Effects of partial mixed rations and supplement amounts on milk production and composition, ruminal fermentation, bacterial communities, and ruminal acidosis

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ABSTRACT

Late-lactation Holstein cows ($n = 144$) that were offered 15 kg dry matter (DM)/cow per day of perennial ryegrass to graze were randomized into 24 groups of 6. Each group contained a fistulated cow and groups were allocated to 1 of 3 feeding strategies: (1) control (10 groups): cows were fed crushed wheat grain twice daily in the milking parlor and ryegrass silage at pasture; (2) partial mixed ration (PMR; 10 groups): PMR that was isoenergetic to the control diet and fed twice daily on a feed pad; (3) PMR+canola (4 groups): a proportion of wheat in the PMR was replaced with canola meal to produce more estimated metabolizable protein than other groups. Supplements were fed to the control and PMR cows at 8, 10, 12, 14, or 16 kg of DM/d, and to the PMR+canola cows at 14 or 16 kg of DM/d. The PMR-fed cows had a lower incidence of ruminal acidosis compared with controls, and ruminal acidosis increased linearly and quadratically with supplement fed. Yield of milk fat was highest in the PMR+canola cows fed 14 or 16 kg of total supplement DM/d, followed by the PMR-fed cows, and was lowest in controls fed at these amounts; a similar trend was observed for milk fat percentage. Milk protein yield was higher in the PMR+canola cows fed 14 or 16 kg of total supplement DM/d. Milk yield and milk protein percentage were not affected by feeding strategy. Milk, energy-corrected milk, and milk protein yields increased linearly with supplement fed, whereas milk fat percentage decreased. Ruminal butyrate and D-lactate concentrations, acetate-to-propionate ratio, (acetate + butyrate)/propionate, and pH increased in PMR-fed cows compared with controls for all supplement amounts, whereas propionate

and valerate concentrations decreased. Ruminal acetate, butyrate, and ammonia concentrations, acetate-to-propionate ratio, (acetate + butyrate)/propionate, and pH linearly decreased with amounts of supplement fed. Ruminal propionate concentration linearly increased and valerate concentration linearly and quadratically increased with supplement feeding amount. The *Bacteroidetes* and *Firmicutes* were the dominant bacterial phyla identified. The *Prevotellaceae*, *Ruminococcaceae*, and *Lachnospiraceae* were the dominant bacterial families, regardless of feeding group, and were influenced by feeding strategy, supplement feeding amount, or both. The *Veillonellaceae* family decreased in relative abundance in PMR-fed cows compared with controls, and the *Streptococcaceae* and *Lactobacillaceae* families were present in only minor relative abundances, regardless of feeding group. Despite large among- and within-group variation in bacterial community composition, distinct bacterial communities occurred among feeding strategies, supplement amounts, and sample times and were associated with ruminal fermentation measures. Control cows fed 16 kg of DM of total supplement per day had the most distinct ruminal bacterial community composition. Bacterial community composition was most significantly associated with supplement feeding amount and ammonia, butyrate, valerate, and propionate concentrations. Feeding supplements in a PMR reduced the incidence of ruminal acidosis and altered ruminal bacterial communities, regardless of supplement feeding amount, but did not result in increased milk measures compared with isoenergetic control diets component-fed to late-lactation cows.

Key words: bacterial community composition, partial mixed ration, protein, ruminal acidosis, supplements

INTRODUCTION

A TMR fed to cattle on a feed pad between grazing periods is termed a partial mixed ration (PMR) (Bargo

Received February 13, 2014.

Accepted May 16, 2014.

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et al., 2002b; Auldist et al., 2013). This feeding strategy increases milk yield and milk fat and protein percentage (Bargo et al., 2002a) and improves marginal milk responses and increased yields of milk fat (Auldist et al., 2013) over those of pasture-fed cows supplemented with grain in the milking parlor and conserved forage fed on the pasture.

Auldist et al. (2013) proposed that a well-formulated PMR that is consumed over a longer period could lead to more stable ruminal fermentation compared with grain fed in the parlor. The risk of ruminal acidosis may then be reduced, a hypothesis that is supported by a higher ruminal pH in PMR-fed compared with control cows fed grain in the parlor, silage, and fresh-cut pasture (Greenwood et al., 2014).

The substitution of some of the wheat for canola meal in a PMR also increased ECM and was associated with a higher concentration and yield of milk fat, and higher pasture DMI (Auldist et al., 2014). Other protein supplements, such as canola meal, have also increased milk yield (Oldham, 1984; Huhtanen et al., 2011; Martineau et al., 2013). Allen et al. (2006) proposed that high protein feeds have a buffering capacity in the rumen, which is consistent with the report of Auldist et al. (2014), in which substituting wheat for canola meal in a PMR decreased the amount of time pH was under 6.0 and increased mean daily ruminal pH, despite having no significant influence on ruminal VFA (Auldist et al., 2014).

Understanding the complex and dynamic ruminal microbial ecosystem (Fernando et al., 2010) is essential to the development of feed management practices that promote optimal production efficiency (de Menezes et al., 2011). Ruminal acidosis is an important example of an interaction between ruminal microbial metabolism and diet that can impair health and production (Tajima et al., 2000; Khafipour et al., 2009). Weimer et al. (2010) suggested that the ruminal microbiome is reasonably resistant to dietary changes, and a change in the microbiome is not always related to the severity of ruminal acidosis (Mohammed et al., 2012). Early understandings of rumen microbiology and bacteria believed to be involved with ruminal acidosis were based on bacterial cultures. More recently, molecular techniques, which are rapidly improving and becoming less expensive, have been adopted in evaluations of rumen microbiology. Integration of knowledge obtained from classical culture-based microbiology and modern molecular techniques is rapidly increasing our understanding of the rumen microbiome and its functions in general (Pers-Kamczyc et al., 2011). This knowledge will potentially facilitate optimal dietary management and reduce the incidence of nutritional disorders such as ruminal acidosis, among other benefits. Despite

constant evolution of methods for studying the rumen ecosystem, only about 10% of the rumen microbiome is known (Pers-Kamczyc et al., 2011). This limited knowledge impedes understanding of the importance of changes in ruminal microbial populations observed during rumen perturbation and ruminal acidosis.

Recent work suggests that the rumen has a core bacterial microbiome that consists primarily of bacteria from the *Firmicutes* and *Bacteroidetes* phyla, which appear to change in cattle fed various ruminal acidosis induction diets or increasing amounts of grain (Khafipour et al., 2009; Callaway et al., 2010; Fernando et al., 2010; de Menezes et al., 2011). Bacteria from the *Proteobacteria* appear to be the third most dominant in the rumen in several (Khafipour et al., 2009; Jami and Mizrahi, 2012), but not all, ruminant studies (Golder et al., 2014b). Increases in *Proteobacteria* have been reported in TMR-fed compared with pasture-fed cattle (de Menezes et al., 2011) and in cattle with grain-induced compared with alfalfa pellet-induced subacute acidosis (Khafipour et al., 2009). Increases in *Streptococcus* and *Lactobacillus* were associated with ruminal acidosis in early work (Hungate et al., 1952; Hungate, 1966); however, as only a small portion of the rumen microbiome is known, other rumen bacteria may have prominent roles in acidosis.

The objectives of this study were to examine relationships between milk measures, ruminal fermentation measures, ruminal acidosis, and ruminal bacterial community composition (**BCC**) in lactating cows offered a restricted pasture allowance and fed supplements using different feeding strategies and at linearly increasing supplement amounts. The aim was to identify feed management systems that improve milk measures, promote optimal conditions in the rumen for digestion, and reduce ruminal acidosis.

The hypotheses tested were (1) that cows fed supplements as a PMR, with or without inclusion of canola meal, would have increased milk measures, altered ruminal measures, distinct ruminal BCC, and decreased ruminal acidosis compared with control cows fed isoen-ergetic diets as grain in the parlor and forage fed on the pasture; (2) that a linear increase in the amount of supplement fed (or increase when 14 and 16 kg of supplement DM are fed) would alter milk and ruminal fermentation measures, create a distinct ruminal BCC, and increase ruminal acidosis.

MATERIALS AND METHODS

The experiment was conducted in late fall at the Department of Environment and Primary Industries (DEPI), Ellinbank Centre, Victoria (**VIC**), Australia (38°14'S, 145°56'E). All experimental procedures were

approved by the DEPI Agricultural Research and Extension Animal Ethics Committee (AEC 2010-18).

Experimental Design

This prospective randomized controlled cohort experiment with an incomplete factorial design included 3 supplement feeding strategies and 5 supplementary feeding amounts offered to 144 Holstein-Friesian cows. These cows were seasonally calving, multiparous cows of mixed ages with an average liveweight of 625 ± 45 kg and were 272 ± 17 DIM. Twenty-four of these cows were rumen fistulated. The cows were milked twice daily at approximately 0700 and 1500 h. The experiment consisted of a 14-d pre-experimental period, during which cows were adapted to the amount of supplement, and a 16-d measurement period, during which DMI and milk production were measured. Ruminal fluid samples were collected on d 7 of the measurement period.

Supplement Feeding Strategies

All 144 cows had been fed either a control or PMR diet before the experiment and were randomized into 24 groups; each group of 6 cows was an experimental unit. Each group was allocated to one of the following feeding strategies:

- (1) In the control strategy (10 groups), cows grazed a perennial ryegrass (*Lolium perenne* L.) pasture supplemented with crushed wheat grain individually hand-fed twice daily in the milking parlor and ryegrass silage fed under an electric wire on the pasture. The ratio of ryegrass silage:wheat grain fed as supplement was 27:73 (DM basis). The pasture allowance was kept constant at 15 kg of DM/cow per day measured to ground level, to provide a target intake of approximately 8 kg of DM/cow per day.
- (2) In the PMR strategy (10 groups), cows grazed perennial ryegrass pasture twice daily at the same allowance as the control cows, were fed approximately 45% of their daily allocation of crushed wheat in the milking parlor, and were fed a PMR in equal proportions on a concrete feed pad immediately after each milking. The PMR comprised crushed wheat grain, crushed maize grain, maize silage, and ryegrass silage mixed and chopped in a feed wagon (model K160, Richard Keenan and Co. Ltd., Co. Carlow, Ireland; Table 1). Water was added to the ration such that the final DM content of the ration approximated 50%. The PMR provided the same estimated ME intake as the supplements fed to the control cows and had the same ratio of forage to grain, but was formulated to ferment more slowly.
- (3) In the PMR+canola strategy (4 groups), cows were fed and managed the same as the PMR cows, except that the ration contained alfalfa hay instead of ryegrass silage and 16% DM of the crushed wheat grain was replaced with solvent-extracted canola meal (Table 1). This ration provided the same ME as the control and PMR diets but had estimated amounts of MP that exceeded requirements (NRC, 2001).

