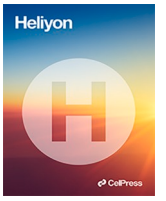
Heliyon 10 (2024) e27991

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Effect of red osier dogwood extract on in vitro gas production, dry matter digestibility, and fermentation characteristics of forage-based diet or grain-based diet

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ARTICLE INFO

Keywords:
Batch culture
Gas production
Feed digestibility
Plant extracts
Fermentation profiles

ABSTRACT

This in vitro batch culture study investigated the effects of red osier dogwood (ROD) extract supplementation on gas production (GP), dry matter disappearance (DMD), and fermentation characteristics in high forage (HF) and high grain (HG) diets with varying media pH level. The experiment was a factorial arrangement of treatments in a completely randomized design with 2 media pH (5.8 and 6.5) × 4 dose rates of ROD extract (0, 1, 3, and 5% of DM substrate). An additional treatment of monensin was added as a positive control for each pH level. The HF substrate consisted of 400 and 600 g/kg DM barley-based concentrate and barley silage, respectively, while the HG substrate contained 100 and 900 g/kg DM barley silage and barley-based concentrate, respectively. Treatments were incubated for 24 h with GP, DMD and fermentation parameters determined. No interaction was detected between the media pH level and ROD extract dose rate on GP, DMD and most of the fermentation parameters. The GP, DMD, and total volatile fatty acid (VFA) concentration were greater ($P = 0.01$) with media pH of 6.5 in both HF and HG diets. The GP were not affected by increasing ROD dose rate, except that GP linearly decreased in the HF ($P = 0.04$) and HG ($P = 0.01$) diets at 24 h; the DMD tended to linearly decrease at pH 6.5 ($P = 0.06$) for both HF and HG diets and at pH 5.8 ($P = 0.02$) for the HG diet. Adding ROD extract to the HF and HG diets linearly ($P = 0.01$) increased the acetate molar proportion at high or low media pH and consequently, the acetate to propionate (A:P) ratio linearly ($P \leq 0.04$) increased. Supplementation of ROD extract to the HF diet linearly ($P = 0.04$) decreased the molar proportion of propionate at pH 6.5 (interaction between pH and ROD extract; $P = 0.05$), but had no effect on propionate proportion when added to the HG diet. Moreover, the proportion of branched-chain fatty acids linearly ($P = 0.03$) decreased with ROD extract supplementation at low pH (interaction, $P < 0.05$) for HF diet and linearly decreased ($P = 0.05$) at pH 6.5 for HG diet (interaction, $P < 0.05$). The NH_3–N concentration was not affected by ROD supplementation in the HF diet but it linearly ($P = 0.01$) decreased with increasing dose rate in the HG diet. Methane concentration tended to linearly ($P = 0.06$) increase with ROD extract

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https://doi.org/10.1016/j.heliyon.2024.e27991
Received 15 July 2022; Received in revised form 12 November 2023; Accepted 10 March 2024
Available online 15 March 2024

supplementation at high pH for HF diet and linearly increased at pH 5.8 (P = 0.06) and pH 6.5 (P = 0.02) for HG diet. These results indicate that the decreased DMD and increased A:P ratio observed with addition of ROD extract may be beneficial to HG-fed cattle to reduce the risk of rumen acidosis without negatively impacting fiber digestion.

Abbreviations

ADF	acid detergent fiber
A:P	acetate to propionate ratio
BCFA	branched-chain fatty acids
CP	crude protein
DM	dry matter
DMD	dry matter disappearance
GP	gas production
HG	high grain
HF	high forage
NH_3 N	ammonia-nitrogen
aNDF	neutral detergent fiber
N	nitrogen
OM	organic matter
ROD	red osier dogwood
VFA	volatile fatty acids

1. Introduction

Increasing concerns over the use of antimicrobial growth promoters in animal production has prompted the need to explore the use of natural alternatives such as phytogenic compounds and probiotics. While these products have been shown to be effective in improving rumen fermentation and nutrient digestibility in some studies [1–3], their beneficial effects are inconsistent and the mode of action is still not well understood [4]. Advancing the understanding of their mechanism of action will help establish these compounds as viable and economical alternatives to conventional antibiotics. Phytogenic compounds derived from herbs, spices or other plants are a group of natural growth promoters and are the subject of considerable research as feed additives for livestock production [4]. These compounds exhibit antimicrobial, anti-inflammatory as well as antioxidant properties, thus presenting potential to be used to reduce or replace for growth promoting antibiotics in livestock animal [5–7]. However, although ionophores, especially monensin, are commonly used in cattle nutrition to improve ruminal fermentation and feed efficiency, they do not illicit the same responses as plant extracts, at least mediated through a direct effect. Furthermore, response to monensin is impacted by dose rate and type of diet, and consequently its rate of inclusion in cattle diets has been increased up to 48 mg monensin/kg diet dry matter (DM) [8] to maintain its effectiveness under high concentrate feeding condition.

