Effects of replacing barley grain in feedlot diets with increasing levels of glycerol on *in vitro* fermentation and methane production

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Abstract

The aim of the study was to assess impacts of increasing dietary levels of glycerol on *in vitro* ruminal fermentation and CH_4 production from a barley based feedlot diet. Glycerol was used as replacement for barley grain at inclusions of 0, 70, 140 and 210 g/kg of diet dry matter (DM) in a diet containing an equal mixture of barley grain and barley silage. Both grain and silage were dried and ground through a 1 mm screen before mixing with glycerol. The experiment was repeated twice using ANKOM® bags in 50 ml sealed batch culture serum vials (*i.e.,* 0.5 g substrate + 25 ml media) with a 3:1 ratio of buffer:rumen liquor (n = 5 bags/treatment/experiment). Rumen liquor was obtained from two cows fed a diet containing 710 g/kg barley silage, 250 g/kg barley grain and 40 g/kg concentrate (DM basis). Gas production was measured by water displacement at 3, 6, 12, 24, 36 and 48 h after inoculation. Volumes corrected for gas released from 15 negative controls (*i.e.*, no substrate) were used to estimate net gas production at 24 and 48 h. Gas samples collected at 24 and 48 h were analyzed for CH_4 concentration. *In vitro* DM disappearance (IVDMD) and culture pH were measured at 48 h. Cumulative gas production as ml/g DM substrate and IVDMD were similar among treatments. Culture pH was higher (P<0.001) in the 210 g/kg glycerol diet compared to other treatments. Total CH_4 production (as mg) did not differ among treatments. However CH_4 expressed as mg CH_4/g digested DM linearly decreased (P=0.02) from 12.5 to 11.3 as the level of glycerol increased from 70 to 210 g/kg. Results suggest that replacing barley grain with glycerol reduces CH_4 production as a function of digested DM.

Keywords: methane, glycerol, *in vitro*

Abbreviations: DM, dry matter; IVDMD, *in vitro* DM disappearance; TCA, trichloroacetic acid; VFA, volatile fatty acids

1. Introduction

The increase of biodiesel production has led to increased stocks of glycerol with a subsequent price reduction, making glycerol a potential high energy feed source for ruminants. Until recently, glycerol was used as a minor component of the diet to prevent or treat ketosis in transition (*i.e.,* immediately before and after calving) and postpartum dairy cows (Rémond et al., 1993; Defrain et al., 2004; Chung et al., 2007). Glycerol

improves glucose status in ruminants as it is readily absorbed through the rumen wall and converted to glucose in the liver (Rémond et al., 1993), or fermented to propionate, a gluconeogenic precursor that increases blood glucose levels after absorption in cattle (Chung et al., 2007) and sheep (Johns, 1953). Bergner et al. (1995) reported that replacement of wheat starch with glycerol increased production of propionate and reduced the acetate:propionate ratio *in vitro*. The same authors found no radioactivity in CH_4, acetic or lactic acid when using C^{14} labelled glycerol, confirming that most glycerol is transformed into propionate *in vitro*.

Although use of glycerol in beef cattle diets has been reported (Schröder and Südekum, 1999; Mach et al., 2008; Parsons et al., 2009), its effects on CH_4 emissions have not been assessed. Among the multitude of strategies suggested to mitigate CH_4 emissions, those that have a positive economic impact on animal production will be the ones which are most likely to be adopted (Beauchemin et al., 2008). As propionate enhancement has been suggested as a means to reduce CH_4 emissions (Boadi et al., 2004), our objective was to assess effects of replacing barley grain with glycerol on *in vitro* CH_4 production using a mixed barley grain and barley silage diet.

2. Materials and method

All procedures and protocols used in this experiment were approved by the Lethbridge Research Centre Animal Care Committee (ACC1008)

2.1 Substrates

The substrate used for incubation was a barley grain:barley silage mixture at the ratio of (500:500; DM basis) left unmodified (Control) or supplemented with (/kg dietary dry matter [DM]) 70, 140 and 210 g of glycerol (99.5 % pure, Sigma-Aldrich, St. Louis, MO, USA) by replacing equivalent amounts of barley grain in the diet. Feed ingredients were dried at 60^{o}C for 24 h and then ground to pass a 1.0 mm screen and mixed to obtain the 4 treatments. Substrates were prepared by mixing barley silage, barley grain and glycerol in ratios of 500:500:0, 500:430:70, 500:360:140 and 500:290:210 for each treatment, respectively. For each incubation, 0.5 g DM of sample was weighed into an ANKOM® bag (model F57) with 5 replicates/treatment and sealed. Even at the 210 g/kg level, the glycerol was fully absorbed onto the feed leaving no free liquid. Each bag was placed

into a 50 ml amber serum bottle fitted with rubber stoppers. The entire incubation procedure was repeated twice (*i.e.,* two incubation runs).