Amount of Supplement

Each group of 6 cows was randomly assigned within their feeding strategy to receive different amounts of supplement, and each group contained a rumen-fistulated cow ($n = 2$ fistulated cows/feeding strategy per supplement feeding amount; Table 1). For the control and PMR cows, 2 groups were assigned to receive 8, 10, 12, 14, or 16 kg of total supplement DM/cow per day (SDM). For the PMR+canola cows, 2 groups were assigned to receive either 14 or 16 kg of SDM. Cows fed the 14 and 16 kg of SDM amounts were introduced gradually to dietary regimens over the first 7 d of the pre-experimental period.

As part of their supplement, all cows received a vitamin and mineral pellet (Nutrified Hi-Milker, Debenham Australia Pty Ltd., Leongatha, VIC, Australia) that contained monensin (110 mg/100 g of pellets) and tylosin (110 mg/100 g of pellets; Table 1). Cows at the highest amount of supplementation (16 kg of SDM) received this supplement at the amount recommended by the manufacturers (250 g of pellets/cow per day), whereas cows receiving lower amounts of supplement received proportionally less (e.g., cows fed 8 kg of SDM received 125 g/cow per day of the vitamin and mineral pellet). Control cows received their vitamin and mineral pellets mixed with their grain at milking time, whereas cows fed PMR and PMR+canola received their pellets mixed into their PMR.

All cows remained in their groups of 6 during feeding and grazing and had several opportunities each day to access water ad libitum. Control cows had access to pasture immediately after each milking, whereas those fed the PMR and PMR+canola diets had access after they had consumed their ration on the feed pad. Each group of 6 cows grazed adjacent areas separated by electric tapes and were prevented from regrazing areas from the previous days.

Table 1. Target DMI of ration composition (kg of total supplement DM/cow per day) and actual DMI of feeding groups

Ration component	Feeding strategy and supplement feeding amount ¹ (kg of total supplement DM/cow per day)											
	Control					PMR					PMR+canola	
	8	10	12	14	16	8	10	12	14	16	14	16
Target DMI												
Crushed wheat ²	5.8	7.3	8.8	10.4	11.7	3.0	3.8	4.6	5.4	6.2	3.1	3.5
Crushed maize	—	—	—	—	—	1.6	2.0	2.4	2.8	3.2	2.8	3.2
Alfalfa hay	—	—	—	—	—	1.0	1.2	1.4	1.6	1.8	1.7	1.9
Maize silage	—	—	—	—	—	2.4	3.0	3.6	4.2	4.8	4.2	4.8
Canola meal	—	—	—	—	—	—	—	—	—	—	2.2	2.6
Ryegrass silage	2.2	2.7	3.2	3.6	4.3	—	—	—	—	—	—	—
Pasture	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Total	16.0	18.0	20.0	22.0	24.0	16.0	18.0	20.0	22.0	24.0	22.0	24.0
Actual DMI												
Crushed wheat	5.3 ± 0.0	6.6 ± 0.0	7.7 ± 0.0	8.8 ± 0.2	10.0 ± 0.3	1.4 ± 0.0	1.8 ± 0.1	2.0 ± 0.1	2.2 ± 0.0	2.6 ± 0.0	2.3 ± 0.0	2.6 ± 0.0
Ryegrass silage	2.5 ± 0.0	3.1 ± 0.0	3.8 ± 0.0	4.4 ± 0.0	5.0 ± 0.0	—	—	—	—	—	—	—
PMR	—	—	—	—	—	7.1 ± 0.2	9.2 ± 0.3	11.1 ± 0.1	12.9 ± 0.1	14.6 ± 0.1	13.2 ± 0.0	14.9 ± 0.0
Pasture	8.8 ± 0.0	9.1 ± 0.3	8.3 ± 0.5	9.0 ± 0.0	7.9 ± 0.2	8.6 ± 0.4	8.7 ± 0.6	7.9 ± 0.5	8.0 ± 0.0	7.8 ± 0.1	8.2 ± 0.3	8.0 ± 0.1
Total supplement	7.8 ± 0.0	9.8 ± 0.0	11.4 ± 0.0	13.2 ± 0.2	15.1 ± 0.3	8.4 ± 0.2	11.0 ± 0.1	13.1 ± 0.0	15.2 ± 0.1	17.2 ± 0.1	15.5 ± 0.0	17.5 ± 0.1
Total	16.6 ± 0.0	18.9 ± 0.3	19.7 ± 0.5	22.2 ± 0.2	23.0 ± 0.5	17.1 ± 0.5	19.8 ± 0.7	21.0 ± 0.5	23.1 ± 0.1	25.0 ± 0.0	23.8 ± 0.2	25.5 ± 0.0

¹PMR = partial mixed ration; PMR+canola = PMR + canola meal. Rations also contained a vitamin and mineral mix (Nutrifeed Hi-Milker pellets; Debenham Australia Pty Ltd., Leongatha, Victoria, Australia) that contained (110 mg/100 g pellets) of monensin and (110 mg/100 g pellets) of tylosin. Cows at the highest amount of supplementation (16 kg of DM/cow per day) received this supplement at the amount recommended by the manufacturers (125 g pellet/cow per day), whereas cows receiving lower amounts of supplement received proportionally less (e.g., cows fed 8 kg of DM supplement/d received 71 g/cow per day of the vitamin and mineral pellet).

²Approximately 45% of the target DMI for crushed wheat for the PMR-fed cows was offered in the milking parlor and the remainder was included in the PMR.

Nutritive Characteristics and Supplement and Pasture Intakes

All ration components were analyzed by near-infrared spectroscopy (method 989.03; AOAC International, 2000; Dairy One Inc., Forage Testing Laboratory, Ithaca, NY; Table 2), and ration chemical composition was estimated in CPM Dairy Ration Analyzer V3.10 (Cornell-Penn-Miner, Cornell University, Ithaca, NY; Table 3). Rations offered and refused were weighed every day of the measurement period and subsamples of ration residuals were collected and analyzed for DM and nutritive characteristics to allow the calculation of daily DMI and estimated ME intake for each group.

Pre- and postgrazing mass was measured daily (Pasturemeter XP1, C-Daz Ltd., Palmerston North, New Zealand) to calculate average pasture DMI for each group. Pre- and postgrazing pasture samples were collected from each new pasture break grazed by cutting pasture at ground level using electric shears. Samples were washed, freeze-dried, and ground through a 0.5-mm sieve, and DM digestibility was analyzed by near-infrared spectroscopy (Dairy One Inc.) to estimate ME.

Milk Sampling Procedure and Laboratory Analysis

Milk yield of every cow was measured at each milking during both the pre-experimental and measurement periods using the DeLaval Alpro milk metering system (DeLaval International AB, Tumba, Sweden). Fat and protein concentrations of the daily milk from each cow were determined twice a week during both periods using inline milk meters (DeLaval International AB) and an infrared milk analyzer (model 2000; Bentley Instruments Inc., Chaska, MN).

Ruminal Fluid Sampling Procedure

On d 7 of the measurement period, ruminal fluid was collected from each rumen-fistulated cow (2 cows per feeding group) at approximately 2.4-h intervals over a 24-h period, totaling 10 samples per cow. This was done by restraining the cows in temporary yards set up in the pasture or in permanent yards next to the feed pad and milking parlor. The first sample was collected after the morning milking at approximately 0820 h. Samples were collected per fistula using a 100-mL plastic syringe connected to a brass pipe inserted into the rumen. Fluid was collected from several sites within the rumen and immediately tested for pH (Hannah HI9023 pH meter, Hannah Instruments, Keysborough, VIC, Australia). An additional untreated sample was stored at -20°C for ruminal bacterial analysis. Ruminal data from sample time 8 were excluded from the data set due to sampling error.

Ruminal Fluid Laboratory Analysis

For VFA analysis, an aliquot of 4 mL of ruminal fluid was dispensed into a tube containing 1 mL of 25% metaphosphoric acid before being stored at -20°C until subsequent analysis by capillary gas chromatography using the method of Packer et al. (2011).

For ammonia analysis, an aliquot of 10 mL of ruminal fluid was dispensed into a tube containing 10 mL of 0.1 M HCl before being stored at -20°C until analysis. Concentrations of ammonia were assayed by a direct enzymatic procedure using a commercially available kit (Boehringer Mannheim; R-Biopharm Laboratory Diagnostics Pty Ltd., Taren Point, NSW, Australia) and a Cobas Mira S autoanalyzer (Roche, Montclair, NJ).

D-Lactate was analyzed using a UV method for D-lactate determination in ruminal fluid using a Boehringer

Table 2. Chemical composition of feed components and pasture¹

Item (% of DM)	Feed component						
	Crushed wheat	Crushed maize	Alfalfa hay	Maize silage	Canola meal	Ryegrass silage	Pasture consumed
CP	13.8	10.0	18.6	10.0	37.4	13.3	22.2
ADF	5.3	4.5	36.3	25.7	21.9	40.1	27.5
NDF	12.9	13.3	44.9	46.0	30.2	61.3	49.9
Lignin	0.7	0.6	10.9	3.3	10.5	3.7	3.4
NFC	74.4	70.8	28.0	36.8	25.5	14.8	17.1
Starch	54.2	59.8	2.6	28.4	1.2	0.7	1.5
Crude fiber	2.0	5.5	2.1	4.0	6.6	3.9	5.8
Ash	1.7	1.9	11.3	4.9	8.9	9.0	10.0
ME (MJ/kg of DM)	14.7	14.6	8.2	10.8	12.7	9.7	11.5

¹Analyzed by near-infrared spectroscopy (Dairy One Inc., Forage Testing Laboratory, Ithaca, NY).

