

Glycerol as an Energy Source for Ruminants: A Meta-Analysis of *in Vitro* Experiments

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ABSTRACT

Glycerol or glycerin is generally recognized as a safe compound to be used in animal feed, especially for ruminants. A number of *in vitro* studies related to glycerol supplementation in ruminant ration have been published but to date the results have not been summarized. The objective of this study was, therefore, to evaluate *in vitro* digestibility, ruminal fermentation characteristics, total gas and methane production through the meta-analysis approach. Meta-analysis was applied to 13 experiments and 42 treatments dealing with glycerol supplementation in ruminants. Data were analyzed by general linear model procedure in which the glycerol levels and the different studies were treated as fixed effects. Results revealed that glycerol supplementation did not affect the *in vitro* digestibility and total VFA production, but significantly decreased molar proportion of acetate and iso-valerate ($P < 0.05$). In contrast, molar proportion of propionate, butyrate, and valerate significantly increased, and thus the ratio of acetate to propionate declined linearly ($P < 0.05$). Methane production decreased linearly and accompanied with an increase of total gas production with increasing levels of glycerol supplementation ($P < 0.05$). It is concluded that the use of glycerol as an energy substitution in animal feed has no detrimental effects in the rumen and environmentally friendly.

Key words: fermentation, glycerol, in vitro, meta-analysis, ruminant

ABSTRAK

Secara umum gliserol aman digunakan di dalam pakan, khususnya untuk ternak ruminansia. Sejumlah eksperimen *in vitro* terkait suplementasi gliserol di dalam ransum ruminansia telah dipublikasikan tetapi hasilnya tidak konsisten. Tujuan penelitian ini adalah untuk mengevaluasi daya cerna *in vitro*, karakteristik fermentasi rumen, serta produksi gas total dan metana menggunakan metode meta-analisis. Meta-analisis secara kuantitatif mengenai suplementasi gliserol pada ruminansia diterapkan pada 13 studi dengan 42 perlakuan. Data untuk masing-masing variabel diestimasi melalui regresi linear dan dianalisis menggunakan metode GLM dengan level gliserol sebagai pengaruh tetapnya. Hasil menunjukkan bahwa suplementasi gliserol tidak berpengaruh terhadap daya cerna *in vitro* dan nilai VFA total, tetapi menurunkan produksi asam asetat dan isovalerat ($P < 0,05$). Sebaliknya, terjadi peningkatan produksi asam propionat, butirrat, dan valerat sehingga dapat menurunkan rasio antara asam asetat dan asam propionat ($P < 0,05$). Produksi metana menurun secara linear dan total produksi gas menunjukkan peningkatan yang signifikan seiring dengan peningkatan level gliserol yang digunakan ($P < 0,05$), tetapi tidak berpengaruh pada produksi protein mikrob. Dapat disimpulkan bahwa penggunaan gliserol sebagai pengganti sumber energi tidak memberikan pengaruh negatif di dalam rumen dan ramah lingkungan.

Kata kunci: fermentasi, gliserol, in vitro, meta-analisis, ruminansia

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INTRODUCTION

Biodiesel industry has grown rapidly along with the increasing demand of renewable and sustainable energy. Biodiesel is a liquid fuel that is obtained from natural lipids such as vegetable oil or animal fat using manufacturing processes of esterification and transesterification (Quispe *et al.*, 2013). These processes produce a main by-product that is glycerol. Generally, glycerol production is up to 10 to 20% from the total volume of biodiesel produced. In the future, the amount of glycerol will be abundantly available and therefore it needs to be re-handled and re-used in order not to create a new environmental problem. On the other hand, high purity glycerol has been included in food, pharmaceutical, and cosmetic industries as a raw material but the inclusion level is still not much. Thus glycerol has an opportunity to be used as feed supplement and may serve as a source of energy. Glycerol or glycerin is generally recognized as safe for use in animal feed (FDA, 2006), especially for ruminant. However, it should be noted that the presence of impurities substances are at the acceptable level, such as the residual of methanol should not exceed 150 ppm, unsaponifiable matter not to exceed 2% (FDA, 2006), and negligible amount of mineral salts, catalysts, and other impurities so that it has no negative influence on animal health (Dasari, 2007).

