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**Review**

# Purified Crude Glycerol Fortification to the Diets of Dairy Cows with Conclusive Impacts: A Review

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## Abstract

*The literature analysis and octennial research carried out in our lab on glycerol, a by-product during biodiesel production, use in dairy cows' diet provides conclusive inferences on dry matter intake (DMI), nutrient digestibility, energy availability, rumen microbial protein (MBP) production, blood profile, blood metabolites and milk production and milk composition. An adaptation period of 1 week is necessary to adjust dairy cows to the glycerol fortification in the diet. The amount of glycerol fed is also important because of its high energy density. Many of the studies and our results showed that glycerol fortification to the diets either unaffected or marginally improved DMI or other nutritional parameters besides rumen MBP. The increased milk yield could be attributed to the significant increase in blood glucose (P< 0.01) to the maximum physiological limits. Milk fat percent or yield was also increased from the 2nd week of glycerol fortification to the diet of dairy cows in early lactation. Glycerol fortification in the diets of close-up cows in the last 4 weeks was found effective in the reduction of NEFA and BHBA in fresh cows because metabolically absorbed glycerol into the blood is channelized 70% to gluconeogenesis and 30% to triglyceride synthesis. Conclusively we recommend glycerol fortification to the diets of dairy cows in early lactation and close-up cows at the rate of 300 mL or 360 g/d for improved health and milk production.*

**Keywords:** By-Product, Energy, Glycogenesis, Lipogenesis, Fermentation, Ruminants, Lactation, Pregnancy

## Introduction

Glycerol or glycerine or 1,2,3–Propanetriol is regulated in the intermediary metabolism based on the substrate requirement and thus, channeled to glycogenesis or glycolysis or lipogenesis (Kholdenko *et al.*, 1995). Glycerol is also the backbone of the phospholipids which are important in cell membrane integrity (Gibellini and Smith, 2010). It is also a precursor in the synthesis of biotin and tryptophan. In ruminants, several bacteria convert glycerol during anaerobic fermentation to 1,3-propanediol by the enzyme dehydrogenase or dehydratase (Doi, 2019).

Glycerol is commercially available as a synthetic or biological source. Crude glycerol is available as a by-product during biodiesel production with a purity of 70 to 80% (Friedrich, 2004). The expansion of the biodiesel market has increased the availability of crude glycerol. The oils from non-edible oilseeds (NEOs) e.g., Pongamia, Simarouba, Jatropha, Neem, Mahua, and, waste oils from kitchens, automobiles, etc., could be used as feedstock for biodiesel production. Biodiesel is considered alternative renewable energy to natural diesel. National policy for biofuels (2018) targeted a 20% blending of biodiesel with fossil-based fuels by 2030. The by-products are recommended to utilization in livestock feeding to compensate for the feedstocks. In the past, glycerol has been used in the treatment of ketosis in dairy cows (Kupczyński *et al.*, 2020). Presently, glycerol is explored extensively in the diet of dairy cattle as an energy source because of its high calorific value (Silva *et al.*, 2014; Cleef van *et al.,* 2014). The advantage of feeding purified crude glycerol (PCG)to ruminants is detoxification of residual impurities like methanol, alkali catalysts, secondary plant metabolites, etc., effectively by the rumen microorganism in the rumen (Srinivas and Chaturvedi, 2019). Hence, the objective of the present review is to highlight the effect of exogenous glycerol as a dietary fortificant to dairy cows with debatable opinions of various research workers but, with a conclusive remark from the octennial research work carried at our lab.

## Glycerol as a By-Product during Biodiesel Production

The biodiesel or fatty acid methyl esters (FAME) are produced from the transesterification of oils or lipids using catalysts like NaOH, $H_2SO_4$, $Na_2CO_3$, etc. Among various catalysis used, NaOH is popular (Wilson and Clark, 2000). The edible and non-edible oils including used or waste cooking or automobile oils are preferred substrates for biodiesel production. Glycerol is available as a by-product during biodiesel production at the rate of 10 to 15% and 85 to 90% is biodiesel (Donkin, 2008). Crude glycerol is separated from biodiesel by either gravity or centrifugation. FDA (2007) recommended 99% purity glycerol as an animal feed ingredient however, it is not cost-effective in the diet of non-productive cows (Donkin and Doane, 2007) but, feeding in the terminal one month of the pregnancy has a beneficial effect on the postpartum performance. The major impediment from crude glycerol feeding to livestock is the presence of residues of catalysts used, salts, and methanol (Donkin, 2008) however, the extensive in vitro gas production kinetics carried over 3000 samples in our lab indicated that even 80 to 88% purified crude glycerol (PCG) can be used in the ruminant diets without any impediment on the rumen fermentation kinetics (KSCST and ICAR-NDRI, 2017). The recommended levels of methanol residues in the glycerol are below 0.5%. Residual methanol above 150 ppm is toxic and deemed unfit for use in livestock diets (FDA, 2007).

