Glycerol inhibition of ruminal lipolysis in vitro¹

H. D. Edwards,* R. C. Anderson,†² R. K. Miller,* T. M. Taylor,* M. D. Hardin,*³ S. B. Smith,* N. A. Krueger,† and D. J. Nisbet†

*Department of Animal Science, Texas A&M University, College Station 77843

†United States Department of Agriculture/Agricultural Research Service, Southern Plains Agricultural Research Center, Food & Feed Safety Research Unit, 2881 F&B Road, College Station, TX 77845

ABSTRACT

Supplemental glycerol inhibits rumen lipolysis, a prerequisite for rumen biohydrogenation, which is responsible for the saturation of dietary fatty acids consumed by ruminant animals. Feeding excess glycerol, however, adversely affects dry matter digestibility. To more clearly define the effect of supplemental glycerol on rumen lipolysis, mixed populations of ruminal bacteria were incubated with 6 or 20% glycerol (vol/vol). After 48-h anaerobic incubation of mixed culture rumen fluid, rates of free fatty acid production (nmol/mL per h) for the 6 and 20% glycerol-supplemented samples were decreased by 80 and 86%, respectively, compared with rates from nonsupplemented control cultures (12.4 ± 1.0 ; mean $\pm$ SE). Conversely, assay of the prominent ruminal lipase-producing bacteria *Anaerovibrio lipolyticus* 5S, *Butyrivibrio fibrisolvens* 49, and *Propionibacterium* species *avidum* and *acnes* revealed no effect of 2 or 10% (vol/vol) added glycerol on lipolytic activity by these organisms. Supplementing glycerol at 6% on a vol/vol basis, equivalent to supplementing glycerol at approximately 8 to 15% of diet dry matter, effectively reduced lipolysis. However, the mechanism of glycerol inhibition of ruminal lipolysis remains to be demonstrated.

Key words: anaerobic lipolysis, biohydrogenation, glycerol, rumen bacteria

INTRODUCTION

Crude glycerol is a major byproduct in the production of biodiesel, and because of its availability

and nutritional properties, it has become an attractive choice for use as a feed supplement for ruminant animals (DiLorenzo and Galyean, 2010). In addition, corn distillers dried solubles used as animal feed have been shown to contain appreciable concentrations of glycerol (Wu, 1994). Feedlot cattle, for instance, consumed more feed and gained more weight when fed diets supplemented with 2 to 8% crude glycerin than when fed diets containing no added glycerin (Parsons et al., 2009). Nevertheless, when cattle were fed diets supplemented with $\geq 12\%$ crude glycerin, rumen digestibility was inhibited (Parsons et al., 2009). An effect was not observed on milk production or feed intake when lactating dairy cows were fed diets supplemented with 5 or 10% refined glycerol, the main component of glycerin, although a slight decrease in intake by cattle fed a diet supplemented with 15% glycerol was observed (Donkin et al., 2009). Gunn et al. (2010), however, reported improved intake and weight gain in finishing lambs fed diets containing up to 15% added glycerin.

Supplementing 2 or 20% glycerol in vitro to mixed populations of ruminal microbes was found to reduce rates of FFA accumulation by 48 and 77%, respectively (Krueger et al., 2010). However, these researchers indicated that when supplemented at 20%, glycerol reduced rates of digestion of the more readily degradable soluble and fibrous fractions (Krueger et al., 2010). Based on these results, glycerol supplementation is being further investigated as a potential strategy to promote incorporation of unsaturated fats into ruminant-produced milk and meat. Additional research is needed to more clearly determine the most efficacious feeding levels, particularly because diet composition and production practices can vary considerably throughout the livestock industry. The primary objective of the present study was to more clearly define the effect of glycerol supplementation on ruminal lipolysis by mixed populations of ruminal bacteria. Additionally, the role of glycerol on individual lipolytic activity expressed by prominent ruminal lipase-producing bacteria was characterized.

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²Corresponding author: Robin.Anderson@ars.usda.gov

³Present address: IEH Laboratories & Consulting Group, 15300 Bothell Way N.E., Lake Forest Park, WA 98155.