Table 3. Predicted chemical composition of consumed rations¹

Item (% of DM)	Feeding strategy and supplement feeding amount ² (kg of total supplement DM/cow per day)											
	Control					PMR					PMR+canola	
	8	10	12	14	16	8	10	12	14	16	14	16
DM	77.6	76.5	74.9	74.5	72.6	75.9	73.9	71.4	70.1	68.6	70.2	68.6
CP	18.1	17.7	17.1	17.0	16.5	17.4	16.7	16.0	15.7	15.4	18.2	18.1
RUP (% of CP)	26.9	27.7	27.6	28.8	28.5	27.5	28.7	29.1	30.1	30.9	35.2	34.6
RDP (% of CP)	73.1	72.3	72.4	71.2	71.5	72.5	71.3	71.0	69.9	69.1	64.8	65.4
RDP	13.2	12.8	12.4	12.1	11.8	12.6	11.9	11.4	11.0	10.7	11.8	11.8
Soluble protein (% of CP)	35.7	36.1	36.9	37.1	37.8	35.2	35.7	36.2	36.5	36.8	35.1	35.2
ADF	22.2	21.6	21.1	21.0	20.4	21.4	20.5	19.6	19.2	18.7	21.0	20.7
NDF	39.6	38.6	37.4	37.2	36.0	38.5	37.0	35.4	34.6	33.8	36.5	35.9
Forage NDF (% of NDF)	89.5	88.1	86.5	86.1	84.2	65.5	59.6	53.1	49.8	46.1	81.7	43.6
Forage NDF (% of DM)	35.4	34.0	32.4	32.1	30.3	25.2	22.0	18.8	17.2	15.6	29.9	15.7
Physically effective NDF	27.8	27.1	26.4	26.3	25.4	27.7	26.7	25.7	25.1	15.6	26.0	25.6
Lignin	2.57	2.48	2.39	2.37	2.27	3.07	3.00	2.93	2.89	2.85	3.80	3.83
NFC ³	33.4	35.1	37.0	37.4	39.5	35.3	37.8	40.4	41.7	43.0	36.8	37.7
Silage acids	0.10	0.10	0.12	0.13	0.14	0.74	0.84	0.94	0.98	1.03	0.98	1.03
Sugar	9.86	9.44	8.85	8.73	8.18	9.23	8.58	7.92	7.60	7.26	8.28	8.01
Starch	17.6	19.3	21.3	21.7	23.8	20.2	22.9	25.6	27.0	28.4	21.3	22.2
Soluble fiber	5.89	6.26	6.74	6.84	7.31	5.15	5.52	5.90	6.08	6.27	6.28	6.47
Total ether extract	4.29	4.14	3.94	3.90	3.71	4.57	4.42	4.25	4.17	4.09	4.65	4.61
Total long-chain FA	2.71	2.63	2.52	2.50	2.40	3.11	3.06	3.00	2.98	2.95	3.33	3.34
Ash	7.55	7.34	7.05	7.00	6.70	7.39	7.06	6.68	6.50	6.31	7.29	7.16
DCAD (mEq/100 g)	50.1	45.8	40.0	38.7	33.2	51.3	45.7	40.0	37.3	34.4	37.5	34.6
Minerals (mg/kg)												
Chloride	6,800	6,900	7,200	7,300	7,400	4,200	4,100	3,900	3,800	3,700	3,700	3,600
Calcium	4,600	4,800	5,000	5,100	5,200	4,100	4,200	4,200	4,200	4,200	5,000	5,100
Copper	9.38	9.68	9.88	9.95	10.1	9.78	10.2	10.4	10.5	10.6	10.4	10.5
Iron	154	155	156	157	157	157	158	158	157	157	182	183
Phosphorus	5,500	5,400	5,200	5,200	5,000	5,200	5,000	4,800	4,700	4,600	5,600	5,500
Potassium	30,300	28,400	25,900	25,400	23,000	29,300	26,600	23,900	22,600	21,300	23,700	22,500
Magnesium	2,100	2,200	2,200	2,200	2,200	2,200	2,200	2,200	2,300	2,300	2,600	2,700
Manganese	46.5	46.4	45.8	45.7	45.3	43.2	42.7	41.8	41.4	41.0	43.8	43.6
Sodium	2,000	2,000	2,200	2,200	2,300	1,000	1,000	1,000	900	900	1,000	1,000
Sulfur	2,700	2,600	2,500	2,500	2,400	2,600	2,400	2,300	2,300	2,200	2,700	2,700
Zinc	44.7	45.7	46.3	46.5	47.1	41.8	42.6	42.8	42.9	43.0	44.9	45.2

¹Estimations were performed using CPM Dairy Analyzer V3.10 (Cornell-Penn-Miner, Cornell University, Ithaca, NY) using DMI data from Table 1 and chemical composition of feed ration components in Table 2 and were based on 625-kg BW cows, 272 DIM, with a BCS of 3.00 and mean milk yield, milk fat and protein percentage for each group.

²PMR = partial mixed ration; PMR+canola = PMR + canola meal.

³NFC = 100 – [(NDF – NDICP) + CP + crude fat + ash], where NDICP = neutral detergent insoluble CP.

Mannheim kit (cat no. 11 112 821 035; R-Biopharm-Laboratory Diagnostics Pty. Ltd.) after deproteinization with perchloric acid according to kit instructions on an Olympus AU400 Autoanalyzer (AHL NTM-56; Olympus, Mount Waverly, VIC, Australia).

DNA Extraction

Bacterial community composition was determined on unfiltered ruminal fluid that was primarily liquid but contained particulate matter, thus representing a mixture of the liquid and particulate phases of the rumen, that was collected at approximately 0820, 1200, and 1640 h (sampling times 1, 3, and 5) on d 7 of the measurement period. Ruminal fluid samples ($n = 72$) were thawed at room temperature and a 1-mL aliquot was centrifuged at $10,000 \times g$ for 1 min and the supernatant discarded. The pellet was resuspended by vigorous vortexing in 200 μ L of ATL buffer (Qiagen GmbH, Hilden, Germany) together with 200 mg of silica-zirconium beads (1:1 mixture of 0.1- and 1.0-mm beads; Biospec, Bartlesville, OK). The mixture was homogenized in a FastPrep-24 homogenizer (MP Biomedicals, Seven Hills, New South Wales, Australia) at maximum speed for 1 min, twice, heated at 70°C for 15 min, and centrifuged at $10,000 \times g$ for 5 min. Supernatant (180 μ L) was removed for digestion with proteinase K at 50°C for at least 3 h, and DNA was extracted according to the QIAmp DNA mini kit protocol (Qiagen GmbH). DNA was quantified using a Nanodrop spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA).

PCR Amplification of 16S Ribosomal DNA Gene Sequences

Genomic DNA from each sample was diluted 1:30 with water and the 16S rRNA gene spanning V1 to V3 was PCR amplified using Platinum Taq polymerase (Invitrogen, Carlsbad, CA) as follows: 1 cycle at 94°C for 2 min, followed by 30 cycles of 94°C for 10 s, 55°C for 45 s, 72°C for 45 s, with a final extension of 72°C for 10 min. Primers used in the reaction were modified universal 8F (Snell-Castro et al., 2005) and 515R (Lane, 1991) primers that included 454 sequencing adapters B and A, respectively. In addition, a unique 8-bp barcode was included in the reverse primer of each amplicon, so that DNA sequence reads could be assigned accurately to each originating sample. The PCR products were visualized on agarose gels and equal amounts of PCR product were pooled and gel extracted (Qiaex gel extraction kit, Qiagen). Approximately 3 μ g of pooled amplicon (~ 40 ng/ μ L) was dispatched to Macrogen (Seoul, Korea) for 454 DNA sequencing using a 454

GS FLX Sequencer with titanium chemistry (Roche, Branford, CT).

Sequence Analyses of Gene Amplicons

Sequence data was processed using the quantitative insights into microbial ecology software package QIIME (Caporaso et al., 2010). Sequences were assigned to their originating sample based on the attached barcode and filtered based on quality and length parameters. Error correction of 454 data was performed using Aca-cia (Bragg et al., 2012). Clustering of sequences to an operational taxonomic unit (OTU) at a 0.97 distance threshold was used. Taxonomic identification was based on similarity to the Greengenes Database (<http://greengenes.lbl.gov>). The OTU table was subjected to α and β diversity measures using QIIME and passed through R (package Ade4; Dray and Dufour, 2007) for nonconstrained principal coordinates and constrained principal coordinates between group analysis, Monte Carlo tests, and co-inertia analysis.

Statistical Analysis

DMI. Dry matter intake data (Table 4) were derived by averaging daily group measures over the measurement period, and data from all feeding amounts ($n = 144$ cows) were analyzed using a linear mixed model in Genstat (14th ed., VSN International Ltd., Hemel Hempstead, UK), using model [1], with the modification of removal of time and interactions with time from the model. Orthogonal polynomial linear and quadratic contrasts were included for feeding amount. The correlation structure of the random error terms was independent:

$$Y_{ijklmn} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\alpha\delta)_{il} \\ + (\alpha\gamma\delta)_{ikl} + (\beta\gamma)_{jk} + (\beta\delta)_{jl} + (\beta\gamma\delta)_{jkl} + (\alpha\beta\gamma)_{ijk} \\ + (\alpha\beta\delta)_{ijl} + (\alpha\beta\gamma\delta)_{ijkl} + X_m + (XY)_{mn} + \varepsilon_{ijklmn}, [1]$$

where Y_{ijklmn} = response at time i ($i = 1$ to 9, or 1 to 3), to protein j (yes or no), for feeding strategy k ($k = 1$ or 2), at supplement feeding amount l ($l = 1$ to 5), for herd m ($m = 1$ to 5), in group n ($n = 1$ to 5); μ = overall mean; α = time; β = protein; γ = feeding strategy nested within time and protein; δ = supplement feeding amount nested within time and protein; X = herd; Y = group nested within herd; and ε = random error.