## Glycerol as an Ingredient in Cattle Feeds

The economic viability of glycerol addition in livestock feeding is dependent on the factors like raw material costs, fuel prices, government policy, cost of livestock feedstuffs, etc. The recent surge in the cost of oilseeds affected the profits in biodiesel production but, the simultaneous increase in the prices of biodiesel opens avenues for biodiesel processing. The PCG demand and supply, and prices highly fluctuate owing to availability, purity, purification cost, supply, demand from other sectors, etc., (Drackley, 2008).

The viscosity of glycerol is 500 centipoises at 30°C and specific gravity is 1.263 at 25 ºC. Glycerol is also an integral metabolite in the animal system produced in the breakdown of triglycerides into glycerol and fatty acid (FA). Glycerol is laxative, mildly antibacterial, and antiviral which often reduces inflammation after 2 hours of application. Glycerol is a hygroscopic compound that is miscible with water. The calorific value of a kilogram of glycerol ranges from 1.98 to 2.26 (Schroder and Sudekum, 1999), 1.91 Mcal net energy for lactation (DeFrain *et al.*, 2004). The calorific value of glycerol is higher than maize starch or jaggery or molasses (Donkin, 2008). The calorific value of glycerol, jaggery, and starch are 3.99, 3.57, and 3.25Mcal/kg, respectively.

Glycerol can replace maize starch to 55% of the ration DM (Schroder and Sudekum, 1999). A caution to substantiate

10% of the crude protein (CP) in the maize grain apart from minerals is required when glycerol is solely replaced with the maize grain (Drackley, 2008; Khattab *et al*., 2012). Glycerol contaminated with 27% methanol (Schroder and Sudekum, 1999) or as low as 1.3% (DeFrain *et al*., 2004) affected the nutritive value of the diet in pre-ruminant calves and monogastric animals but, metabolized to great extent by rumen microflora in mature ruminants (Drackley, 2008).

## Glycerol Metabolism in the Ruminants

The glycerol fed to cows has three routes of utilization: 1) Ferment in the rumen, 2) Absorbed across the rumen epithelium, and 3) Escapes the rumen to the omasal orifice. According to Phanthavong *et al*., (2017) 60% of the glycerol fed to the ruminants is absorbed from the rumen into the blood, 30% metabolizes in the rumen, and 10% reaches to the intestine with no or little chance are there to reach the colon (Werner Omazic *et al*., 2013). The 3 different routes available in the utilization of glycerol in ruminants make it too difficult to quantify different pools. An 85% of glycerol infused in the rumen has been noticed to absorb within 2 hours and is evident from increased plasma glycerol levels (Kijora*et al*.,1998). On contrary, Remond *et al.*, (1993) suggested the disappearance of glycerol at an optimum rate of 1.2 to 2.4 g/h and only 10% absorbed from the rumen from the infusion of large therapeutic doses of 925 g/d (Kristensen and Raun, 2007). The ruminal fermentation of glycerol is influenced by the concentration and rate at which glycerol disappearance in the rumen. Glycerol is metabolized to propionate and butyrate during ruminal fermentation (Silva *et al.*, 2014) by 4 h of postprandial (Linke *et al*.,2004). An increase in valeric acid and caproic acid production *in vitro* fermentation was also reported (Trabue *et al.*, 2007).

Since the glycerol is absorbed from the rumen by passive diffusion and no carriers are required (Werner Omazic *et al*., 2015), its absorption into blood is beneficial to the host animal. Glycerol enters the blood and is converted to glycerol-3-phosphate principally in the liver and kidney. Both organs have a high activity of glycerol kinase to conserve preferentially for gluconeogenesis followed by triglycerides in ruminants (Guo and Jensen, 1999). The glycerol entering directly into the blood has the advantage of converting to glucose by gluconeogenesis in the liver because the liver is not a significant site of triacylglycerol synthesis in bovines (Bionaz *et al*., 2020) and skeletal muscle and adipose tissue have low activity of glycerol kinase (Guo and Jensen, 1999). Glycerol from the systemic circulation is phosphorylated and Glycerol-3-Phosphate participates in the esterification of FAs however, this is dependent on the equilibrium between Dihydroxyacetone 3-Phosphate where glycerol 3-Phosphate is the precursor of carbon 4, 5, and 6 of glucose formed via gluconeogenesis. Thus, the systemic glycerol either diffusing from the rumen or released during lipolysis in adipose tissue or skeletal muscle during energy imbalance can be dominantly used in gluconeogenesis. When dihydroxyacetone 3-Phosphate is in equilibrium, there is scope to synthesize into triacylglycerol (Jensen *et al*., 2001).

The glycerol escaping to the omasum is converted to monoacylglycerol and its absorption across the intestine was reported negligible in sheep (Bionaz *et al*., 2020). The droplets of tri, di, and mono acyl glycerol are attached to the apical part of the enterocyte in monogastric animals but, lipid droplets storage in enterocytes in ruminants is rare owing to the constant flow of digesta besides low fat in their diet. Triacylglycerol synthesis in the jejunum of ruminants is remarkable when compared to the mammary gland and least in the liver (Bionaz *et al*., 2020).

