Glycerol Supplementation in Dairy Cows and Calves

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Cover: Cow and calf grazing by a rapeseed field.
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Abstract

The production of biodiesel from rapeseed oil methyl ester leaves glycerol (synonym: glycerine, 1,2,3-propanetriol) as a valuable by-product and a promising feed supplement for farm animals. This thesis provides information about the supplemental feeding with glycerol to dairy cows in early lactation and to young calves, and describes the fate of glycerol entering the rumen and the impact of glycerol on enteric methane production and gastrointestinal microbial populations. This was achieved by investigating the effects of two glycerol products of different purity - crude glycerol (88.1%) and glycerol (>99%) - on milk production, feed intake and metabolic parameters in 42 dairy cows in early lactation. Furthermore, different routes of the disappearance of glycerol from the rumen were quantified. These were *i*) fermentation in the rumen, *ii*) absorption across the rumen epithelium and *iii*) rumen outflow through the omasal orifice. The effect of glycerol on enteric methane production, rumen volatile fatty acid profiles and microbial population in rumen fluid was investigated in a gas *in vitro* system. Finally, the effects of oral rehydration solutions (ORS) with added glycerol on metabolic parameters and intestinal microbiota were examined in young calves.

Glycerol supplementation, irrespective of purity, did not significantly affect milk yield or composition, the total intake of dry matter or metabolic parameters in dairy cows in early lactation. It was estimated that approximately 70% of the glycerol was absorbed mainly from the rumen, but probably also from the small intestine. A smaller fraction disappeared from the rumen compartment by microbial digestion. These findings indicate that glycerol is an available gluconeogenic substrate which might efficiently contribute to glucose synthesis in the liver. Addition of glycerol in the gas *in vitro* system indicated no reduction in methane production. Furthermore, bacterial and archaeal community structures subjected to additional glycerol followed a similar pattern as the *in vitro* control with no feed additive. Glycerol was also shown to be a suitable component in ORS for young calves since it ameliorated the effects of dehydration on feed and fluid deprived calves. In calves, glycerol was rapidly absorbed, presumably in the small intestine, and thus most likely not available to the intestinal microbiota.

Keywords: glycerol, cattle, feed intake, milk production, NEFA, BHBA, oral rehydration solution, microbial population, enteric methane production

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Dedication

To my grandfather, a man of great knowledge whit a love of research

Just because it's black in the dark doesn't mean there's no color.
Laleh

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Werner Omazic, A., Tråvén, M., Bertilsson, J. Holtenius, K. (2013). High and low purity glycerine supplementation to dairy cows in early lactation – effects on silage intake, milk production and metabolism. *Animal* 7(9), 1479-1485.

II Werner Omazic, A., Kronqvist, C., Lu, C., Martens, H. and Holtenius, K. (2013). The fate of glycerol entering the rumen of dairy cows and sheep (Submitted).

III Danielsson, R., Werner Omazic, A., Ramin, M., Dicksved, J., Bertilsson, J., Griinari, M. and Schnürer, A. Effects on methane production and bacterial and archaeal communities by the addition of cashew nut shell liquid or glycerol – an in vitro evaluation (Manuscript).

IV Werner Omazic, A., Tråvén, M., Roos, S., Mellgren, E. and Holtenius, K. (2013). Oral rehydration solution with glycerol to dairy calves: Effects on fluid balance, metabolism and intestinal microbiota. *Acta Agriculturae Scandinavica, Section A – Animal Science* 63(1), 47-56.

Abbreviations

BCS	body condition score
BHBA	β-hydroxybutyrate
BW	body weight
CNSL	cashew nut shell liquid
DM	dry matter
DMI	dry matter intake
ECM	energy corrected milk
FRA	fractional rate of absorption
ME	metabolisable energy
NDF	neutral detergent fibre
NEB	negative energy balance
NEFA	non-esterified fatty acids
ORS	oral rehydration solution
TPP	total plasma protein
VFA	volatile fatty acids

1 Introduction

There is increased interest in biofuels made from renewable resources. Europe currently produces and consumes 80% of global biodiesel (Biodiesel 2020, 2008). The biodiesel industry generates large quantities of the by-product glycerol (synonym: glycerine, 1,2,3-propanetriol), and the annual production of biodiesel in Europe has been estimated to be 11.2 million tonnes in 2010 (Khanna *et al.*, 2012). In a review by Friedrich (2004), the approximate proportions of the chemical reaction involved in the production of biodiesel are as follows: 100 l of oil + 10 l of methanol yield 100 l of biodiesel + 10 l of glycerol. Glycerol is generated from the trans-esterification of natural oils and fats, with rapeseed oil mainly being used in European countries (Körbitz, 1999). The expansion of the biodiesel industry has provided prospects for the use of glycerol as a feed additive for livestock. Feeding crude glycerol to farm animals is currently not common in Sweden. However, different supplemental feeds containing glycerol with a purity of >99 % are available on the Swedish feed market. Furthermore, glycerol is used in the food, pharmaceutical and cosmetics industries. At present, the price of glycerol (>99%) in Sweden is around 0.6 €/kg.

As far back as 1954, glycerol was used as a treatment for metabolic disorders around parturition, *e.g.* ketosis (Johnson, 1954). Therapeutic effects were further investigated in the 1970s by Fischer *et al*. (1973). A positive effect on total energy intake was also reported when glycerol was added to concentrate (Fisher *et al.*, 1971). In early lactation most dairy cows experience a shortage of glucose and negative energy balance (NEB) due to copious milk secretion which is not matched by nutrient intake. In order to increase the supply of glucose and improve the metabolic status, by acting as a substrate for gluconeogenesis, glycerol can be used as a feed additive in cows' diets (Ingvartsen, 2006; Seal & Reynolds, 1993; Leng, 1970). In recent years, several studies have focused on the use of glycerol as a supplement to ameliorate NEB during calving and to increase milk production, but results

have been inconsistent (Wang *et al.*, 2009b; Osman *et al.*, 2008; DeFrain *et al.*, 2004). However, Chung *et al*. (2007) conclude that glycerol may be beneficial by improving energy status, although the authors point out that it is not always matched by increased production.

Increasing atmospheric concentrations of methane are an issue since this is one of the potent greenhouse gases to which cattle contribute over 40% of anthropogenic methane emissions (IPCC, 2007). The production of methane from ruminants also causes energy losses for the animal, corresponding to approximately 2 to 12% of gross energy intake (Johnson & Johnson, 1995). Enteric methane is produced in the rumen by methanogens (archaea). During the degradation of carbohydrates to volatile fatty acids (VFA), hydrogen is released by different fermentative microorganisms. Archaea use hydrogen as an energy source and hydrogen reacts with carbon dioxide to form methane. Methane production can be influenced by different factors. One such factor is the rate of the digestion and the production of different VFA, where increasing the proportion of propionate relative to acetate has been shown to reduce methane production (Beauchemin *et al.*, 2008; Johnson & Johnson, 1995; Blaxter & Clapperton, 1965). Previous studies *in vivo* and *in vitro* have shown the impact of glycerol on VFA profiles, with higher proportions of propionate relative to acetate (Avila *et al.*, 2011; Carvalho *et al.*, 2011; Wang *et al.*, 2009a; DeFrain *et al.*, 2004). The inverse relationship between propionate production and enteric methane formation indicates that glycerol might have the potential to reduce methane production, as shown *in vitro* by Lee *et al*. (2011).