To incorporate the effect of protein, data from the groups fed total supplement at amounts of 14 and 16 kg of DM/cow per day were termed the high supplement feeding amounts and were analyzed using model [2],

Table 4. Main effects and interaction of feeding strategy (S) and supplement feeding amount (A), and means $\pm$ SE of the difference (SED) of feeding strategy for DMI, milk yield, and milk composition of the herd including fistulates (n = 144 cows)

Measure	Amount ¹	Feeding strategy means ²			SED	P-value			
		Control	PMR	PMR+canola		S	A ³		
							Lin	Quad	S × A
DMI (kg/d)									
Pasture	All	8.62	8.21		0.20	0.068	0.011	0.495	0.666
	High	8.44 ^a	7.89 ^b	8.10 ^b	0.14	0.024	0.004		0.041
Total supplement	All	11.5 ^a	13.0 ^b		0.07	<0.001	<0.001	0.076	<0.001
	High	14.2	16.2	16.5	0.15	<0.001	<0.001		0.696
Total	All	20.1 ^a	21.2 ^b		0.24	<0.001	<0.001	0.266	0.340
	High	22.6 ^a	24.1 ^b	24.6 ^c	0.22	<0.001	<0.001		0.088
Milk									
Yield (kg/cow per day)	All	17.0	17.1		0.65	0.847	<0.001	0.152	0.995
	High	19.7	19.6	22.6	1.40	0.120	0.673		0.556
ECM ⁴ (kg/cow per day)	All	17.5	18.3		0.50	0.179	<0.001	0.055	0.534
	High	19.0 ^a	20.0 ^a	24.0 ^b	1.14	<0.001	0.542		0.537
Fat (%)	All	4.17	4.41		0.15	0.137	<0.001	0.958	0.403
	High	3.56	4.02	4.32	0.24	0.052	0.854		0.422
Fat (kg/cow per day)	All	0.68	0.74		0.03	0.058	0.221	0.092	0.270
	High	0.68 ^a	0.76 ^b	0.96 ^c	0.05	0.003	0.324		0.481
Protein (%)	All	3.76	3.75		0.04	0.654	0.505	0.477	0.198
	High	3.73	3.75	3.71	0.06	0.795	0.024		0.090
Protein (kg/cow per day)	All	0.63	0.64		0.02	0.844	<0.001	0.132	0.804
	High	0.73 ^a	0.73 ^a	0.84 ^b	0.04	0.025	0.948		0.551

^{a-c}Means within a row not sharing a common superscript differ significantly ($P < 0.05$).

¹All = supplement feeding amounts 8, 10, 12, 14, and 16 kg of DM total supplement/cow per day; High = supplement feeding amounts 14 and 16 kg of DM total supplement/cow per day, to incorporate the effect of canola meal substitution.

²PMR = partial mixed ration; PMR+canola = PMR + canola meal.

³Lin = linear polynomial contrast; Quad = quadratic polynomial contrast.

⁴Standardized to 4.0% fat and 3.3% protein, was calculated using the following formula: ECM (kg/cow per day) = milk yield kg $\times$ (376 $\times$ fat % + 209 $\times$ protein % + 948)/3,138 (Tyrrell and Reid, 1965).

with the modification of removal of time and interactions with time from the model. Orthogonal polynomial linear and quadratic contrasts were not included in this analysis as the high supplement feeding amounts comprised only 2 feeding amounts. The correlation structure of the random error terms was independent:

$$Y_{ijklm} = \mu + \alpha_i + \gamma_j + \delta_k + (\gamma\delta)_{jk} + (\alpha\gamma)_{ij} + (\alpha\delta)_{ik} + (\alpha\gamma\delta)_{ijk} + X_m + (XY)_{mn} + \varepsilon_{ijklm}, \quad [2]$$

where Y_{ijklm} = response at time i ($i = 1$ to 9), for feeding strategy j ($j = 1$ to 3), at supplement feeding amount k ($k = 1$ to 5), for herd l ($l = 1$ to 5), in group m ($m = 1$ to 5); μ = overall mean; α = time; γ = feeding strategy; δ = supplement feeding amount; X = herd; Y = group nested within herd; and ε = random error.

Milk Measures. Milk measures (Table 4) were derived by averaging daily individual measures over the measurement period and were analyzed for the herd (n = 144 cows) and for the fistulates only (n = 24 cows) for all supplement feeding amounts and the high supplement feeding amounts, using models [1] and [2], respectively, with the removal of time and interactions

with time from each model. Orthogonal polynomial linear and quadratic contrasts were included for supplement feeding amount in model [1].

Ruminal Measures. Ruminal data (Table 5) from 9 sampling times from the fistulated cattle were analyzed for all supplement feeding groups (n of samples = 216) and the high supplement feeding amount groups (n of samples = 108) using models [1] and [2], respectively. Orthogonal polynomial linear and quadratic contrasts were included for feeding amount in model [1].

Bacterial Prevalence. The relative abundance of bacterial families in ruminal fluid collected at approximately 0820, 1200, and 1640 h (sampling times 1, 3, and 5) with a mean relative abundance of >0.2% for at least one feeding strategy were analyzed for all supplement feeding amounts (n of samples = 72) and the high supplement feeding amounts (n of samples = 36) using the same linear mixed models used for the ruminal data. The relative abundances of the following bacterial genera were also analyzed using the same models: *Prevotella*, *Lactobacillus*, *Butyrivibrio*, *Megasphaera*, *Selenomonas*, *Veillonella*, and *Streptococcus*.

Evaluation of Ruminal Acidosis. The incidence of ruminal acidosis was diagnosed using eigenvalues

Table 5. Main effects and their interactions of feeding strategy (S), supplement feeding amount (A), and sample time (T) and means $\pm$ SE of the difference (SED) of feeding strategy for acidosis eigenvalues and ruminal measures

Item	Amount ¹	Feeding strategy means ²				P-value							
		Control	PMR	PMR+canola	SED	S	A ³		T	S $\times$ A	S $\times$ T	A $\times$ T	S $\times$ A $\times$ T
							Lin	Quad					
Acidosis eigenvalue	All	0.26 ^a	0.06 ^b		0.07	0.012	<0.001	0.014	<0.001	0.075	0.030	<0.001	0.154
	High	0.63	0.14	0.06	0.16	0.066	0.104		<0.001	0.654	0.027	0.259	0.393
Ruminal (mM)													
Total VFA	All	120.4	113.9		3.51	0.088	0.389	0.679	<0.001	0.205	<0.001	0.007	0.439
	High	123.0	113.4	122.7	6.47	0.313	0.846		<0.001	0.902	0.022	0.098	0.175
Acetate (A)	All	71.4	70.3		2.02	0.601	0.021	0.085	<0.001	0.045	0.034	0.152	0.624
	High	67.2	67.8	74.9	3.50	0.129	0.107		<0.001	0.801	0.236	0.474	0.215
Propionate (P)	All	30.2 ^a	22.3 ^b		1.79	<0.001	<0.001	0.110	<0.001	0.098	<0.001	<0.001	0.019
	High	39.9	26.2	26.9	3.58	0.071	0.049		<0.001	0.606	<0.001	<0.001	0.197
A:P	All	2.74 ^a	3.39 ^b		0.16	0.002	<0.001	0.186	<0.001	0.083	<0.001	0.002	0.742
	High	1.84	2.82	3.01	0.30	0.067	0.031		<0.001	0.438	<0.001	0.063	0.038
Butyrate (B)	All	13.5 ^a	16.2 ^b		0.80	0.006	<0.001	0.197	<0.001	0.025	<0.001	0.640	0.865
	High	10.3	14.5	15.4	1.57	0.125	0.143		<0.001	0.855	0.025	0.836	0.619
(A+B)/P	All	3.28 ^a	4.17 ^b		0.22	0.001	<0.001	0.218	<0.001	0.097	<0.001	<0.001	0.816
	High	2.11	3.41	3.63	0.40	0.077	0.033		<0.001	0.467	<0.001	0.053	0.126
Valerate	All	2.29 ^a	1.77 ^b		0.16	0.007	<0.001	0.006	<0.001	0.018	<0.001	<0.001	<0.001
	High	2.94	2.00	2.14	0.35	0.070	0.013		<0.001	0.067	<0.001	<0.001	0.001
D-Lactate	All	0.06 ^a	0.44 ^b		0.09	0.002	0.377	0.194	<0.001	0.637	<0.001	0.988	0.990
	High	0.04 ^a	0.60 ^b	0.29 ^a	0.14	<0.001	0.132		<0.001	0.657	<0.001	0.406	0.948
Ammonia	All	7.07	8.02		0.99	0.358	0.001	0.221	<0.001	0.255	<0.001	<0.001	0.051
	High	3.79 ^a	5.59 ^a	11.36 ^b	1.34	0.011	0.071		<0.001	0.566	<0.001	0.099	0.104
pH	All	6.07 ^a	6.25 ^b		0.06	0.009	0.007	0.473	<0.001	0.645	<0.001	<0.001	0.926
	High	5.94	6.18	6.13	0.09	0.092	0.893		<0.001	0.935	<0.001	0.108	0.840

^{a,b}Means within a row not sharing a common superscript differ significantly ($P < 0.05$).

¹All = supplement feeding amounts 8, 10, 12, 14, and 16 kg of DM total supplement/cow per day; High = supplement feeding amounts 14 and 16 kg of DM total supplement/cow per day, to incorporate the effect of canola meal substitution.

²PMR = partial mixed ration; PMR+canola = PMR + canola meal.

³Lin = linear polynomial contrast; Quad = quadratic polynomial contrast.

obtained using discriminant analysis of standardized variates of ruminal concentrations of the individual VFA: acetate, butyrate, propionate, valerate, isovalerate, isobutyrate, and caproate, and total lactate and concentrations of ruminal ammonia and ruminal pH defined according to the methods of Bramley et al. (2008). Acidosis eigenvalues were based on the statistical distance of each sample from the centroid for known cases of ruminal acidosis identified in the data set of Bramley et al. (2008). Eigenvalues that approached 1 are in the center of the acidosis category and those approaching 0 are not acidotic.

RESULTS

A linear decrease in milk fat percentage with increased supplement amount fed was a clinical sign consistent with ruminal acidosis; however, diarrhea and lameness were not observed in any of the cows during the experiment. Acidosis eigenvalues showed that control cows fed 16 kg of SDM had ruminal acidosis (Table 5; Figure 1). Milk data were not recorded for a control cow fed 12 kg of SDM during the measurement period, as she ceased production.

DMI

Total DMI was within 1 kg of DM of the target total DMI for the controls, but was up to 1.8 kg of DM higher than the target DMI in the PMR and PMR+canola cows (Table 1). Differences between target and actual DMI of total supplement did not exceed 1 kg of DM for the controls, regardless of supplement feeding amount. The DMI of total supplement for the PMR and PMR+canola cows exceeded the target intakes for all supplement feeding amounts by between 0.4 to 1.5 kg of DM/cow per day. Pasture DMI for the controls was higher for all supplement feeding amounts except the 12 and 16 kg of DM feeding groups compared with the target DMI and was similar to the target DMI for pasture in the PMR and PMR+canola cows. The DMI of crushed wheat was lower than the target DMI for all control cows, regardless of supplement feeding amount. The controls had from 0.3 to 0.8 kg of DM higher DMI of ryegrass silage than the target DMI (Table 1).

All Supplement Feeding Amounts. Pasture DMI was not influenced by feeding strategy; however, the total daily DMI and DMI of total supplement were higher in the PMR compared with control cows. Pasture DMI, total supplement DMI, and total daily DMI increased linearly with supplement feeding amount (Table 4).