## Glycerol and Rumen Microflora

Forages contain about 2% fatty acids in the form of galactosyl diglycerides and the most abundant are C18:3 (60 to 70%) and C18:2 (20%). Seed-derived feedstuffs used in concentrate supplements (CS) mostly contain triglycerides. Lipids in ruminant diets are recommended to be less than 5% of the total diet. Glycerol moiety is separated from FA in the rumen by microbial lipases thus; it is too treated like exogenous sources. The increased propionic and butyric acids on glycerol feeding to ruminants ought to reduce rumen pH and adversely affect rumen microbial protein (MBP) production (Roger *et al*., 1992; Kijora *et al*., 1998). Glycerol feeding appears to affect cellulolytic bacterial species viz., *Ruminococcus flavefasciens*, and *Fibrobacter succinogenes* in rumen causing reduced fiber digestibility. The glycerol supplementation decreases the intraluminal concentration of acetic acid but increases the propionic acid (Kijora*et al*., 1998). However, rumen microflora can quickly adapt to glycerol feeding (Porcu *et al*., 2018). Selective bacteria can utilize glycerol by their cellular lipases. Glycerol uptake by bacteria is facilitated by intrinsic glycerol facilitator protein. Glycerol is transported into microbial cells and then becomes phosphorylated and catabolized in Embden–Meyerhoff pathway (BlötzandStülke, 2017). The 2 important glycerol dissimilation pathways in bacteria are; 1. Dehydrogenation pathway in which glycerol is oxidized to dihydroxyacetone mediated by the glycerol dehydrogenase followed by phosphorylation by the dihydroxyacetone kinase enzyme, and 2. Phosphorylation pathway where glycerol kinase is phosphorylates the glycerol to glycerol-3-phosphate. Both the pathways culminate to yield the dihydroxyacetone phosphate and then metabolize itd (Doi, 2019).

## Glycerol Channels in Intermediary Metabolism

Glycerol is an intrinsic metabolite in the system (Bionaz *et al*., 2020). It is produced during the hydrolysis of dietary fat in the rumen (Fig. 1). The white adipocytes produce large amounts of glycerol and their ability to reuse glycerol is limited hence, it is the main source of gluconeogenesis in the liver and energy or triacylglycerol synthesis in other tissues (Rotondo *et al*., 2017). The acyl glycerol moiety during lipogenesis in the liver can naturally come from glucose, glycerol, or substrates involved in the citric acid cycle those used in gluconeogenesis. Glycerol when supplemented in the diet, glucose input for the new synthesis of glycerol backbone has been reported to be more than 50% while 35% input happened after glucose passes through the citric acid cycle (Jin *et al*., 2013). Alternatively, glycerol is the backbone to phosphatidic acid where saturated FA, unsaturated FA, and a phosphate group are attached to carbon; C1, C2, and C3, respectively where phosphatidylglycerol is an important cellular membrane constituent (Gibellini and Smith, 2010).