Glycerol is indeed a normal component of animal metabolism that is produced through lipolysis of adipose tissue or blood lipoprotein, and there is no agreement on metabolic implications of exogenous supplementation of glycerol in the diet (Silva, 2014). It had been observed that glycerol supplementation improved glucose status in ruminants by acting as a gluconeogenic precursor that increased blood glucose level (Chung *et al.*, 2007) and/or converted to glucose in the liver (Rémond *et al.*, 1993). Glycerol is also readily absorbed through the rumen wall (Rémond *et al.*, 1993) or

fermented to propionic acid (Chung *et al.*, 2007). Some *in vitro* studies related to glycerol supplementation in ruminant ration have been published, but the results are inconsistent in digestibility, total volatile fatty acid (VFA), $\text{NH}_3\text{-N}$, total gas, and methane (CH_4) production. The clearly resembled data is typically acetate to propionate ratio which is being lowered with the increasing level of glycerol in the diet (Rémond *et al.*, 1993; Abo El-Nor *et al.*, 2010; Krueger *et al.*, 2010; Lee *et al.*, 2011; Avila-Stagno *et al.*, 2014). Many inconsistent outcomes between individual studies may be resulted from specific differences in experimental conditions.

Various data can be combined and analyzed by meta-analysis quantitatively in order to deal with such inconsistency. The objective of this study was, therefore, to evaluate the effect of glycerol supplementation on *in vitro* digestibility, ruminal fermentation characteristics, total gas and methane production through the meta-analysis approach.

MATERIALS AND METHODS

Description of Database

Database was constructed from published experiments listed in Scopus. The search used the following combination of two or more keywords: glycerol, glycerin, rumen, ruminant, and *in vitro*. A total of 11 publications, comprised of 13 studies and 42 treatments were obtained and used for the present meta-analysis (Table 1). All publications contained two or more glycerol supplementation levels which were studied in *in vitro* system (either batch or continuous culture system) with steer, cow or sheep as the donors of rumen fluid. Rumen fluid was obtained from the fistulated animals fed with forage and concentrate mixture with the ratio of 50:50 to 80:20 in all studies, with an exception of three studies fed with a single feed only. They were Krueger

Table 1. *In vitro* experiments included in the meta-analysis of the effect of glycerol levels on rumen fermentation

Exp no.	Reference	<i>In vitro</i> type	Basal Feed	Glycerol content (%)	Glycerol level (g/kg DM)
1	Ramos & Kerley 2012	Continuous culture	Concentrate	-	0-200
2	Rico <i>et al.</i> 2012	Continuous culture	Tmr	65	0-124
3	Avila-Stagno <i>et al.</i> 2014	Rusitec	Brome hay and maize silage	99.5	0-150
4	Danielsson <i>et al.</i> 2014	Automated gas-production system	Silage and concentrate	99.5	0-163
5	Abo el Nor <i>et al.</i> 2010	Continuous culture	Alfalfa hay and concentrate	99.5	0-108
6	Krueger <i>et al.</i> 2010	Gas-production system	Alfalfa hay	-	0-400
7	Avila <i>et al.</i> 2011	Gas-production system	Barley grain and barley silage	99.5	0-210
8	Lee <i>et al.</i> 2011	Gas-production system	Alfalfa hay	99	0-182
9	Lee <i>et al.</i> 2011	Gas-production system	Corn grain	99	0-188
10	Meale <i>et al.</i> 2013	Gas-production system	Total mixed ration (tmr)	99.2	0-120
11	AbuGhazaleh <i>et al.</i> 2010	Continuous culture	Total mixed ration (tmr)	99.5	0-108
12	Rémond <i>et al.</i> 1993	Fermenter	Wheat starch	-	0-13.3
13	Rémond <i>et al.</i> 1993	Fermenter	Microgranular cellulose	-	0-13.3

