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INCLUSION OF RED OSIER DOGWOOD IN HIGH-FORAGE AND HIGH-GRAIN DIETS AFFECTED *IN VITRO* RUMEN FERMENTATION*

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Abstract

Red osier dogwood (ROD) is an abundant shrub plant in Canada and other places in the world. It is rich in antioxidants such as quercetin, gallic acid and tyrosol. The objective of this study was to evaluate the effects of substituting barley silage with ROD in high-forage (HF) or high-grain (HG) diets on gas production (GP), dry matter (DM) disappearance (DMD) and fermentation characteristics in ruminal batch cultures. The study was a randomized design with 2 media pH (5.8 vs. 6.5) × 4 doses of ROD. An additional treatment of monensin and tylosin was added as a positive control for each pH level. The basic diet consisted of 60% barley silage and 40% barley grain for HF or 15% silage and 85% grain for HG diet. The barley silage was partly replaced with ROD at 0, 3, 6 or 12% in both diets (DM basis). Each diet was incubated for 24 h in culture bottles with three replicates for each treatment combination, and three runs on different days. The GP and DMD were greater (P<0.01) with media pH 6.5 vs. pH 5.8. The DMD linearly (P<0.01) decreased at pH 5.8 with increasing levels of ROD. Increasing ROD levels also linearly (P<0.01) decreased total VFA concentration and the proportion of propionate, and increased (P<0.01) the acetate to propionate ratio (A:P) at pH 5.8. Compared to the antibiotic treatment, the inclusion of ROD resulted in lower (P<0.02) DMD at pH 5.8, and a greater (P<0.01) proportion of acetate but a lower (P<0.01) proportion of propionate. These results indicated that the DMD of diets and the fermentation pattern were adversely affected by ROD at pH 5.8. However, the increased A:P along with the decreased DMD at pH 5.8, suggested a lower impact on fibre digestion than on starch digestion by ROD. Feeding ROD may therefore potentially reduce the incidence of rumen acidosis resulting from feeding HG diets to ruminants by decreasing starch digestion in the rumen.

Key words: batch culture, gas production, fermentation, red osier dogwood

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Red osier dogwood (ROD; *Cornus sericea*) is a native shrub plant across North America and other places in the world and is abundant in low wetlands, pasture land and areas where crops and forages do not grow well (Isaak et al., 2013). The ROD is rich in bioactive compounds with total phenolic concentrations varying between 40 to 220 mg/g depending on the season. The phenolics include anthocyanins, gallic acid, ellagic acid, quercetin, kaempferol and cyanin (Isaak et al., 2013). These phenolic compounds have antioxidant and antimicrobial properties (Rasool et al., 2010). Gallic acid has been shown to have antioxidant capacity in mice (Nair and Nair, 2013), whereas ellagic acid induces apoptosis of cancer cells (Hagiwara et al., 2010), and has antimicrobial and immunomodulatory activity (BenSaad et al., 2017). Quercetin reduced inflammation and oxidation damage caused by *Helicobacter pylori* in the mucosa of guinea pigs (Gonzalez-Segovia et al., 2008; Abuelsaad et al., 2014). Administration of quercetin may also have positive effects on the metabolic adaption of high-producing cows to early lactation due to insulin release and sensitivity (Gohlke et al., 2013).

In vivo studies demonstrated that feeding ROD may reduce the use of antibiotics in weaned pigs (Scales, 2015), and reduce the incidence of diarrhea and the mortality of rabbits (Schafer, 2011). Recently, the potential of ROD as a ruminant feed ingredient has been evaluated. It was shown that ROD could be fed to ruminants to replace silage with higher feed value (Scales, 2015). A farm demonstration carried out using 95 control heifers and 95 heifers fed 0.9 kg/d of ROD showed that average daily body weight gain increased by 25% (Scales, 2015). The mechanism by which feeding ROD improved growth performance is unknown.