Information relating to glycerol as a feed additive for young calves is limited. Ebert *et al*. (2008) have shown that at least 38% of the total lactose in milk replacers can be replaced by glycerol without any adverse effects on calf performance, findings confirmed by Raeth-Knight *et al*. (2009). Oral rehydration solution (ORS) is often used to maintain the fluid and electrolyte balance in calves with diarrhoea (Constable *et al.*, 2001). However, there appears to be a lack of studies addressing the possible effects of ORS containing glycerol on calves.

Lactobacillus and members of the *Enterobacteriaceae* family - *Citrobacter*, *Klebsiella*, *Clostridium* and *Enterobacter* - are the main bacteria able to utilise glycerol in their metabolism, as recently reviewed by Khanna *et al*. (2012). *Lactobacillus reuteri* resides in the gastrointestinal tract of mammals and birds, and has the ability to convert glycerol into an antimicrobial substance termed reuterin (Axelsson *et al.*, 1989). Reuterin has the capability to inhibit the growth of pathogenic bacteria including enterotoxigenic *Escherichia coli* and *Salmonella*, as shown in vitro (Spinler *et*

al., 2008; Axelsson *et al.*, 1989). Furthermore, *Lactobacillus* is one of the dominant bacterial groups found in the intestinal microbiota and faeces of calves (Karney *et al.*, 1986) and *Lactobacillus reuteri* strains have been recovered directly from the calf intestine (Busconi *et al.*, 2008). However, there is so far no information as to whether glycerol increases the production of reuterin in calves.

This thesis focuses on supplementation of glycerol in order to evaluate whether it is a favourable nutrient source in diets for dairy cows in early lactation and in ORS for young calves. The fate of glycerol entering the rumen is addressed. In addition, the impact of glycerol supplementation on enteric methane production and microbial populations in the rumen is investigated to evaluate further the perspectives of glycerol as a feed additive for dairy cows.

2 Aims of the thesis

The overall aim of this thesis was to provide more information about supplementation of glycerol in diets for dairy cows in early lactation and in ORS for young calves. The specific aims of the studies described in **Papers I-IV** were to:

- Evaluate the effect of supplemental feeding with crude glycerol (purity 88.1%) and glycerol (purity >99%) on milk production, silage intake and parameters reflecting the metabolic status in early lactating cows.

- Elucidate the fate of glycerol entering the rumen.

- Investigate the effects of glycerol on enteric methane production along with changes in bacterial and archaeal community structures in rumen fluid.

- Assess the effects of ORS containing glycerol on plasma constituents in calves, under feed and fluid deprived conditions *vs*. normal state.

- Examine whether ORS containing a glycerol/glucose mix influences the faecal numbers of *Lactobacillus* and *Enterobacteriaceae* and the production of reuterin in calves.

3 Experimental design overview

All animal experimental procedures have been approved by the Ethical Committees in Uppsala (**Papers I, II and IV**) and Umeå (**Paper III**). The *ex vivo* experiment with sheep tissue in **Paper II** was performed in accordance with German law regarding the care and use of experimental animals. All cows and calves were of the Swedish Red breed (**Papers I – IV**). In addition, rumen epithelial tissues were prepared from the rumen tissues of sheep immediately following slaughter. The sheep were of the German Dairy breed. In all *in vivo* and *in vitro* studies in **Papers II – IV**, glycerol with a purity of 99.5% was used. In **Paper I**, cows received glycerol (purity 99.5%) or crude glycerol (purity 88.1%). Subsequently, glycerol refers to glycerol with a purity of >99%. Different purities of crude glycerol are given in brackets. A brief overview of the experimental designs used in this thesis is provided below. More detailed descriptions of the materials and methods used in the different studies are provided in **Papers I-IV**.

Paper I – The effects were evaluated of supplementation with glycerol of different purities on milk production, silage intake and metabolic parameters of dairy cows in early lactation. Forty-two cows entered the experiment two days after parturition and continued for four weeks. The cows were randomised into three groups and assigned to one of three dietary treatments: supplementation with 0.5 kg crude glycerol/day (7.3 MJ metabolisable energy (ME)/day), supplementation with 0.5 kg glycerol (7.4 MJ ME/day) and the control diet without additional energy.

Paper II – The fate of glycerol entering the rumen was elucidated by assessing the overall disappearance rate of glycerol, the rate of ruminal glycerol absorption and the rate of the rumen microbial degradation of glycerol. The study was divided into four experiments:

1. An *ex vivo* experiment where rumen epithelia from sheep were used to investigate glycerol transport properties by means of the Ussing chamber technique.

2. An *in vivo* experiment where rumen-fistulated cows were given a bolus dose of 500 g glycerol and a fluid marker (CoLi-EDTA) dissolved in 100 ml of water in order to estimate the overall glycerol disappearance rate. The fractional outflow rate of the fluid marker CoLi-EDTA and disappearance were described by first-order kinetics, assuming neglected net fluid transport across the rumen epithelium and uniform distribution of CoLi-EDTA in rumen fluid.

3. Microbial degradation of glycerol was studied *in vitro*. Samples of rumen digesta from two rumen-fistulated cows were collected and studied in an *in vitro* system consisting of two polyethylene drainage pipes kept in a box with a warming system. Six l buffer and 140 g glycerol dissolved in 280 ml water were added to each tube. No absorption or outflow could occur from the tubes and consequently the fractional rate of disappearance was assumed to reflect microbial degradation.

4. An *in vivo* experiment where the rumen of rumen-fistulated cows was completely emptied in order to study the rate of glycerol absorption from the rumen compartment and the rumen fluid was replaced by buffer solution with different glycerol concentrations (15, 30 and 45 mmol/l) and then the disappearance of glycerol was estimated.

Paper III – The effect of different feed additives – glycerol and cashew nut shell liquid (CNSL) – and their impact on methane production and bacterial and archaeal community structures in the rumen were evaluated. In addition, the microbial community structures *in vitro* versus *in vivo* were compared in order to evaluate whether the *in vitro* system reflects the situation *in vivo*. Information and results regarding cashew nut shell liquid is only presented in **Paper III**. Rumen fluid collected from three rumen-fistulated lactating cows was used in a gas *in vitro* system developed by Ramin and Huhtanen (2012). In the *in vitro* system, 1,000 mg of substrate, a mixture of 600 mg silage and 400 mg concentrate was used. The substrate was equal to the diet given to the cows used as rumen donors. The substrate was added to the fermentation unit with two levels of glycerol concentrations: 15 mmol/l and 30 mmol/l and a control

with no feed additive. Samples were incubated for 48 h in two consecutive runs. The levels of glycerol used were approximately 8% and 16% respectively of the dry matter (DM).

Paper IV – The effects of different ORS containing glycerol on calves' plasma constituents and intestinal microbiota were investigated. The study was divided into two experiments:

1. Short-term experiment – Five calves were subjected to two ORS treatments, glycerol and glucose in a change-over design, where each treatment period lasted for one day followed by a seven-day wash-out period.

2. Long-term experiment – Nineteen calves were randomly assigned to one of three treatments: ORS containing glycerol/glucose mix, ORS containing glucose and the control (receiving no ORS). After 10 days of adaptation, calves were deprived of fluid and feed for 24 h.

