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Effects of hops on *in vitro* ruminal fermentation of diets varying in forage content

N. Narvaez, Y. Wang *, Z. Xu, T. McAllister

Lethbridge Research Centre, Agriculture and Agri-Food Canada, 5403 1 Ave S., Lethbridge, AB, Canada

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ABSTRACT

Four *in vitro* incubations were conducted to assess the potential of hops (*Humulus lupulus*) as an alternative to antimicrobials for improving ruminant production. Ground whole hops (*var.* Teamaker) pellets were included in batch culture ruminal incubations (500 mg substrate + 40 mL inoculum) of pure forage, barley grain and total mixed rations (TMR) of growing (GD) and finishing (FD) diets for feedlot cattle. The TMR contained (DM basis) barley silage:barley grain in ratios of 55:40 (GD) or 9:86 (FD). Hops were included in cultures with pure forage and barley grain at 0, 50, 100, 200 and 400 μg/mL and with GD or FD at 0, 200, 400, 800 and 1600 μg/mL. Incubations with GD or FD were prepared with and without polyethylene glycol (PEG) to define the effects of hop condensed tannins (CT) on ruminal fermentation. Adding PEG did not alter *in vitro* fermentation suggesting that hops CT at the concentrations used did not influence microbial activity. With hops inclusion, gas production from barley grain was linearly increased ($P<0.05$) but it linearly decreased ($P<0.001$) for all the other substrates over the entire incubation period (24 or 48-h). True DM disappearance (DMD) from pure forage, starch true digestibility (STD) from barley grain and apparent DMD from FD at the end of the respective incubation were linearly increased ($P<0.05$) with increasing hops content. A quadratic ($P<0.001$) reduction of MN in pure forage was observed with hops, but it was linearly increased ($P<0.001$) from barley grain. Hops linearly increased ($P<0.001$) volatile fatty acids production from FD but it was linearly reduced ($P<0.001$) in the GD. The acetate:propionate ratio was quadratically reduced ($P<0.001$; lowest at 400 μg/mL) with GD and with FD ($P<0.001$; lowest at 800 μg/mL) as the concentration of hops increased. Hops quadratically ($P<0.05$) decreased methane production as per unit of digested DM irrespective of the type of substrate incubated. Effects of whole hops on ruminal fermentation were diet and dose dependent. Inclusion of hops in ruminants may offer a means of decreasing ruminal methane emissions without compromising fermentability of feed. However, its efficacy on *in vivo* rumen fermentation and animal performance deserves further research.

1. Introduction

There is a growing trend to restrict the use of in-feed antibiotics in livestock production as was aptly demonstrated by the ban on the use of antibiotic growth promoters in the European Union (OJEU, 2003). Natural plant compounds that possess antimicrobial activity have been proposed as a potential alternative to the use of antibiotics in livestock production (Wallace, 2004). Hops (*Humulus lupulus*) are primarily used in the brewery industry as they confer the bitterness and aroma to beer. However, hops have also been used for culinary, medicinal, and cosmetic purposes. A number of studies have demonstrated that hops possess antimicrobial activity against a range of bacterial species (Batchvarov and Marinova, 2001). The antimicrobial properties of hops are thought to arise from a mixture of α- and β-acids, essential oils, and polyphenolics including tannins (Moir, 2000). It has been shown that α- and β-acids inhibit most Gram positive

* Corresponding author. Tel.: +1 403 317 3498; fax: +1 403 382 3156.
E-mail address: Yuxi.Wang@agr.gc.ca (Y. Wang).

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(Schmalreck et al., 1975; Langezaal et al., 1992; Simpson and Smith, 1992), but not Gram negative bacteria (Oshugi et al., 1997; Srinivasan et al., 2004). This specific activity against Gram positive bacteria is a property shared with monensin, an antimicrobial feed additive that is widely used in feedlot cattle in North America. Purified ß-acids extracted from hops affected rumen fermentation in a dose- and diet-dependent manner (Schmidt and Nelson, 2006; Schmidt et al., 2006; Drouillard et al., 2009). Although the impact of hop extracts on fermentation has been investigated, the effect of whole hops on ruminal fermentation has not been explored. Cornelison et al. (2006) reported that broilers supplemented with 227 mg/kg of whole hops had improved growth and feed utilization. Our laboratory found that inclusion of hops in a barley grain–barley silage growing diet at 476 mg/kg DM and 952 mg/kg DM in finishing diet improved rumen fermentation, but did not affect the growth of feedlot cattle (Wang et al., 2010). The results suggest that higher levels of hops in the diet may be required to alter feed utilization or growth in feedlot cattle. The objective of the present series of experiments was to use an *in vitro* system to assess the effects of hops on ruminal fermentation at concentrations higher than those previously included in forage and concentrate diets.

2. Materials and methods

2.1. Hops and preparation

Hops (*H. lupulus*, cv. Teamaker®) were supplied as pellets (John Segal Hop Ranch—Grandview, WA) and contained 79 g of ß-acids, 11 g of α-acids (Washington State Hop Lab — Yakima, WA), and 44 g of extractable condensed tannins (CT) per kg dry matter (DM). Extractable CT concentration in hops was estimated by the Butanol/HCl method (Terrill et al., 1992) using purified tannins from Sainfoin (*Onobrychis viciifolia*) as a standard. Hops were stored in a sealed container in the dark at 4 °C and ground to pass a 1.0 mm screen just prior to use in *in vitro* experiments.