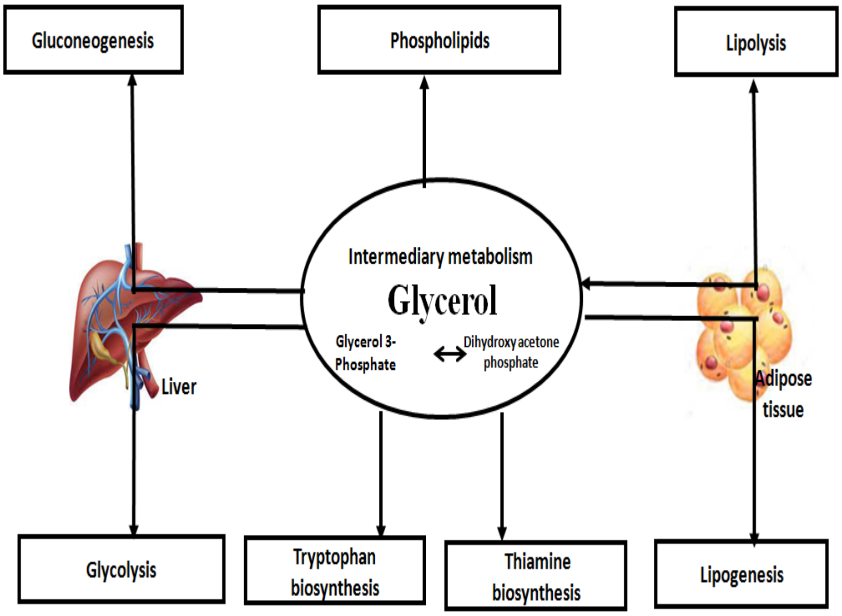

**Fig 1:** Glycerol channels in intermediary metabolism

## Glycerol in the Dairy Animal Feeding

### *Dry Matter Intake and Digestibility*

Glycerol has a dose-dependent effect on feed intake. Increased DMI was reported when diets for milking cows were supplemented with glycerol (Ogborn *et al*., 2006) even compared to corn (Bajramaj *et al*.,2017). If no increase in DMI, other workers reported unaffected DMI when lambs were supplemented with a diet containing crude glycerine of about 0, 10, 20, and 30% (Pellegrin 2012). Bensimon *et al*., (2011) also opined the inclusion of glycerol by 30% in the basal diet did not affect the DMI in lambs. Ezequiel *et al*., (2015) reported shallow DMI when the diet of prepartum cows top-dressed glycerol at the rate of 0, 430, or 860 g/d from 14 days before delivery, but the same diet fed in the postpartum period for 21 days had no impact on the DMI. The shallow DMI intake in prepartum cows can’t be attributed to the glycerol in the diet because the DMI of prepartum cows, in general, diminishes by 32% before 14 days and even 89% just 5 days before calving because of homeorhetic factors (Grummer *et al*., 2004). Probably, the level of glycerol in the diet may matters as its energy density is high. Many workers are fed different levels of glycerol in the diet as low as 15 to 300 g/kg DM (Syanhiar *et al*., 2021). Most studies dealing with glycerol supplementation in ruminant diets found that increasing the amount of glycerol resulted in a decrease in feed consumption (DeFrain *et al*., 2004, Paiva *et al*., 2016). Gunn *et al*., (2010) also reported declined feed intake when lambs were supplemented with a diet consisting of 30% and 45% glycerol. A decrease in DMI on glycerol feeding was not only observed in cattle but also in other ruminant species (Paiva *et al.* 2016, Saleem *et al*., 2018, Andrade *et al*., 2018) and pigs (Pellegrin 2012). Ezequiel (2015) reported a 15% decreased DMI in lactating cows when crude glycerol inclusion was more than 10 to 30% (Ezequiel *et al*., 2015).

Diminishing DMI on higher quantities of glycerol feeding could be due to increased hepatic fuel oxidation which sends signals to the hypothalamus to activate the satiety center and translates into reduced DMI (Forbes,1992; Allen *et al*., 2005). The increased dietary energy with the inclusion of glycerol in the diet up-regulates the Krebs cycle in the liver and yields more ATP (Allen *et al*., 2009). Hence, cows reach their satiety earlier because of energy regulated intake mechanism. Our studies indicated that supplementation of PCG did not affect the green fodder intake rather marginal increase was observed. We noticed improved ragi straw intake (P< 0.10) from the 6th week of its feeding. The choice of consumption of concentrate supplement remains the same even when glycerol was fed by mixing. The total diet intake indeed increased insignificantly when the basal diet was fortified with 300 ml/d or 360 g/d of glycerol (Fig. 2). The total DMI was increased with glycerol feeding from the 2nd week because glycerol is rapidly undergoing fermentation in the rumen, the animal needs few days of adaptation when fed the first time (Paiva *et al.* 2016).

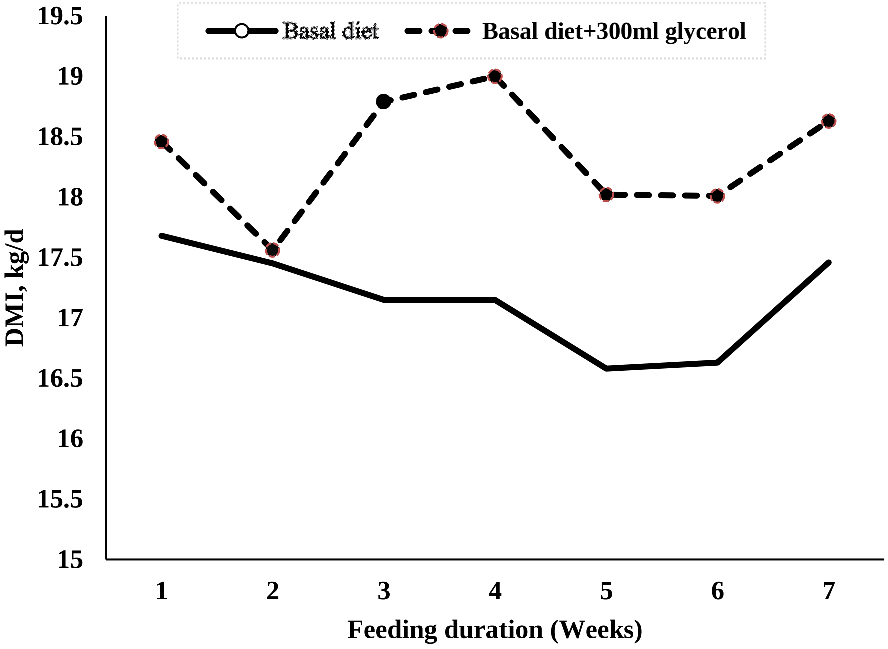

**Fig.2:** Total diet intake pattern in the dairy cows' diet fortified with glycerol

Increased OM, NDF, and CP digestibility were reported when bullocks were fed with a basal diet consisting of crude glycerol at the rate of 200 g/day (Wang *et al*., 2009a) but decreased NDF digestibility (Paiva *et al*., 2015). Syahniar *et al*. (2019) conclude from the meta-analysis that glycerol feeding did not affect nutrient digestibility but decreased NDF digestibility (P< 0.001) and reduced ADF digestibility (P< 0.10). Khalili (1997) even reported declined OM apart from the decreased fiber digestibility in dairy cows. The effect of glycerol feeding on the NDF digestibility is divisive (Donkin, 2009, Saleem *et al*., 2018). A basal diet supplemented with glycerol of about 100, 200, and 300 g/day improved the digestibility of CP, OM, and fiber linearly (Saleem,2017). Schroder and Sudekum (1999) reported no impact on the intake and digestibility of nutrients when milking cows fed with 20% purified crude glycerol in the diet. Our study showed that the digestibility of OM, CP, NDF, ADF, and cellulose was unaffected with the glycerol fortification to a diet of 300 mL/day or 360 g/day. The non-fibrous carbohydrates digestibility was increased significantly with glycerol feeding (P = 0.02) which is essential for the maintenance and yield ATP of the rumen microflora (Russel, 2007).