The rumen is the primary site to digest feeds and provide nutrients to ruminants. To our best knowledge, there is little information available on the digestibility of ROD in the rumen, and the effect of feeding ROD on ruminal microbial activity. Red osier dogwood is especially interesting for its antioxidant and antimicrobial properties. Plant antioxidants may play an important role in the elimination of oxidative stress within the rumen microbial ecosystem (Cattani et al., 2012). Hino et al. (1993) reported that ruminal microbial growth and fibre digestibility were affected by adding antioxidants in a dose-dependent manner. Other *in vitro* studies (Busquet et al., 2006; Macheboeuf et al., 2008) also reported that high dosages of phenols inhibited feed degradability and fermentation acid production but inhibition did not occur with low dosages of phenols. Increasing concentrations of a blend of natural phenols extracted from red chicory could have induced a shift in the partition of energy, with a greater proportion of nutrients channeled towards microbial protein synthesis (Cattani et al., 2012). These phenolic compounds are able to bind and precipitate macromolecules, such as dietary proteins, thereby reducing protein degradability in the rumen (Bravo, 1998). A decrease in protein degradability in the rumen is beneficial to improve protein efficiency by increasing postruminal protein digestion. Although several *in vitro* studies (Hino et al., 1993; Vázquez-Añón and Jenkins, 2007) have reported increased rumen microbial activity and efficiency of diet utilization by adding natural and synthetic antioxidants, information regarding the effects of synthetic or natural phenols with antioxidant properties on rumen fermentation and microbial growth is limited (Makkar et al., 2007; Cattani et al., 2012).

The objective of this study was to evaluate whether ROD rich in phenolic compounds affects rumen fermentation in a pH- or dose-dependent manner by measuring the gas production (GP) kinetics, dry matter (DM) disappearance (DMD) and fermentation characteristics in batch cultures with either high-forage (HF) or high-grain (HG) diets.

Material and methods

Sources and preparation of ROD

A sample of ROD was provided by Red Dog Enterprise Ltd. (Winnipeg, MB, Canada). The ROD was immature and grown for less than one year. The sample consisted of approximately 75% leaves, 10% bark and 15% stem. The samples were oven-dried at 55°C for 48 h (standard model 4, Arthur Thomas Co., Philadelphia, PA, USA) and ground through 1-mm screen for chemical analysis and incubation in batch culture.

Table 1. Ingredient and chemical composition of the experimental diets

Item	ROD (% of DM)				Antibiotics*
	0	3	6	12	
High forage					
Ingredient (%)					
barley silage	60	57	54	48	60
red osier dogwood	0	3	6	12	0
barley grain, dry-rolled	35	35	35	35	35
supplements**	5	5	5	5	5
Chemical composition (% DM)					
DM (%)	55.6	57.6	59.5	63.3	55.6
OM	93.8	93.8	93.8	93.9	93.8
NDF	34.9	34.3	33.7	32.4	34.9
ADF	21.6	21.6	21.6	21.6	21.6
Starch	34.1	33.6	33.0	31.9	34.1
CP	14.3	14.2	14.2	13.9	14.3
High grain					
Ingredient (%)					
barley silage	15	12	9	3	15
red osier dogwood	0	3	6	12	0
barley grain, dry-rolled	82	82	82	82	82
supplements**	3	3	3	3	3
Chemical composition (% DM)					
DM (%)	84.7	86.6	88.5	92.3	84.7
OM	96.6	96.7	96.7	96.8	96.6
NDF	24.0	23.3	22.7	21.4	24.0
ADF	10.0	10.0	10.0	10.0	10.0
Starch	51.9	51.3	50.8	49.7	51.9
CP	15.1	15.1	15.0	14.8	15.1

*Antibiotics were supplemented with monensin and tylosin/kg diet DM.

** Supplements consisted of 54.7% ground barley, 9.7% canola meal, 24.3% calcium carbonate, 2.3% molasses, 5.0% salt, 1.0% feedlot premix, 2% urea, 0.07% vitamin E (500,000 IU/kg), and 1% canola oil.

Experimental design, substrate and inoculum

Two experiments were conducted with each using substrates representing either a growing-based diet (HF; 60% barley silage + 40% barley grain) or finishing-based diet (HG; 15% barley silage + 85% barley grain) typical of western Canadian feedlots (Table 1). Each experiment was arranged in a complete randomized design with a factorial arrangement of 2 pH values × 4 dosages of ROD. Dosages of ROD were 0, 3, 6 and 12% (total DM basis), respectively, in the place of barley silage. In addition to the test diets, individual feed ingredients including barley grain, barley silage and ROD were also tested as substrates in batch cultures. Also, an antibiotic treatment consisting of monensin and tylosin was used as a positive control for each pH level because monensin and tylosin are widely used to decrease risk of ruminal acidosis and liver abscess in beef cattle. Monensin and tylosin were added, respectively, at a dose of 0.17 mg and 0.07 mg per serum bottle which was calculated based on a daily dose of 300 mg monensin and 110 mg tylosin per head in beef cattle. Anaerobic buffer medium was prepared according to the method described by Goering and Van Soest (1970). Media of low (5.8) and high (6.5) pH were achieved by adjusting the amount of sodium bicarbonate in the buffer solution. Inoculum for the batch cultures was obtained from two ruminally fistulated beef heifers fed a growth diet and two beef heifers fed finishing diet, for incubating HF and HG substrates, respectively. All animal procedures followed the guidelines of the Canadian Council on Animal Care (2009).