2.2 Inoculum

Inoculum for the *in vitro* incubation was obtained from two ruminally cannulated cows fed a mixed diet consisting of 250 g/kg barley grain, 40 g/kg feedlot supplement and 710 g/kg barley silage. Rumen fluid was collected 2 h after feeding from 4 distinct sites in the rumen, filtered through 4 layers of cheesecloth, combined in equal portions from each animal and transported in a prewarmed Thermos® flask to the laboratory. Inoculum was prepared by mixing rumen fluid and a mineral buffer with 0.5 ml of cysteine sulphide solution (Menke et al., 1979) in a ratio of 1:3. The inoculum was then transferred (25 ml) into pre-loaded pre-warmed (39^{o}C) vials under a stream of O_2-free N gas. Vials were sealed and placed on an orbital shaker rack set at 90 oscillations/min in an incubator at 39^{o}C.

2.3 Determination of total gas, methane concentration and IVDMD

Net gas production of each vial was measured at 24 and 48 h of incubation with a water displacement apparatus (Fedorak and Hrudey, 1983). Headspace gas was sampled from each vial prior to gas measurement with a 20 ml syringe and immediately transferred into a 5.9 ml evacuated Exetainer (Labco Ltd., High Wycombe, Buckinghamshire, UK), which was then analyzed for CH_4 concentration by gas chromatography (Holtshausen et al., 2009). Methane was expressed as mg of CH_4/g DM incubated which disappeared, and total net gas production as ml/g of incubated DM.

After 48 h of incubation, and after gas was sampled for CH_4 and total gas production was measured, the fermentation vials were opened and the pH of the culture was measured using a pH meter (Orion Model 260A, Fisher Scientific, Toronto, ON, Canada). The ANKOM® bags with the residues were then removed from the bottles, rinsed thoroughly with distilled water, dried at 55°C for 48 h to constant weight and weighed to estimate *in vitro* dry matter disappearance (IVDMD).

2.4 Determination of ammonia-N and volatile fatty acids

The liquid fraction of the fermentation at the beginning of the incubation at 0 h and after removal of the filter bag at the end of the 48 h incubation was sub-sampled for determination of ammonia and volatile fatty acids (VFA). Two subsamples (1.6 ml) of

each vial were transferred to 2 ml micro-centrifuge tubes containing 150 µl of TCA (0.65; vol/vol) and centrifuged at 14,000 × *g* for 10 min at 4°C (Spectrafuse 16M, National Labnet Co., Edison, NJ, USA) to precipitate particulate matter and protein. The supernatant was transferred into 2 ml micro-centrifuge tubes (Fisher Scientific, Ottawa, ON, Canada) and frozen at -20°C until analyzed for ammonia N.

In addition, two subsamples (1.5 ml) of each vial were collected, acidified with 300 µl of metaphosphoric acid (0.25; wt/vol), and centrifuged as described for ammonia N analysis. The supernatant was frozen at -20°C until analyzed for VFA concentrations. The 0 h samples were also analyzed for ammonia N and VFA to calculate net ammonia-N and net total VFA production (Holtshausen et al., 2009).

2.5 Statistical analyses

The univariate procedure of SAS was used to test for normal distribution of the data. *In vitro* data were analyzed using average values of both *in vitro* runs for each replicate and analyzed as a randomized complete block design using the PROC mixed procedure of SAS Inc. (2010), with treatment as fixed effects. Planned polynomial contrasts were made to determine linear and quadratic effects of increasing levels of glycerol in the substrates. As no significant quadratic responses occurred, only linear responses are reported. Differences among means were tested using the least squares mean linear hypothesis test with significance declared if $P<0.05$. .

3. Results and Discussion

3.1 Gas production and DM disappearance

Cumulative gas production at 48 h as ml/g incubated DM was similar among treatments. Krueger et al. (2010) reported a linear increase in gas production when glycerol was added to alfalfa hay (at 100, 200 and 400 g/kg DM) *in vitro*, but others have found lower gas production from pure glycerol compared to alfalfa or corn silage (Ferraro et al., 2009).