Recently, an abundant native shrub in Canada, red osier dogwood (ROD), has been evaluated in vitro and in vivo for its potential as a feed additive to improve production efficiency in ruminant and monogastric livestock [3,9,10]. The ROD is characterized by its rich phenolic compounds such as anthocyanins, quercetin, gallic and ellagic acids, cyanin, and kaempferol [11]. Gomaa et al. [9] suggested that inclusion of ROD in beef heifer high-grain (HG) diets would improve protein efficiency as a result of decreasing in situ ruminal protein degradability. Wei et al. [12] reported that partial substitution of ROD for barley silage in a HG diet reduced in vitro starch disappearance without affecting fiber digestion, suggesting that supplementation of ROD to a HG diet may help reduce the incidence of ruminal acidosis. A ROD extract with a high concentration of phenolics was recently developed and fed to broiler chicks [13] resulting in improved feed digestibility, intestinal morphology, mRNA levels of nutrient transporters, and a reduction in mortality. Although several in vitro and in vivo studies have investigated the effects of raw ROD material on rumen fermentation, little information exists regarding the impact of ROD extract supplementation on gas production (GP) and rumen fermentation [14] in diets differing in chemical composition and a range of ruminal pH. Ruminal pH is one of key factors that alters the microbial population and fermentation end products. In western Canadian feedlots, cattle are typically fed diet contain moderate level grain in growth period, and high-grain diet in finishing period, where mean rumen pH has been reported, respectively, as 6.2 [15] and 5.8 [16].

We hypothesize that GP, rumen fermentation characteristics, in response to ROD extract supplementation, will improve with increased rumen pH and varying with type of diet in a dose-depending manner. The objective of this study was to evaluate the effect of ROD extract, and its dose rate, on DM disappearance (DMD), GP, and ruminal fermentation characteristics using either high forage (HF) or high grain (HG) diets at two media pH in batch culture.

2. Materials and methods

2.1. Preparation of ROD extract

The ROD extract was provided by Red Dog Enterprise Ltd. (Winnipeg, MB, Canada), and the extraction was conducted using hydrothermal technology [11]. In brief, the ROD samples, comprised of leaves and stems, were freshly harvested from Swan River (MB, Canada), air-dried and pulverized into tiny particles. The ground samples were subject to extraction by hydrothermal treatment at 98 °C for 1 h. The extraction procedure consisted of steeping samples in water, solid separation, purification, concentration and spray drying, as described by Apea-Bah et al. [11]. The total phenolic content (g/kg DM) of ROD extract was 218 (expressed as gallic acid equivalents) with 27.5 rutin, 16.7 gallic acid, 7.0 ellagic acid, 0.1 quercetin, and 3.2 quercetin malonyl glucoside [11].

2.2. Experimental design, substrate and inoculum

Experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee at the Lethbridge Research and Development Centre (ACC#1830). Animals were cared and managed according to the guidelines of the Canadian Council on Animal Care [17].

This experiment was a completely randomized design with 2 media pH (5.8 and 6.5) × 4 dose rates of ROD extract (0, 1, 3, 5% of substrate DM). An additional treatment of monensin at a dose of 0.17 mg per bottle was used as a positive control for each pH level due to its antibiotic properties and wide use to decrease risk of ruminal acidosis in beef cattle [18]. The dose rate of monensin was calculated based on a daily dose of 300 mg monensin per head in beef cattle [19]. Two basal diets (HF and HG) were evaluated in two in vitro batch culture experiments. The HF diet consisted of 600 g/kg barley silage and 400 g/kg barley-based concentrate, while the HG consisted of 100 g/kg barley silage and 900 g/kg barley-based concentrate (DM basis; Table 1).

Four ruminally cannulated beef heifers were used as rumen inoculum donor, with two heifers fed the HF diet and two heifers fed the HG diet to provide inoculum for the incubation using HF and HG substrates, respectively. Rumen fluid (approximately 1.8 L/heifer) was collected before morning feeding from four different sites within the rumen, pooled, squeezed through four layers of cheesecloth, kept in a thermos flask (39 °C), and transported to the lab immediately. Upon arrival to the lab, the pH of the rumen fluid was measured using a pH meter (B20 PI, 98 Symphony Benchtop Meters; VWR, Edmonton, AB, Canada).

2.3. In vitro batch culture procedure

The diet substrates were dried at 55 °C for 48 h, ground through 1-mm sieve (standard model 4, Arthur Thomas Co., Philadelphia, PA, USA), and weighed into ANKOM bags (0.5 g substrate/bag, DM basis). The ANKOM bags (ANKOM F57 filter bags, Fairport, NY, USA) were prepared, washed in acetone, oven-dried at 55 °C for 24 h, and weighed before the ROD extract and monensin were added at the desired dose rate, and mixed thoroughly. All bags were sealed and inserted into 125 mL glass bottles, with three replicate bottles prepared for each treatment [2].

The buffer was prepared based on the formulation of Goering and Van Soest [20]. The low (5.8) and high pH (6.5) of fermentation media were achieved by adjusting the amount of sodium bicarbonate in the buffer solution. On the incubation day, all bottles and freshly prepared buffer were kept in an incubator at 39 °C for 1 h. Pre-warmed buffer (45 mL) and strained rumen fluid (15 mL) were mixed (3:1) and dispensed into each bottle. The bottles were flushed with CO_2 to induce anaerobic conditions and sealed tightly with

Table 1
Ingredient and chemical composition of the experimental diets.

