat hay, while the opposite was found for NDF and ADF (Table 1). The protein content of forage is negatively correlated with the content of cellulose, hemicellulose, and lignin, each of which increases with maturity (Van Soest et al. 1978). For the high ADF content of forage, more concentrate was required to compensate available energy for TMR formulation (Cleale and Bull 1986). In experiment 1, milk yield and composition were not significantly different between the Pangola and Bermuda groups, except for SCCs, C18:0, and C18:1. Moreover, the mean relative abundances of bacterial taxa in the rumen were not significantly different between the Pangola and Bermuda groups. Xue et al. (2018) showed that some taxa occupy particular ecosystems within the rumen and play a crucial role in influencing lactation performance. They found that milk fat was positively correlated with the relative abundance of *Butyrivibrio, Pseudobutyrivibro,* and *Clostridium*. However, they were not the most abundant in our study.

Garnsworthy et al. (2006) suggested that milk yield varies with the stage of lactation, which influences the total yield of fatty acids and the relative proportions of individual fatty acids. In experiment 1, the variable fatty acid composition between the groups might be due to the average days in milk rather than to forage effects, which showed no significant differences in the levels of any bacterial taxa. In experiment 2, the oat group had a significantly higher percentage of milk CP and SNF than the Pangola group ($P < 0.05$). On the contrary, in the Pangola group, the MUN density was significantly higher than that of the oat group. These findings may indicate that the

https://doi.org/10.17221/86/2024-CJAS

CP digestibility of Pangola hay was poorer than that of oat hay, rendering lower levels of available amino acids in the Pangola group. However, there were no significant differences in milk fatty acid composition. The oat group had significantly higher levels of CP and SNF, mainly because the digestibility of oat hay is higher than that of Pangola hay; thus, cattle in the oat group might gain more amino acids and energy. In general, rations containing higher fibre promote higher acetic acid production, while those containing lower fibre produce more propionic acid, benefitting the synthesis of milk CP and SNF. The proportion of roughage and concentrates also affected the acetic to propionic acid ratio in the rumen. The NDF and ADF content in the diet of the oat group was lower than in the Pangola group, which could be unfavourable for rumen cellulolytic bacteria. High-yielding cows have more significant daily energy requirements to supply lactose for milk production than lower-yielding cows with the same milk composition. Thus, milk yield may explain the association, due to its link with increased body tissue mobilisation at early lactation (Morton et al. 2016). Table 7 shows that the TSFA and preformed FA were higher in experiment 2 than in experiment 1, likely due to the metabolic differences between lactation stages. Meanwhile, the TMR of experiments 1 and 2 had the same EE content. There was no deliberate increase in EE content in the TMR of experiment 2; thus, the milk fatty acid composition in early lactation cows was not significantly different between the groups under the influence of body fat mobilisation.

CONCLUSION

Different forage sources and proportions in the TMR of milking cows affected rumen microbiota and milk production performance. Under high TMR forage level conditions (experiment 1), a lower proportion of forage (Pangola *vs* Bermuda hay) caused only minor changes in rumen microbiota diversity (Simpson index, beta diversity) and milk production performance (milk SCCs, C18:0, and C18:1 concentration). On the other hand, a switch to a higher proportion of forage (Pangola *vs* oat) under a low TMR forage condition (experiment 2) resulted in greater changes in rumen microbiota diversity (beta diversity, relative abundances of bacterial taxa and *Prevotella brevis*) and milk production performance (milk CP, SNF, and MUN).

Acknowledgement

The authors thank the Dairy Association of Taiwan for their technical support and datasets.

Conflict of interest

The authors declare no conflict of interest.

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Received: May 31, 2024
Accepted: September 11, 2024
Publihsed Online: September 30, 2024