2.2. Substrates, experimental design and treatments

Four batch culture incubations were conducted to study the effects of hops on ruminal fermentation of pure forage (Exp 1), barley grain (Exp 2), and two total mixed rations (TMR) of growing (GD; Exp 3) and finishing (FD; Exp 4) diets for feedlot cattle. The composition and chemical analysis of the experimental diets are shown in Table 1. The pure forage substrate and TMRs were freeze-dried and ground through a 1.0 mm screen before use. With the purpose of getting a uniform particle size to reduce variation in the fermentation within treatment, barley grain was first ground through a 2.0 mm screen, then sieved with a 0.5 mm screen. The particles between 0.5 and 2.0 mm were used.

The treatments in Exps 1 and 2 (pure substrates) were control (no hops) and hops addition at the concentrations of 50, 100, 200 and 400 μg/mL. Treatments in Exps 3 and 4 (TMR substrates) were control and hops supplied at 200, 400, 800, and 1600 μg/mL, with and without polyethylene glycol (PEG; 250 μg/mL, MW 3,350). Inclusion of PEG in the fermentation inactivates CT, enabling their effects on fermentation to be assessed (Jones and Mangan, 1977).

Table 1
Diet composition and chemical characterization of the experimental diets.

	Experimental diets			
	Barley grain	Pure forage	Growing	Finishing
Diet composition; kg/100 kg DM				
Barley silage	–	25	55	9
Alfalfa hay	–	25	–	–
Grass hay	–	50	–	–
Barley grain	100	–	40	86
Mineral supplement [a]	–	–	5	5
Chemical composition; g/kg DM [b]				
Organic matter	97.8	92.8	937	953
Total Nitrogen	25.2	25.2	18.2	20.1
Neutral detergent fiber	227.4	397.5	359	292.5
Acid detergent fiber	65.2	262.6	150	–
Starch	475	–	374	477

[a] Supplement contained (per 1000 kg): 653 kg ground barley, 237 kg limestone, 50 kg salt, 40 kg Dynamate (Pitman-Moore Inc., Oakville, ON), 10 kg urea, and 10 kg trace mineral mix containing (per kg): sodium chloride (926 g), zinc sulphate (11 g), Dynamate (50 g), manganese sulphate (9.4 g), copper sulphate (3.2 g), cobalt sulphate (0.005 g), canola oil (as carier of $CoSO_4$; 0.04 g), sodium selenite (0.044 g), and ethylenediaminedioidic acid (80%; 0.012 g), Dynamate contains 22% S; 18%K; 11% Mg; 0.1% Fe; 0.0005 Pb (max).
[b] Average values of 3 replicates for each of the four treatments.

2.3. Inoculum, hops addition, and incubation

In each experiment, substrate was weighed into 125-serum vials (500 mg/vial). In Exps 1 and 2, ground hops were introduced into vials as a 1.0 mL suspension in distilled water (dH_2O) just prior to addition of rumen fluid inocula. In Exps 3 and 4, ground hops were weighed directly into the vials at the designated concentrations. Four replicate vials were prepared for each treatment and incubated for 0, 6, 12, 24 and 48 h in Exp 1 and for 0, 3, 6, 12 and 24 h in Exp 2. Eight replicate vials were prepared for each concentration of hops and incubated for 0 or 48 h in Exp 3 and 0 or 24 h in Exp 4. In Exps 3 and 4, 10 mg of PEG in 0.5 mL of dH_2O were added to four of the vials, with 0.5 mL of dH_2O added to the remaining four vials in each incubation time.

In each experiment, microbial inoculum was prepared by using two parts of mineral buffer (Menke et al., 1979) and one part of mixed rumen fluid (two donors). The rumen fluid in each experiment was collected 2 h after feeding from two cows fed a diet (DM basis) containing alfalfa–grass hay (50:50) for Exp 1, barley silage and barley grain (40:60) for Exp 2, a TMR containing barley silage, alfalfa hay, barley grain and supplement (74:5:17:4) for Exp 3, and barley silage, barley grain and supplement (31.3: 65:3.7) for Exp 4. All animals used in this study were cared for in accordance with standards of the Canadian Council on Animal Care (CCAC, 1993).

Inoculum for Exps 1 and 2 also contained 0.5 g/L of ^{15}N-labeled ammonium sulphate (10.01 atom% minimum enrichment; Sigma Chemical Company, St. Louis, MO, USA) as a marker for microbial protein synthesis. Inoculum was dispensed anaerobically (40 mL/vial) under a stream of O_2-free CO_2, followed immediately by sealing and affixing to a rotary shaker platform (120 rpm) within a temperature controlled incubator at 39 °C. Triplicate vials containing no substrate were also prepared as blank controls for each incubation time in each experiment.

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2.4. Sample collection and processing

Gas produced in each vial after 3 (Exp 2 only), 6, 12, 24 and 48 (Exp 1 only) h in Exps 1 and 2, and at 4, 8, 12, 24 and 48 (Exp 3 only), and methane concentration of the gas sampled at these time points were determined using methods described by Wang et al. (2008). After incubation, vials for each treatment (n = 4) in each experiment were withdrawn from the incubator, pH of the whole contents was measured and subsequently processed for determinations of volatile fatty acids (VFA), NH_3-N, microbial N (MN; Exps 1 and 2), *in vitro* true dry matter disappearance (TDMD; Exps 1 and 2), *in vitro* starch true digestibility (STD; Exp 2) and *in vitro* apparent dry matter disappearance (ADMD; Exps 3 and 4) as described by Wang et al. (2008).