High Supplement Feeding Amounts. Pasture DMI was higher in the control compared with PMR and PMR+canola cows, highest in the control cows fed

14 kg of SDM, and lower in the cattle fed 16 kg of SDM. The DMI of total supplement was lower in the control compared with the PMR and PMR+canola cows and higher in cows fed 16 compared with 14 kg of SDM. Total daily DMI was lower in the control compared with the PMR cows and highest in the PMR+canola cows, and was higher in the cows fed 16 compared with 14 kg of SDM (Table 4).

Milk Measures

Greater variation in milk measures occurred in the fistulates compared with the herd (data not shown). The feeding strategy $\times$ supplement feeding amount interaction was not significant for any milk measures (Table 4).

All Supplement Feeding Amounts. Yields of fat tended to increase in the PMR cows compared with control cows, but feeding strategy had no other effects on milk measures. Yields of milk, ECM, and protein increased linearly with supplement feeding amount, and fat percentage decreased linearly with supplement feeding amount (Table 4).

High Supplement Feeding Amounts. Yield of ECM and protein were higher in the cows fed PMR+canola compared with the control and PMR cows. Fat yield was higher in the PMR cows than the control cows and was highest in the PMR + canola-fed cows. A similar trend was observed for fat percentage. Protein percentage was higher in the cows fed 16 compared with 14 kg of SDM (Table 4).

Ruminal Measures

For all supplement feeding amounts and the high supplement feeding amounts, total and individual VFA and ammonia concentrations had a biphasic pattern over time, with peaks in concentrations occurring at times 3 and 6. The opposite pattern was observed for ruminal pH over time. Concentrations of D-lactate were relatively stable over time except for an increase in concentration in the PMR and PMR+canola cows at time 5 (the first sampling time after the afternoon feed).

All Supplement Feeding Amounts. Acidosis eigenvalues were lower by 0.2 ± 0.07 units in the PMR-fed cows compared with the controls, and had a linear and quadratic increase with supplement feeding amount (Table 5). Eigenvalues were higher in the controls from times 3 to 9 (Figure 1B) and were higher in the cattle fed 16 kg of SDM at times 2 to 10, and the cows fed 14 kg of SDM at times 3 and 4, compared with those fed 8 to 12 kg of SDM (Figure 1C). Peaks in acidosis eigenvalues occurred at times 4 and 7 (Figure 1B).

Figure 1. Mean ($\pm$ SEM) acidosis eigenvalues for dairy cows from all feeding groups showing interactions between (A) feeding strategy and supplement feeding amount, (B) feeding strategy and sample time, and (C) supplement feeding amount and sample time. Mean ($\pm$ SEM) acidosis eigenvectors for dairy cows from the high supplement feeding amount groups only (14 and 16 kg of DM of total supplement/cow per day) showing interactions between (D) feeding strategy and supplement feeding amount, (E) feeding strategy and sample time, and (F) supplement feeding amount and sample time. Sample times were approximately 2.4 h apart over a 24-h period. Sample time 1 was approximately 0820 h and milking was at 0700 and 1500 h (black arrows). PMR = partial mixed ration; PMR + canola = PMR + canola meal; amount = kg of DM of total supplement/cow per day.

Concentrations of D-lactate and butyrate, the acetate to propionate ratio, the lipogenic to gluconeogenic VFA ratio [(acetate + butyrate)/propionate], and pH were higher in the PMR cows compared with the controls, whereas propionate and valerate concentrations were lower in the PMR cows compared with control cows. Total VFA, acetate, and ammonia concentrations were not influenced by feeding strategy (Table 5).

The acetate to propionate ratio, the lipogenic to gluconeogenic VFA ratio, pH, and acetate, butyrate, and ammonia concentrations linearly decreased with supplement feeding amount. Propionate concentration

linearly increased with supplement feeding amount and valerate concentration linearly and quadratically increased with supplement feeding amount (Table 5).

Propionate and valerate concentrations were the only ruminal measures with a 3-way interaction between feeding strategy, supplement feeding amount, and sample time. Acetate concentration was higher in the control cows fed 8 and 10 kg of SDM and lower in control cows fed 12 kg of SDM compared with the PMR cows. Butyrate concentrations were increased in the PMR cows fed 12 to 16 kg of SDM compared with the controls. Valerate concentration was higher in the

Figure 2. Mean interactions for (A) total VFA concentrations between feeding strategy and sample time from all feeding groups ($n = 24$ cows), (B) total VFA concentrations between supplement feeding amounts and sample time from all feeding groups ($n = 24$ cows), (C) acetate to propionate ratio between feeding strategy and sample time from all feeding groups ($n = 24$ cows), (D) total VFA concentrations for between feeding strategy and sample time from cattle fed only 14 and 16 kg of total supplement/cow per day ($n = 12$ cows), (E) acetate to propionate ratio between supplement feeding amount and sample time from all feeding groups ($n = 24$ cows), and (F) acetate to propionate ratio between feeding strategy and sample time from cattle fed only 14 and 16 kg of total supplement/cow per day ($n = 12$ cows). Sample times were approximately 2.4 h apart over a 24-h period. Sample time 1 was approximately 0820 h and milking was at 0700 and 1500 h (black arrows). PMR = partial mixed ration; PMR + canola = PMR + canola meal; amount = kg of DM of total supplement/cow per day.

control cows fed 16 kg of SDM compared with others (Table 5).

Total VFA (Figure 2A), acetate, propionate, and valerate concentrations were higher in control cows at times 3 and 6 and lower at time 5. The acetate to propionate ratio (Figure 2C), the lipogenic to gluconeogenic VFA ratio, pH, and butyrate concentrations were greater over time in the PMR-fed cattle (Table 5).

Total VFA concentrations were lower in cows fed 16 kg of SDM at times 9 and 10 compared with cattle fed 8 and 12 kg of SDM, and were higher in cattle fed 16 compared with 8 and 10 kg of SDM at time 6 (Figure 2B). Propionate and valerate concentrations were higher for cows fed 16 kg of SDM than for all other supplement feeding amounts at times 3 to 6. The acetate to propionate (Figure 2E) and lipogenic to

gluconeogenic VFA ratios decreased with supplement feeding amount across time, with the highest ratios at times 1 and 10. Ruminal pH decreased with supplement feeding amount and was lowest at times 3 and 6. Ammonia concentration was greater in the PMR cows at times 2, 3, and 5, and lower at time 7.

High Supplement Feeding Amounts. Acidosis eigenvalues were higher in the control compared with PMR and PMR+canola cows between times 3 and 9 (Figure 1E) and peaked at times 4 and 7. D-Lactate concentration was increased in PMR cows compared with control and PMR+canola cows, and ammonia concentration was increased in the PMR+canola cows. Propionate and valerate concentrations were increased in the cows fed 16 compared with 14 kg of SDM, and the acetate to propionate and lipogenic to gluconeogenic VFA ratios were decreased in the cows fed 16 kg of SDM compared with 14 kg of SDM. A 3-way interaction of feeding strategy $\times$ supplement feeding amount $\times$ time occurred for valerate concentration, but no 2-way interaction of feeding strategy $\times$ supplement feeding amount was significant for any ruminal measures (Table 5).

Total VFA (Figure 2D), propionate, and valerate concentrations, the acetate to propionate ratio (Figure 2F), the lipogenic to gluconeogenic VFA ratio, and pH had similar patterns across time for each feeding strategy when all supplement feeding amounts were analyzed. The PMR+canola cows produced similar results to the PMR cows over time for these measures, except for an increase in pH in PMR+canola cows compared with control and PMR cows at time 3. Ammonia concentrations were higher in the PMR+canola cows from times 1 to 7 compared with the control and PMR cows and peaked at times 3 and 6. Propionate and valerate concentrations were higher in the cows fed 16 kg of SDM over time compared with those fed 14 kg of DM, similar to when all supplement feeding amounts were analyzed (Table 5).

Bacterial Diversity Analysis

Rarefaction analysis (Hughes et al., 2001), which is used to estimate the depth of coverage of diversity of ruminal bacteria within ruminal fluid samples (Kim et al., 2011), indicated that coverage of bacterial diversity appeared sufficient to evaluate BCC. Sampling saturation of OTU had not yet been met. The PMR cows fed 10 kg of SDM had the highest number of observed OTU (722 ± 50) and the control cows fed 16 kg of SDM had the lowest (254 ± 29 ; Figure 3).

The overall microbiomes of the cows were not distinctly different in their BCC, with only 6 and 4.5% of the variance being explained, which could not be

attributed to any of the groupings (data not shown). However, distinct variation (Monte Carlo $P = 0.001$) in the 16S rDNA-estimated BCC occurred among feeding strategies at the species level in a between-group constrained principal component analysis (PCoA) that captured 100% of the sample variation (Figure 4A). The first principal component (PC) of the between-group analysis accounted for 57.8% of the variation between feeding strategies, and the second PC accounted for 42.2% (Figure 4A). Bacterial community composition was the most distinct between the control and PMR+canola cows. Spatial heterogeneity among cows was relatively large and was greatest in the control-fed cows (Figure 4A).

The between-group constrained PCoA identified variation in 16S rDNA-estimated BCC among groups from different feeding strategy and supplement feeding amount combinations (Monte Carlo $P = 0.001$). This variation was not as distinct as that between feeding strategy groups alone, which accounted for 37.4% of the variation (Figure 4B). The first PC accounted for 22.4% of the variation between feeding strategies, and the second PC accounted for 15.0% (Figure 4B). Bacterial community composition was not different among control cows fed 8 and 10, PMR cows fed 8, 10, 12, and 14, and PMR+canola cows fed 14 kg of SDM (Figure 4B); however, the BCC of these tightly clustered groups differed from that of the remaining groups. Bacterial community composition was similar between PMR cows fed 16 kg of SDM and PMR+canola cows fed 16 kg of SDM, and these 2 groups had the greatest spatial heterogeneity (Figure 4B). The control cows fed 12 and 14 kg of SDM also had a relatively large spatial heterogeneity and similarity in BCC. The control cows fed 16 kg of SDM had the most distinct BCC with, uniquely, no overlap of composition observed (Figure 4B).

Distinct BCC occurred among each of the 3 ruminal fluid sample times (Figure 4C; Monte Carlo $P = 0.001$). A total of 100% of the variation was accounted for by the first 2 PC: PC1 accounted for 63.9% and PC2 accounted for 36.2% of the variation (Figure 4C).