Procedures of batch culture

Glass bottles (125 mL) fitted with rubber stoppers to prevent the escape of fermentation gases were used for incubations. Substrate (0.5 g) ground through a 1-mm sieve was weighed into a filter bag to be added into a gas-tight culture vial in three replications for each combination of treatments. Anaerobic buffer medium (45 mL) and rumen fluid (15 mL) were added into each bottle purged with CO_2 to remove air from the headspace. All the bottles were sealed with a 14 mm butyl rubber stopper plus aluminum crimp cap immediately after loading, and incubated at 39°C for 24 h (Cattani et al., 2016). The batch culture was repeated in 3 runs on different days. Three additional substrate-free bottles served as blanks by adding the same fermentation media for each batch culture.

Gas pressure was measured at 3, 6, 9, 12, and 24 h post-inoculation by inserting a 23-gauge (0.6 mm) needle and a pressure transducer (model PX4200-015GI, Omega Engineering, Inc., Laval, QC, Canada) connected to a visual display device (Data Track, Christchurch, UK). Once pressure value was recorded, the transducer was then removed leaving the needle in place to permit venting (Rymer et al., 2005). Pressure values, corrected for the gas released from negative controls (blanks), were used to generate volume estimates using the following equation of Mauricio et al. (1999):

$$Gas\ volume = 0.18 + (3.697 \times gas\ pressure) + (0.0824 \times gas\ pressure^2)$$

Kinetic parameters of GP were calculated using the equation of France et al. (2000) as follows:

$$V = A \times [1 - e^{-C(t-Lag)}]$$

where:

V is the cumulative volume of GP at time t (h),
A is the asymptotic GP (mL/g DM),
C is the rate of GP (%/h),
Lag (*h*) was the discrete lag time prior to gas produced.

After 24 h of incubation, the bottles were placed in cold water to stop fermentation, and pH of the culture media was determined. Feed bags were removed from each vial and washed under cold running water until the water was clear. Bags were then oven-dried at 55°C for 48 h, and weighed to determine DMD. Samples of 5 mL culture fluid were preserved with 1 mL of 25% (wt/vol) HPO_3 solution and with 1 mL of 1% (vol/vol) H_2SO_4 at –20°C for determining volatile fatty acid (VFA) and NH_3-N, respectively.

Chemical and fermentation variable analyses

The substrate was analyzed (AOAC, 2005) for DM (method 930.15), organic matter (method 942.05), crude protein (CP; method 968.08), and acid detergent fibre (ADF; method 973.18). Neutral detergent fibre (NDF) was determined as described by Van Soest et al. (1991) using heat-stable α-amylase (Termamyl 120 L, Novo Nordisk Biochem, Franklinton, NC) without sodium sulfite. Starch was determined by enzymatic hydrolysis of α-linked glucose polymers (Li et al., 2011). Total phenolic contents of the ROD sample after a methanolic extraction, were determined colorimetrically using Folin-Ciocalteu reagent, following a procedure described by Isaak et al. (2013). Results were expressed as percentage of gallic acid equivalents per gram DM of ROD, using gallic acid as a standard. Volatile fatty acid was quantified using a gas chromatograph (model 5890; Hewlett-Packard Lab, Palo Alto, CA, USA) with a capillary column (30 m by 0.32 mm i.d.; 1-µm phase thickness; Zebron ZB-AAP; Phenomenex, Torrance, CA, USA) and flame ionization detection, while crotonic acid (trans-2-utenoic acid) was used as an internal standard. The NH_3-N was analyzed using the method described by Rhine et al. (1998).

Statistical analysis

The two experiments were analyzed separately using the MIXED model procedure of SAS (SAS Inst. Inc. Cary, NC) including fixed effects of pH, dosages of ROD or antibiotics, and two way interactions, and the random effects of incubation day. Contrasts were generated to compare the average of three dogwood doses vs. antibiotic supplementation, and control (i.e., 0% ROD) vs. antibiotics. The effect of increasing ROD inclusion was examined through linear and quadratic orthogonal contrasts using the CONTRAST statement of SAS. The data for fermentation variables of the three individual ingredients (i.e. barley grain, barley silage, and

ROD) were also analyzed using the MIXED model procedure of SAS including fixed effects of ingredient and the random effects of incubation day. The PDIFF option adjusted by the Tukey method was included in the LSMEANS statement to account for multiple comparisons among ingredients. Differences were declared significant at $P \leq 0.05$. Trends were discussed at $0.05 < P \leq 0.10$ unless otherwise stated.