MATERIALS AND METHODS

Microbial Preparation and Handling Procedures

The mixed bacterial population used in this study was contained in fresh rumen fluid collected from a cannulated cow grazing on predominantly rye grass pasture. Fluid was strained through a nylon paint strainer (Leyendecker et al., 2004) into a prewarmed insulated container that had been flushed with warm water before sample collection. The container was filled completely (approximately 500 mL), capped, and transported to the laboratory. Upon arrival at the laboratory, CO₂ was bubbled through the rumen fluid to keep it in an anaerobic state until its use as inocula (within 30 min of collection). The cow was cared for according to procedures approved by the Southern Plains Agricultural Research Center Animal Care and Use Committee (College Station, TX; protocol #2010005).

Pure cultures of *Anaerovibrio lipolyticus* 5S and *Butyrivibrio fibrisolvens* 49 were obtained from Jay Yankee, Agriculture and Agri-Food Canada (Ottawa, ON, Canada). Strains of *Propionibacterium avidum* and *Propionibacterium acnes* were previously isolated from the rumen of a pastured cow (Krueger et al., 2008). For long-term preservation of pure cultures, bacteria were stored in 20% anaerobic glycerol at -80°C. Upon removal from storage, each bacterium was revived via 2 consecutive 24- to 48-h cultures in 10 mL of standard anaerobic broth supplemented with 2% presterilized olive oil. Each was then grown for a third consecutive culture in 2 or 4 mL of standard anaerobic broth supplemented with presterilized olive oil. Tubes were incubated (39°C) and agitated horizontally at 35 rpm in an Innova model 4000 shaking incubator (New Brunswick Scientific Co. Inc., Edison, NJ) for 24 to 96 h to achieve late logarithmic phase upon harvest for assay of lipolytic activity.

Standard Anaerobic Medium Formulation

Standard anaerobic broth medium contained (per liter): 292 mg of K₂HPO₄, 292 mg of KHPO₄, 480 mg of (NH₄)₂SO₄, 480 mg of NaCl, 100 mg of MgSO₄·7H₂O, 64 mg of CaCl₂·2H₂O, 4,000 mg of Na₂CO₃, 600 mg of cysteine hydrochloride, 10 g of Trypticase (BBL Microbiology Systems, Cockeysville, MD), 2.5 g of yeast extract, branched-chain FA (1 mmol each of isobutyrate, isovalerate, and 2-methylbutyrate), 0.1 g of D-glucose, 2 µg of hemin, vitamin mix (20 mg each of thiamine, pantothenate, nicotinamide, pyridoxine HCl, and riboflavin, 1 mg of *p*-aminobenzoic acid, 0.5 mg of biotin, 0.5 mg of folic acid, 0.2 mg of vitamin B₁₂, and 0.5 mg

of lipoic acid), and trace minerals (Cotta and Russell, 1982). All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI) unless otherwise noted. Medium was boiled to displace dissolved oxygen and then cooled on ice while being saturated with a continuous flow of O₂-free CO₂ for 20 min. Media was then distributed to 18 × 150-mm glass-walled tubes using the anaerobic Hungate technique described previously (Bryant, 1972). For mixed culture studies, 6 mL of medium was added to tubes containing 0.1 mL of olive oil and 21 g of glass beads (4-mm diameter), which served as a solid support matrix to promote dispersion of the lipid substrate. For pure cultures, 2 to 4 mL of medium was added to tubes as indicated. Once the media was added, tubes were closed with rubber stoppers and sterilized by autoclaving.

Effect of Glycerol Concentration on Inhibition of Mixed Culture Lipolysis

Sterilized tubes were cooled to room temperature and assigned to treatments, in triplicate, and presterilized glycerol was administered by syringe injection to obtain final concentrations of 0, 6, or 20% glycerol (vol/vol; Mallinckrodt Baker Inc., Paris, KY). All tubes were inoculated with 2 mL of mixed-culture rumen fluid and incubated at 39°C while being agitated at 35 rpm for 48 h as described above. At the end of the incubation period, tubes were opened and 0.5 mL of concentrated HCl was added to stop bacterial growth and lipase activity. A separate set of tubes was prepared as described above, except that bacterial growth was stopped by addition of 0.5 mL of concentrated HCl before incubation (0 time). Total FFA were extracted from all tubes and concentrations determined colorimetrically according to methods described by Kwon and Rhee (1986); results were analyzed for differences in FFA between 0 and 48 h as a function of glycerol concentration.