Item	High forage (HF)	High grain (HG)
Ingredient, g/kg DM		
Barley silage[a]	600	100
Barley grain, ground[b]	340	889
Canola meal	40	…
Calcium carbonate	15.3	7.7
Feedlot premix[c]	0.4	0.4
Molasses	0.5	0.6
Salt	1.5	1.5
Urea	2.0	0.4
Canola oil	0.3	0.4
Chemical composition, g/kg DM		
Dry matter (DM), g/kg	541	845
Organic matter (OM)	915	951
Crude protein (CP)	134	123
Neutral detergent fiber (aNDF)	347	219
Acid detergent fiber (ADF)	222	92

[a] Composition (DM basis): 296 g/kg DM, 912 g/kg OM, 116 g/kg CP, 447 g/kg aNDF, and 318 g/kg ADF.
[b] Composition (DM basis): 905 g/kg DM, 967 g/kg OM, 124 g/kg CP, 196 g/kg aNDF, and 68 g/kg ADF.
[c] Feedlot premix supplied per kilogram of dietary DM: 15 mg Cu, 65 mg Zn, 28 mg Mn, 0.7 mg I, 0.2 mg Co, 0.3 mg Se, 6000 IU vitamin A, 600 IU vitamin D, and 47 IU vitamin E).

14 mm rubber stoppers and aluminum crimp caps. The sealed bottles were placed on a shaker (Lab-Line Instruments Inc., Melrose Park, IL, USA) in an incubator (39 °C) for 24 h according to the method described by Cattani et al. [21]. Three bottles containing ruminal fluid and buffer without substrates were added as blanks to correct for gas release. Three incubation runs were carried out within 3 weeks (i.e., one run/week) and the run was used as experimental unit.

Gas pressure was recorded at 3, 6, 9, 12, and 24 h post-incubation using a pressure transducer connected to a 23-gauge needle (PX 4200-015GI, Omega Engineering, Inc., Laval, QC, Canada). To allow gas venting after pressure recording, the transducer was disconnected and the needle was left on the top of the rubber stopper for several seconds [22]. Gas volume was estimated for each pressure recording using the equation of Romero-Perez and Beauchemin [23]:

$$\text{Gas volume} = 4.7047 \times (\text{gas pressure}) + 0.0512 \times (\text{gas pressure}^2)$$

At the end of the incubation of 24 h, bottles were removed from the incubator and cooled with ice for 5 min to stop the fermentation, and then the pH (Orion model 260A, Fisher Scientific, Toronto, ON, Canada) was recorded. The filter bags were removed from the bottles using tweezers, rinsed with cold water until the water became clear, oven-dried at 55 °C for 48 h, and weighed for calculation of DMD.

2.4. Chemical analysis

Culture fluid was sampled for VFA and NH_3–N determination after 24 h of incubation. For VFA analysis, 5 mL of fluid was preserved with 1 mL of 25% (wt/vol) HPO_3 in a screw-capped vial and frozen (−20 °C) until further analysis. Samples were thawed at room

Table 2
Effects of media pH level and red osier dogwood (ROD) extract dosage on gas production (GP at 3, 6, 9, 12, and 24 h of incubation, mL/g DM) and dry matter disappearance (DMD at 24 h of incubation, %) of high-forage (HF) or high-grain (HG) diet.

Item	Dose of ROD, % of substrate						*P*-Value[a]			
	0	1	3	5	MON[b]	SEM	pH	L	Q	R vs M
HF										
GP, 3 h										
5.8	24.4	23.6	24.9	25.9	21.0	1.54	0.01	0.82	0.53	0.16
6.5	28.9	28.3	30.6	29.1	32.6	2.56		0.61	0.62	0.22
GP, 6 h										
5.8	42.3	39.5	40.3	41.5	38.1	2.45	0.01	0.42	0.44	0.27
6.5	56.7	52.1	54.2	51.0	59.3	3.84		0.80	0.54	0.14
GP, 9 h										
5.8	60.6	56.1	56.2	56.7	54.4	3.57	0.01	0.25	0.49	0.53
6.5	84.0	78.5	79.1	73.1	88.8	5.53		0.54	0.66	0.08
GP, 12 h										
5.8	103.5	95.8	95.0	94.0	96.7	5.93	0.01	0.12	0.47	0.29
6.5	160.0	151.4	149.7	136.5	165.1	8.17		0.38	0.74	0.06
GP, 24 h										
5.8	141.3	132.6	134.1	131.0	143.3	4.90	0.01	0.04	0.09	0.01
6.5	222.9	209.9	211.4	202.8	232.1	9.43		0.28	0.43	0.01
DMD										
5.8	43.7	40.4	41.5	41.3	40.0	1.8	0.01	0.51	0.41	0.62
6.5	53.1	51.3	51.1	48.0	50.1			0.06	0.91	0.98
HG										
GP, 3 h										
5.8	26.6	26.2	24.4	23.6	25.1	2.73	0.01	0.36	0.74	0.84
6.5	46.6	46.3	46.6	43.3	44.8	1.92		0.99	0.87	0.74
GP, 6 h										
5.8	43.6	42.8	39.8	37.7	40.4	4.67	0.01	0.21	0.68	0.88
6.5	81.4	77.7	77.8	69.9	78.4	3.22		0.37	0.59	0.32
GP, 9 h										
5.8	59.3	57.8	53.5	51.1	55.3	6.30	0.01	0.10	0.63	0.66
6.5	115.2	108.0	107.0	96.1	111.9	5.17		0.20	0.57	0.12
GP, 12 h										
5.8	103.1	98.7	94.2	98.1	95.9	11.10	0.01	0.07	0.99	0.56
6.5	206.1	191.1	190.8	175.2	205.7	11.83		0.14	0.41	0.03
GP, 24 h										
5.8	154.7	149.7	141.7	139.8	139.9	17.72	0.01	0.01	0.65	0.21
6.5	295.7	282.9	285.8	265.3	294.3	24.40		0.08	0.11	0.01
DMD										
5.8	56.2	52.3	51.2	45.6	52.4	2.7	0.01	0.02	0.93	0.39
6.5	68.5	65.5	63.7	60.7	66.9			0.06	0.73	0.25