Results

Chemical composition and *in vitro* fermentation of individual feeds

Although statistical analysis on the chemical composition of feed ingredient was unable to be performed due to single samples being analyzed, compared with barley silage, the ROD obviously had lower concentrations of NDF and CP without differing in ADF concentration (Table 2). A lower NDF concentration but similar ADF concentration in ROD compared to the silage suggested that there was a lower hemicellulose concentration in ROD. The ROD used in the present study consisted of 12.3% total phenolics including 0.54% gallic acid, 0.92% methyl gallate, 0.15% cathechin, 0.80% epicatechin, 0.83% rutin, 0.45% ellagic acid, and 0.72% quercetin (DM basis).

Table 2. Chemical composition and *in vitro* fermentation of experimental feed ingredients

Item	Barley grain	Barley silage	Dogwood	SEM[1]	P<
Chemical composition (%)					
dry matter (DM)	94.3	29.6	93.3		
organic matter	97.6	91.2	92.1		
neutral detergent fibre	20.3	44.7	23.6		
acid detergent fibre	6.2	31.8	31.7		
crude protein	15.4	13.6	10.8		
starch	57.8	18.3	…		
Rumen fermentation					
gas production (mL/g DM)	145 a	138 a	99 b	8.4	0.01
DM disappearance (%)	65.0 a	61.5 b	55.0 c	3.11	0.01
volatile fatty acid (mM)	84.8 a	85.1 a	72.8 b	3.72	0.01
acetate (A, %)	52.3 c	53.7 b	61.7 a	0.66	0.01
propionate (P, %)	23.1 a	21.1 b	15.5 c	0.58	0.01
butyrate (%)	14.4 a	13.8 a	12.2 b	0.22	0.01
A:P	2.29 c	2.55 b	3.98 a	0.088	0.01
NH_3-N (mM)	18.9 a	19.1 a	14.1 b	0.51	0.01
pH	6.17	6.23	6.33	0.084	0.36

a, b, c – values in rows with different letters differ significantly ($P \leq 0.01$).
[1]SEM = standard error of the mean.

When comparing ruminal fermentation of individual diet ingredients, gas production, DMD and VFA were lower ($P<0.05$) with ROD compared to barley grain or silage (Table 2). Although DMD was lower ($P<0.05$) with silage than grain, GP

and total VFA concentration did not differ. The molar proportion of propionate was highest, intermediate and lowest, respectively, with grain, silage and ROD substrates (P<0.01), whereas the opposite was observed for the molar proportion of acetate and the ratio of acetate to propionate (P<0.01). A lower (P<0.01) molar proportion of butyrate and NH_3-N concentration was observed with ROD compared to the grain and silage.

High-forage diet

Asymptotic GP, rate constant of GP and DMD of HF diet were consistently greater (P<0.01) with high media pH, compared to the low media pH, regardless of increasing ROD proportion or the addition of antibiotics (Table 3). However, there was interaction between media pH and ROD inclusion rate on GP. Increasing ROD in place of silage in the HF diet did not affect GP kinetics at a low media pH, but it quadratically (P<0.01) changed asymptotic GP and the rate constant of GP with greater values at 3% or 6% of ROD at the high media pH. The DMD linearly decreased at low (P<0.01) or high media pH (P<0.02) with the addition of ROD. In comparison with the antibiotic treatment, diets containing ROD tended to have a greater asymptotic GP (P<0.06), greater rate constant of GP (P<0.02), longer lag time (P<0.03) at low pH, and less (P<0.02) DMD at both pH levels.