## Effect of Glycerol on Ruminal Fermentation

The major impediment to the inclusion of glycerol in dairy diets is the change in rumen pH when included at higher levels (Kijora *et al*., 1998). Van Cleef *et al*., (2018) suggested feeding crude glycerol up to 30% but cautioned the risk of increasing ruminal pH with increased glycerol in the diet. Glycerol fermentation in the rumen is faster than the starch from the wheat (Schröder and Südekum, 1999). Variation in the rumen pH due to feeding of glycerol is because of the quantity fed (Ariko *et al*., 2015) and we opine that the decrease in rumen pH is a rider in the quantity of glycerol feeding to the dairy cows. The inclusion of glycerol in the diet of ruminants affects the TVFAs production and pH of the rumen (Shin *et al*., 2009b, Khalili *et al*., 1997). The pattern of pH decrease (P< 0.10) was quadratic (Syahniar *et al*., 2020). Syahniar *et al.*, (2020) suggested an intercept of 6.53 and a slope of -1.67 thus the predicted pH after feeding 300 mL glycerol could be about 6.0. Hence, beyond 300 mL/d or 360 g/d of glycerol feeding reduces pH below 6.0 which is detrimental to the rumen microflora. Glycerol supplementation in higher quantities was observed to inhibit cellulose digestibility in many ways (Roger *et al.*, 1992). Those are;

- Abridged substrate accessibility by cellulolytic bacteria
- Affect the fungal adhesion on the fiber
- Reduces the permeability of microbial cell walls
- Inhibition of microbial cellulase enzyme production
- Obstruct cellulase enzyme action by either modifying the enzyme affinity to the substrate or blocking the enzyme's active site.

Drenching 1 L/d glycerol to HF dairy cows lowered acetate with increased propionate and butyrate concentration (Linke *et al*., 2014). Kristensen and Raun (2007) noted that increased butyrate and reduced acetate concentration when milking cows infused with glycerol however, as the level of glycerol in the diets increased, TVFAs and individual VFA in the rumen were decreased (Van clef *et al*., 2018). The meta-analysis from 47 studies indicated that the mean ratio of acetate, propionate, and butyrate (A: P: B) in glycerol-based diets was 60:25:15, respectively (Syahniar *et al*., 2019). The ratio of A: P: B was 50:30:20 with a greater increase in propionate and butyrate (DeFrain *et al*., 2004). The glycerol metabolizes by 30 to 69%to propionate during ruminal fermentation, thus again contributing to gluconeogenesis in lactating cows (Remond *et al*., 1993). Kristensen and Raun (2007) noted that increased butyrate and reduced acetate concentration when milking cows infused with glycerol and it could be the reason for increased fat content in milk.

The effect of glycerol on ruminal ammonia and microbial protein (MBP) synthesis are inconclusive but, many workers reported increased ruminal ammonia concentration (Shin *et al*., 2012; Boyd *et al*., 2013) and range from 1.68 to 16.9 mM (Syahniar *et al*., 2019) depending on the quantity of glycerol fed. The rumen microbial protein (MBP) production estimated in our lab using purine derivatives excretion in urine as markers were 347 g/d in prepartum/close-up cows and 327 g/d in fresh cows/early lactation in contrast to 342 g/d and 318 g/d, respectively on control diets. We can conclusively infer that glycerol feeding 300 mL/d had no impact on MBP production.

## Effect of Glycerol Supplementation on Nutrient Digestibility

According to Schroder (2008), glycerol improves nutrient digestibility by elevating the rumen environment in a similar way to maize. Wang *et al*., (2009a) observed increased OM, NDF, and CP digestibility when bullocks were fed with a basal diet consisting of crude glycerol about 0.2 kg/day but in the contrary Schroder and Sudekum (1999) reported no impact on intake and digestibility of nutrients when milking cows fed with 20% purified crude glycerol in the diet. The addition of crude glycerol in the diets of milking cows about 0.21 kg/day enhanced the digestibility of DM, CP, CF and decreased NDF (Paiva *et al*., 2015) while some experiments reported increased fiber digestibility (Paiva *et al*., 2016; Saleem *et al*., 2018). Similarly, Donkin (2009) observed higher DM, OM, and lower NDF digestibility when nursing cows fed with 15% glycerol. Contrary to that Khalili (1997) reported declined OM and fiber digestibility when dairy cows were supplemented with glycerol. Lower DMI was linked to higher energy output and fullness as a result of superior production of total volatile fatty acids (TVFAs) and their increased flow to the liver (Trabue *et al*., 2007).