3.1 Additional experiments

3.1.1 *In sacco* degradability and microbial digestion

An *in vivo* study was performed with the aim of examining whether the rumen micro-organisms adapted to glycerol during a 12-day adaptation period. Four rumen-fistulated lactating cows were included in the study, and 250 g crude glycerol (88.1 %) or glycerol (>99 %) dissolved in 200 ml water was added through the rumen fistula twice daily. Each experimental period lasted for two weeks and one of the three periods was a control period. Samples of rumen fluid from cows fed glycerol of different purities were collected at different time points on day twelve: 0 (before glycerol was added), and 2, 4, 8 and 12 h after glycerol load in order to examine the VFA profiles in rumen fluid. The impact of the two purities of glycerol on the *in sacco* degradability of DM, neutral detergent fibre (NDF) and crude protein fractions in the silage after 2, 4, 8, 16, 24 and 48 h was investigated.

At the end of the experimental period, rumen fluid from each cow was collected and then strained separately through a double layer of cheesecloth into pre-warmed thermos flasks. An *in vitro* study, described in detail in Sveinbjornsson *et al*. (2007), was performed. In brief, propylene centrifuge tubes (50 ml) with 14 ml of 39ºC *in vitro* medium were added to each tube. Tubes were gassed with CO_2 and closed using rubber stoppers with gas outlets, and then placed in a 39ºC water bath. Reducing solution (1 ml) and 7 ml

strained rumen fluid and 4 g glycerol/l were added to each tube under CO_2 gassing. During incubation (24 h), samples were obtained at different time points: 1, 3, 6, 9, 12 and 24 h. Finally, the glycerol concentration for each cow at each time point was determined.

3.1.2 Infusion of glycerol

A changeover experiment with three rumen-fistulated cows that were not pregnant or lactating was set up in order to investigate the effects of infusion of 0.6 g glycerol/kg BW (approximately 400 g glycerol/day) in either the rumen or the abomasum, and a control with no infusion, over a three-day period. Glycerol was infused into the abomasum by means of a tube anchored in the abomasum as described by Mogodiniyai Kasmaei and Holtenius (2013). Rumen fluid was collected and blood samples were drawn from the coccygeal or jugular vessel twice daily. The plasma concentrations of glycerol, glucose, insulin and non-esterified fatty acids (NEFA) were determined to distinguish differences in the metabolism.

4 Main results

Taken together, the results presented in this thesis have provided new knowledge about glycerol supplementation in dairy cows and young calves. A summary of the main results of the studies in this thesis is presented below. More detailed descriptions of the results can be found in **Papers I – IV**.

4.1 Supplemental feeding of glycerol with different purities (Paper I)

Milk yield, calculated as kg energy corrected milk (ECM)/day, tended to be higher in cows receiving glycerol (>99%) than the control group with no extra energy supplementation in their diet. Cows receiving glycerol (>99%) had a higher protein concentration in their milk and tended to have higher yield of milk fat + protein than cows receiving crude glycerol (88.1%). Conversely, daily lactose production in cows receiving crude glycerol and glycerol (>99%) was similar.

Daily silage intake and total dry matter intake (DMI) were not influenced by the different glycerol purities. Thus methanol and other compounds in the crude glycerol obviously did not reduce feed intake. No main effects of treatment on the concentrations of glycerol, glucose and insulin in plasma were observed. The energy status was monitored indirectly by determining NEFA and β-hydroxybutyrate (BHBA) concentrations in blood plasma. However, these plasma compounds were not affected by treatment nor were there any differences in loss of body condition during the four-week experimental period. Taken together, these results indicate that the treatment did not affect the energy balance.

4.2 Rumen metabolism (Paper II)

Glycerol can be absorbed from the rumen in substantial amounts, approximately 70%, and the fractional rate of absorption (FRA) of glycerol was not affected by variations in glycerol concentration (*in vivo*). Glycerol absorption apparently occurred largely by passive diffusion and was probably not facilitated by carriers (*ex vivo*). Isolated sheets of ovine rumen epithelium exhibited no net transport under classical Ussing chamber conditions and showed linear transport with increasing glycerol concentration. The transport did not correlate with tissue conductance. Therefore, paracellular transport is probably negligible and glycerol passes through the rumen epithelium predominantly via the cellular pathway. These findings are favourable, especially for high-yielding dairy cows, as the absorbed glycerol can be efficiently converted into glucose via gluconeogenesis in the liver. Glycerol also disappeared from the rumen compartment by means of microbial digestion and through outflow via the omasal orifice, but apparently to a lesser extent than absorption across the rumen epithelium. Microbial digestion was estimated to account for approximately 30% of the total glycerol disappearance. The corresponding figure for the outflow through the omasal orifice was approximately 10% in this study. The fate of glycerol entering the rumen of dairy cows and sheep in the present study is illustrated in Figure 1.

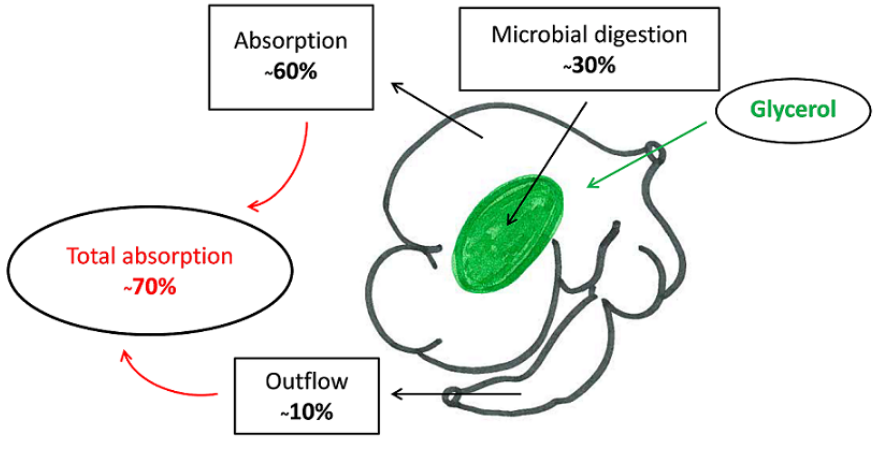

Figure 1. The fate of glycerol entering the rumen of dairy cows and sheep.

4.3 Enteric methane production and rumen microbiology (Paper III)

Reduction of methane production was not observed when glycerol was added to the substrate in the gas *in vitro* system. The concentration of VFA was numerically higher when glycerol was added compared to the control with no feed additive. The same applied for propionate and butyrate production. The pH declined during incubation, but remained above pH 6.0.

The community structure for bacteria at genus level and archaea at species level subjected to additional glycerol *in vitro* appeared to follow the same pattern as the *in vitro* control without feed additive. However, differences at genus level within the bacterial community structure responded to added glycerol with an increase in the relative abundance of unclassified *Ruminococcaceae* and *Anaerovibrio*. The dominating sequences within the archaeal structure were related to *Methanobrevibacter thaueri* and the result was consistent with the *in vitro* control.

Furthermore, the outcomes of **Paper III** showed the importance of relating *in vitro* results with the *in vivo* situation in the cow, and that diverse systems may affect the bacterial and archaeal community structures in different ways. For instance, the transfer of rumen fluid to the *in vitro* system appeared to have an effect on the distribution of different species within the genus *Methanobrevibacter*, already after 8 h of incubation.

4.4 Oral rehydration solutions with glycerol (Paper IV)

Plasma concentrations of glycerol and glucose were already elevated 15 min after the ORS load with glycerol or glucose, and peaked at 60 min. The result indicates that glycerol was absorbed from the gastrointestinal tract at least as rapidly as glucose. Glycerol concentration and VFA pattern in faeces was similar among treatments. Taken together, these results showed that glycerol was rapidly absorbed from the gastrointestinal tract and most likely not available for intestinal microbiota.