Table 3. Effects of red osier dogwood (ROD) inclusion rate in a high-forage (HF) diet[1] on gas production (GP) kinetics (A, asymptotic gas; C, rate constant), dry matter disappearance (DMD) and fermentation characteristics in batch cultures with different media pH (5.8 vs 6.5)

Item	ROD (% of DM)[2]				Ant	SEM	P <[3]			
	0	3	6	12			pH	L	Q	ROD vs Ant
1	2	3	4	5	6	7	8	9	10	11
GP										
A (ml/g DM)										
5.8	126	125	131	123	106	30.1	0.01	0.83	0.65	0.06
6.5	185 b	221	219	203	222 a			0.44	0.01	0.48
C (%/h)										
5.8	6.75 a	6.49	6.81	6.23	4.81 b	1.88	0.01	0.56	0.77	0.02
6.5	9.18	11.15	11.14	9.94	10.27			0.66	0.01	0.46
Lag (h)										
5.8	2.40	2.27	2.04	1.75	1.64	0.46	0.01	0.05	0.57	0.03
6.5	2.39	2.34	2.36	2.38	2.50			0.96	0.83	0.46
DMD (%)										
5.8	55.0	52.5	53.0	48.8	55.1	3.81	0.01	0.01	0.76	0.02
6.5	60.5 b	58.0	59.3	55.4	65.5 a			0.02	0.72	0.01
Fermentation										
VFA (m*M*)										
5.8	60.7 a	61.2	61.0	59.5	55.3 b	2.66	0.01	0.51	0.60	0.01
6.5	69.4 a	69.7	71.1	65.6	64.9 b			0.08	0.09	0.04
Acetate (A, %)										
5.8	51.1 a	51.7	51.3	52.2	49.7 b	0.98	0.05	0.15	0.70	0.01
6.5	53.4	52.8	53.7	53.2	52.4			0.94	0.86	0.16

Table 3 – contd.

1	2	3	4	5	6	7	8	9	10	11
Propionate (P, %)										
5.8	22.7	22.0	21.8	21.4	23.1	0.87	0.05	0.01	0.39	0.01
6.5	22.8 b	22.7	22.4	22.3	24.4 a			0.16	0.89	0.01
Butyrate (%)										
5.8	14.5	14.7	15.4	15.0	14.1	0.57	0.02	0.15	0.06	0.01
6.5	13.5 a	14.2	14.1	14.3	12.6 b			0.06	0.25	0.01
A:P										
5.8	2.26	2.37	2.38	2.47	2.15	0.133	0.99	0.01	0.55	0.01
6.5	2.35 a	2.33	2.40	2.41	2.16 b			0.28	0.98	0.01
NH_3-N (m*M*)										
5.8	19.3 a	17.7	17.2	17.1	17.4 b	1.19	0.01	0.01	0.07	0.96
6.5	16.9 a	16.3	17.0	14.8	14.7 b			0.01	0.20	0.05

[1]HF diet consisted of 60% of barley silage and 40% barley concentrate (DM basis).

[2]ROD substituted for silage at 0, 3, 6 and 12%, respectively; and Ant was supplemented with 0.17 mg monensin and 0.07 mg tylosin per bottle.

[3]pH = pH 5.8 vs. 6.5; L, Q = linear or quadratic effect of increasing ROD level; ROD vs Ant = contrast between average of ROD and antibiotics.

Interactions: A, pH×ROD, P<0.05.

a, b – means with different letter within the same row differ significantly (P<0.05).

Consistent with the results of GP and DMD, total VFA concentration and molar proportion of acetate and propionate were linearly (P<0.01) greater, and NH_3-N concentration was lower (P<0.05) when the media pH was high, compared to when it was low (Table 3). Increasing the inclusion rate of ROD in the HF diet linearly (P<0.01) decreased the proportion of propionate without affecting that of acetate, and as a result, the acetate to propionate ratio linearly (P<0.01) increased at a low media pH. In contrast, there were only trends of a quadratic effect (P<0.09) for VFA concentration and a linear (P<0.06) effect for butyrate proportion when the media pH was high. The concentration of NH_3-N was linearly (P<0.01) reduced by increasing ROD levels at both media pH levels. Compared with adding antibiotics, the inclusion of ROD overall resulted in a greater (P<0.05) VFA concentration and proportion of acetate and butyrate but a lower (P<0.01) propionate proportion regardless of media pH.

High-grain diet

Elevating the media pH from 5.8 to 6.5 consistently enhanced (P<0.01) asymptotic GP, rate constant of GP, lag time, and DMD for the HG diet (Table 4). Increasing ROD in replacement of silage in the HG diet affected neither GP kinetic at low and high media pH nor the DMD at a high media pH. In contrast, there was a media pH × ROD inclusion rate effect on DMD, which linearly (P<0.01) decreased at low media pH. Compared to the antibiotic treatment, DMD was also lower for ROD (P<0.02) when the media pH was low. Overall, GP was not different between treatments, except for greater (P<0.01) asymptotic GP being observed with ROD compared with the antibiotic treatment at a high media pH.