2.5. Calculations and statistical analysis

Gas production, TDMD, STD and MN accumulation in Exps 1 and 2 were calculated according to the method described by Wang et al. (2008). Apparent DMD from Exps 3 (after 48-h incubation) and 4 (after 24-h incubation) was calculated as the difference between dry weight of the substrate incubated and the dry weight of the residue (*i.e.*, undegraded substrate and residual feed particle associated microbial mass) corrected for corresponding blank residue weight. Methane produced during ruminal fermentation was calculated as mL per g of truly digested DM (TDDM; Exps 1 and 2) or apparent digested DM (ADDM; Exps 3 and 4).

All data were analyzed statistically by analysis of variance using PROC MIXED (SAS Institute Inc., 2007). Individual vial was used as the random factor for all experiments. Exps 3 and 4 were first analyzed as 2 × 5 factorial design. As inclusion of PEG was found to have no effect on measured parameters, data from PEG and non-PEG treatments were pooled for each concentration of hops and analyzed as a completely randomized design. The model used for analysis of time-course (repeated measures) data included time and the time × treatment interaction. When these effects (time or time × treatment interaction) were significant (*i.e.* $P<0.05$), means of the treatments were compared at each time point. Differences among treatments were tested using LSMEANS with the PDIFF option in SAS (2007). Polynomial contrasts were used to determine linear (L) and/or quadratic (Q) responses to the hops concentration in all experiments. In all the analyses, significant effects were declared at $P<0.05$.

3. Results

3.1. Pure forage — Exp 1

Treatment × time interactions ($P<0.05$) occurred for all studied fermentation products and therefore they were presented at each time point (Tables 2 and 3).

Table 2
Effect of hops on *in vitro* ruminal fermentation characteristics of pure forage substrate during 48 h of incubation (Exp 1).

	Hop concentration, μg/mL					SEM [a]	Main effect	Contrast [b]	
	0	50	100	200	400			L	Q
Gas production (mL/g DM)									
6	78.6	81.0	83.4	85.3	85.7	2.06	0.086	0.014	0.151
12	120.2	119.2	120.2	119.0	119.8	1.50	0.969	0.894	0.685
24	161.3	159.9	157.9	155.3	154.8	1.76	0.058	0.007	0.195
48	188.1	187.9	187.4	183.8	180.3	1.78	0.029	0.002	0.941
True dry matter disappearance (mg/g)									
6	460.3	476.7	492.4	502.0	479.5	15.30	0.415	0.468	0.082
12	576.3	566.5	619.3	643.1	591.2	12.09	0.007	0.154	0.002
24	736.0	750.4	754.4	741.7	727.2	4.56	0.011	0.014	0.018
48	796.5	780.2	806.5	812.7	830.5	5.11	<0.001	<0.001	0.894
Methane (mL/g Truly digested DM)									
6	25.6	24.0	24.2	23.2	24.1	0.65	0.201	0.224	0.059
12	30.1	29.2	27.5	26.1	28.2	0.75	0.032	0.085	0.006
24	32.6	30.5	29.2	29.6	31.0	0.61	0.020	0.411	0.003
48	34.4	34.4	32.5	31.6	31.6	0.59	0.011	0.003	0.048
Microbial ^{15}N (μg/g Truly digested DM)									
6	551.8	558.6	503.9	445.1	417.8	8.00	<0.001	<0.001	0.003
12	647.2	625.9	554.7	482.9	484.4	6.98	<0.001	<0.001	<0.001
24	551.0	521.4	545.8	507.4	455.3	4.55	<0.001	<0.001	0.060
48	402.9	404.2	414.0	411.6	362.7	2.79	<0.001	<0.001	<0.001
Ammonia-N accumulation (μm/mL)									
6	1.2	−0.2	−0.78	−2.8	−3.4	0.48	<0.001	<0.001	0.021
12	1.4	2.2	−4.41	−2.0	−1.0	0.44	<0.001	0.002	<0.001
24	2.5	1.8	0.24	−1.2	0.6	0.54	0.006	0.018	0.001
48	6.8	10.1	8.19	9.3	7.8	0.40	0.001	0.853	0.203

Incubation time and incubation time × treatment for all parameters listed were significant ($P<0.05$).
[a] SEM, standard error of the mean.
[b] Hop effects: L, linear; Q, quadratic.

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Table 3
Effect of hops on volatile fatty acids (VFA) production and their molar percentages (mM/100 mM total VFA) during 48 h of *in vitro* ruminal incubation of pure forage substrate (Exp 1).