et al. (2010) and the two studies of Rémond et al. (1993) in which the cows were fed with Bermuda grass, maize silage or natural grassland hay, respectively. Glycerol used in the studies was crude glycerol with percentage of purity between 65% to 99.5% and in powder or liquid form. Glycerol was supplemented to substitute a main energy source such as corn (AbuGhazaleh et al., 2010; Abo el Nor et al., 2010; Ramos & Kerley, 2012; Rico et al., 2012), wheat (Meale et al., 2013), barley grain (Avila et al., 2011), or maize silage (Avila-Stagno et al., 2014) or as an additive in ration (Danielsson et al., 2014). There was also glycerol addition into single feed such as alfalfa hay (Krueger et al., 2010; Lee et al., 2011), corn grain (Lee et al., 2011), and wheat starch or micro-granular cellulose (Rémond et al., 1993).

Parameters tabulated in this study were *in vitro* digestibility, ruminal fermentation characteristics, as well as total gas and methane production. The *in vitro* digestibility parameters were dry matter (DM) digestibility, organic matter (OM) digestibility, and neutral detergent fiber (NDF) digestibility. The ruminal fermentation characteristics included were total VFA; molar percentage of acetic (C₂), propionic (C₃), butyric (C₄), isobutyric (isoC₄), valeric (C₅), and isovaleric (isoC₅) in total VFA; ratio of C₂ to C₃ by calculation; pH and ammonia concentration, total gas, methane, and microbial N production. Methane production values were taken from each paper and in case the parameter was not measured, it was calculated by using the stoichiometric formula outlined by Moss et al. (2000) since the formula has been proven to be accurate against direct measurement using a standard infrared methane analyzer (Jayanegara et al., 2015a).

Statistical Analysis

The analysis of data constructed in the database was conducted using a statistical meta-analysis approach (Sauvant et al., 2008; Jayanegara et al., 2014). The number of publication included in the database reflected the population of such *in vitro* study on glycerol supplementation from all period. Therefore, this present study assumed a fixed effect of various studies (Sauvant et al., 2008). The general linear model (GLM) procedure of SAS 9.1.3 was employed with the following model:

$$Y_{ij} = B_0 + B_1X_{ij} + s_i + e_{ij}$$

where Y_{ij}= dependent variable, B₀= overall intercept

across all experiments (fixed effect), B₁= linear regression coefficient of Y on X (fixed effect), X_{ij}= value of the continuous predictor variable (glycerol supplementation level), s_i= fixed effect of experiment i, and e_{ij}= the unexplained residual error. The variable of experiment was declared in the class statement as it did not contain any quantitative information. In addition, the regression equations are also presented with p-value and root mean square error (RMSE).

RESULTS

In Vitro Digestibility

The equations of linear regressions between glycerol supplementation levels and *in vitro* digestibility parameters are presented in Table 2. Increasing levels of glycerol supplementation did not significantly affect *in vitro* dry matter digestibility (IVDMD), *in vitro* organic matter digestibility (IVOMD), and *in vitro* neutral detergent fiber digestibility (IVNDFD).

Ruminal Fermentation Characteristics

The effect of glycerol supplementation level on ruminal fermentation characteristics are presented in Table 3. Total VFA production was not changed by the increasing level of glycerol. Molar proportion of acetate and iso-valerate declined linearly (P<0.05) with the increasing level of glycerol, while on the contrary, molar proportion of propionate, butyrate, and valerate increased (P<0.05) linearly. The ratio of acetate to propionate thus significantly decreased (P<0.05) as the level of glycerol increased. Ruminal pH and ammonia concentration with the glycerol inclusion in the diet decreased linearly (P<0.05) with small and high magnitude effects relatively to their intercept values, respectively.