[a] pH = pH 5.8 vs. 6.5; L, Q = linear or quadratic effect of increasing dose of ROD extract from 0, 1, 3–5% within pH 5.8 or 6.5; R vs. M = contrast between average of ROD extract (1, 3, and 5%) and monensin; No interactions between media pH and ROD dose ($P > 0.15$).
[b] Monensin (MON) was supplemented at 0.17 mg/bottle.

temperature and analyzed via gas chromatography (model 5890, Hewlett-Packard Lab, Palo Alto, CA, USA) with a capillary column (30 m × 0.32 mm i.d., 1-μm phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA, USA), flame ionization detector, and crotonic acid as an internal standard [24]. For NH_3–N analysis, 5 mL of fluid was preserved with 1 mL of 1% (wt/vol) H_2SO_4 in a screw-capped vial and kept at −20 °C until analyzed according to the method of Rhine et al. [25].

Dry matter, acid detergent fiber (ADF) and ash content of experimental substrates were determined using AOAC [26] methods 930.15, 942.05 and 973.18, respectively. Neutral detergent fiber was analyzed as described by Van Soest et al. [27] using heat-stable alpha-amylase (aNDF). The samples for N determination were ground using a ball mill (Mixer Mill MM2000; Retsch, Haan, Germany) to a fine powder and a sample was weighed and transferred to a tin capsule. The total N content was analyzed (method no. 990.03) using a combustion analyzer (NA 2100, Carlo Erba Instruments, Milan, Italy). Methane concentration in fermentation media was calculated using the equation: $CH_4 = 0.45 \times C2 - 0.275 \times C3 + 0.40 \times C4$ [28], and the C2, C3 and C4 were molar proportion of acetate, propionate and butyrate, respectively.

2.5. Statistical analysis

Data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, USA) [29] with the model including the fixed effects of media pH, ROD dose rate, and their interaction, with experiment run as a random effect. Contrasts were used to compare between ROD dose rate and monensin. Linear and quadratic orthogonal polynomial contrasts were used to examine the effect of increasing ROD extract dose rate using the CONTRAST statement of SAS. Significant differences and trends were reported at $P \leq 0.05$ and $0.05 < P \leq 0.10$, respectively.

Table 3
Effects of media pH level and red osier dogwood (ROD) extract dosage on volatile fatty acid (VFA), NH_3–N and CH_4 concentration of a high forage (HF) diet.

Item	Dose of ROD, % of substrate						*P*-Value[a]			
	0	1	3	5	MON[b]	SEM	pH	L	Q	R vs M
Total VFA, m*M*										
5.8	53.2	51.1	53.7	55.6	51.9	2.74	0.01	0.35	0.58	0.58
6.5	61.2	60.3	54.1	58.0	54.5			0.27	0.16	0.31
Mol/100 mol										
Acetate (A)										
5.8	49.3	50.5	50.9	52.9	48.2	0.75	0.01	0.01	0.95	0.03
6.5	51.5	52.0	52.6	53.8	51.6			0.01	0.91	0.05
Propionate (P)										
5.8	20.4	20.3	20.6	20.2	21.3	0.38	0.01	0.71	0.71	0.04
6.5	23.3	23.2	22.8	22.2	23.7			0.04	0.87	0.25
Butyrate										
5.8	17.1	15.9	15.8	14.8	17.1	0.48	0.01	0.01	0.25	0.02
6.5	14.3	14.1	13.9	13.7	13.7			0.10	0.79	0.93
BCFA[c]										
5.8	7.1	7.2	6.9	6.6	6.6	0.18	0.01	0.03	0.39	0.21
6.5	6.3	6.2	6.2	6.0	6.3			0.27	0.89	0.16
Valerate										
5.8	5.1	5.0	4.9	4.6	5.9	0.12	0.01	0.01	0.71	0.01
6.5	3.9	3.8	3.8	3.6	4.0			0.09	0.81	0.04
Caproate										
5.8	1.03	0.99	0.97	0.93	0.91	0.112	0.01	0.16	0.87	0.46
6.5	0.70	0.68	0.69	0.66	0.67			0.60	0.90	0.92
A:P										
5.8	2.41	2.49	2.48	2.62	2.26	0.068	0.01	0.03	0.75	0.01
6.5	2.21	2.25	2.31	2.43	2.17			0.02	0.87	0.15
NH_3–N, m*M*										
5.8	26.6	26.3	25.1	25.4	26.4	1.11	0.09	0.40	0.58	0.55
6.5	24.8	25.2	25.3	22.3	26.0			0.09	0.21	0.18
CH_4, mg/g DM										
5.8	17.5	17.7	17.7	18.2	17.8	0.23	0.01	0.69	0.90	0.99
6.5	16.9	17.0	17.3	17.7	16.9	0.19		0.06	0.79	0.01

[a] pH = pH 5.8 vs. 6.5; L, Q = linear or quadratic effect of increasing dose of ROD extract from 0, 1, 3–5% within pH 5.8 or 6.5; R vs. M = contrast between average of ROD extract (1, 3, and 5%) and monensin; Interactions of pH × ROD dose: proportion of propionate ($P < 0.05$), butyrate ($P = 0.03$), and BCFA ($P < 0.05$).