Effect of Glycerol Concentration on Inhibition of Pure Culture Lipase Activity

Short-term anaerobic assay of lipase activity by *A. lipolyticus* 5S, *B. fibrisolvens* 49, *P. avidum*, and *P. acnes* were performed in 18 × 150-mm glass tubes preloaded with 21 g of glass beads, 0.6 mL of olive oil, and anaerobic buffer to achieve a final reaction volume of 6 mL. The anaerobic buffer contained minerals and cysteine HCl as in the standard broth medium but lacked the other ingredients. The entire contents of cultures grown in 2- or 4-mL volume standard anaerobic medium were anaerobically transferred to assay tubes so as to ensure

transfer of bacterial suspensions containing soluble and cell-associated lipase enzymes. Final reaction volumes of resulting 1× and 2× crude enzyme suspensions were achieved upon addition of anaerobic buffer and 0, 2, or 10% added glycerol. Two sets of tubes were prepared, each containing treatments prepared in triplicate, with reactions in tubes of one set being stopped at time 0 by addition of 0.5 mL of concentrated HCl. The other set of tubes were stopped in identical fashion after they were incubated horizontally at 39°C in an incubator shaker at 35 rpm for 6 h. Free fatty acid extractions and measurements were performed as described earlier. Protein concentrations were determined using the Modified Lowry Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL) according to manufacturer instructions.

Statistical Analysis

Tests for the effects of the different treatments were done using a 2-way general ANOVA (Statistix9 Analytical Software, Tallahassee, FL) with a Tukey separation of means ($P < 0.05$). The model included main effects of glycerol, assay level, and their interaction: $Y_{ijk} = \mu + \text{glycerol}_i + \text{assay level}_j + (\text{glycerol} \times \text{assay level})_{ij} + \varepsilon_{ijk}$, where Y_{ijk} is the dependent variable, μ is the overall mean, glycerol and assay level are the fixed effects of the experimental treatments ($i = 1$ to 3; $j = 1$ to 2), glycerol $\times$ assay level is their interaction, and ε_{ijk} is the error term.

RESULTS AND DISCUSSION

Glycerol Inhibition on Mixed Ruminal Cultures

Nutritional approaches are sought to naturally fortify milk and meat with health-promoting unsaturated FA and PUFA (Antongiovanni et al., 2003; Decker and Park, 2010). Microbial biohydrogenation within the rumen, however, rapidly saturates dietary unsaturated FA and PUFA consumed by the host, which can preclude the incorporation of unsaturated fats into ruminant-produced milk and meat (Harfoot and Hazlewood, 1997). Glycerol was recently reported to inhibit in vitro ruminal lipolysis by 48 to 77% (Krueger et al., 2010) and because lipolysis is a prerequisite for biohydrogenation, it was proposed that glycerol supplementation may be a useful method to promote rumen passage and assimilation of MUFA and PUFA into milk and meat. Accumulation of certain intermediates such as *trans*-10, *cis*-12 and *trans*-9, *cis*-11 conjugated linoleic acids produced during incomplete ruminal biohydrogenation and their subsequent postruminal absorption have also been reported to lead to a depression in milk fat synthesis in dairy cattle fed high-fat diets (Perfield et al.,

Figure 1. Rate of FFA accumulation when mixed culture is incubated with varying levels of added glycerol incubation. Bars depict means of 3 replications of experiments with error bars depicting 1 standard deviation from the mean. Bars with differing letters are significantly different ($P < 0.05$).