	Hop concentration, μg/mL					SEM [a]	Main effect	Contrast [b]	
	0	50	100	200	400			L	Q
Total VFA (mM)									
6	32.2	34.6	35.3	36.0	31.5	2.15	0.521	0.194	0.431
12	47.5	50.7	47.3	50.3	52.4	1.09	0.033	0.012	0.766
24	66.1	66.9	66.2	64.8	59.9	4.11	0.627	0.172	0.668
48	79.4	71.2	65.0	65.1	71.5	2.54	0.017	0.197	0.002
VFA (mM/100 mM)									
Acetate									
6	64.9	64.6	65.6	65.7	66.6	0.34	0.018	0.002	0.855
12	64.6	65.2	65.9	65.7	66.5	0.31	0.012	0.002	0.202
24	64.8	65.1	65.8	66.8	68.4	0.36	<0.001	<0.001	0.605
48	65.1	67.6	66.7	67.2	67.9	0.46	0.012	0.009	0.176
Propionate									
6	20.1	20.4	19.4	18.6	18.0	0.20	<0.001	<0.001	0.104
12	20.1	20.3	19. 9	18.9	17.8	0.15	<0.001	<0.001	0.466
24	20.0	19.9	20.1	19.1	17.7	0.19	<0.001	<0.001	0.090
48	19.4	18.9	19.4	18.7	17.7	0.20	<0.001	<0.001	0.243
Butyrate									
6	10.1	10.2	10.2	10.7	10.9	0.20	0.059	0.008	0.542
12	10.3	9.9	9.6	10.3	10.8	0.21	0.019	0.013	0.066
24	9.9	10.0	9. 5	9.4	9.3	0.17	0.067	0.017	0.172
48	9.7	8.7	9.1	9.3	9.5	0.18	0.026	0.290	0.110
Branched-chain VFA									
6	4.4	4.5	4.4	4.6	4.2	0.15	0.648	0.394	0.274
12	4.8	4.2	4.3	4.7	4.7	0.12	0.031	0.247	0.325
24	5.0	4.8	4.4	4.4	4.3	0.14	0.016	0.006	0.030
48	5.6	4.6	4.7	4.6	4.7	0.13	0.001	0.022	0.009
Acetate:propionate									
6	3.2	3.2	3.4	3.5	3.7	0.05	<0.001	<0.001	0.289
12	3.2	3.2	3.3	3.5	3.8	0.03	<0.001	<0.001	0.745
24	3.2	3.3	3.3	3.5	3.9	0.05	<0.001	<0.001	0.192
48	3.4	3.6	3.5	3.6	3.8	0.06	0.002	<0.001	0.762

Incubation time and incubation time × treatment for all parameters listed were significant ($P<0.05$).
[a] SEM, standard error of the mean.
[b] Hop effects: L, linear; Q, quadratic.

Inclusion of hops with pure forage at levels from 0 to 400 μg/mL linearly increased ($P<0.01$) gas production at 6 h, but linearly decreased it ($P<0.01$) at 24 and 48 h of incubation. A quadratic response ($P<0.05$) in TDMD to hops was observed at 12 and 24 h, but it linearly increased ($P<0.001$) at 48 h of incubation. In contrast, methane produced per g TDDM was quadratically decreased ($P<0.05$) at 12, 24 and 48 h of the incubation. Microbial N production (as per g of TDDM) decreased quadratically ($P<0.05$) at 6, 12 and 48 h and linearly ($P<0.001$) at 24 h of incubation. The response of NH_3-N to hops at concentrations from 50 to 400 μg/mL was linear and/or quadratic depending on the incubation time (Table 2). Total VFA was linearly increased ($P<0.05$) at 12 h, but decreased quadratically ($P<0.01$) at 48 h of incubation. Moreover, increasing hops resulted in a linear ($P<0.01$) increase in the molar proportion of acetate and the acetate:propionate (A:P) ratio, whereas the proportion of propionate linearly decreased ($P<0.001$). From 6 to 12 h of incubation, butyrate molar proportions linearly increased ($P<0.05$) with hops. Molar proportions of branched chain VFA (BCVFA) decreased quadratically at 24 ($P<0.05$) and 48 h ($P<0.05$) of incubation.

3.2. Barley grain — Exp 2

In this experiment, treatment × time interactions ($P<0.05$) occurred for gas production, methane production, TDMD, STD, MN, NH_3-N, total VFA and their molar proportions, with the exception of molar proportions of propionate (Tables 4 and 5).

Including hops in the barley grain substrate quadratically increased ($P<0.01$) gas production and TDMD (highest at 50–200 μg/mL of hops, respectively) at 3 (TDMD only), 6 and 12 h of incubation. Starch disappearance was also quadratically increased ($P<0.01$) over 24-h of incubation (highest at 200 μg/mL). These results were accompanied by a quadratic ($P<0.05$) decrease in the amount of methane produced per g TDDM over the 24-h incubation (Table 4). As concentration of hops increased, MN per g TDDM increased in a quadratic fashion ($P<0.01$) up to 24 h and in a linear fashion ($P<0.001$) at 48 h of incubation. Ammonia-N concentration linearly increased ($P<0.01$) at 12 h, but was quadratically reduced ($P<0.006$) at 24 h of incubation (lowest at 200 μg/mL) with increasing concentrations of hops. Production of total VFA was increased ($P<0.05$) by hops at the concentrations of 200 and 400 μg/mL at 3 h only (Table 5). Incubations with hops at

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Table 4
Effect of hops on *in vitro* ruminal fermentation characteristics of barley grain substrate during 24 h of incubation (Exp 2).