Total Gas, Methane, and Microbial Protein Production

The linear regression analyses between glycerol supplementation levels and total gas, methane, and microbial protein production are presented in Table 4. Glycerol inclusion in the ruminant diets increased the *in vitro* total gas production (P<0.05) at simultaneously lowered enteric methane emission (P<0.05). Glycerol supplementation had no effect on microbial protein production.

Table 2. Equations for linear regression of *in vitro* digestibility on dietary glycerol level

Response variables	n	Variable estimates						
		Intercept	SE intercept	p intercept	Slope	SE slope	p slope	RMSE
IVDMD (%)	19	72.5	2.42	<0.001	0.0077	0.0160	0.637	3.84
IVOMD (%)	8	56.1	1.47	<0.001	-0.0251	0.0140	0.128	2.38
IVNDFD (%)	13	49.7	1.56	<0.001	0.0003	0.0070	0.965	2.55

Note: IVDMD= *in vitro* dry matter digestibility, IVOMD= *in vitro* organic matter digestibility, IVNDFD= *in vitro* neutral detergent fiber digestibility, n= number of treatment, SE= standard error, RMSE= residual mean square error.

Table 3. Equations for linear regression of ruminal fermentation characteristics on dietary glycerol level

Response variables	n	Variable estimates						RMSE
		Intercept	SE intercept	p intercept	Slope	SE slope	p slope	
Total VFA (mmol/L)	42	33.2	14.3	0.028	0.0559	0.0430	0.204	20.2
C2 (% total VFA)	42	64.9	4.31	<0.001	-0.0589	0.0130	<0.001	6.10
C3	42	25.1	1.63	<0.001	0.0404	0.0050	<0.001	2.31
C4	42	7.86	1.26	<0.001	0.0132	0.0040	0.002	1.78
iso-C4	23	0.69	0.086	<0.001	0.00004	0.0004	0.928	0.17
C5	30	2.57	0.365	<0.001	0.0037	0.0020	0.039	0.71
iso-C5	23	1.93	0.099	<0.001	-0.0012	0.0005	0.028	0.19
C2/C3	42	2.04	0.137	<0.001	-0.0047	0.0006	<0.001	0.27
pH	33	6.58	0.065	<0.001	-0.0008	0.0004	0.048	0.12
N-NH ₃ (mmol/L)	28	5.70	1,076	<0.001	-0.0201	0.0060	0.006	2.04

Note: VFA= volatile fatty acid, C2= acetate, C3= propionate, C4= butyrate, C5= valerate, N-NH₃= nitrogen ammonia, n= number of treatment, SE= standard error, RMSE= residual mean square error.

Table 4. Equations for linear regression of total gas, methane, and microbial protein production on dietary glycerol level

Response variables	n	Variable estimates						RMSE
		Intercept	SE intercept	p intercept	Slope	SE slope	p slope	
Total gas production (mL/g DM)	15	109	12.5	<0.001	0.2546	0.080	0.013	20.1
CH4 (mmol/L)	42	8.55	3.00	0.008	-0.0258	0.009	0.008	4.24
MPP (g/d)	8	37.9	0.094	<0.001	0.0002	0.0002	0.491	0.04

Note: CH4= methane, MPP= microbial protein production, n= number of treatment, SE= standard error, RMSE= residual mean square error.

DISCUSSION

Glycerol can be used to substitute or replace some parts of the energy source in ruminant diets without causing any detrimental effect on the digestibility. These results were in agreement with some previous studies in which glycerol as an energy substitution in diets had no effects on total tract digestibility of DM, OM (Khalili *et al.*, 1997), and NDF (Schröder & Südekum, 1999; Ramos & Kerley, 2012). Similarly, no difference in OM digestibility was observed when glycerol was added or supplemented to wheat starch substrate (Rémond *et al.*, 1993) and in NDF digestibility determined after 48 h incubation (Krueger *et al.*, 2010).