Increased OM and CF digestibility with an increasing amount of crude glycerine about 0, 2.5 or 5% in the diets has been observed in the pigs (Madrid *et al*., 2013). Glycerol treatment at 100, 200, and 300 g/d improved the DM and fiber digestibility reflecting higher ruminal microbial activity (Wang *et al*., 2009a). Andrade *et al*., (2018) noticed higher digestible DM, CP, and fiber when finishing lambs were supplemented with 10.9% glycerol. Saleem (2017) supplemented the basal diet with 100, 200, and 300 g/day glycerol and noticed greater digestibility of CP, OM, and fiber with higher addition of glycerol. Syahniar *et al*., (2019) conclude from the meta-analysis that glycerol feeding did not affect nutrient digestibility but decreased NDF digestibility (P< 0.001) and reduced ADF digestibility (P<

0.10). Our experiments on dairy cows also indicated no adverse effect on tutrient digestibility.

## Effect of Glycerol Supplementation on Serum Biochemical Parameters

Glycerol fortification to the diet had no adverse effect on the RBC parameters, erythrocyte indices, WBC parameters, and platelet parameters. Supplementation of glycerol increases blood glucose and reduces non-esterified fatty acids (NEFA) in serum (Osman *et al*., 2006). Drenching milking cows with 500 mL/d glycerol for a fortnight after calving improves energy status and reduces NEFA in the systemic circulation (Osman *et al*., 2006) hence, glycerol has therapeutic properties in treating ketosis in dairy animals (Kupczyński *et al*., 2020, Goff and Horst (2001). Glycerol is as effective as polyethylene glycol (Lokesha *et al*., 2018). Using gluconeogenic substances is recommended in the treatment of ketosis hence, glycerol is the best option (Goff and Horst, 2001, Kupczyński *et al*., 2020). Glycerol metabolism to propionate production in the rumen and glycerol absorbed across the rumen wall both are used in glucose production in ruminant's liver (Kupczyński*et al*.,2020) . The physiological limits of blood glucose in ruminants are 45 to 75 mg/dL. We observed 75 mg/dL glycerol in the HF cows' diet fortified with 300 mL/d glucose in contrast to 58 mg/dL on a basal diet without glycerol. Glucose levels in blood appear to be increased between 30 to 60 min after glycerol intake. Ferraro *et al*., (2016), confirmed that direct entry of glycerol into the blood is a dominant route (Phanthavong *et al*., 2017). Even feeding glycerol included in ORS to calves was reported to increase blood glucose and insulin levels (Omazic *et al*., 2013b). The summation from different studies on glycerol supplementation indicated that the blood glucose ranges from 2.82 to 5.45 mM, triglycerides from 60 to 180 µM, NEFA from 68 to 639 µM, and blood urea nitrogen (BUN) from 11 to 51 mg/dL depending on the level of glycerol in the diet (Syahniar *et al*., 2020). Our studies also indicated increased glucose ($P< 0.01$) and the alanine transaminase (ALT) enzyme ($P = 0.06$) in the blood. Goff and Horst (2001) observed 16%, 20%, and 25 % increased blood sugar levels in transition dairy cows treated with 1, 2, or 3 L/d glycerol, respectively. Few studies reported no influence of glycerol feeding on plasma glucose, NEFA, BHBA, or triglycerides (Ogborn *et al*., 2004, Kass *et al*.,2013, Omazic *et al*., 2013a). We observed an increase of 35% blood glucose when HF cows were fed 300 mL/d glycerol compared to control but serum creatinine, BUN, Triglycerides, and AST were unaltered.

The feeding of a glucogenic mixture containing glycerol, polypropylene glycol, and water in the ratio of 70:20:10 increased plasma osmolality and blood volume. Since glycerol is a highly diffusible molecule that can permeate the RBC membrane against concentration gradient thus, above 200 mg/dL circulating glycerol may cause RBC osmotic stress (Pasciu *et al*., 2021). Glycerol supplementation in the diet of lactating buffaloes influenced the total serum protein such as globulin and albumin or glucose accumulation as well as reduced β-hydroxybutyric acid (BHBA) and NEFA levels (Saleem *et al*., 2018). Glycerol supplementation in the diet also increased blood glucose ($P< 0.001$), and insulin ($P< 0.01$) with a simultaneous decrease in plasma NEFA and urea (Porcu *et al*., 2018).