	Hop concentration, μg/mL					SEM [a]	Main effect	Contrast [b]	
	0	50	100	200	400			L	Q
Gas production (mL/g DM)									
3	61.5	65.9	63.1	67.0	65.9	1.45	0.061	0.071	0.141
6	139.0	148.7	142.4	147.2	142.0	1.61	<0.001	0.980	0.004
12	202.3	209.8	207.4	209.2	205.7	1.15	<0.001	0.590	<0.001
24	242.0	248.4	241.3	244.6	238.2	1.71	0.010	0.029	0.139
In vitro starch disappearance (mg/g)									
3	249.5	253.4	251.2	319.5	247.0	16.8	0.053	0.663	0.019
6	697.8	716.7	678.3	750.3	658.5	9.52	<0.001	0.022	0.001
12	948.1	970.1	969.2	974.1	945.2	0.79	<0.001	<0.001	<0.001
24	990.3	1001.1	1000.3	1000.6	999.6	1.63	0.004	0.035	0.005
In vitro true DM disappearance (mg/g)									
3	41.9	45.6	47.1	44.7	34.8	2.04	0.011	0.007	0.014
6	57.5	72.3	70.7	70.4	62.4	2.30	0.004	0.721	0.001
12	75.1	78.7	78.6	82.4	77.4	0.62	<0.001	0.063	<0.001
24	88.2	88.7	87.4	86.6	86.3	0.84	0.282	0.070	0.524
Methane (mL/g Truly digested DM)									
3	17.4	16.9	15.3	17.5	20.7	0.66	0.003	0.001	0.018
6	26.4	21.8	20.7	22.1	22.3	0.76	0.003	0.071	0.003
12	30.3	27.5	26.4	25.5	25.5	0.72	0.004	0.002	0.008
24	31.6	29.5	27.0	28.0	25.7	0.55	<0.001	<0.001	0.036
Microbial ^{15}N (μg/g Truly digested DM)									
3	353.1	341.9	291.1	332.3	376.9	8.40	<0.001	0.008	<0.001
6	547.1	650.3	658.8	675.2	696.9	14.83	<0.001	<0.001	0.003
12	649.5	623.4	633.2	627.8	677.8	9.05	0.010	0.008	0.008
24	441.6	424.4	536.5	478.5	541.1	9.09	<0.001	<0.001	0.116
Ammonia-N accumulation (μm/mL)									
3	3.6	4.6	3.0	5.0	5.9	0.80	<0.001	0.001	<0.001
6	8.0	5.3	9.5	9.9	8.6	0.90	0.035	0.640	0.013
12	10.7	11.3	11.8	13.1	14.3	0.72	0.035	0.003	0.504
24	19.4	17.5	17.5	14.2	16.9	0.86	0.022	0.055	0.006

Incubation time and incubation time × treatment for all parameters listed were significant ($P<0.05$).
[a] SEM, standard error of the mean.
[b] Hop effects: L, linear; Q, quadratic.

400 μg/mL produced more ($P<0.05$) total VFA than that of control at 12 h of incubation. Molar proportions of acetate linearly decreased ($P<0.001$) and those of propionate linearly increased ($P<0.001$) with increasing concentrations of hops at 12 h of incubation. A quadratic response ($P<0.05$) was observed at 12 h of incubation in molar proportions of butyrate and at 24 h in those of BCVFA. The A:P ratio linearly decreased ($P<0.001$) at 12 h of incubation with increasing concentrations of hops.

3.3. Growing diet — Exp 3

Incubation time × treatment interactions ($P<0.001$) occurred for total gas and methane production (Table 6). A linear ($P<0.001$) decrease in total gas produced at 12, 24, and 48 h of incubation and in ADMD measured at the end of 48-h incubation was observed as hops increased from 0 to 1600 μg/mL. The amount of methane produced per mL/g DM over the entire incubation period as well as that produced per g of ADDM at the end of the fermentation was quadratically ($P<0.05$) decreased. A linear decrease ($P<0.001$) in NH_3-N concentration and total VFA production was observed in response to increasing hops after 48-h incubation. Molar proportions of acetate and propionate quadratically increased ($P<0.05$), whereas those of butyrate and BCVFA quadratically decreased ($P<0.001$) as the amount of hops increased. A quadratic decrease ($P<0.001$) was observed in the A:P ratio with increasing hops, with the lowest ratio observed between 400 and 800 μg hops/mL.

3.4. Finishing diet — Exp 4

Significant effects ($P<0.01$) of hops on gas production, ADMD, methane production, NH_3-N concentration, total VFA, molar proportions of individual VFA and on the A:P ratio were observed for the FD (Table 7). Likewise, a treatment × time interaction ($P<0.001$) was found for total gas and methane production.

Increasing concentrations of hops in FD reduced (L, $P<0.001$; Q, $P<0.01$) total gas production throughout the fermentation process and decreased (L, Q; $P<0.001$) methane production (as per g DM) at 8, 12 and 24 h of the incubation. In contrast, ADMD and concentrations of TVFA and NH_3-N in the fermentation solution were increased (L, $P<0.01$; Q, $P<0.05$) at 24-h, whereas the amount of methane produced per g ADDM was decreased (L, Q, $P<0.001$) as the concentration of hops

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Table 5
Effect of hops on volatile fatty acids (VFA) production and their molar percentages (mM/100 mM total VFA) during 24 h of *in vitro* ruminal incubation of barley grain substrate (Exp 2).