Glycerol fermentation in the rumen has a propiogenic property (Rémond *et al.*, 1993; Avila-Stagno *et al.*, 2014) and produces a high level of butyrate (Czerkawski & Breckenridge, 1972) at the expense of acetate. The increasing molar proportion of propionate and towards the decreased ratio of acetate to propionate responses were also in agreement with a number of previous publications on addition of glycerol into starch-rich ration (Rico *et al.*, 2012; Meale *et al.*, 2013), or forage substrates (Krueger *et al.*, 2010; Lee *et al.*, 2011), as well as *in vivo* in the beef cattle (Wang *et al.*, 2009) and dairy cows (Carvalho *et al.*, 2011). Ramos & Kerley (2012) concluded that glycerol was selectively used by rumen microbes for producing propionate rather than acetate. It was also supported by Bergner *et al.* (1995) who showed the most

of 14C labeled glycerol was found in propionate form. Propionate is one of the main glucogenic precursors thus increasing the glucose availability as an energy supply for animal. The increase of valerate with the increasing level of glycerol is apparently related to an enhancing proportion of propionate in the rumen since the former is synthesized from the latter (Hungate, 1966).

The mean of ruminal pH showed a normal rumen environment. The lower ruminal pH due to higher glycerol level was probably due to the tendency for a higher total VFA concentration (Wang *et al.*, 2009; Rico *et al.*, 2012) although it was not significant in our present study. However, it has to be noted that a declining ruminal pH depends on the proportion of glycerol-starch substitution and the types of basal diet. The decrease of ammonia concentration can be explained by a lower proteolysis and/or deamination occurred during ruminal fermentation. Lower deamination due to glycerol is supported by the decrease in iso-valerate as a product of branched-chain amino acids fermentation (Andries *et al.*, 1987).

The increase of gas production with glycerol inclusion in the diets was in agreement with the study of Krueger *et al.* (2010) that reported a linear increase in gas production when glycerol was added to alfalfa hay. In contrast, the increasing level of glycerol supplementation in the diet had no effect on gas production kinetics or cumulative gas production at 24 h (Meale *et al.*, 2013) and a reduction in gas production occurred when glyc-

erol is used to substitute alfalfa hay or corn grain (Lee *et al.*, 2011). It was possibly because the glycerol fermentation had a long lag time and a slow rate of kinetics of gas production (Ferraro *et al.*, 2009; Lee *et al.*, 2011). However, it is difficult to explain how the total gas production can be increased when no differences for *in vitro* digestibility and total VFA, lower enteric CH₄ and less value for ratio of acetate to propionate. Propionate production as the end product of fermentation deliver less gas than acetate (Blümmel *et al.*, 1997). In addition, we did not measure a quantitatively meta-analysis of kinetics of gas production.

Lower methane production by the increasing level of glycerol was similarly observed by Lee *et al.* (2011) who declared that glycerol reduced enteric CH₄ production from feeds while maintaining animal production. It is associated with the decline ratio of acetate to propionate. Glycerol is fermented towards more propionate in the rumen and serves as a hydrogen sink, a main substrate for methanogenesis (Jayanegara *et al.*, 2015b). Propiogenic substrates have been proposed as methane abatement strategy (Beauchemin *et al.*, 2008; Jayanegara *et al.*, 2014). Depression of methanogenesis may be related to a long lag time for glycerol fermentation (Lee *et al.*, 2011) so that rumen microbes especially methanogens need more time to get adapted. When they have settled and successfully adapted, there is a possibility that they would ferment glycerol and produce methane. So far there is no recommendation on supplementing glycerol *in vivo* for a longer time period or a whole life time of animal.

CONCLUSION

Glycerol is apparently appropriate to be used as an energy source in the ruminant diets. Ruminal fermentation of glycerol inclusion was identified to have no detrimental effects in the rumen and environmentally friendly as shown by the increase in molar proportion of propionate and the decrease in acetate without affecting the *in vitro* digestibility and microbial protein production, and concomitantly decrease methane production. However, further studies are needed to investigate the effects of glycerol supplementation for microbial adaptation and its usage in a long term *in vivo* study.

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