## Effect of Glycerol Supplementation on Body Weight

The effect of glycerol feeding on the body weight and (BW) and body condition score (BCS) has been reported as either no effect (Defrain *et al*., 2004, Kass *et al*., 2013, Porcu *et al*., 2018) or positive change (Donkin *et al*., 2007). Similarly, Wang *et al*., (2009b) also mentioned that the BW of dairy cows was increased when glycerol was administered at 100, 200, and 300 g/d per cow. The researchers who reported increased BW on glycerol feeding are attributed to increased feed intake. Trabue *et al*., (2007) suggested that the advantage due to glycerol feeding to the ruminant animal is greater energy availability for maintenance and production in addition to changes in the ratio of VFA. Increased feed input, changes in the VFA composition and production in the rumen, and energy availability on glycerol feeding are primary elements to ameliorate BW and BCS (Kholif, 2019). Zijlstra (2009) observed that the inclusion of crude glycerol about 8% in the diet of pigs increased feed intake and BW gain. On contrary, Lammers (2007) reported unaffected DMI and growth in pigs with the addition of crude glycerol in the diet by 0, 5, and 10%. Glycerol feeding to buffaloes exhibited a negligible impact on the BW and BCS (Saleem *et al*., 2018). Our laboratory studies showed that the scope of improved BW is possible only when the concomitant availability of dietary protein is ascertained. Glycerol supplementation provides glycogenic energy in growing ruminants therefore BW change or BCS changes remain unchanged or at the most marginally positive.

## Milk Production and Composition

The lactation performance of cows enhances when the dietary energy density is improved because of the effect on blood insulin and glucose (Lomander *et al*., 2012; Bajramaj *et al*., 2017). Kass *et al*., (2013) concluded that cows given crude glycerol orally produced more milk (82.6%). The glycerol had an equal impact to polypropylene glycol on milk yield when both supplemented at 450 and 300 g/d, respectively in the early lactation, however; blood insulin was comparatively lesser on glycerol-fed cows (Lomander *et al*., 2012). The feeding of glycerol to lactating buffaloes at the rate of 300 mL/d has been reported to increase total milk yield or 3.5% fat-corrected milk yield but milk composition is unaltered compared to 150 mL/d glycerol feeding (Saleem *et al*., 2018). In goats, feeding 5% glycerol to goats increased milk yield from 2.38±0.12 to 2.64±0.23 kg/d (Thoh *et al*., 2017). The glycerol is a preferentially glycogenic compound in ruminants, and has a positive impact on the milk yield but not on the milk fat (Porcu *et al*., 2018).

Feeding dairy cows with 1.1% of sodium butyrate on a DM basis has been reported to increase milk fat from 4.37% to 4.58% and milk fat yield was increased from 1.42 kg/d to 1.51 kg/d (Izumi *et al*., 2019). The increased butyrate during rumen fermentation may enhance the milk fat%. Kupczyński *et al*., (2020) also reported that glycerol feeding increased the odd-chain fatty acids and conjugated linoleic acid in milk. The low or high level of supplementation of glycerol appears to influence the lactating cows beside the stage of lactation. For example, the supplementation of glycerol at the rate of 5% of the diet DM had a positive impact but 10% supplementation reduced milk fat (Thoh *et al*., 2017). Total milk fat contains 96% triacylglycerol and 3% of diacylglycerol plus monoacylglycerol. Cholesterol and its esters constitute 0.5%, and phospholipids 1% (Jensen, 1999). A few research workers also opined that increased availability of glycerol for gluconeogenesis in the liver spares propionate which is therefore available in enhanced quantities at the mammary gland as a precursor for short-chain FA (Maxin *et al*., 2011).

**Table 1:** Nutritional impact of glycerol feeding to dairy cows

| **Parameter** | **Basal diet** | | **S.E.M** | **P value** |
|---|---|---|---|---|
| | **No glycerol** | **300ml/d glycerol** | | |
| Total DMI (kg/d) | 10.06 | 10.51 | 0.06 | 0.02 |
| **Digestibility coefficient (%)** | | | | |
| Dry matter | 57.77 | 63.17 | 2.38 | 0.12 |
| Organic matter | 45.39 | 52.34 | 3.08 | 0.12 |
| Crude protein | 41.69 | 46.99 | 2.61 | 0.10 |
| Neutral detergent fiber | 39.36 | 46.07 | 3.36 | 0.17 |
| Acid detergent fiber | 26.68 | 34.26 | 4.17 | 0.19 |
| **Energy partition (Mcal)** | | | | |
| Gross energy | 38.05 | 38.39 | 0.24 | 0.48 |
| Digestible energy | 19.27 | 21.79 | 1.25 | 0.16 |
| Metabolizable energy | 10.30 | 12.28 | 1.49 | 0.26 |
| Heat increment | 4.49 | 5.48 | 1.09 | 0.39 |
| Energy retained | 5.81 | 6.80 | 0.16 | 0.05 |
| **Rumen microbial protein production** | | | | |
| PD Excretion (mM/d) | 437.12 | 449.96 | 63.83 | 0.88 |
| Duodenal flow of MBP (g/d) | 317.81 | 327.14 | 46.40 | 0.88 |
| Efficiency of MBP (g/kg) | 47.30 | 45.17 | 8.69 | 0.85 |
| **Blood metabolites** | | | | |
| Blood glucose (mg/dL) | 52.70 | 32.13 | 1.40 | 0.01 |
| Triglycerides (mg/dL) | 10.15 | 9.85 | 1.42 | 0.79 |
| Blood urea nitrogen (mg/dL) | 19.30 | 23.75 | 3.14 | 0.24 |
| AST: ALT* | 2.25 | 2.56 | 0.13 | 0.35 |
| **Milk yield and composition** | | | | |
| 4% FCMY (kg/d) | 10.46 | 12.39 | 0.22 | 0.004 |
| Milk total solids % | 11.84 | 12.25 | 0.06 | 0.01 |
| Milk Fat % | 3.42 | 3.98 | 0.01 | 0.001 |