The 1,3-propanediol concentration in faeces did not increase in calves that had received ORS containing glycerol, indicating that there was no elevated synthesis of the bioactive substance reuterin. This finding is supported by the 16S rRNA sequencing of bacteria confirming that *Lactobacillus reuteri* was present in faeces, but differences among treatments were not observed.

Control calves deprived of feed and fluid for 24 h tended to respond with reduced plasma glucose levels. Calves receiving ORS containing glycerol avoided the hyperglycaemia and hyperinsulinaemia shown by calves receiving ORS containing glucose, suggesting a more favourable metabolic response to

glycerol. Furthermore, calves receiving ORS containing glycerol tended to show a less pronounced increase in the concentration of total plasma protein (TPP) following feed and fluid deprivation for 24 h than the control calves and calves receiving ORS with glucose. This result suggests that ORS containing glycerol might have ameliorated the dehydration effects of the deprivation period.

5 Glycerol as feed additive

Glycerol is an essential structural component of triglycerides and phospholipids. The chemical structures of glycerol, propylene glycol and propionic acid show similarities (Figure 2). Glycerol is an energy-rich component, with an estimated value of 16.2 MJ ME/kg of DM for ruminants (Mach *et al.*, 2009), and the glucogenic property of glycerol is well established (Cori & Shine, 1935). Recently, Lomander *et al*. (2012) reported that glycerol has comparable effects on milk production to propylene glycol in early lactating cows. Both glycerol and propylene glycol have long been used to treat ketosis (Fisher *et al.*, 1973; Johnson, 1954). However, unlike glycerol, propylene glycol is not a substance that is part of the animals' natural metabolism.

Even though glycerol was evaluated as an aid for the treatment of ketosis in dairy cows during the transition period in the 1960s and 1970s, the high cost of glycerol meant that there was no demand for the product in farm animals (Fisher *et al.*, 1973; Sauer *et al.*, 1973). In the late 1990s the expansion of the biofuel industry provided new sources of glycerol as a by-product of biodiesel production, which reduced the cost of glycerol (Schröder & Südekum, 1999). The cost of crude glycerol is approximately half the price of glycerol (>99%) and thus makes it more attractive as a feed additive. The increased availability of glycerol created a renewed interest in the use of glycerol as a potential feed supplement for dairy cows, especially in early lactation. The implications of using glycerol in cattle diets were reviewed by Südekum (2008). During the 2000s different feeding strategies as well as diverse levels and purities of the glycerol product have been used in diets for dairy cows in early lactation. All these studies have aimed to determine the effects of diets supplemented with glycerol on milk production and composition, feed intake, metabolic and rumen parameters, and fertility in transition dairy cows. The upper limit for glycerol inclusion in the diet of dairy cows without negative effects on the

production performance is unclear. Donkin *et al.* (2009) reported that up to 15% of the total ration DM for dairy cows can be replaced by glycerol without negatively affecting milk production or milk composition. In addition, Schröder and Südekum (1999) have shown that different purities of glycerol can replace starch in diets for ruminants at levels up to 10% of the diet DM without any negative effects on feed and water intake and nutrient digestibility.

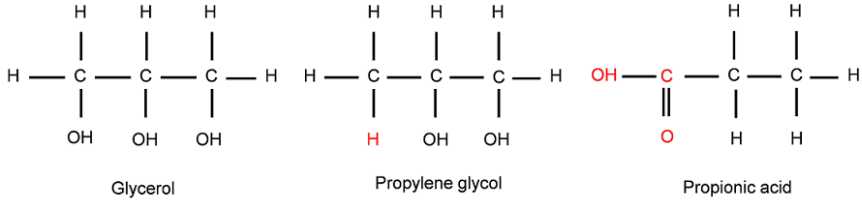

Figure 2. Chemical structure of glycerol, propylene glycol and propionic acid.

5.1 Digestibility

Providing ruminants with glycerol supplementation in their diets appears to have no negative effects on the total tract digestibility of organic matter, NDF or starch, as recently reviewed by Südekum (Südekum, 2008). However, Paggi *et al*. (1999) reported that proteolytic activity reduced by approximately 20% when an increased concentration of glycerol (50, 100, 200 and 300 mmol/l) was added to bovine rumen fluid *in vitro*. Wang *et al*. (2009a) also showed a decrease in crude protein degradability of concentrate but with an increase in nutrient digestibility in the total tract with increasing levels of glycerol (100, 200 and 300 g glycerol/day). Furthermore, Wang *et al*. (2009a) suggested that the improved total digestibilities of DM, NDF and ADF were to some extent triggered by enhanced ruminal degradation when glycerol was supplemented in the diet with an optimum glycerol level of 200 g glycerol/steer/day. In contrast, Shin *et al*. (2012) showed a 30% reduction in the apparent total tract digestion of dietary NDF in dairy cows when replacing concentrate with crude glycerol (80.3%) at 10% of DMI. The latter result is in agreement with Donkin *et al*. (2009) who reported lower total tract digestibility of NDF in dairy cows when replacing corn grain with glycerol at 5, 10 or 15% of DMI. Furthermore, Shin *et al*. (2012) suggested that glycerol feeding at this level (10% of DMI) in diets with low amounts of effective fibre may reduce the milk fat concentration.

In sacco degradability of silage on DM, NDF and crude protein fraction was similar in dairy cows provided with 250 g crude glycerol (88.1%) or glycerol (>99%) through the rumen fistula twice daily, during a 12-day period (Figure 3). However, glycerol appeared to inhibit the degradability of NDF initially, and a significant difference was observed between cows provided with glycerol, regardless of purity, and the control at 4 h of incubation, thus indicating that the lag phase was marginally longer in cows provided with glycerol.

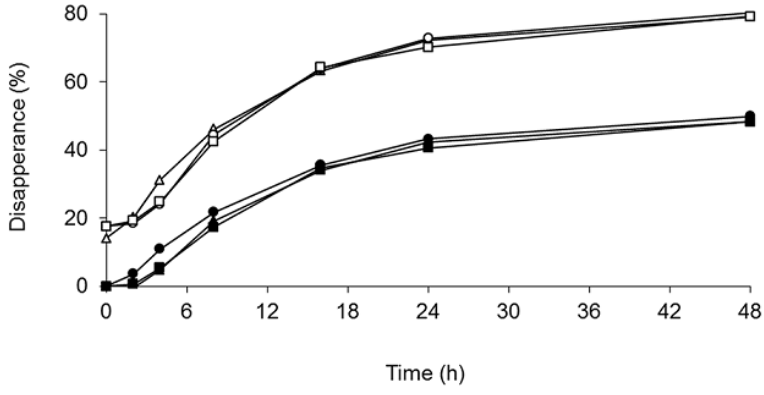

Figure 3. In situ disappearance of dry matter (%) in dairy cows fed the control diet (n =4; open triangles), crude glycerol (88.1%; n = 4; open circle) and glycerol (>99%; n = 4; open squares), and neutral detergent fibre (%) in dairy cows fed the control diet (n =4; filled triangles), crude glycerol (88.1%; n = 4; filled circle) and glycerol (>99%; n = 4; filled squares), added to the fistula twice daily for 12 days.