[b] Monensin (MON) was supplemented at 0.17 mg/bottle.

[c] BCFA = isobutyrate + isovalerate.

3. Results

3.1. Gas production and dry matter disappearance

No interactions between pH and dose rate of ROD extract on GP (mL/g DM) at 3, 6, 9, 12, and 24 h, and DMD at 24 h were observed for the HF diet (Table 2). Increasing media pH from 5.8 to 6.5 increased (P = 0.01) the GP, and increased (P = 0.01) average DMD from 417 to 509 g/kg at 24 h of incubation. In the HF diet, increasing ROD extract supplementation did not overall affect the GP regardless of media pH level or incubation time. However, there was a tendency for DMD to linearly decrease (P = 0.06) at pH 6.5 at 24 h. The GP was greater (P = 0.01) with monensin than with ROD extract at 24 h under either media pH 5.8 or pH 6.5, whereas there were no differences in DMD between ROD extract and monensin supplementation.

There were no significant interactions between media pH and ROD extract supplementation on GP and DMD for the HG diet (Table 2). Increasing media pH from 5.8 to 6.5 consistently increased (P = 0.01) GP (mL/g substrate) at all incubation time, and increased (P = 0.01) average DMD from 513 to 646 g/kg at 24 h of incubation. Increasing ROD extract dose rate in the HG diet linearly (P = 0.01) decreased 24 h GP at pH 5.8, and also tended to linearly (P = 0.08) decrease 24 h GP at pH 6.5. Similarly, increasing ROD extract dose rate linearly (P = 0.02) decreased DMD at pH 5.8, and also tended (P = 0.06) to decrease DMD at pH 6.5. Overall, no differences in GP and DMD were observed when comparing ROD extract with monensin, except for lower 12 h GP (P = 0.03) and 24 h GP (P = 0.01) at pH 6.5 with ROD extract.

3.2. Fermentation characteristics

Effects of media pH and ROD dose rate on total VFA concentrations, individual molar proportion of VFA, and NH_3–N concentration with HF substrate at 24 h of incubation are reported in Table 3. Significant interactions were observed between media pH and ROD extract dose rate for total VFA concentration (P < 0.05) and molar proportion of propionate (P < 0.05), butyrate (P = 0.03) and branched-chain VFA (BCFA; P < 0.05). Increasing dose rate of ROD extract linearly decreased proportion of propionate at pH 6.5 (P =

Table 4
Effects of media pH and red osier dogwood extract (ROD) dosage on volatile fatty acid (VFA), NH_3–N, and CH_4 concentration of a high-grain (HG) diet.

Item	Dose of ROD, % of substrate						*P*-Value[a]			
	0	1	3	5	MON[b]	SEM	pH	L	Q	R vs M
Total VFA, m*M*										
5.8	76.9	74.5	74.4	69.8	76.2	3.12	0.01	0.11	0.88	0.34
6.5	83.2	81.9	86.6	80.6	81.9			0.67	0.36	0.74
Mol/100 mol										
Acetate (A)										
5.8	43.3	44.2	45.3	46.5	40.4	1.53	0.01	0.01	0.57	0.03
6.5	46.9	47.7	48.5	49.7	45.1			0.01	0.85	0.09
Propionate (P)										
5.8	27.1	26.7	26.4	25.6	28.1	1.35	0.52	0.12	0.97	0.10
6.5	26.6	26.4	26.0	25.5	26.8			0.25	0.96	0.81
Butyrate										
5.8	16.5	16.2	15.9	15.7	16.5	0.88	0.02	0.32	0.73	0.37
6.5	15.5	15.2	15.1	14.9	15.3			0.48	0.89	0.77
BCFA[c]										
5.8	6.7	6.8	6.6	6.6	7.2	0.28	0.01	0.57	0.92	0.10
6.5	6.2	6.1	6.0	5.7	6.3			0.05	0.73	0.09
Valerate										
5.8	5.8	5.6	5.3	5.1	5.9	0.25	0.01	0.01	0.42	0.01
6.5	4.6	4.3	4.1	3.9	4.8			0.01	0.39	0.01
Caproate										
5.8	0.53	0.52	0.49	0.49	0.56	0.069	0.01	0.38	0.75	0.34
6.5	0.31	0.30	0.31	0.32	0.41			0.74	0.81	0.81
A:P										
5.8	1.68	1.76	1.81	1.93	1.44	0.156	0.01	0.02	0.91	0.06
6.5	1.90	1.93	2.00	2.10	1.68			0.04	0.92	0.07
NH_3–N, m*M*										
5.8	31.7	31.4	31.0	29.6	32.7	1.27	0.94	0.01	0.47	0.01
6.5	32.1	31.4	30.6	29.9	32.5			0.01	0.53	0.01
CH_4, mg/g DM										
5.8	14.0	14.3	14.6	15.1	14.3	2.05	0.01	0.06	0.95	0.11
6.5	15.0	15.2	15.5	16.0	15.2	2.41		0.02	0.74	0.02

[a] pH = pH 5.8 vs. 6.5; L, Q = linear or quadratic effect of increasing dose of ROD extract from 0, 1, 3–5% within pH 5.8 or 6.5; R vs. M = contrast between average of ROD extract (1, 3, and 5%) and monensin; Interactions of pH × ROD dose: BCFA, P < 0.05.
[b] Monensin (MON) was supplemented at 0.17 mg/bottle.
[c] BCFA = isobutyrate + isovalerate.