Bacterial Prevalence

A total of 12 phyla and 1 candidate phyla were identified within the ruminal bacterial population from 16S rDNA gene pyrosequences. The majority of sequences were represented by the *Firmicutes* or *Bacteroidetes* phyla, accounting for a combined total of 84.2% of sequences; 49.9 and 34.3% of total sequences on average, respectively. The candidate phyla TM7 represented 6.3% and the phylum *Tenericutes* represented 5.4% of the total sequences on average. The remaining microbiota

Figure 3. Rarefaction curves for each feeding group consisting of the mean number of observed operational taxonomic units (OTU) $\pm$ SEM. The OTU were calculated with a 3% disparity. Numbers 8, 10, 12, 14, and 16 denote the amount (kg) of DM of total supplement fed/cow per day. PMR = partial mixed ration; PMR + canola = PMR + canola meal.

was composed of phyla with low relative abundances. A total of 44 bacterial families were identified, and the *Prevotellaceae*, *Ruminococcaceae*, and *Lachnospiraceae* families and the unclassified *Clostridiales* had the largest relative abundances, at 30.9, 21.3, 11.3, and 11.7%, respectively. In total, 64 genera were identified, with *Prevotella* being the predominant genus. These represented approximately 30.1% of the relative abundance of bacterial sequences, ranging from an average of 13.0 to 50.7% among groups.

When all supplement feeding groups were analyzed, members of the *Prevotella* genus were lower in relative abundance in the PMR compared with control cows (mean $\pm$ SED of relative abundance = 30.0 and 38.6% $\pm$ 4.77; $P = 0.03$). Relative abundance of members of the *Lactobacillus*, *Streptococcus*, and *Butyrivibrio* genera were higher in the PMR (0.12% $\pm$ 0.03, 0.05% $\pm$ 0.01, and 3.42% $\pm$ 0.57; respectively), compared with control cows (0.00% $\pm$ 0.03, 0.01% $\pm$ 0.01, and 1.52% $\pm$ 0.57; $P = 0.001$, 0.001, and 0.006, respectively). The relative abundance of the *Megasphaera*, *Selenomonas*, and *Veillonella* genera were not influenced by feeding strategy. The *Butyrivibrio* linearly increased in relative abundance with supplement feeding amount ($P = 0.05$);

however, feeding amount had no effect on the relative abundance of the *Prevotella*, *Lactobacillus*, *Streptococcus*, *Megasphaera*, *Selenomonas*, and *Veillonella* genera. When only the high supplement feeding amount groups were analyzed, feeding strategy and supplement feeding amount did not affect the *Prevotella*, *Lactobacillus*, *Butyrivibrio*, *Megasphaera*, *Selenomonas*, or *Veillonella* genera, but *Streptococcus* was lower in control cows compared with PMR- and PMR+canola-fed cows (0.06, 0.05, and 0.00% $\pm$ 0.02, respectively; $P = 0.039$).

The main effects and the interactions of feeding strategy, supplement feeding amount, and sample time for bacterial families with a mean relative abundance of $>0.2\%$ in at least one feeding strategy for all cows are displayed in Table 6. These main effects and interactions are also displayed for cows fed 14 and 16 kg of SDM, incorporating the effect of canola substitution (Table 6). The *Streptococcaceae* and *Lactobacillaceae* were among the bacterial families that had a mean relative abundance of $>0.2\%$ in at least one feeding strategy.

All Supplement Feeding Amounts. Relative abundances of the following bacterial families were increased in the control compared with PMR cows:

Figure 4. Constrained between-group principal coordinates (PC) analysis of bacterial 16S rDNA gene sequences at the species level from ruminal fluid samples from dairy cattle (A) fed 1 of 3 feeding strategies: control ($n = 10$ cows; $n = 30$ samples), partial mixed ration (PMR; $n = 10$ cows; $n = 30$ samples), or PMR+canola (PMR + canola meal; $n = 4$ cows; $n = 12$ samples), (B) fed 1 of 3 feeding strategies: control ($n = 10$ cows), PMR ($n = 10$ cows), or PMR+canola ($n = 4$ cows) at one of the following supplement feeding amounts 8, 10, 12, 14, or 16 kg of DM of total supplement/cow per day (2 cows per amount from each feeding strategy; $n = 6$ samples per amount from each feeding strategy), and (C) collected at 1 of 3 sample times: 0820, 1200, and 1640 h. All ruminal fluid samples were collected on d 19 of diet supplementation. Each point on the plot represents the bacterial community composition of a single sample (cow by feeding strategy by supplement feeding amount by sample time combination) with a greater distance between points indicating a greater difference in bacterial community composition. Color version available in the online PDF.

Prevotellaceae, *Erysipelotrichaceae*, *Veillonellaceae*, and *Fibrobacteraceae*. Relative abundances of the following bacterial families decreased in the control compared with the PMR cows: *Ruminococcaceae*, unclassified *Clostridiales*, *Anaerolineaceae*, *Clostridiales* Family XIII *incertae sedis*, *Bacillaceae*, and *Acetobacteraceae*. Relative abundances of *Lachnospiraceae*, unclassified *Bacteroidales*, F16, *Coriobacteriaceae*, unclassified RF39, and *Catabacteriaceae* were not influenced by feeding strategy.

The relative abundance of *Ruminococcaceae* and unclassified *Clostridiales* linearly and quadratically decreased with supplement feeding amount. The relative abundance of *Lachnospiraceae*, unclassified *Bacteroidales*, *Fibrobacteraceae*, and *Acetobacteraceae* linearly increased with supplement feeding amount, whereas that of *Erysipelotrichaceae* was both linearly and quadratically increased. *Anaerolineaceae* and *Catabacteriaceae* linearly decreased in relative abundance with supplement feeding amount.

The relative abundances of F16, *Coriobacteriaceae*, *Anaerolineaceae*, *Clostridiales* Family XIII *incertae sedis*, *Bacillaceae*, and *Acetobacteraceae* increased, whereas *Veillonellaceae* and *Fibrobacteraceae* decreased over the sample times. The relative abundance of *Ruminococcaceae*, unclassified *Clostridiales*, *Lachnospiraceae*, and *Catabacteriaceae* increased in samples collected at 1200 h compared with those collected at 0820 and 1640 h, and the inverse occurred for *Prevotellaceae* and unclassified *Bacteroidales*.

The *Ruminococcaceae* linearly and quadratically decreased in relative abundance with supplement feeding amount in the control and PMR cows, respectively. The relative abundance of the unclassified *Clostridiales* and *Veillonellaceae* linearly and quadratically decreased in the controls with supplement feeding amount, respectively. The *Erysipelotrichaceae* increased linearly and quadratically in relative abundance in the controls with supplement feeding amount. The relative abundance of the unclassified *Bacteroidales* linearly and quadratically increased in the PMR cows and quadratically decreased in the controls with supplement feeding amount. The *Anaerolineaceae* linearly and quadratically decreased in relative abundance with supplement feeding amount in the control and PMR cows, respectively. The *Acetobacteraceae* linearly and quadratically increased in the PMR cows with supplement feeding amount (Table 6).

High Supplement Feeding Amounts. The relative abundance of F16 was higher in the PMR and PMR+canola cows and similar between the control and PMR cows. *Veillonellaceae* and *Acetobacteraceae* increased and decreased in relative abundance in the control compared with PMR and PMR+canola cows, respectively. The relative abundance of the unclassified

Bacteroidales was higher in the PMR compared with control and PMR+canola cows. Relative abundances of *Ruminococcaceae*, unclassified *Clostridiales*, and *Anaerolineaceae* decreased in cows fed 16 kg of SDM, and increases in *Erysipelotrichaceae* and *Acetobacteraceae* occurred for these cows.

Prevotellaceae, *Ruminococcaceae*, *Lachnospiraceae*, unclassified *Clostridiales*, *Anaerolineaceae*, *Bacillaceae*, and *Acetobacteraceae* had a consistent pattern of relative abundance over time compared with when all supplement feeding amounts were analyzed. The relative abundance of *Veillonellaceae* was decreased at 1200 h, compared with at 0820 and 1640 h, and the *Catabacteriaceae* were decreased in relative abundance at 1640 h.

The *Erysipelotrichaceae* increased in relative abundance in the controls fed 16 kg of SDM compared with the PMR and PMR+canola cows fed 14 or 16 kg of SDM, and relative abundance of this bacterial family decreased in the PMR cows fed 16 kg of SDM compared with control and PMR+canola cows fed 16 kg of SDM. The unclassified *Bacteroidales* were increased in relative abundance in the controls fed 14 kg of SDM compared with the other feeding strategies fed at this amount and the control and PMR+canola cows fed 16 kg of SDM. The relative abundance of unclassified *Bacteroidales* was lower in the control cows fed 16 kg of SDM compared with cows fed by all feeding strategies at 14 and 16 kg of SDM except the PMR+canola cows fed 14 kg of SDM (Table 6).

Co-Inertia Analysis

Co-inertia analysis showed that supplement feeding amount had the largest association with BCC. Bacterial community composition had the largest association with concentrations of the fermentation products ammonia, butyrate, valerate, and propionate, whereas time, pH, and total VFA concentration were less associated (Figure 5). Bacterial community composition in PMR+canola cows fed 14 kg of SDM and PMR cows fed 12 kg of SDM was associated with concentrations of ruminal ammonia, butyrate, lactate, acetate, and pH. An association was also observed between BCC of the controls fed 14 or 16 kg of SDM and ruminal propionate concentration. Valerate concentration was associated with BCC of controls fed 16 kg of SDM (Figure 5).