	Hop concentration, μg/mL					SEM [a]	Main effect	Contrast [b]	
	0	50	100	200	400			L	Q
Total VFA (mM)									
3	18.7	20.2	20.1	25.6	24.2	1.31	0.008	0.002	0.045
6	46.3	49.2	45.0	45.8	52.1	1.59	0.075	0.056	0.120
12	70.9	68.6	68.0	70.8	72.4	2.42	0.688	0.347	0.521
24	92.5	96.4	80.9	88. 6	87.9	3.39	0.034	0.248	0.146
VFA (mM/100 mM)									
Acetate									
3	49.0	46.7	47.0	46.6	46.5	0.63	0.093	0.061	0.099
6	44.3	44.7	44.4	44.5	45.1	0.57	0.873	0.398	0.699
12	45.0	44.6	44.7	44.0	41.8	0.57	0.015	0.001	0.356
24	41.6	42.9	45.0	43.6	43.3	0.89	0.192	0.313	0.106
Propionate									
3	39.7	42.4	41.9	42.2	41.8	0.75	0.161	0.287	0.085
6	43.5	44.1	44.0	44.0	43.3	0.31	0.328	0.274	0.116
12	41.7	42.5	43.0	43.1	44.4	0.29	<0.001	<0.001	0.481
24	41.7	39.8	40.9	40.4	41.4	1.16	0.793	0.815	0.466
Butyrate									
3	5.4	5.7	5.9	5.9	6.2	0.15	0.045	0.007	0.346
6	7.0	6.7	6.9	7.0	7.2	0.15	0.314	0.153	0.396
12	7.8	7.6	7.5	7.6	8.5	0.16	0.007	0.002	0.009
24	9.2	8.9	8.2	8.6	8.8	0.18	0.025	0.515	0.012
Branched-chain VFA									
3	4.8	4.9	5.0	5.1	5.3	0.23	0.419	0.067	0.794
6	4.8	4.2	4.1	4.3	4.1	0.16	0.058	0.056	0.117
12	5.1	5.0	4.5	5.0	5.0	0.15	0.06	0.990	0.111
24	8.0	8.0	5.6	6.9	5.8	0.24	<0.001	<0.001	0.016
Acetate:propionate									
3	1.17	1.10	1.12	1.10	1.11	0.03	0.366	0.265	0.169
6	1.01	1.01	1.01	1.01	1.04	0.03	0.761	0.339	0.484
12	1.08	1.05	1.04	1.02	0.94	0.02	0.007	<0.001	0.777
24	1.11	1.08	1.10	1.08	1.05	0.02	0.186	0.091	0.886

Treatment × time interactions ($P<0.05$) occurred for total VFA and their molar proportions, except propionate.
[a] SEM, standard error of the mean.
[b] Hop effects: L, linear; Q, quadratic.

increasing up to 1600 μg/mL. Molar proportions of acetate and BCVFA linearly decreased ($P<0.001$), whereas those of propionate and butyrate quadratically increased ($P<0.001$) with increasing hops. The A:P ratio quadratically decreased ($P<0.001$) as hops were added to the FD.

4. Discussion

The purpose of using pure mixed forages or grain as substrate in this study was to define the effects of hops on ruminal fermentation of diets with wide range of forage: concentrate ratios. However, as pure forage or grain diet is rarely used in feedlot industry, we consider that comparing the effects of hops on rumen fermentation of forage-concentrate combinations (TMR) is perhaps more meaningful in the real world of animal feeding. Additionally, inclusion of PEG in Exps 3 and 4 was designed to assess the effect of hop CT on rumen fermentation. Similar responses between PEG and non-PEG treatments suggest that hop CT had negligible effects on ruminal fermentation, likely due to its low level in the fermentation culture and/or low inhibitory potential of its structural compounds on rumen microbial activity. Hop cones are rich in secondary metabolites classified as resinous bitter acids (α- and β-acids), volatile oils (terpenoids), and a wide range of phenolic acids, and flavonoid glycosides (Stevens et al., 1998; Moir, 2000; Cleemput et al., 2009). All of these compounds possess antimicrobial activity (Krishna et al., 1986; Langezaal et al., 1992; Schmidt et al., 2006; Siragusa et al., 2008), and α- and ß-acids seem to account for the majority with β-acids having greater antimicrobial activity than the α-acids (Michener and Anderson, 1949; Hough et al., 1957). Therefore, results obtained from this study represent the combined effects of all these bioactive compounds (including CT) present in whole hops.

In this study, rumen fermentation response of the different substrates to hops was feed type- and dose-dependent. When TMRs were used as substrates, effects of hops at 200 to 1600 μg/mL concentrations on ruminal fermentation were diet dependent in terms of its impact on DMD and VFA production, being negative with GD but favourable with FD. However, methane production and A:P ratio were all reduced by hops with both TMR as substrates and the overall extent of this reduction appeared to be greater for concentrate-based substrates than for forage-based substrates. All these results suggest that hops exert a greater favorable effect on diets high in starch as compared to those high in fiber. On the contrary, Wang et al. (2010) reported a more pronounced response in *in vitro* ruminal fermentation from forage-based diets than from concentrate-based diets when hops were added at levels up to 476 or 952 mg/kg DM, respectively. Dissimilarity between

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Table 6
Effect of hops on *in vitro* ruminal fermentation characteristics of mixed barley silage/barley grain growing diet during 48 h of incubation (Exp 3).