0.04) and butyrate (P = 0.01) and BCFA at pH 5.8 (P = 0.03) but did not impact VFA proportion of these three VFAs at the alternate pH. Overall, increasing media pH from 5.8 to 6.5 increased (P = 0.01) total VFA concentration and proportion of acetate and propionate, but decreased (P = 0.01) the proportion of other VFA and acetate to propionate ratio (A:P). Concentration of NH_3–N tended (P = 0.09) to decrease with increasing pH level or with increasing ROD extract dose rate at pH 6.5. Methane concentration was reduced (P = 0.01) by increasing media pH from 5.8 to 6.5, and tended to linearly (P = 0.06) increased with increasing ROD extract at pH 6.5. Compared with monensin, supplementation of ROD extract had greater proportion of acetate at pH 5.8 (P = 0.03) and pH 6.5 (P = 0.05), and lower proportion of propionate (P = 0.04) and butyrate (P = 0.02), as well as lower A:P ratio (P = 0.01) at pH 5.8. The methane concentration was also greater (P = 0.01) with ROD extract than monensin at pH 6.5.

Significant interactions between media pH and ROD extract dose rate on fermentation characteristics were observed for BCFA with the HG diet where increasing ROD extract linearly decreased (P < 0.05) BCFA proportion at pH 6.5, with no impact at pH 5.8 (Table 4). Increasing media pH increased (P = 0.01) total VFA concentration, proportion of acetate, A:P ratio, and methane concentration, but decreased (P = 0.01) the molar proportion of butyrate, BCFA, valerate and caproate. The molar proportion of propionate and NH_3–N concentration were not affected by the media pH. Additionally, the total VFA concentration was not affected by increasing ROD extract dose rate, however the proportion of acetate, A:P ratio, and methane concentration linearly (P = 0.01) increased. The NH_3–N concentration was not affected by media pH but linear (P = 0.01) increases at both pH was observed with increasing ROD extract dose rate. Methane concentration was increased (P = 0.01) by increasing media pH from 5.8 to 6.5, and linearly increased at media pH 5.8 (P = 0.06) and at pH 6.5 (P = 0.02) with increasing ROD extract.

In comparison with monensin, supplementation of ROD extract resulted in a higher (P = 0.03) proportion of acetate at low pH, with a tendency for lower (P = 0.10) propionate proportion and as a result, higher (P = 0.06) A:P ratio. At high media pH, the acetate proportion (P = 0.09) and A:P ratio (P = 0.07) also tended to be higher with ROD than monensin. There were lower valerate proportion (P = 0.01) and NH_3–N concentration (P = 0.01) with ROD than monensin at both low and high pH. The methane concentration was greater (P = 0.02) with ROD extract than monensin at pH 6.5.

4. Discussion

This in vitro study was designed to evaluate ROD extract for its effects on GP, DMD and fermentation characteristics in various combinations with media pH, and dose rate when supplemented to HF or HG diets. The HF and HG diets used in the present study represent those that are typically offered to growing and finishing beef cattle, respectively, in western Canadian feedlots. It is well established that feeding HF and HG diets to cattle results in differing rumen pH [15,30] which, as such, may respond differently to ROD extract supplementation. Cardozo et al. [31] reported that some plant extracts such as allicin, cinnamaldehyde, carvacrol, thymol, have a greater impact on rumen VFA profiles at low rumen pH, suggesting that the status of the phytogenic compound molecules (i.e., dissociated or undissociated) is dependent of rumen pH. This study is novel in that it illustrates the various modes of action of a newly-developed ROD extract in the rumen under different fermentation conditions and although impacts on microbial profiles that could not be measured in the current study due to the large numbers of samples and the short incubation period. This study provided novel information on a range of digestive parameters.

4.1. Effect of in vitro media pH

The end products of rumen fermentation can be altered by type of diet used and its effects on rumen pH [2]. Studies suggest that the mean rumen pH ranges from 5.84 for beef cattle offered HG finishing diets [16] to 6.25 for cattle offered HF growing diets [15], thus pH of 5.8 and 6.5 were chosen for evaluation in the current study to reflect the differing ruminal pH conditions that may be anticipated in a feedlot environment. At the end of the incubation period, the final media pH values (data not shown) of 5.79 and 6.43 for HF, and 5.69 and 6.39 for HG for the 2 pH, respectively, were similar to the values recorded at the beginning of the incubation because of high buffering media in the batch culture. In the current study, the GP, DMD, and total VFA concentrations were lower at pH 5.8 compared to pH 6.5, reflecting the negative impact of lowering ruminal pH on ruminal microbial activity, which was not unexpected [32]. Similarly, Wei et al. [12] and Jiao et al. [2] reported that GP, DMD and VFA concentrations of HG diet in batch culture were adversely affected by a reduction in media pH from 6.5 to 5.8. Ruminal pH < 6 has been shown to adversely affect cellulolytic bacteria and consequently impaired fiber digestion may occur [33]. However, in the current study, the HF diet had lower A:P ratio, but the HG diet had higher A:P ratio with high media pH compared to low media pH. These differences may be attributed to an increase in the proportion of both acetate (+3%) and propionate (+12%) with the HF diet at high pH, but only an increase in the proportion of acetate (+7%) in the HG diet. Wei et al. [12] reported that increasing media pH from 5.8 to 6.5 in batch cultures had no influence on A:P ratio with the HF diet, whereas it increased the A:P ratio with the HG diets. These results suggest that the in vitro rumen fermentation pattern may be particularly sensitive to media pH when the HG diet is incubated.