Table 4. Effects of red osier dogwood (ROD) inclusion rate in a high-grain (HG) diet[1] on gas production (GP) kinetics (A, asymptotic gas; C, rate constant), dry matter disappearance (DMD) and fermentation characteristics in batch cultures with different media pH (5.8 vs 6.5)

Item	ROD (% of DM)[2]						P < [3]			
	0	3	6	12	Ant	SEM	pH	L	Q	ROD vs Ant
GP										
A (ml/g DM)										
5.8	114	102	101	95	98	14.5	0.01	0.18	0.63	0.92
6.5	222 a	235	222	213	187 b			0.31	0.43	0.01
C (%/h)										
5.8	5.77	5.69	5.44	4.99	5.58	0.76	0.01	0.31	0.90	0.77
6.5	11.60 a	11.46	11.20	10.66	10.03 b			0.22	0.89	0.12
Lag (h)										
5.8	0.58	0.80	0.64	0.34	1.01	0.19	0.01	0.26	0.28	0.05
6.5	1.46	1.28	1.49	1.23	1.48			0.46	0.75	0.45
DMD (%)										
5.8	64.6 a	54.3	55.3	52.5	59.2 b	3.32	0.01	0.01	0.02	0.02
6.5	62.2	64.2	62.4	62.6	62.9			0.91	0.74	0.96
Fermentation										
VFA (m*M*)										
5.8	90.9 a	87.2	85.7	84.1	83.7 b	3.84	0.01	0.01	0.29	0.33
6.5	97.5 a	98.3	95.0	96.4	89.4 b			0.49	0.60	0.01
Acetate (A, %)										
5.8	41.5	42.3	42.1	43.0	41.0	1.14	0.01	0.01	0.97	0.05
6.5	43.9 a	43.0	43.5	43.9	42.6 b			0.53	0.12	0.02
Propionate (P, %)										
5.8	34.6 b	34.1	34.1	33.4	35.8 a	0.76	0.13	0.02	0.90	0.01
6.5	33.7 b	34.1	33.6	33.6	35.5 a			0.51	0.68	0.01
Butyrate (%)										
5.8	12.1 a	11.6	12.0	11.9	11.2 b	0.33	0.02	0.77	0.36	0.05
6.5	11.2 a	11.6	11.8	11.8	10.3 b			0.05	0.17	0.04
A:P										
5.8	1.20 a	1.24	1.24	1.29	1.15 b	0.058	0.05	0.01	0.98	0.05
6.5	1.31 a	1.26	1.30	1.31	1.21 b			0.40	0.22	0.05
NH_3-N (m*M*)										
5.8	19.5	19.7	17.9	18.5	19.9	2.51	0.01	0.23	0.46	0.20
6.5	28.0	27.4	26.8	26.3	26.2			0.14	0.76	0.51

[1]HG diet consisted of 10% of barley silage and 90% barley concentrate (DM basis).

[2]ROD substituted for silage at 0, 3, 6 and 12%, respectively; and Ant was supplemented with 0.17 mg monensin and 0.07 mg tylosin per bottle.

[3]pH = pH 5.8 vs. 6.5; L, Q = linear or quadratic effect of increasing ROD level; ROD vs Ant = contrast between average of ROD and antibiotics.

Interactions: DMD, pH×ROD, P<0.01; butyrate, pH×ROD, P<0.05.

a, b – means with different letter within the same row differ significantly (P<0.05).

Total VFA concentration, molar proportion of acetate, and acetate to propionate ratio were greater (P<0.05) with a high versus low media pH (Table 4). Increasing ROD in the HG diet linearly decreased total VFA concentration (P<0.01) and the

molar proportion of propionate (P<0.02). In contrast, an increase in ROD linearly (P<0.01) increased the proportion of acetate, and consequently, linearly (P<0.01) increased the acetate to propionate ratio when the media pH was low. However, at a high media pH, VFA concentrations and the molar proportion of individual VFA were largely unchanged with increasing ROD concentrations. Compared with the control antibiotics, the inclusion of ROD resulted in a greater (P<0.01) VFA concentration at high pH, and a greater (P<0.05) proportion of acetate, and butyrate, but a decreased (P<0.01) proportion of propionate at either low or high media pH. No treatment effects were observed on NH_3-N concentration.