	Hop concentration, μg/mL					SEM [a]	Main effect	Contrast [b]	
	0	200	400	800	1600			L	Q
Gas production (mL/g DM)									
8	116.9	121.5	122.6	119.8	110.4	0.97	<0.001	<0.001	<0.001
12	162.8	163.0	159.7	154.5	147.2	0.75	<0.001	<0.001	0.816
24	213.3	211.8	204.0	191.6	175.1	0.96	<0.001	<0.001	0.084
48	253.6	253.7	244.5	223.9	200.0	1.05	<0.001	<0.001	0.521
Methane (mL/g DM)									
8	29.9	30.6	30.3	29.9	28.2	0.29	<0.001	<0.001	0.015
12	37.3	37.5	35.1	33.7	32.1	0.33	<0.001	<0.001	0.004
24	50.3	50.0	46.9	43.6	39.2	0.45	<0.001	<0.001	0.030
48	63.0	62.2	58.2	52.3	45.7	0.34	<0.001	<0.001	<0.001
Apparent DM disappearance (mg/g)	646.4	652.6	654.6	628.2	621.5	1.67	<0.001	<0.001	0.639
Methane (mL/g Digested DM)	97.4	95.3	88.9	83.2	73.5	0.48	<0.001	<0.001	<0.001
Total VFA [c] (mM)	72.2	63.8	59.6	61.1	53.3	2.31	<0.001	<0.001	0.139
VFA (mM/100 mM)									
Acetate	60.0	60.1	61.4	62.9	65.3	0.22	<0.001	<0.001	0.246
Propionate	17.2	18.3	20.6	21.3	18.9	0.15	<0.001	<0.001	<0.001
Butyrate	16.5	15.4	12.4	10.9	11.3	0.20	<0.001	<0.001	<0.001
Branched-chain VFA	6.4	6.2	5.7	4.9	4.6	0.08	<0.001	<0.001	<0.001
Acetate:propionate	3.5	3.3	3.0	3.0	3.5	0.03	<0.001	0.299	<0.001
Ammonia-N (μm/mL)	15.2	15.5	13.2	10.9	10.0	0.52	<0.001	<0.001	0.097

Incubation time × treatment interactions ($P<0.001$) occurred for all parameters.
[a] SEM, standard error of the mean.
[b] Hop effects: L, linear; Q, quadratic.
[c] VFA, volatile fatty acids.

these studies might be related to the lower doses of hops used by Wang and collaborators.

Addition of hops to all diets consistently decreased the amount of methane produced per unit of DDM. This together with its effect on reducing A:P from TMR fermentation, suggests that supplementation of whole hops could be an efficient manipulation strategy to reduce methane production and increase energy efficiency for ruminant production. The mechanism by which supplementation whole hops decreased ruminal methane production is not known. However, the fact that methane production from all substrates used was decreased by whole hops and that response of other fermentation products to hops addition was diet dependent suggest that whole hops might contain compounds that specifically inhibit the ruminal methanogenesis. Methane is produced in the rumen mainly by *Archae* using H_2, CO_2 and formate as substrate (Miller, 1995). In consequence, the increased propionate proportion in the total VFA observed in 3 of the 4 experiments in this study by inclusion of whole hops and concomitant decrease of methane production suggest that greater proportion of H_2 was partitioned into propionate sink rather than methane pathway. Plant secondary compounds such as ß-acids in hops have been shown to be more inhibitory to Gram positive bacteria than to Gram negative bacteria (Teuber and Schmalreck, 1973). However, its effects on rumen bacteria and on *Archae* have not been assessed. Identifying the effective compounds in hops and characterizing the reactions of these compounds with different rumen microbes would be crucial to elucidate the mechanism by which hops decrease ruminal methane production.

The concentrations of hops used in this study (50 to 1600 μg/mL) equals to an inclusion level of 0.5–16 g/kg DM in a feedlot cattle diet based on the rumen fluid flowing out of the rumen (100 L). This amount of hops (50 to 1600 μg/mL) provided α- and β-acid concentrations from 5.5 to 176 and 40 to 1264 mg/kg DM, respectively. Cornelison et al. (2006) reported that in the absence of growth promoting antibiotics the inclusion of hop pellets into growing broiler diets at 227 mg/kg DM (21.1 mg/kg DM of ß-acid) resulted in improved growth rate and feed utilization. However, Wang et al. (2010) found that the inclusion of whole hops (Teamaker) at concentrations that provided β-acid concentrations up to 40 mg/kg DM in growing diet and up to 80 mg/kg DM in finishing diet had no influence on the growth or feed efficiency of feedlot cattle. These results indicated that ruminants could need higher concentration of hops to overcome potential losses in the biological activity of active compounds in hops as a result of their metabolism by ruminal microorganisms. In this study, positive effects of hops on *in vitro* fermentation of forage- and concentrate-based substrates were achieved with hops concentrations at 400–800 and 800–1600 μg/mL, respectively. At these concentrations, a feedlot animal consuming 10 kg of diet DM per day would need 40 to 80 or 80 to 160 g of whole hops per day for forage or concentrate diet, respectively. These amounts of whole hops would provide 0.4 to 0.9 or 0.9 to 1.8 g of α-acids and 3.2 to 6.3 or 63 to 125 g of β-acids per day for forage or concentrate diet, respectively. However, a controlled *in vivo* experiment is needed to verify this assumption.