4.2. Effect of increasing dose rate of ROD extract

4.2.1. Effect on GP, DMD and VFA concentration

Both ROD raw material and its extract have the ability to improve protein efficiency, nutrient digestibility, and ruminal microbial activity, resulting in enhancement of beef cattle performance and health [9,10,14]. In the present study, increasing the dose rate of ROD extract to the HG diets significantly or tended to linearly decrease the GP and DMD at 24 h at either pH 5.8 or pH 6.5, whereas, it did not affect total VFA concentration. Similarly, Wei et al. [12] demonstrated that replacement of barley silage with ROD up to 6% in a

similar diet did not affect GP or total VFA concentration at low media pH, however it linearly decreased DMD at both pH 5.8 and 6.5. The decreased DMD could be due to the negative effects of phytogenic compounds on protein and starch degradation due to its selective action on certain rumen microorganisms [34]. The effect of feed additives containing phenolic compounds including rutin, gallic acid, ellagic acid, and quercetin and their adverse impact on potential DMD and fermentation parameters is also dose-dependent [35,36]. Wei et al. [12] conducted an in vitro study with similar treatment arrangements to the present study, but with supplementation of raw plant ROD, and reported that the concentration of total phenolic compounds (gallic acid, methyl gallate, cathechin, epicatechin, rutin, ellagic acid and quercetin) from raw plant ROD were estimated to be 11, 22 and 44 mg/L of fermentation media, respectively, for 3, 6 and 12% ROD inclusion, while the total phenolics were 31, 62 and 124 mg/L (i.e., 12.3% of ROD, DM basis). In the present study, the determined phenolic compounds from ROD extract were approximately 5, 14 and 23 mg/L, respectively, for 1, 3 and 5% of ROD extract inclusion, while the doses of total phenolics were 18, 55 and 91 mg/L, respectively. These results indicate that a lower dose of phenolic compounds would be needed with ROD extracts compared to raw plant ROD to decrease the DMD, particularly for HG diet. Gomaa et al. [14] suggested that the different DMD response may be due to difference in phenolic compound profiles between raw plant ROD and its extract because the extraction rate varies among individual phenolic compounds [11]. Bravo [37] suggested compromised nutrient digestion and fermentation as a consequence of the presence of phenolic compounds may be primarily attributed to their ability to bind with large molecules like proteins and carbohydrates.

It is well-known that fiber is essential for stimulating ruminal motility and maintaining a healthy ruminal microbial ecosystem [38,39]. In this study, the molar proportion of acetate increased with increasing ROD extract dose rate. The observed increase in acetate proportion at pH 5.8 with increasing ROD extract supplementation in the HF diet suggests that fiber digestion would be enhanced by the ROD extract. Ruminal fermentation and the microbial ecosystem can be affected by either the raw plant ROD or its extract but this effect may differ with their phenolic compound concentration and composition [11]. Although, supplementation of the HG diet with ROD extract did not affect total VFA concentration, the GP and DMD at 24 h linearly decreased which was partly in agreement with Wei et al. [12] who reported that adding ROD to a HG diet linearly decreased both DMD and total VFA concentration without altering GP at low media pH (5.8). The inconsistency of GP and VFA concentration with the decreased DMD in our study was unexpected but may be explained by a variation of microbial mass production, however this was not measured. In rumen batch culture, truly digested substrates are converted among VFA, gas and microbial biomass and the DMD is an indication of true rumen digestibility [40]. The linearly decreased DMD was indicative of a reduction in the activity of rumen microbes with increasing ROD extract. Low dose rates (2.5–5 mg/L) of antioxidant (α-tocopherol and β-carotene) in ruminant diets has been shown to improve ruminal microbial activity and fiber digestion, however, high doses (30–40 mg/L) negatively impacted both parameters [41]. The antioxidant activity was reported in beef cattle fed raw plant ROD [10]. Recent research at this institution also reported that adding ROD extract to a HG diet at a low dose rate of 1% (diet DM basis) did not affect the DMD nor total VFA concentration when using a RUSITEC system [14]. The variation in responses among studies may also be attributed to the differences in techniques used, diet composition, phenolic concentrations and composition in ROD and its extract, or the dose rates used.

4.2.2. Effect on fermentation efficiency

Energy efficiency and utilization in ruminants is associated with fermentation patterns as A:P ratio is an indicator of fermentation efficiency [42]. In this study, the fermentation pattern favoured acetate production and consequently increased A:P ratio, especially at low pH in the HG diet with increasing ROD extract supplementation. This was in agreement with previous research at this institution which evaluated ROD in batch culture [12] and in a RUSITEC system [14]. The latter authors reported that adding ROD extract to a HG diet increased NDF digestibility but decreased starch digestibility, which was supported by changes in the ruminal microbial activity at the genus level where *Treponema* was more abundant with diets containing ROD extract. Kudo et al. [43] reported that fiber digestion occurs only when *Treponema bryantii* are present with cellulolytic bacteria such as B. succinogenes. These results show that cellulolytic bacteria interact with noncellulolytic *Treponema* to promote the digestion of fiber. *Treponema*, along with *Fibrobacter*, are important in lignocellulose degradation and their presence support enhanced feed digestion [44]. In that, the decreased rumen starch digestibility of the HG diet with ROD supplementation suggests the potential to alleviate the incidence of ruminal acidosis in cattle fed HG diet. The reduction in starch digestibility could be due to the decrease of relative abundance of *Prevotella* at genus level [14], as they are able to utilize various nutrients such as starch, proteins and non-cellulosic polysaccharides [45]. Furthermore, although the increased A:P ratio indicates lower fermentation efficiency, it suggested fiber digestion was improved [12]. Improved fiber digestion in ruminants offered HG diets is of interest in terms of rumen health as pH is often less than 5.8 under such feeding strategies [30,39], thus increasing the risk/incidence of acidosis. Given that cellulolytic bacteria are sensitive to ruminal pH, this could have a negative impact on their activity at low pH and decrease fiber digestion in the rumen [46,47].