5.2 Purity and methanol content

Biodiesel is produced by a chemical reaction involving vegetable oil or animal fat with an alcohol, usually methanol: oil + alcohol → biodiesel + glycerol. Methanol and free fatty acids are major impurities of crude glycerol (Thompson & He, 2006). Residues of methanol in crude glycerol are due to the excess methanol used when the chemical transesterification is driven to completion and not all the methanol is recovered. Free fatty acids originate from a reaction between the free fatty acid present in the initial feedstock and the catalyst (base). In addition, crude glycerol also contains water and a variety of mineral salts. Depending on the different glycerol purification methods and parent feed stocks (e.g. rapeseed and soybean) used in the biodiesel production, a wide range of purity values of glycerol are obtained (Südekum, 2008; Thompson & He, 2006). Schröder and Südekum (1999) have defined the purity of glycerol as low (63%), medium (85%) and high (>99.5%). This definition reflects different stages of the process of rapeseed oil methyl ester production. However, the term crude glycerol appears to be used in general for glycerol purities <99% and the chemical composition of glycerol is often stated.

Glycerol with a purity below 85% is rarely fed to dairy cows. However, studies by Kass *et al*. (2013), Shin *et al*. (2012) and DeFrain *et al*. (2004) have used glycerol supplementation with purities of 82.6%, 80.3% and 80.2% respectively in the diets of lactating cows. Glycerol supplementation with purity of 88.1% was fed to dairy cows postpartum in **Paper I**.

Methanol should be removed from glycerol in the diets of all farm animals as far as technically possible in order to eliminate the potential negative effects on feed intake, performance and metabolism (Südekum, 2008). In Germany, the threshold value of methanol content of glycerol in farm animal feed is 0.5% (Südekum, 2008) (Table 1). However, in ruminants methanol is produced by rumen bacteria, particularly when the animals consume diets containing pectins (Neumann *et al.*, 1999; Vantchev *et al.*, 1970). Methanol is released by the hydrolysis of methyl esters from pectins and then efficiently metabolised to methane by rumen microorganisms (Neumann *et al.*, 1999; Pol & Demeyer, 1988). Furthermore, findings by Pol and Demeyer (1988) showed that methanol was rapidly metabolised mainly to methane when 15 g methanol/day was infused into the rumen of sheep for one month. In **Paper I**, dairy cows were fed crude glycerol with a methanol content of 0.8% (Table 1), thus the intake was approximately 4 g methanol/cow/day and probably only induced a marginal increase in rumen fluid methanol concentration.

Paper 1 appears to be the first to evaluate and compare different purities of glycerol, *e.g.* crude glycerol (synonym: low purity glycerine) and >99% glycerol (synonym: high purity glycerine), added to the diet of dairy cows in early lactation.

Table 1. *Standard composition (%) of different glycerol qualities according to the Normenkommission für Einzelfuttermittel im Zentralausschuss der Deutchen Landwirtschaft (2006), and crude glycerol (AArhusKarlshamn, Karlshamn, Sweden) used in* ***Paper I***

Item	Glycerol	Glycerol, crude	Crude glycerol (**Paper 1**)
Glycerol	≥ 99.0	≥ 80	88.1
Water	0.5 - 1	10 -15	9.3
Ash	≤ 0.1	≤ 10	0.9
Methanol	0[1]	≤0.5	0.8
Other[1]	-	No value stated	0.9

[1]Numbers indicates concentrations below detection limit.
[2]Other = sodium, calcium, magnesium, potassium, phosphorous, sulphur *etc*.

6 Glycerol and production performance

6.1 Feed intake

In **Paper I**, it was hypothesised that no negative effect on feed intake would follow when dairy cows in early lactation were fed glycerol supplementation. Results showed that neither of the two glycerol products affected total DMI (Figure 4). Thus methanol (0.8%) and other compounds in the crude glycerol did not obviously reduce feed intake. This result confirms the findings of DeFrain *et al*. (2004) and Kass *et al*. (2013) who reported no negative effects on feed intake when dairy cows received crude glycerol with a methanol content of 1.3% and 0.4% respectively during early lactation.

The experimental diets in **Paper I** were designed to make comparisons between glycerol products of different purities, supplemented on top of the concentrate. Silage was fed *ad libitum*. Differences in silage intake were not observed (Figure 4), suggesting that none of the glycerol products induced any metabolic satiety effect in the animals. Furthermore, the intake of concentrate was similar among treatments and there were virtually no concentrate refusals, indicating that palatability was not affected by glycerol.

Results from **Paper I** are consistent with most of the recent studies that have reported no influence of glycerol on feed intake in cows in early lactation (Carvalho *et al.*, 2011; Donkin *et al.*, 2009; Wang *et al.*, 2009b). In contrast, Fisher *et al*. (1971) and Bodarski *et al*. (2005) reported higher feed intake when dairy cows received glycerol within the concentrate mixture or total mixed ratio (TMR) respectively. Furthermore, Shin *et al*. (2012) replaced concentrate with crude glycerol (80.3%) at 5% of dietary DM to cows in mid-lactation, and found that DMI increased without improved milk yield. Ogborn (2006) showed a depressive effect of crude glycerol (80.6%) on feed intake in lactating cows. Osborne *et al*. (2009) also reported a decrease in feed intake when glycerol was added to the drinking water of dairy cows during the transition period. Water

intake in cows provided with glycerol in drinking water was lower compared to cows without additional glycerol prepartum (Osborne *et al.*, 2009). However, water intake was similar between treatments postpartum.

The shifting quality of the glycerol product and glycerol feeding level, as well as the different administrations strategies and the ratio of forage to concentrate in the diets might be the reason for the inconsistent results.

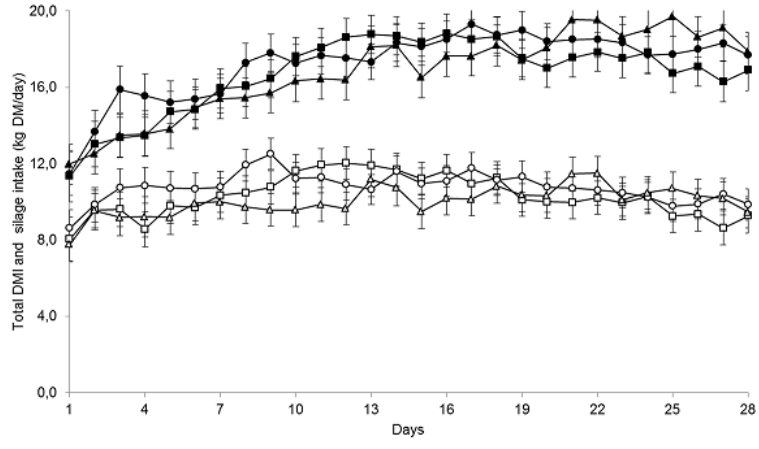

Figure 4. Total dry matter intake (kg DM/day) of dairy cows fed control (n =10; filled triangles), crude glycerol (88.1%; n = 12; filled circle) and glycerol (>99%; n = 10; filled squares) diet and the silage intake (kg DM/d) of dairy cows fed control (n =10; open triangles), crude glycerol (88.1%; n = 12; open circle) and glycerol (>99%; n = 10; open squares) diet during the first four weeks of lactation. Data are shown as least squares means ± standard error of the mean.