In vitro DM disappearance tended to increase ($P=0.08$) with higher levels of glycerol. Previous research (Rémond et al., 1993) found no difference in fermented organic matter when glycerol was added to a starch substrate, but did measure a slight increase in digestibility when the substrate was cellulose. Krueger et al. (2010) and Schröder and

Südekum (1999) reported no differences in nutrient digestibility when glycerol replaced alfalfa or wheat grain under *in vitro* or *in vivo* conditions, respectively. The lack of a difference in IVDMD with increasing glycerol levels in our study suggests that glycerol was closely associated with the feed and that disappearance reflected digestion as opposed to loss of glycerol via diffusion through the porous bag.

3.2 Fermentation characteristics

Total VFA production was not affected by glycerol inclusion in the diet (Table 1). Effects of glycerol on fermentation profiles seem to differ according to the degradability of the diet. For example, glycerol increased total VFA production when mixed with cellulose, but not when mixed with starch (Rémond et al., 1993). Wang et al. (2009) recorded increased VFA concentration in steers by adding low amounts of glycerol (*i.e.*, 1.1, 2.2 and 3.3 g/kg DM) to high forage diets, which was mainly attributed to increased concentration of propionate and butyrate in total VFA.

Substituting increasing levels of glycerol for barley grain linearly increased propionate ($P<0.01$) and reduced acetate ($P<0.01$) concentrations resulting in a decline in the acetate to propionate ratio. This fermentation pattern is consistent with other *in vitro* (Rémond et al., 1993, Bergner et al., 1995; Trabue et al., 2007) and *in vivo* (Schröder and Südekum, 1999; DeFrain et al., 2004; Wang et al., 2009) studies, and confirms the propioneogenic properties of glycerol. Butyrate proportions were slightly reduced with increasing levels of glycerol. This result contrasts with others who reported increased proportions of butyrate in total VFA with inclusion of glycerol *in vitro* (Rémond et al., 1993; Trabue et al., 2007). In contrast, *in vitro* (Krueger et al., 2010) and *in vivo* (DeFrain et al., 2004; Mach et al., 2009) studies found no effects on butyrate proportions of total VFA with increased levels of glycerol. Johns (1953) reported that almost all glycerol is fermented to propionate in *in vitro* incubations.

3.3 Methane production

Total CH_4 production (mg/g DM) did not differ among treatments (Table 1). However, CH_4 expressed as mg/g DMD linearly decreased ($P=0.02$) from 12.5 to 11.3 with increasing levels of glycerol. This corroborates that propionate is a H_2 sink and associated with lower levels of CH_4 production (Wolin, 1960; ∅rskov et al., 1968, Janssen, 2010).

4. Conclusions

Replacing barley grain with glycerol in a feedlot diet increased propionate concentration in ruminal fluid and reduced CH_4 production as a function of digested DM *in vitro*. Results suggest that glycerol has the potential to reduce CH_4 emissions in ruminants if used as replacement of grains in feed lot diets.

Acknowledgements:

The research was supported by Canada – Norway Greenhouse Gas Project and the SAGES program of Agriculture and Agri-Food Canada. J.S. Avila was supported by a Conicyt-Chile Scholarship. The authors acknowledge the assistance of Darrell Vedres and Zhong Jun Xu.

References

Bergner, H., Kijora C. , Ceresnakova Z., Szakacs J., 1995. *In vitro* studies on glycerol transformation by rumen microorganisms. Arch. Tierernahr. 48, 245-256.

Beauchemin, K.A., Kreuzer, M., O'Mara, F., McAllister, T.A., 2008. Nutritional management of enteric methane abatement: a review. Aust. J. Exp. Agric. 48, 21-27

Boadi, D., Benchaar, C., Chiquette, J., Massé, D., 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. Can. J. Anim. Sci. 84, 319–335.

Chung, Y.H., Rico, D.E., Martinez, C.M., Cassidy, T.W., Noirot, V., Ames, A., Varga, G. A.., 2007. Effects of feeding dry glycerin to early postpartum Holstein dairy cows on lactational performance and metabolic profiles. J. Dairy Sci. 90, 5682- 5691.

DeFrain, J.M., Hippen, A.R., Kalscheur, K.F., Jardon., P.W., 2004. Feeding glycerol to transition dairy cows: effects on blood metabolites and lactation performance J. Dairy Sci. 87, 4195-4206

Fedorak, P.M., Hrudey, S.E., 1983. A simple apparatus for measuring gas production by methanogenic culture in serum bottles. Environ. Technol. Lett. 4 , 425–432.