4.2.3. Effect on protein degradation

The decrease in NH_3–N concentration observed with increased ROD extract supplementation in the HG diet was consistent with the response observed by Wei et al. [10] when raw plant ROD was supplemented at 0, 3, 7, and 10% (DM basis) to a grain-based diet offered to beef heifers. Decreased NH_3–N concentration may be due to the binding of phenolics in ROD or its extract with protein molecules, resulting in decreased ruminal protein degradation and increased by-pass protein, thereby enhancing protein efficiency [12]. In an in situ study, Gomaa et al. [9] demonstrated that increasing feeding raw plant ROD to heifers offered a HG diet consistently decreased the ruminal protein degradation rate of barley silage. Furthermore, as deamination of branched-chain amino acids during protein degradation forms BCFA, the linear decrease in BCFA with ROD extract supplementation in the current study also supports the theory that ROD phenolic compounds have the capacity to bind protein molecules and decrease protein degradation.

4.3. Effect of supplementation of ROD extract vs. monensin

Monensin was used in this study as a positive control, with the primary justifications for feeding monensin to cattle being the resultant reduction of ruminal protein degradation, increase in ruminal propionate production, and the improvement of feed efficiency when it is offered in vivo [48]. Monensin can inhibit ruminal H_2-producing ruminal bacteria that are more apt to produce acetate and butyrate than those that produce succinate and propionate; therefore monensin can shift the fermentation from acetate to propionate which explains, partially, improvements in feed efficiency [49]. In the present study, there was an consistent effect of monensin. It numerically decreased DMD, total VFA concentration and A:P ratio compared to the control. This was in agreement with Tedeschi et al. [48] who reported reduced A:P ratio partially in agreement with Ponce et al. [50] who reported a decrease in total VFA concentration in grain-based diets. Compared with monensin, supplementation of the HF and HG diets with ROD extract increased molar proportion of acetate and decreased valerate. Additionally, in the HG diet, DMD, the molar proportion of BCFA, and NH_3–N concentrations were lower with ROD supplementation than with monensin. Wei et al. [12] compared the effect of adding raw plant ROD vs. monensin on in vitro rumen fermentation and also reported decreased DMD but observed increased total VFA concentrations and GP with ROD, suggesting that rumen fermentation and energy partition was directed toward decreasing microbial biomass and more toward GP and VFA. Differences in fermentation patterns between ROD extract and monensin indicate possible differences in the modes of action between ionophores and phenolic compounds in the rumen. However, that hypothesis appeared to be not supported in the present study due to lack of differences in DMD and total VFA concentration between ROD extract and monensin, and the GP at 24 h was actually lower with ROD extract, rather than greater.

5. Conclusions

Increasing the supplementation rate of ROD extract in HF or HG diets may demonstrate adverse effects on in vitro DMD, but the effects varied with media pH and type of diet. Furthermore, consistent differences in GP, DMD and fermentation parameters between low and high media pH confirmed the critical role of rumen pH in rumen microbial activity. However, the increased A:P ratio, without systematically changing proportion of propionate, that occurred regardless of media pH and diet, with increasing ROD extract supplementation suggests that the effects were targeted towards fiber digestion. This finding may be of particular interest in the HG diet of finishing cattle where rumen pH is often below 5.8 and rumen fibrolytic activity is compromised. The medium dose of ROD extract is recommended as the high dose appeared to have a more adverse impact on DMD without affecting the fermentation profile.

Ethics statement

Study procedures were approved by Lethbridge Research and Development Centre Institutional Animal Care and Use Committee (Protocol#2124), and the experiment was performed according to the guidelines of the Canadian Council on Animal Care [17].

Funding

The research was funded by Ag Action Manitoba Program-Research and Innovation, Manitoba Agriculture, Winnipeg, MA, Canada; Red Dog Enterprises Ltd., Winnipeg, MA, Canada; Manitoba Beef Producers, Winnipeg, MA, Canada; Beef Farmers of Ontario, Guelph, ON, Canada.

CRediT authorship contribution statement

W.M.S. Gomaa: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **A.M. Saleem:** Data curation, Investigation, Methodology. **E.J. McGeough:** Writing – review & editing, Funding acquisition. **K. Ominski:** Funding acquisition, Writing – review & editing. **L.Y. Chen:** Funding acquisition, Writing – review & editing. **W.Z. Yang:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that no conflict of interest, financial or other, exists.

Acknowledgments

We acknowledge Alastair Furtado, Derrell Vedres, and Pamela Caffyn for their assistance during laboratory work and the metabolism barn staff for their management and care of animals during the experiment.

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