2007; Shingfield and Grinari, 2007). Accordingly, it is reasonable to hypothesize that decreased availability of FFA resulting from glycerol's inhibition of lipolysis may alleviate the accumulation and resultant milk fat-depressing affects of these intermediates. In the present incubations, rates of FFA accumulation were decreased ($P < 0.05$) more than 80% by glycerol supplementation, relative to nonsupplemented controls, thus confirming the earlier work of Krueger et al. (2010). The inhibited rates did not differ ($P > 0.05$) whether supplemented with 6 or 20% (vol/vol) glycerol (Figure 1).

Supplementing ruminant diets with extruded oilseeds provides about 15% protection from in vitro biohydrogenation (Gulati et al., 1997). Calcium-protected fat sources and whole oil seeds provide about 50% protection to ruminally consumed fats, as determined by in vitro assays (Gulati et al., 1997). Therefore, feeding glycerol has the potential to make an immediate impact on the FA composition of dairy and meat products. High levels of glycerol, however, may negatively affect ruminal digestive function. For instance, when supplemented at 20% of added DM, glycerol reduced digestibility of the soluble and fibrous fractions of feed by approximately 22 and 33%, respectively (Krueger et al., 2010). Roger et al. (1992) showed that the growth of important cellulolytic rumen bacteria and fungi was inhibited in pure cultures supplemented with 5% (vol/vol) glycerol.

Numerous animal feeding studies have likewise suggested that higher levels of glycerol, supplied as crude glycerin, reduce DMI and OM digestibility. In feedlot cattle, optimal feeding levels were reported to be between 2 to 8% (Parsons et al., 2009; Gilbery et al., 2010; MacDonald and Bondurant, 2011). In finishing lambs, up to 15% crude glycerin was found to improve performance (Gunn et al., 2010). Adverse effects were

not observed on milk production or feed intake when prepartum and postpartum dairy cattle were fed diets supplemented with up to 11.5% refined glycerol, although a slight and transient (during the first week of a 56-d study) decrease was observed in intake by lactating cattle fed a diet supplemented with 15% glycerol (Donkin et al., 2009; Carvalho et al., 2011).

In the case of lactating dairy cows as in the study of Donkin et al. (2009), consumption of 24 kg of a diet supplemented with 15% glycerol would deliver 0.072 or 0.06 g of glycerol/mL of rumen fluid (assuming 50- to 60-L rumen fluid volume). For dairy-type feeder calves such as those used by Gilbery et al. (2010), the consumption of approximately 9 kg of a diet supplemented with 12% glycerol would deliver 0.077 to 0.049 g of glycerol/mL (assuming 14- to 22-L rumen volumes). Assumptions of rumen fluid volumes for lactating cows and feeder calves were derived from reported values of similar-type cattle (Galyean et al., 1979; Reynolds et al., 2004). Based on a specific gravity of 1.26 g/mL, we can calculate that the inclusion of 2% glycerol (vol/vol) to in vitro cultures as in the study of Krueger et al. (2010) or the inclusion of 6% (vol/vol) glycerol in the present study delivered 0.025 to 0.076 g of glycerol/mL of culture fluid, respectively. Thus, depending on rumen volume, rate of DMI, and rate of glycerol clearance (via metabolism, absorption, or passage), it seems reasonable to hypothesize that 46 to 80% inhibition of ruminal lipolysis may be achieved by feeding diets supplemented with optimal amounts as reported above. Research is needed, however, to test this hypothesis in vivo.

Tests for Potential Displaced Equilibrium Effect

Whereas glycerol has long been known to be metabolized by bacteria within the rumen (Garton et al., 1961), less is known on the effects of glycerol on lipolytic activity by individual rumen bacteria considered to be major contributors to ruminal lipolysis (Harfoot and Hazlewood, 1997). Extracellular lipases expressed by bacteria can catalyze the hydrolysis of mono-, di-, and triacylglycerols to FFA and glycerol (Pandey et al., 1999; Aravindan et al., 2007). Moreover, lipases may catalyze the esterification of FFA to glycerol, particularly in low-water activity environments (Aravindan et al., 2007). In the present study, lipolytic activity of both *P. avidum* and *P. acnes* was qualitatively comparable to, or greater than, that of *A. lipolyticus* and *B. fibrisolvens* (Table 1). In the case of *B. fibrisolvens* 49, *P. avidum*, and *P. acnes*, a main effect of enzyme concentration was observed, with increased activity observed in assays containing 2× bacterial suspension compared with 1× bacterial suspension. Because olive