*AST= Aspartate transaminase; ALT=Alanine transaminase*

Omazic *et al*., (2013a) reported that feeding glycerol of more than 99% purity to dairy cows for 4 weeks was accelerated milk production but crude glycerol with a purity of 88.1% had no effect. Replacing maize grain at 5% increments up to 15% of the total diet hardly showed any relationship in milk yield, milk fat, milk protein, or milk urea-N content (Donkin *et al*., 2007). Syahniar *et al*., (2020) summarized from the 52 studies that glycerol supplementation did not reduce milk production in dairy cows. The transitional cows fed with dry glycerine of 0.25 kg/day had no significant effect on nutrient intake and milk production (Chung *et al*., 2007).

Any heterogeneity in findings could be driven by glycerol purity, supplementation duration, stage of lactation, and the plane of nutrition (Porcu *et al*., 2018). Glycerol supplementation to cows in mid-lactation at the rate of 3.6% of the diet had no significant impact on milk production and composition (Khalili *et al*.,1997). The dominant route of glycerol metabolisms such as rumen microflora or liver or small intestine also matters for the difference reported in glycerol impact on milk production in various studies (Paiva *et al*., 2016). The changes in the milk yield and milk fat on glycerol feeding are proportional to the TVFAs production and their ratio (Ariko *et al*., 2015).

Paiva *et al*., (2016) reported 21% lesser milk yield in dairy cows fed crude glycerol over extended periods. In goats, feeding crude glycerol at the rate of 5 and 10% of the diet DM reduced any variation in the day-to-day milk yield (Thoh *et al*., 2017). Bajramaj *et al*., (2017) reported a decrease in milk fat due to glycerol supplementation because of a decrease in the molar proportion of acetic acid. The glycerol being hygroscopic may reduce available water for the dilution of solid milk components synthesized *de novo* in the mammary gland (Porcu *et al*., 2018). Gaillard *et al*., (2018) reported when glycerol was fed to lactating cows during mid and late lactation at the rate of 6% (x) besides incremental increases of 2x and 3x linearly decreased saturated (mainly caproic and palmitic), -mono, and -polyunsaturated (Palmitoelic and Myristoleic) FAs. The increased energy yield by glycerol supplementation appears to affect milk fat more than milk yield because of the production of conjugated $C18:2^{\Delta9,12}$ in the rumen that has an antagonistic effect on the expression of lipogenesis gene in the *de novo* synthesis of milk fat in the mammary gland (Kupczyński *et al*., 2020).

Our study indicated that fortification of PCG improved milk yield significantly ($P<0.01$) from the 3rd week onwards. Significant ($P<0.01$) Increase in 4% FCM yield was observed from the 3rd week. The total solids % was significant in the 3rd ($P<0.05$), 4th($P<0.05$), 5th ($P<0.10$), and 6th ($P<0.10$) week. Milk fat% increased from 1st week significantly throughout the trial period. The milk fat yield was significant from the 2nd week. Milk SNF % was non-significant but, the yield increased from the 3rd week onwards. The efficiency of 4% FCM yield production was significantly higher in the 5th ($P<0.05$) and 6th ($P<0.01$) week, respectively. The efficiency of total solids yield was significant in the 6th week ($P<0.05$). The efficiency of milk fat yield was also increased from the 4th ($P<0.10$), 5th ($P<0.01$), and 6th ($P<0.01$) week. The efficiency of milk SNF only increased in the 6th week ($P<0.10$).

## Conclusion

The literature analysis and octennial research carried out at our lab conclusively infer glycerol feeding unaffected the DMI, water intake, digestibility of macronutrients, and cell wall components. Energy balance is also unaltered because the dominant route of its utilization in lactating cows is absorption into the blood and its utilization in gluconeogenesis as evident from the increased blood glucose. Glycerol fortification to the di*et* also unaffected the rumen MBP rather marginal increase was observed. Blood RBC, WBC, platelet parameters, and erythrocyte indices were seldom affected rather marginal improvements were noticed. Serum triglycerides, BUN, total protein, albumin, and AST were also within the physiological thresholds. Blood glucose increase by 35% to maximum physiological limits on 300 mL/d glycerol feeding is the oblivious impact that translated into increased milk yield by 33% in early lactation. The milk fat% is also improving with the glycerol feeding because of increased butyric acid in rumen besides its use in 70:30 ratios for gluconeogenesis and lipogenesis. Few meta-analyses suggested its level is important hence, we recommend 300 mL/d glycerol feeding to dairy cows in early lactation and terminal pregnancy. The benefits accrue from glycerol feeding to dairy cows compensate for its higher cost with increased milk yield, milk fat, and health of the cows.

## Contribution by authors

All the authors contributed equally to writing the manuscript. The final manuscript was read by all others and consented to publication.

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## Conflict of Interests

There is no conflict of interest.

## Publisher Disclaimer

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