6.2 Milk yield and milk composition

The hypothesis was that glycerol supplementation, irrespective of purity, would enhance milk yield and reduce the milk fat concentration in dairy cows during early lactation (**Paper I)**. The results showed a tendency for higher milk yield (kg ECM/day) in cows fed glycerol (>99%) for four weeks after parturition, but cows fed crude glycerol (88.1%) did not respond with an increased milk yield. An improved milk yield has previously been reported in

cows receiving crude glycerol (82.6%) by oral drenching for three weeks of lactation (Kass *et al.*, 2013) and in cows fed glycerol for ten weeks of lactation (Bodarski *et al.*, 2005). Also, in a field study by Lomander *et al*. (2012), a higher milk yield was observed during the first 90 days of lactation in cows fed 450 g glycerol/day during three weeks postpartum compared to control cows. In other studies, cows receiving glycerol with different purities in the diet did not respond with an increased milk yield during early lactation (Carvalho *et al.*, 2011; Wang *et al.*, 2009b; Chung *et al.*, 2007; DeFrain *et al.*, 2004) or at mid lactation (Coskun *et al.*, 2012; Shin *et al.*, 2012; Khalili *et al.*, 1997). However, in some of these studies, concentrate was replaced by glycerol while in **Paper I** and in studies by Kass *et al*. (2012) and Lomander *et al*. (2012) glycerol was provided in addition to concentrate.

Results from **Paper I** showed that cows receiving glycerol (>99%) had a higher protein concentration and tended to have a higher yield of milk fat + protein than cows receiving crude glycerol (88.1%). No difference in lactose yield was observed between the two purities of glycerol. This finding suggests that the two glycerol products used in **Paper 1** might be an equally good supply of glucose, thus supporting lactose synthesis in cows during the first four weeks of lactation. However, it cannot be ruled out that there might be components in crude glycerol (88.1%) that have a negative effect on the yield of milk fat and protein.

In general, the effects of glycerol on milk composition are moderate and earlier studies have shown diverse results. Several recent studies have reported no effects of glycerol on milk composition (Coskun *et al.*, 2012; Carvalho *et al.*, 2011; Chung *et al.*, 2007; Ogborn, 2006). In contrast, Wang *et al*. (2009b) observed a trend towards lower milk fat yield and a tendency for an overall linear decrease in milk protein content with increasing glycerol supplementation (100, 200 and 300 g glycerol/day) in cows during early lactation. Also, DeFrain *et al*. (2004) reported a tendency for a decrease in the milk fat yield in cows fed glycerol (430 and 860 g glycerol (80.2%)/day) for three weeks postpartum compared to control cows. Findings by Bodarski *et al*. (2005) showed an increased milk protein content with an increased amount of glycerol (300 ml and 500 ml glycerol/day) fed to cows during the first 70 days of lactation. Kass *et al*. (2013) observed an increase in milk lactose content in cows that received 500 ml glycerol/day as an oral drench during three weeks of lactation compared to control cows receiving no additional feed additive.

7 Metabolism

7.1 Energy status during the transition period

The transition period comprises the final growth of the foetus, calving and the start of milk production. The extensive increase in energy demand by the mammary gland for milk secretion at the onset of lactation is enabled partly by an increase in feed intake and partly by mobilisation of fat from adipose tissue (Grummer *et al.*, 2004). However, the increase in DMI is rather slow compared with the increased energy demand for lactation, and results in a period of NEB in early lactation in almost all high-yielding cows. This stage is characterised by low levels of blood glucose and insulin, and high levels of blood NEFA and BHBA (Ingvartsen & Andersen, 2000). Furthermore, the high demand of glucose and insufficient gluconeogenesis imposes increased ketogenesis that is reflected by an increased level of ketone bodies, primarily BHBA, in blood plasma (Holtenius & Holtenius, 1996). Some cows develop clinical ketosis, a metabolic disorder, with varying frequency and severity primarily during the transition period (Ingvartsen, 2006). Glycerol supplementation in the diet during early lactation might contribute to higher plasma concentrations of glucose and lower concentrations of NEFA and BHBA, and thus result in improved metabolic status. Previous studies have also suggested that glycerol may alleviate the risk of ketosis during the transition period (Fisher *et al.*, 1973; Johnson, 1954).

7.1.1 NEFA, BHBA and body condition

The energy status in dairy cows during early lactation is reflected indirectly by plasma NEFA and BHBA concentrations (Herdt, 2000). At early lactation plasma NEFA and BHBA levels above the cut-off values of ≥0.7 mmol/l and ≥1.4 mmol/l respectively, there is an increase in the risk of metabolic diseases,

e.g. displaced abomasum and clinical ketosis, leading to substantial loss of milk yield (Oetzel, 2004; Whitaker, 2004).

In **Paper I**, plasma concentrations of NEFA and BHBA were not affected by glycerol supplementation during four weeks of lactation. This finding is consistent with Kass *et al*. (2013), Chung *et al*. (2007), Ogborn *et al*. (2006) and DeFrain *et al*. (DeFrain *et al.*, 2004). The average plasma BHBA concentration ranged from 1.4-1.7 mmol/l among cows in **Paper I**, and might be high as an effect of the experimental design since the cows in early lactation were fed a restricted amount of concentrate. This suggests that the limited concentrate allowance may have aggravated the NEB and increased liver ketogenesis. However, Fall *et al*. (2008) compared indicators of energy balance in early lactation in cows managed according to organic rules and conventionally-managed cows under field conditions. They reported that organically-managed cows did not show a greater extent of mobilisation of body tissue than conventionally-managed cows, even though they were fed diets with a lower content of concentrate. It cannot be ruled out that the relatively high BHBA concentration emanates from rumen butyrate metabolised to BHBA in rumen epithelium. Differences in loss of body condition were not observed nor were there any differences in the feed conversion rate (kg ECM/kg DM) among treatments (**Paper I**). Taken together, these results indicate that energy metabolism was not affected by supplemental feeding of glycerol, irrespective of purity. A lack of effects on average BW and BCS in cows receiving glycerol in early lactation have been reported in previous studies (Kass *et al.*, 2013; Carvalho *et al.*, 2011; Chung *et al.*, 2007; DeFrain *et al.*, 2004). In contrast, Wang *et al*. (2009b) reported that plasma NEFA and BHBA linearly decreased with increasing glycerol supplementation (100, 200 and 300 g glycerol/day), and cows fed glycerol tended to increase BW at a higher rate relative to cows fed the control diet in early lactation.

7.1.2 Glucose and insulin

The plasma concentrations of insulin, a key hormone involved in the regulation of glucose and energy metabolism within the animal, declines abruptly at calving and remains low during the first weeks of lactation (Ingvartsen, 2006). Regardless of the improved availability of glycogenic substrates in **Paper I**, neither the plasma levels of insulin nor the plasma levels of glucose were affected by glycerol supplementation. This result is in agreement with previous studies (Kass *et al.*, 2013; Chung *et al.*, 2007; Ogborn, 2006; DeFrain *et al.*, 2004). In addition, an infusion of 0.6 g glycerol/kg BW (approximately 400 g glycerol/day) to either the rumen or the abomasum, and a control with no

infusion, in cows for three days showed no differences in the plasma concentrations of glucose, insulin and NEFA. These results indicate that glycerol feeding has a minor effect on the insulin concentration in plasma in dairy cows in early lactation. However, DeFrain *et al*. (2004) reported a decrease in the plasma concentration of glucose between days 14 and 21 postpartum in cows fed 860 g crude glycerol (80.2%)/day. In contrast, Wang *et al*. (2009b) observed a linear increase of plasma glucose with increasing glycerol supplementation (100, 200 and 300 g glycerol/day). Furthermore, several studies have shown that glycerol can elevate plasma glucose levels (Osman *et al.*, 2008; Linke *et al.*, 2004; Goff & Horst, 2001).