215 Ferraro, S.M., Mendoza, G.D., Miranda, L.A., Gutierrez, C.G., 2009. In vitro gas
216 production and ruminal fermentation of glycerol, propylene glycol and molasses. Anim.
217 Feed Sci. Technol. 154, 112-118.
218 Holtshausen, L., Chaves, A.V., Beauchemin, K.A., McGinn, S.M., McAllister, T.A.,
219 Odongo, N.E., Cheeke, P.R., Benchaar, C., 2009. Feeding saponin-containing Yucca
220 schidigera and Quillaja saponaria to decrease enteric methane production in dairy cows.
221 J. Dairy Sci. 92, 2809–2821.
222 Johns, A.T., 1953. Fermentation of glycerol in the rumen of sheep. NZ. J. Sci. Technol.
223 35, 262-269.
224 Krueger, N.A., Anderson, R.C., Tedeschi, L.O., Callaway, T.R., Edrington, T.S., Nisbert,
225 D.J., 2010. Evaluation of feeding glycerol on free-fatty acid production and
226 fermentation kinetics of mixed ruminal microbes in vitro. Bioresource Technol. 101,
227 8469-8472.
228 Mach, N., Bach, A and Devant, M.. 2009. Effects of glycerin supplementation on
229 performance and meat quality of young Holstein bulls fed high-concentrate diets. J.
230 Anim. Sci. 87:632.
231 Menke, K.H., Raab, L., Salewski, A., Steingass, H., Fritz, H., Schrieder, W., 1979. The
232 estimation of the digestibility and metabolizable energy content of ruminant feeding
233 stuffs from the gas production when they are incubated with rumen liquor *in vitro*. J.
234 Agric. Sci. (Camb.) 93, 217-222.
235 Ørskov E.R., Flatt, W.P., Moe, P.W., 1968. Fermentation balance approach to estimate
236 extent of fermentation and efficiency of volatile fatty acid formation in ruminants, J.
237 Dairy Sci. 51, 1429–1435
238 Rémond, B., Souday, E., Jouany, J.P., 1993. *In vitro* and *in vivo* fermentation of glycerol
239 by rumen microbes. Anim. Feed Sci. Technol. 41:121–132
240 Schröder A, Südekum K-H, 1999. Glycerol as a by-product of biodiesel production in
241 diets for ruminants. In: Wratten, N., Salisbury, P.A. (Eds.) New Horizons or an Old
242 Crop. Proc. 10th International Rapeseed Congress, Canberra, Australia, Paper No. 241
243 SAS Inc., 2010. SAS Online Doc 9.1.3. Cary, NC, USA.

244 Trabue, S., Scoggin, K., Tjandrakusuma, S., Rasmussen, M.A., Reilly, P.J., 2007.
245 Ruminal fermentation of propylene glycol and glycerol. J. Agric. Food Chem. 55,
246 7043–7051.
247 Wang, C., Lui, Q., Huo, W.J., Yang, W.Z., Dong, K.H., Huang, X.Y., Guo. G., 2009.
248 Effects of feeding glycerol on rumen fermentation, urinary excretion of purine
249 derivatives and feed digestibility in steers. Livestock Sci. 121, 15–20
250 Wolin M.J., 1960. A theoretical rumen fermentation balance. J. Dairy Sci. 43, 1452–
251 1459.

252 Table 1

253 Effects of increasing levels of glycerol as replacement of barley grain on 48 h fermentation characteristics and *in vitro* methane
254 production.

	Glycerol level (g/kg DM)					*P*
	0	70	140	210	SEM	Linear
Gas production						
Gas, ml/g DM	163.3	163.5	157.6	154.4	2.96	ns
Methane, mg/g DM	7.5	7.4	7.5	7.1	0.19	ns
Methane, mg/g DMD	12.4	12.0	12.4	11.3	0.03	0.02
Fermentation characteristics						
Culture pH	5.85	5.76	5.71	6.25	0.08	0.01
Total VFA, m*M*	91.5	97.4	93.0	97.2	2.99	ns
VFA, mol/100 mol						
Acetate (A)	39.4	35.3	32.6	28.3	0.68	<0.01
Propionate (P)	34.0	38.3	42.1	47.3	0.29	<0.01
Butyrate	17.9	18.2	16.6	16.2	0.34	<0.01
A:P ratio	1.16	0.92	0.78	0.60	0.02	<0.01
Ammonia N, mmol	13.7	12.6	10.9	11.3	1.00	ns
IVDMD, g/kg DM	643.2	660.4	654.2	669.7	7.25	ns

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256 ns, *P*>0.10
257 IVDMD, in vitro dry matter disappearance;