DISCUSSION

This experiment examined the effects of PMR and linearly increasing supplement feeding amounts on milk production, milk composition, and ruminal fermentation, ruminal acidosis, and ruminal BCC and their associations in late-lactation cows. Many of the feeding

Table 6. Main effects and their interactions of feeding strategy (S), supplement feeding amount (A), and sample time (T), and their interactions, and means $\pm$ SE of the difference (SED) for feeding strategy for bacterial families identified from 16S rDNA sequences with mean relative abundances of $>0.2\%$ in at least one feeding strategy

Family	Amount ¹	Feeding strategy means ² (relative abundance, %)				P-value							
		Control	PMR	PMR+canola	SED	S	A ³		T	S $\times$ A	S $\times$ T	A $\times$ T	S $\times$ A $\times$ T
							Lin	Quad					
<i>Prevotellaceae</i>	All	38.9 ^a	27.2 ^b		4.81	0.032	0.178	0.361	<0.001	0.123	0.018	0.063	0.324
	High	46.8	29.6	22.6	8.28	0.122	0.061		<0.001	0.811	0.764	0.648	0.108
<i>Ruminococcaceae</i>	All	18.0 ^a	24.5 ^b		2.58	0.026	0.001	0.010	<0.001	0.027	0.682	0.017	0.211
	High	8.73	18.8	20.1	4.43	0.264	0.014		0.002	0.943	0.223	0.030	0.059
Unclassified <i>Clostridiales</i>	All	10.35 ^a	13.20 ^b		1.13	0.026	<0.001	0.038	<0.001	0.009	0.251	0.148	0.440
	High	4.66	12.77	10.86	2.11	0.114	0.044		<0.001	0.077	0.124	0.059	0.014
<i>Lachnospiraceae</i>	All	12.9	10.6		2.13	0.296	0.001	0.074	0.001	0.877	0.002	0.018	0.009
	High	17.8	14.9	9.24	4.69	0.369	0.082		0.001	0.671	0.004	0.078	0.019
F16	All	2.30	6.89		2.52	0.094	0.265	0.882	0.041	0.876	0.222	0.410	0.727
	High	3.27 ^a	5.85 ^{ab}	12.4 ^b	3.88	0.009	0.074		0.077	0.357	0.173	0.222	0.357
<i>Erysipelotrichaceae</i>	All	5.16 ^a	3.06 ^b		0.73	0.019	<0.001	0.003	0.061	<0.001	<0.001	0.385	0.813
	High	8.86	3.53	6.68	2.02	0.051	0.003		0.143	0.009	0.499	0.119	0.709
<i>Veillonellaceae</i>	All	4.63 ^a	2.60 ^b		0.61	0.006	0.771	0.253	<0.001	0.043	0.007	0.118	0.220
	High	4.98 ^a	2.25 ^b	1.58 ^b	1.07	0.042	0.424		<0.001	0.061	<0.001	0.150	0.458
Unclassified <i>Bacteroidales</i>	All	3.27	3.52		0.47	0.603	0.029	0.430	0.002	<0.001	0.002	0.113	0.193
	High	3.89 ^a	4.85 ^b	2.91 ^a	0.86	0.044	0.701		0.598	<0.001	0.030	0.669	0.156
<i>Coriobacteriaceae</i>	All	0.97	2.28		0.48	0.272	0.614	0.300	<0.001	0.082	0.001	0.574	0.178
	High	0.32	1.96	4.15	2.63	0.438	0.244		0.062	0.328	0.229	0.395	0.492
<i>Anaerolinaceae</i>	All	0.87 ^a	1.39 ^b		0.23	0.044	<0.001	0.509	0.002	0.035	0.116	0.911	0.581
	High	0.09	0.82	1.25	0.45	0.164	0.005		0.028	0.209	0.229	0.783	0.835
<i>Clostridiales</i> Family XIII <i>incertae sedis</i>	All	0.82 ^a	1.34 ^b		0.22	0.035	0.262	0.615	<0.001	0.218	0.032	0.876	0.606
Unclassified RF39	High	0.45	1.28	1.59	0.44	0.253	0.581		0.083	0.691	0.579	0.731	0.746
	All	0.39	0.64		0.16	0.155	0.709	0.578	0.583	0.775	0.373	0.881	0.885
<i>Catabacteriaceae</i>	High	0.31 ^a	0.65 ^a	1.38 ^b	0.38	0.031	0.409		0.632	0.179	0.840	0.770	0.669
	All	0.49	0.48		0.14	0.945	0.030	0.924	0.047	0.228	0.020	0.033	0.452
<i>Bacillaceae</i>	High	0.33	0.36	0.42	0.20	0.904	0.051		0.022	0.184	0.010	0.021	0.009
	All	0.05 ^a	0.46 ^b		0.15	0.018	0.381	0.816	0.001	0.688	0.014	0.576	0.771
<i>Fibrobacteraceae</i>	High	0.02	0.52	0.44	0.35	0.504	0.503		0.016	0.792	0.240	0.291	0.864
	All	0.27 ^a	0.08 ^b		0.08	0.039	0.008	0.275	0.037	0.275	0.116	0.270	0.070
<i>Acetobacteraceae</i>	High	0.56	0.13	0.10	0.18	0.084	0.600		0.074	0.843	0.122	0.774	0.267
	All	0.00 ^a	0.28 ^b		0.05	<0.001	<0.001	0.043	<0.001	0.005	<0.001	<0.001	0.002
	High	0.00 ^a	0.55 ^b	0.32 ^b	0.12	0.019	0.006		<0.001	0.094	<0.001	0.004	0.101

^{a,b}Means within a row not sharing a common superscript differ significantly ($P < 0.05$).

¹All = supplement feeding amounts 8, 10, 12, 14, and 16 kg of DM total supplement/cow per day; High = supplement feeding amounts 14 and 16 kg of DM total supplement/cow per day, to incorporate the effect of canola meal substitution.

²PMR = partial mixed ration; PMR+canola = PMR + canola meal.

³Lin = linear polynomial contrast; Quad = quadratic polynomial contrast.

Figure 5. Duality diagram of co-inertia analysis of ruminal bacterial communities from 16S rDNA 454 pyrosequences, measures of ruminal fermentation, sample time, and amount of total supplements fed in dairy cows fed 1 of 3 feeding strategies: control ($n = 10$ cows), partial mixed ration (PMR; $n = 10$ cows), or PMR+canola (PMR + canola meal; $n = 4$ cows) at amounts 8, 10, 12, 14, or 16 kg of DM of total supplement/cow per day (2 cows per amount at 3 times from each feeding strategy). On the bi-plot, the ruminal fermentation measures are represented as arrows. The direction of the arrow of each ruminal fermentation measure indicates an increasing magnitude of that measure. The angle between the arrows indicates their degree of correlation. The magnitude of the arrows indicates the importance of a measure on bacterial community composition. Measures with long arrows are more strongly correlated with the ordination axes than short arrows and have a greater influence on the pattern of variation (Carberry et al., 2012).

strategies and feeding amount combinations evaluated in this study are consistent with those fed on commercial farms (Bramley et al., 2012). The control feeding strategy mimics traditional twice-daily feeding of concentrates in the milking parlor, which is the dominant supplement feeding system on dairy farms in southeastern Australia (Bramley et al., 2012) and many other countries. Feeding supplements as a PMR is the second most dominant feeding system for dairy farming in this region (Bramley et al., 2012) and is designed to provide energy over a longer period compared with feeding an isoenergetic component-fed control diet (Bargo et al., 2003; Auldist et al., 2013).

It should be noted that although the control and PMR feeding strategies evaluated in this experiment were isoenergetic, they contained different feed components. The substitution of some wheat for canola meal in the PMR was designed to increase MP supply above NRC (2001) requirements. The removal of starch and the addition of protein, some of which provides ammonia and peptides, may result in better ruminal conditions and increased milk production. Although cows in the current study consumed a similar total DMI to

target intakes, the control cows consumed less wheat and more ryegrass silage than targeted and were at a lower risk of ruminal acidosis than anticipated. These interventions were initiated in very late lactation, and milk production levels were consequently low.

The effects of feeding groups on the incidence of ruminal acidosis was assessed by the generation of acidosis eigenvalues using the model described by Bramley et al. (2008), which has been used to assess other data sets (O'Grady et al., 2008; Golder et al., 2012, 2014a). The eigenvalues are based on ruminal fermentation measures associated with health and production measures from 800 cows from 100 dairies that included pasture only, pasture and concentrate, PMR, and TMR feeding systems (Bramley et al., 2008, 2012). Thus, acidosis eigenvalues are a more comprehensive measure to diagnose ruminal acidosis compared with single measures such as ruminal pH. The acidosis eigenvalues indicate that ruminal acidosis or rumen perturbation was present at different severities in the feeding groups. The acidosis eigenvalues in this study are consistent with expected physiological outcomes for the feeding groups and are consistent with the milk measures. The co-

inertia analysis also supports the Bramley et al. (2008) model, with the strength and direction of relationships between feeding groups, ruminal fermentation, and ruminal BCC data coinciding with eigenvalues for feeding groups.

The acidosis eigenvalues of the control cows fed 14 or 16 kg of SDM indicate that these cows had the greatest incidence of ruminal acidosis. The control cows fed 16 kg of SDM also had the most distinct BCC in the constrained PCoA and co-inertia analysis and the lowest number of observed OTU in the rarefaction analysis. The linear and quadratic increase in acidosis eigenvalues with feeding amount is not surprising, as increasing the feeding amount provided an increase in dietary starch content. The quadratic decrease in yield of milk fat with feeding amount provides further support for this. Supplement feeding amount also had the largest influence on BCC of the measures tested in the co-inertia analysis, supporting these findings.

The PMR+canola cows had very low eigenvalues in cows fed both 14 and 16 kg of SDM, suggesting that these cows had the most favorable ruminal conditions of all feeding groups at higher feeding amounts. This favorable environment coincided with increased yields of ECM and milk fat in these groups of cows. Increased formation of MP may have contributed to the increase in yield of milk fat and ECM in the PMR+canola cows; however, in contrast, yields of milk and milk fat were not influenced by RUP content in the majority of studies quantitatively reviewed (Bargo et al., 2003). The involvement of dietary protein in ruminal acidosis and protein and carbohydrate synchrony requires further investigation.

The lower eigenvalues in cows fed the PMR compared with the controls indicate more favorable ruminal conditions and a lower incidence of ruminal acidosis in the PMR-fed cows. This finding may in part reflect the slower rate of fermentation of maize starch fed in the PMR compared with the rapid rate of fermentation of wheat starch, which predisposes cows to digestive disorders (Sutton, 1984; Khorasani et al., 2001). The more stable fermentation pattern that occurred in the PMR cows across the day, which may be more favorable, may also be partly because the maize grain and maize silage included in the PMR were consumed over a longer period than the wheat consumed by the control cows. Partial mixed ration feeding also allows simultaneous delivery of fermentable carbohydrates and physically effective fiber and better synchronization of starch and protein fermentation compared with feeding concentrates in the milking parlor followed by silage access on the pasture.