Discussion

Effect of media pH

The pH value of rumen fluid is primarily impacted by the rate and extent of diet fermentation and can alter rumen microbial fermentation. The HF and HG diets that were used in this study represent typical growing and finishing diets, respectively, that are used for beef cattle in Western Canadian feedlots. The mean rumen pH was reported as 6.3 in the rumen of heifers fed the HF diet (Li et al., 2013) and 5.85 in cattle fed the HG diet (Yang et al., 2010). Therefore, the purpose of adjusting the media pH to 5.8 or 6.5 was to determine whether the rich phenolic ROD responded differently to rumen pH. Final pH values were 5.89 and 6.57 (low and high pH treatments, respectively) for the HF, and 5.64 and 6.33 (low and high pH treatments, respectively) for the HG diet, which were similar to the initially set low (5.8) or high (6.5) media pH. The reduction in GP, DMD and total VFA concentration with media pH 5.8 compared to pH 6.5 was expected because when rumen pH is less than 5.8, various microbial activities can be significantly compromised (Russell and Wilson, 1996). Particularly, the cellulolytic bacteria have been shown to be sensitive to a low pH and as a result, fibre digestion can decrease (Russell and Wilson, 1996). In the present study, the magnitude of decreasing DMD and VFA concentration due to reducing the media pH from 6.5 to 5.8 was slightly greater on average for the HF (–11% for DMD, –12% for VFA concentration) versus the HG (–9% for DMD, –9% for VFA concentration) diets. The acetate to propionate ratio of the HF diet was not affected by media pH level, whereas the acetate to propionate ratio of the HG diet was slightly lower at a low compared to a high media pH (1.22 vs. 1.28). This indicated that there was a reduction in the acetate proportion with a low pH. These results suggested that altering media pH in the batch cultures appeared to have more impact on DMD of the HF diet than that of HG diet, while the media pH had limited influence on fermentation patterns for both HF and HG diets.

Effect of increasing ROD in the diet

Throughout modern livestock production, animals are often exposed to stressful conditions resulting from nutrition (e.g. placed on high-grain diets), the environment, and management. Stressful events have been implicated in promoting oxi-

dative stress through excessive reactive oxygen species production or decreased antioxidant defenses (McGuffey et al., 2001). Excessive reactive oxygen species production can overwhelm the antioxidant defenses leading to oxidative damage of biological molecules, disrupting normal metabolism and physiology (He et al., 2012). Hence, improving the antioxidant capacity through feeding substrates rich in phenolic compounds with antioxidant properties, such as ROD, is expected to enhance animal health (Toumi et al., 2016). In addition, although the rumen is recognised as an anaerobic environment (Hungate, 1966), some oxygen can enter the rumen via feeds, water, saliva, or by diffusion from blood (Williams and Coleman, 1992). Holovska et al. (2002) suggested that plant antioxidants may play an important role in the elimination of oxidative stress within the rumen microbial ecosystem. However, in the present study, increasing ROD inclusion in diets decreased DMD and VFA concentration, which indicated reduced rumen microbial activity. Hino et al. (1993) compared two antioxidants (α-tocopherol and β-carotene) at high (30–40 mg/L) or low (2.5–5 mg/L) doses, and found a reduction in microbial growth and fibre digestibility at the high dose but an improvement at the low dose. The doses of phenolic compounds (gallic acid, methyl gallate, cathechin, epicatechin, rutin, ellagic acid and quercetin) from ROD were estimated to be 11, 22 and 44 mg/L, respectively, for 3, 6 and 12% of ROD inclusion. If the total phenols are taken into account (i.e., 12.3%), the doses of phenol would have been 31, 62 and 124 mg/L, respectively, for 3, 6 and 12% of ROD. Therefore, the doses of phenolic compounds from ROD were much higher than the low dose used by Hino et al. (1993), which may explain the reduction in DMD and VFA concentration observed in our study when dietary inclusion of ROD increased. Similarly, other *in vitro* studies (Busquet et al., 2006; Macheboeuf et al., 2008) reported that a low dose of phenols did not decrease the GP, DMD and VFA concentration, but a higher dose of phenols impaired the GP, DMD and VFA concentration. The deleterious effects of phenolic compounds resulting from the binding and precipitating of macromolecules, such as dietary proteins, carbohydrates and digestive enzymes, can reduce feed digestibility (Bravo, 1998).