7.2 Metabolic pathway

Glycerol can enter the gluconeogenic pathway at the triose phosphate level (Leng, 1970) and glycerol is therefore more metabolically favourable than propionate (Figure 5). Glycerol fed to ruminants may be fermented in the rumen, be absorbed across the rumen epithelium or escape the rumen by outflow through the omasal orifice. It has generally been assumed that glycerol is mainly fermented in the rumen of ruminants and that the rate of absorption from the gastrointestinal tract is low (Südekum, 2008). However, the result of the *in vivo* and *in vitro* studies in Paper II indicate that approximately 70% of the glycerol escapes rumen fermentation and may be an available gluconeogenic substrate. Consequently, glycerol might contribute efficiently to glucose synthesis in the liver. This result is in agreement with Remond *et al*. (1993) where approximately 43% of the glycerol consumed was calculated to be absorbed across the rumen epithelium. Also, Kijora *et al*. (1998) showed that ruminants receiving an intra-ruminal glycerol load of 200 g twice daily over a period of 6 days responded with a three-fold increase in plasma glycerol concentration compared to the control (without glycerol intake). Furthermore, low glycerol flux into the duodenal digesta was reported, indicating absorption of glycerol across the rumen epithelium (Kijora *et al.*, 1998). Glycerol also disappears from the rumen compartment through outflow via the omasal orifice (**Paper II**) and is most likely rapidly absorbed from the small intestine and then utilised as a gluconeogenic substrate. The latter is confirmed by observations in calves in **Paper IV**, where glycerol was absorbed at least as rapidly as glucose. However, cows infused with approximately 400 g glycerol/day either to the rumen or the abomasum for three days showed no differences in glycerol concentration in plasma compared with the control without infusion.

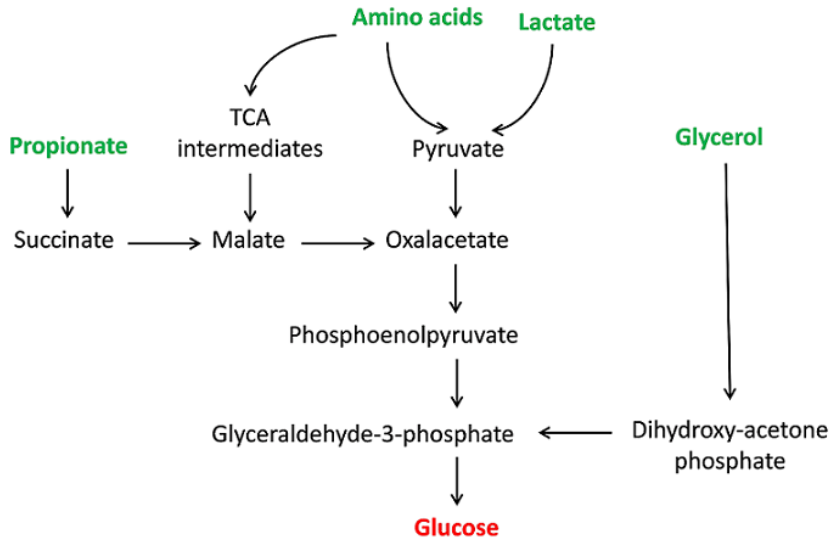

Figure 5. Major pathways in gluconeogenesis in cows (Mc Donald *et al.*, 2011).

Results showed a microbial digestion rate of 6.6% and 10.8%/h in the two tubes respectively (Figure 2 in **Paper II**), and are consistent with observations made by Traube *et al*. (2007). Assuming that the *in vitro* system used in **Paper II** accurately reflected the microbial degradation of glycerol *in vivo*, the observed rapid rate of glycerol disappearance from the rumen could scarcely be explained by rumen microbial digestion. Ferraro *et al*. (2009) showed that glycerol (90%) had a slow rate of degradation, with a lag phase of 10 to 12 h, using ruminal fluid from sheep with the addition of glycerol, 320 or 640 µl, *in vitro*. The latter result is consistent with Lee *et al*. (2011). However, Remond *et al*. (1993) reported a rapid *in vitro* fermentation of glycerol, 4 to 6 h, in animals adapted to glycerol (240 g/day) for two weeks. The differences in the fermentation pattern of glycerol may be due to the different administration strategies of drenching versus being fed continuously in the diet, and thus it is assumed that the adaptation of the rumen microbiota might have changed the fermentation kinetics, particularly the lag phase.

The rate of *in vitro* microbial digestion of glycerol was not elevated by the daily supplementation of glycerol of different purities through the rumen fistula for 12 days (Figure 6). This result suggests that rumen microbes were not able to increase their digestion of glycerol regardless of adaptation. In contrast, Kijora *et al*. (1998) showed a more rapid disappearance of glycerol in ruminants with an infusion of 200 g glycerol twice daily into the rumen during

a seven-day experimental period. The concentration of total VFA declined until day 4 of the experiment and then rose towards a concentration similar to that observed on day 1 (Kijora *et al.*, 1998).

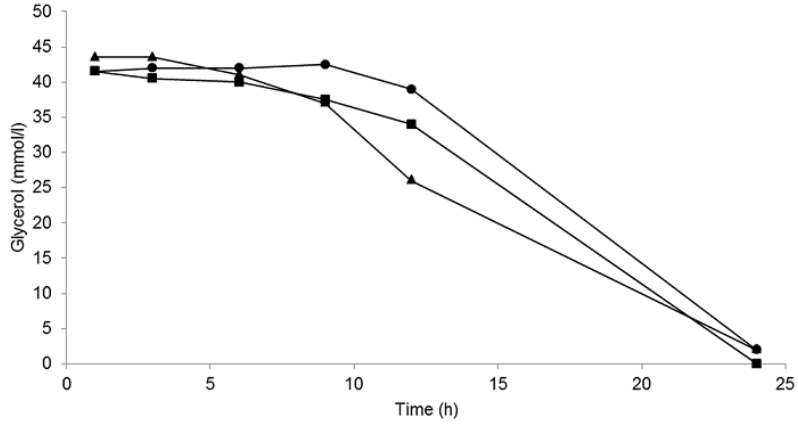

Figure 6. In vitro disappearance rate of glycerol during 24 h of incubation in dairy cows provided with daily glycerol supplementation of different purities - control (n =4; triangles), crude glycerol (88.1%; n = 4; circle) and glycerol (>99%; n = 4; squares) - through the rumen fistula for a period of 12 days.

7.3 VFA profiles

Rumen fermentation of different diets causes changes in the yield of total VFA and type of VFA formed, which means that the utilisation of the absorbed nutrients, and thus milk yield and composition, will be affected (Aschenbach *et al.*, 2010; Ingvartsen, 2006; Leng, 1970). The FRA of glycerol did not appear to be reduced and acetate and propionate were not affected by increasing glycerol concentration (15, 30 and 45 mmol/l) in the experimental solution (Table 4 in **Paper II**). The FRA was calculated using exponential equations assuming that absorption occurred according to first-order kinetics. The latter result is in line with that of Otha *et al*. (2006) who showed that glycerol is transported passively across the intestine when the glycerol concentration in the lumen of rats is high. However, the FRA results shown in **Paper II** must be viewed with caution as the relationship is based on data from only three levels of glycerol. Furthermore, the FRA values for acetate and propionate in **Paper II** were lower than those observed by Dijkstra *et al.* (1993). The pH of the

buffer experimental solutions used in **Paper II** to predict the FRA of glycerol and VFA was about 7.7. Around 99.9% of short-chain fatty acids are present in dissociated form in the rumen at pH 7.7, according to the Henderson-Hasselbach equation. Dissociated short chain fatty acids do not diffuse across the cell membrane (Sehested *et al.*, 1999), and the overall passage of VFA from the rumen across the epithelium would thereby be reduced. However, the rumen pH is generally lower than 7.7 in fed ruminants, thus the VFA absorption was probably lower in the present experimental set-up (**Paper II**). Also, Dijkstra *et al*. (1993) showed that the rate of absorption of short-chain fatty acids increased markedly when rumen pH decreased in lactating dairy cows. Furthermore, glycerol does not dissociate in the slightly acidic rumen pH prevailing when ruminants are fed, and therefore it is suggested that the glycerol absorption rate is not affected by rumen pH (**Paper II**).