oil was present in excess during the assay, it would be expected that more enzyme capacity in the crude whole-cell reaction mixture would yield higher activity. Rates of FFA accumulation did not differ between the 1× and 2× crude suspensions of *A. lipolyticus* 5S, suggesting that factors other than enzyme concentration may be limiting this reaction. Only in the case of *B. fibrisolvens* 49 was a glycerol × enzyme level interaction observed (Table 1).

Based on results from this study, it was hypothesized that supplementing lipase-producing bacterial populations with glycerol may displace the equilibrium of the lipase reaction to favor esterification over de-esterification. However, further analysis of findings does not support the displaced equilibrium hypothesis, as lipolytic rates were unaffected by glycerol, regardless of the amount supplemented to the crude whole-cell assays (Table 1). It is also known that glycerol adsorbs onto matrixes supporting the lipases in immobilized systems, thereby presenting a hydrophilic barrier that limits access of hydrophobic substrates such as triacylglycerols to the enzyme's active site (Dossat et al., 1999; Xu et al., 2011). However, this mechanism does not adequately explain why lipolysis was markedly inhibited by glycerol in cultures of mixed populations of ruminal microbes but not in pure cultures. It is possible that expression of lipase activity may have been suppressed by glycerol during culture of the mixed population, but this would conflict with previous reports that *A. lipolyticus* and *B. fibrisolvens* are likely the main contributors to ruminal lipolysis (Prins et al., 1975; Harfoot and Hazlewood, 1997). Lipase is reported to be constitutively expressed in *A. lipolyticus* (Prins et al., 1975). Similarly, *B. fibrisolvens* strains may be FA auxotrophs (Cotta and Hespell, 1986) and likely express lipase constitutively so that they may acquire FFA required for membrane synthesis. *Propionibacterium avidum* and *P. acnes* also are prominent rumen anaerobes, but only recently have lipolytic strains been identified as important contributors to ruminal lipolysis (Krueger et al., 2008).

CONCLUSIONS

Based on these results, supplementing glycerol at or near 8 to 15% of diet DM would be expected to achieve effective reductions in lipolysis without adversely affecting rumen DM digestion. However, results obtained during culturing of mixed populations of ruminal microbes and from assays of pure cultures of rumen bacteria have not adequately established the mechanism by which glycerol inhibits ruminal lipolysis. Additional research is warranted to determine the effects of long- and short-term glycerol supplementation regimens for ruminants reared under differing production practices,

Table 1. Least squares means of the specific lipase activity of olive oil-grown cells assayed in the presence of olive oil (10%) with 0, 2, or 10% added glycerol as a potential inhibitor of lipolysis

Item	Lipase activity (nmol of FFA/mg of protein per hour)					
	<i>Anaerobivibrio lipolyticus</i> 5S		<i>Butyrivibrio fibrisolvens</i> 49		<i>Propionibacterium avidum</i>	
	1× suspension	2× suspension	1× suspension	2× suspension	1× suspension	2× suspension
Amount of added glycerol (vol/vol)						
0%	0.0031	0.0024	0.0230	0.0302	0.0219	0.0878
2%	0.0052	0.0060	0.0424	0.0378	0.0459	0.0758
10%	0.0009	0.0051	0.0063	0.0531	0.0115	0.1356
Main effect	<i>P</i> -value		<i>P</i> -value		<i>P</i> -value	
Glycerol	0.49		0.34		0.74	
Assay level	0.46		0.049		0.0033	
Interaction	0.58		0.040		0.19	
SEM	0.0024		0.0092		0.024	
					<i>P</i> -value	
					0.40	
					0.0014	
					0.81	
					0.014	
					0.0579	
					0.0302	
					0.0170	
					0.0663	
					0.0535	

and to gain a better understanding of the role(s) of ruminal microorganisms on rumen lipolysis and gastro-intestinal function.

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