The effects of feeding strategy (Bargo et al., 2002b; Auldist et al., 2013, 2014) and increasing supplement

tary feeding amount (Stockdale et al., 1987; Leddin et al., 2010; Auldist et al., 2013) on ruminal pH, total and individual VFA measures, and ammonia concentrations were generally consistent with similar studies. However, in contrast, propionate and valerate concentrations decreased and acetate to propionate ratio and butyrate concentrations increased in the PMR cows compared with control cows in our study. Ruminal pH was decreased with increased supplement feeding amount in the current study, but was not influenced in other studies (Stockdale et al., 1987; Leddin et al., 2010); however, those authors fed supplements only up to 10 kg of DM. Supplementation of the ionophore monensin and the antibiotic tylosin in the current study to all cows may also have reduced the generation of lactic acid (Dennis et al., 1981; Weimer et al., 2008) and influenced BCC. The spike in D-lactate concentration at sample time 5 in the PMR cows likely corresponded to the feeding of maize silage containing preformed lactic acid.

Concentrations of ammonia, butyrate, valerate, and propionate had a large influence on ruminal BCC, after feeding amount, supporting Bramley et al. (2008), who found that these are good predictors of ruminal acidosis. Ruminal pH had little influence on BCC in the co-inertia analysis (Figure 5), consistent with findings that cows with different pH profiles had similar BCC (Palmonari et al., 2010). In contrast, ruminal pH was a large vector in the study by Carberry et al. (2012), which examined associations between bacterial community diversity patterns and ruminal fermentation measures.

Protein supplementation did not influence total VFA concentration and molar proportions of acetate, propionate, and butyrate in the majority of studies in a quantitative review (Bargo et al., 2003), a finding consistent with this study. A higher ammonia concentration was associated with BCC in the PMR+canola cows fed 14 kg of SDM in the co-inertia analysis (Figure 5). These findings may have resulted from an increase in RUP availability and increased microbial growth in the PMR+canola cows; however, these measures were not directly assessed in the current study.

A decrease in the lipogenic to gluconeogenic VFA ratio (Auldist et al., 2013) and increases in *Butyrivibrio fibrisolvens* and *Megasphaera* spp., which are regarded as major biohydrogenating bacteria in the rumen (Or-Rashid et al., 2009), have been associated with a lower yield of milk fat. The relative abundance of *Butyrivibrio* spp. increased and the lipogenic to gluconeogenic VFA ratio decreased with increased feeding amount, along with decreased milk fat percentage, which supports this association, but the relative abundance of *Megasphaera* spp. was not influenced by feeding amount. We hypothesized that *B. fibrisolvens* and *Megasphaera* spp. would

be increased in relative abundance in the controls fed 14 or 16 kg of SDM compared with the PMR and PMR+canola cows fed at these amounts, as they had a lower yields of milk fat and tended to have a lower milk fat percentage. However, the relative abundance of *Butyrivibrio* and *Megasphaera* spp. was not different in these cows. Ramirez et al. (2012) found no difference in biohydrogenating bacteria in the rumen, despite differences in milk fat percentage and yield. Perhaps these bacteria do not always have the most substantial role in biohydrogenation.

The large among- and within-group variation in BCC, which was evident in the constrained between-group PCoA, is consistent with considerable diversity in bacterial populations in ruminants fed identical diets (Brulc et al., 2009; Li et al., 2009; Chen et al., 2012). The apparent host specificity of ruminal bacteria may pose a challenge when examining interventions or management changes that affect the rumen and emphasizes the need for large sample sizes for in vivo studies.

The ruminal BCC in the current study was consistent with known bacterial communities in dairy cattle, as bacteria from the *Bacteroidetes* (predominately gram-negative) and *Firmicutes* (predominately gram-positive) phyla appeared to dominate the core bacterial microbiome, regardless of feeding group, consistent with other studies (Khafipour et al., 2009; de Menezes et al., 2011; Jami and Mizrahi, 2012). Similarly, the *Prevotella* were the dominant ruminal bacterial genus in the current study and others (Tajima et al., 2000; Fernando et al., 2010; Jami and Mizrahi, 2012). However, the relative abundance of bacteria from the *Proteobacteria* phylum, which have highly diverse metabolic functions (Brenner et al., 2005), was much lower in the current study than generally reported in dairy cows. Knowledge of substrate utilization and end products of members of the candidate phylum TM7, which had the third highest relative abundance in the current study, is limited, but members have been identified in dairy cows (Khafipour et al., 2009; Kong et al., 2010; Hook et al., 2011).

The predominance of the *Prevotellaceae*, *Ruminococcaceae*, and *Lachnospiraceae* bacterial families, regardless of feeding group, is consistent with other studies (de Menezes et al., 2011; Thoetkiattikul et al., 2013; Zened et al., 2013); however, Thoetkiattikul et al. (2013) also reported a large relative abundance of *Flavobacteriaceae*, which were not observed in the current study. The members of the *Prevotellaceae* family are gram-negative bacteria that belong to the *Bacteroidetes* phylum and can utilize a range of substrates (Boone et al., 2011), whereas members of the *Ruminococcaceae* and *Lachnospiraceae* families belong to the *Firmicutes* phylum and are largely cellulolytic and fibrolytic bacteria, respectively (Thoetkiattikul et al., 2013).

Nagaraja and Titgemeyer (2007) state that the relative abundances of gram-negative bacteria are replaced with abundances of gram-positive bacteria during acute ruminal acidosis. However, in our study, the relative abundance of the *Prevotellaceae*, which represent the dominant gram-negative bacteria, was increased, whereas the abundances of the *Ruminococcaceae* and the unclassified *Clostridiales* (gram-positive bacteria) were lower in control cows compared with PMR cows. Zened et al. (2013) also reported a dietary effect on BCC of members of the *Prevotellaceae* and *Ruminococcaceae* families, but in contrast to the current study, those authors reported an influence on *Lachnospiraceae* in cows fed maize silage diets with high or low starch content. A higher relative abundance of *Prevotellaceae* has been reported in cows fed pasture compared with TMR (de Menezes et al., 2011), a finding consistent with their higher relative abundance in the control cows in the current study. Khafipour et al. (2009) reported a decrease in *Prevotella* spp. with induced subacute ruminal acidosis, whereas, in the current study, *Prevotella* were higher in relative abundance in the control cows, which had higher acidosis eigenvalues compared with the PMR cows; Mohammed et al. (2012) found that *Prevotella* were not directly associated with ruminal acidosis.

The relative abundances of the ruminal bacteria *Streptococcus* and *Lactobacillus*, which are associated with ruminal acidosis, and *Megasphaera*, which are associated with protection against ruminal acidosis, were <1% in the current study. The involvement of *Streptococcus bovis* in ruminal acidosis is unclear; other studies have reported varied effects of starch on the relative abundance of or total bacterial rDNA copies contributed by *Strep. bovis* (Tajima et al., 2000; Fernando et al., 2010; Palmonari et al., 2010). The cows in the current study were adapted to their feeding groups before rumen sampling and were also supplemented with monensin and tylosin, which may account for the low abundance of *Streptococcus* and *Lactobacillus*.

The higher relative abundance of the *Veillonellaceae* family in the controls is similar to the higher abundance of these bacteria identified in pasture-fed compared with TMR-fed cows (de Menezes et al., 2011). The *Veillonellaceae* family includes members that produce propionate as their major fermentation product (Strobel and Russell, 1991), which is consistent with the current study. Some bacterial species belonging to the *Veillonellaceae* family utilize lactate (Stewart et al., 1997).

The *Erysipelotrichaceae* family, which had a higher relative abundance in control cows, is more abundant in pasture-fed compared with TMR-fed cows (de Menezes et al., 2011), possibly reflecting the higher pasture DMI

of the control cows in the current study, although the role of this family in ruminal microbial fermentation is unknown (Verbarg et al., 2004; de Menezes et al., 2011). Literature on the involvement of bacteria from the following families in ruminal acidosis is limited: F16, *Coriobacteriaceae*, *Anaerolinaceae*, *Clostridiales* Family XIII *incertae sedis*, *Catabacteriaceae*, *Bacillaceae*, and *Acetobacteraceae*, but these ruminal bacteria warrant further investigation.

The increase in dietary starch content with increasing supplement feeding amount was expected to decrease the number of fibrolytic bacteria, including members of the *Ruminococcaceae* and *Fibrobacteraceae* families (Tajima, 2001; Zened et al., 2013). This finding was consistent with observations for the *Ruminococcaceae* but in contrast to the increase in relative abundance of bacteria from the *Fibrobacteraceae* family.

Studies of the effects of protein on ruminal BCC are limited. The relative abundance of bacteria belonging to the *Prevotella*, *Butyrivibrio*, and *Streptococcus* genera that contain proteolytic bacteria were not affected by increased dietary protein in the current study. The only ruminal bacterial families that were influenced in the high supplement feeding amounts analysis that incorporated the effect of protein were the F16, *Veillonellaceae*, and *Acetobacteraceae*, and further research is required to investigate their involvement in ruminal function.

Assessment of the microbiome by meta-transcriptomics, as suggested by Mullins et al. (2013), would allow examination of metabolic shifts occurring during dietary changes and may be beneficial in broadening our understanding of ruminal responses to dietary changes. Investigations into the interactions of ruminal bacteria, protozoa, archaea, bacteriophages, and fungi, and associations with ruminal acidosis may be beneficial.

CONCLUSIONS

First, as hypothesized, cows fed diets as a PMR, with or without canola meal, had improved ruminal measures, distinct ruminal BCC, and a decreased incidence of ruminal acidosis compared with controls fed isoenergetic diets as individual feed components. However, milk measures were only increased in the cows fed PMR+canola. Second, as hypothesized, a linear increase in supplement feeding amount from 8 to 16 kg of DM/cow per day of total supplement altered milk and ruminal measures, created a distinct ruminal BCC, and increased the incidence of ruminal acidosis. These measures were also associated with each other. In conclusion, feeding diets as a PMR may benefit ruminal conditions and reduce the incidence of ruminal acidosis, regardless of feeding amount up to 16 kg of DM of total

supplement/cow per day but did not result in increased milk measures compared with isoenergetic control diets fed as components in late-lactation cows. Feeding protein above dietary requirements may provide milk production and milk composition benefits; however, further research is warranted.

ACKNOWLEDGMENTS

The authors acknowledge staff at the Department of Environment and Primary Industries (Ellinbank, VIC, Australia) for provision of ruminal fluid samples and milk measures data. This work was supported by Department of Environment and Primary Industries (VIC, Australia), Dairy Australia (Southbank, VIC, Australia), SBS*Scibus* (Camden, NSW, Australia), DairyNSW (Mudgee, NSW, Australia), The CSIRO Animal, Food and Health Services (St. Lucia, QLD, Australia), and The University of Sydney (Camden, NSW, Australia). The authors also acknowledge the staff at CSIRO Animal, Food and Health Services (St. Lucia, QLD, Australia) for laboratory assistance, in particular Vicki Whan.

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