It needs to be pointed out that although chemical analysis of hop pellet was not performed in this study, nutrients from added hops could partly contribute to the observed results. O'Rourke (2003) reported that beside of secondary compounds hop pellets contained (per kg DM) approximately 400 g of fiber (cellulose and lignin) and 150 g of protein. However, it is difficult to quantify the contribution of these nutrient to the overall ruminal fermentation due to the anti-microbial nature

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Table 7
Effect of hops on *in vitro* rumen fermentation characteristics of mixed barley silage/barley grain finishing diet during 24 h of incubation (Exp 4).

	Hop concentration, μg/mL					SEM [a]	Main effect	Contrast [b]	
	0	200	400	800	1600			L	Q
Gas production (mL/g DM)									
4	87.3	90.0	87.9	87.3	54.5	1.05	<0.001	<0.001	<0.001
8	152.0	152.9	144.3	142.3	112.0	0.97	<0.001	<0.001	<0.001
12	184.5	179.5	168.6	165.0	146.7	0.81	<0.001	<0.001	0.004
24	210.0	202.4	192.6	183.8	171.4	0.83	<0.001	<0.001	<0.001
Methane (mL/g DM)									
4	20.6	19. 5	18.3	18.5	14.3	0.25	<0.001	<0.001	0.057
8	32.2	30.6	27.2	25.9	23.2	0.28	<0.001	<0.001	<0.001
12	38.8	35.4	31.3	28.7	28.4	0.43	<0.001	<0.001	<0.001
24	47.6	43.9	39.6	36.4	35.1	0.61	<0.001	<0.001	<0.001
Apparent DM disappearance (mg/g)	553.2	565.0	599.1	603.6	622.1	7.26	<0.001	<0.001	0.010
Methane (mL/g Digested DM)	86.2	77.9	66.1	60.3	56.4	1.34	<0.001	<0.001	<0.001
Total VFA [c] (mM)	145.6	150.8	152.1	163.1	160.8	3.43	0.004	0.001	0.040
VFA (mM/100 mM)									
Acetate	54.1	53.5	53.4	52.5	51.4	0.24	<0.001	<0.001	0.417
Propionate	24.5	25.1	26.0	27.4	26.4	0.26	<0.001	<0.001	<0.001
Butyrate	15.4	15.5	14.8	14.4	16.8	0.23	<0.001	<0.001	<0.001
Branched-chain VFA	6.0	6.0	5.9	5.7	5.4	0.08	<0.001	<0.001	0.877
Acetate:propionate	2.2	2.1	2.1	1.9	2.0	0.03	<0.001	<0.001	<0.001
Ammonia-N (μm/mL)	6.5	7.5	10.3	9.6	6.1	0.25	<0.001	0.007	<0.001

Incubation time and incubation time × treatment for all parameters listed were significant ($P<0.001$).
[a] SEM, standard error of the mean.
[b] Hop effects: L, linear; Q, quadratic.
[c] VFA, volatile fatty acids.

of the secondary compounds when whole hops is used (*i.e.* nutrients and antimicrobial compounds are co-existing in whole hops). Nevertheless, the fact that ruminal fermentation responded to hops in both diet- and dose-dependent manner in this study indicated that the main effect of hops on ruminal fermentation was attributable to their secondary compounds as opposed to their other nutrients.

The substrate-related differences in the effects of hops on rumen fermentation likely reflect the specific inhibition or enhancement of a group(s) of microorganisms by secondary compounds present in hops. It is well recognized that populations of rumen bacteria vary when different diets are fed to ruminants. The species specific activity of plant secondary compounds against rumen bacteria has also been well observed (Jones et al., 1994; Wallace et al., 1994; Wang et al., 2000, 2009). In general, rumen cellulolytic bacteria (most of them are Gram-positive) are more sensitive to secondary compounds than non-cellulolytic bacteria. The reduced microbial N production with pure forage as substrate versus the increased microbial N production with barley grain as substrate, as well as the alterations in VFA profiles by the addition of whole hops indicate that antimicrobial compounds in hops are more inhibitory to rumen cellulolytic bacteria than to non-cellulolytic bacteria. It has also been shown that ß-acids, one of the major anti-microbial compounds in hops, inhibit Gram-positive bacteria more than Gram-negative bacteria (Langezaal et al., 1992; Simpson and Smith, 1992), which is consistent with the observations in this study.

5. Conclusions

Addition of hops to *in vitro* fermentation of mixed forage-concentrate diets decreased methane production and the A:P ratio with these effects greater for concentrate diets as compared to forage diets. These effects were dose-dependent, with optimal hop concentrations between 800 and 1600 μg/mL (632 to 1264 mg/kg DM of β-acids) for concentrate-based diets and between 400 and 800 μg/mL (316 to 632 mg/kg DM of β-acids) for forage-based diets. Including hops in feedlot diets has the potential to enhance feed efficiency by decreasing methane emissions without compromising the ruminal fermentability of the diet. Further research is needed to assess the effects of hops on *in vivo* rumen fermentation and animal performance.

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