Increasing ROD concentrations appeared to switch fermentation patterns to more acetate production, at the expense of propionate (i.e., increased the ratio of acetate to propionate) at a low pH rather than at a high pH with the HG diet in the current study. The increased acetate proportion relative to that of propionate suggests a potential to improve fibre digestion in the rumen by inclusion of ROD in the diet. The finding of potentially improved fibre digestion by ROD at pH 5.8 is of interest, particularly in high-grain fed animals where the rumen pH is typically below 5.8 (Li et al., 2011). In fact, fibre digestion can be significantly compromised when the pH is ≤5.8 because cellulolytic bacteria are sensitive to low pH values (Russell and Wilson, 1996). Furthermore, considering the DMD of the HG diet decreased at the low pH, and the fact that there was an increase in acetate and decrease in propionate proportion, this would suggest that the adverse impact of DMD when feeding ROD was attributed primarily to a reduction in starch digestion rather than fibre digestion in the rumen. Reducing starch digestion in the rumen may potentially reduce the incidence of rumen acidosis when high-grain diets are fed to ruminants.

The NH_3-N concentration in rumen fluid reflects a dynamic process where NH_3-N is utilized by rumen microbes and released from feed protein degradation. The observation that increasing dietary ROD decreased the NH_3-N concentrations in batch cultures with the HF diet can likely be attributed to the protein-binding capacity of phenols (Cattani et al., 2012). The reduction of rumen NH_3-N concentration by adding ROD is of interest because the HF diet containing barley silage had high soluble protein, which is highly degradable and absorbable through rumen wall. High levels of soluble protein consequently reduce protein efficiency and increase urinary excretion of ammonia nitrogen. A lack of effect of ROD supplementation on media NH_3-N concentration in the HG diet is not clear. The HG diet would have lower soluble protein than the HF diet since the proportion of soluble protein in the total protein is much higher in barley silage (70%) than in barley grain (17%; NRC, 1996). Based on data from our study, we speculated that phenols in ROD may have greater protein-binding capacity with soluble than unsoluble protein.

Effect of ROD vs. monensin/tylosin

Adding monensin and tylosin versus control diet (i.e., 0% ROD) had no conclusive effects on DMD of HF or HG diets, but consistently lowered the VFA concentration and the ratio of acetate to propionate. These results are supported by Ponce et al. (2012) who observed decreased total VFA concentrations *in vitro* with the inclusion of monensin in a high corn grain diet. Monensin was supplemented as positive control in this study, and the result of a reduced ratio of acetate to propionate is accordance with the generally accepted modes of action of monensin (i.e. reduced acetate:propionate), thereby validating the measurements. In comparison with the ROD, addition of monensin and tylosin altered the *in vitro* ruminal fermentation with an overall increase in DMD, but lower total VFA concentration and asymptotic GP. In a typical batch culture using rumen inoculum, truly digested substrates (i.e., DMD) are divided among VFA, gas and microbial biomass. Thus, greater DMD in conjunction with lower VFA production from adding monensin/tylosin suggested a change of energy partition toward increased production of microbial biomass, compared to ROD. Furthermore, the reduction in the acetate to propionate ratio as a result of the decreased acetate and increased propionate proportions from supplementing monensin/tylosin, are consistent with the well-established effects of monensin shifting ruminal fermentation by selecting for bacterial species that produce propionate (McGuffey et al., 2001; Han et al., 2002). The differences in fermentation patterns between ROD phenolic compounds and monensin/tylosin indicated possible different modes of actions in the rumen. However, overall similar NH_3-N concentrations between ROD (particularly the high dose of ROD) and monensin/tylosin suggest inhibited proteolytic activity by both ROD (Bravo, 1998) and monensin/tylosin. A decrease in protein degradability and increase in ruminal by-pass protein is beneficial by either improving protein efficiency or decreasing urinary nitrogen excretion, the latter is more volatile than the nitrogen excreted in feces.

Conclusions

Increasing the substitution of barley silage with ROD either in HF or HG diets showed to have adverse effects on *in vitro* rumen DMD, but the results varied ac-

cording to media pH. However, the increased ratio of acetate to propionate in conjunction with the decreased DMD that resulted from ROD supplementation at pH 5.8, suggested that the adverse effects of ROD were targeted more towards starch than fibre digestion. The finding may be of interest, particularly in high-grain fed animals where the rumen pH is consistently below 5.8 and fibrolytic bacteria are significantly compromised. Adding ROD to a HF diet may be also beneficial to improve protein efficiency because of the protein-binding capacity of ROD phenols. Both ROD and monensin/tylosin can inhibit ruminal proteolytic activity but they have different effects on feed digestion and fermentation patterns in the rumen.

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