The total VFA production and acetate to propionate ratio in rumen fluid was not influenced by different glycerol purities when the dairy cows were provided with 250 g glycerol with crude glycerol (88.1%) or glycerol (>99%) through the rumen fistula twice daily for 12 days. Adding glycerol to the substrate in the gas *in vitro* system resulted in numerically higher values for total VFA production and propionate proportion over time (48 h), compared to the control (Table 3 in **Paper III**). The levels of glycerol used in **Paper III** were equal to the rumen concentrations in **Paper II**, at 15 and 30 mmol/l respectively. Presumably the glycerol was used as a substrate by rumen microorganisms in the *in vitro* rumen model, a closed system with no outflow, suggesting that the total VFA production and propionate proportion may have increased slightly.

Several *in vivo* studies (Wang *et al.*, 2009a; DeFrain *et al.*, 2004; Kijora *et al.*, 1998) have observed an impact of glycerol on VFA profiles in ruminants. These studies indicated that glycerol was fermented in the rumen, and gave rise to lower acetate to propionate ratio. The change was primarily a result of increased proportions of propionate. Furthermore, Avila *et al*. (2011) reported a linear increase in propionate and reduced acetate concentrations, resulting in a decline in the acetate to propionate ratio when barley grain was replaced by increasing levels of glycerol *in vitro*. Other studies have shown that the proportion of propionate and butyrate generally increases at the expense of acetate when diets are supplemented with glycerol (Shin *et al.*, 2012; Carvalho *et al.*, 2010; Kristensen & Raun, 2007; Linke *et al.*, 2004). Furthermore, results from an *in vitro* study with pure cultures of *Propionibacterium acidipropionici* showed an efficient production of propionate from glycerol without acetate formation (Coral *et al.*, 2008).

7.3.1 Enteric methane

Enteric methane production in ruminating animals has received increased attention in recent years. However, there are still no answers to questions concerning the factors that regulate enteric methane levels and how to mitigate enteric methane production (Hook *et al.*, 2009; McAllister & Newbold, 2008; Wright *et al.*, 2007). Lee *et al*. (2011) reported that addition of glycerol resulted in reduced CH_4 production during *in vitro* incubation (48 h) of strained rumen fluid, suggesting that glycerol may have a positive impact on the efficiency of use of dietary energy. However, a reduction in methane production was not observed in **Paper III** when rumen fluid was supplemented with glycerol and incubated *in vitro* for 48 h. Total gas production was similar among treatments, but differences in methane production were observed (Table 2 in **Paper III**). Addition of glycerol increased methane production by 12% at most. The energy loss through methane production was calculated, assuming a gross energy concentration of 18 MJ/kg DM, resulting in a predicted methane concentration of 8.2% of gross energy in glycerol treatment. Methane production may have been amplified with the addition of glycerol, suggesting that glycerol was probably used as a substrate in the closed *in vitro* system, and thus increased total VFA and the proportion of propionate. However, results from **Paper II** showed that glycerol was mainly absorbed across the rumen epithelium, and thus used as a substrate for VFA synthesis to a lesser extent. Instead glycerol can act as a nutrient source available for gluconeogenesis in the liver without generating methane.

The lack of an inhibiting effect of glycerol on methane production is consistent with findings by Avila *et al*. (2011) and Avila-Stagno *et al*. (2013). Bizukojc *et al*. (2010) reported that glycerol can be converted to methane by *Methanosarcina mazei* (*in vitro*), a methane-producing microorganism in the rumen, and thus increase methane production. However, most of these studies were performed *in vitro*, and it is important to underline that the effects shown cannot be directly translated to *in vivo* conditions in the ruminant.

8 Bacterial and archaeal community structures

Results from **Paper III** showed that the bacterial community structure had a similar pattern *in vivo* as the *in vitro* control with no feed additive, thus indicating that the transfer of the rumen fluid to the *in vitro* system had little if any impact on the bacterial community structure. However, the transfer of rumen fluid to the *in vitro* system appeared to have an effect on the archaeal community structure, with differences in the distribution of species within the genus *Methanobrevibacter* already after 8 h of incubation. The change was probably triggered by the shift from the continuous rumen system to the more static batch culture, probably giving some species a competitive advantage. Furthermore, the effects on the archaeal community were more noticeable as they were dominated by one single genus, *Methanobrevibacter*, while the bacterial community was composed of more than 100 different genera. Thus, the effect of transfer of rumen fluid to the *in vitro* system should be considered when evaluating the effects of glycerol addition to the substrate. On the other hand, the impact of time on the bacterial community was shown *in vitro*. For instance, the relative abundance of *Prevotella* decreased and this genus almost completely disappeared during incubation in all treatments. Furthermore, the decrease was at a similar proportion in all treatments, and consequently was not associated with treatment effects.

The rumen bacteria before additives were dominated by *Bacteroidetes* and *Firmicutes*. The result is consistent with previous studies using pyrosequencing approaches (Zened *et al.*, 2013; Hristov *et al.*, 2012) and culture-based techniques (Stewart *et al.*, 1997), suggesting that *Bacteroidetes* and *Firmicutes* play a major role in ruminal metabolism. At family level *Prevotellacea*, *Bacteriodales*, *Lachonspiracea* and *Ruminocaccae* were dominant, which is in agreement with earlier studies (Zened *et al.*, 2013; Kong *et al.*, 2010). According to the discussion in Bekele *et al*. (2010), the decrease in *Prevotella*

could inhibit fibre digestion and decrease the formation of methane. The archaeal structure was dominated by *Methanobrevibacter* at genus level, both *in vitro* and *in vivo*. This result is consistent with several studies where *Methanobrevibacter* represents the common genus structure in rumen (St-Pierre & Wright, 2013; King *et al.*, 2011; Hook *et al.*, 2009)

In general, the addition of glycerol *in vitro* appeared to have a similar pattern as the *in vitro* control without feed additive in the community structure of both bacteria and archaea (**Paper III**). Thus the results indicate that the impact of additional glycerol on the *in vitro* system was inferior on bacteria at genus level and on archaea at species level. The principal component analysis of treatment effects showed that the addition of glycerol *in vitro* gave rise to differences at genus level within the bacterial structure, *e.g.* an increased relative abundance of unclassified *Ruminococcaceae* at 24 h of incubation and *Anaerovibrio* at all time points compared to the other treatments *in vitro* and *in vivo* (Figure 2 in **Paper III**). Furthermore, Stewart and Bryant (1988) reported that *Anaerovibrio Lipolytica* is one of the major bacteria species fermenting glycerol. The dominating sequences within the archaeal structure were related to *Methanobrevibacter thaueri* when adding glycerol *in vitro*, and the result was consistent with the control without feed additive *in vitro*. However, the most dominating sequences within the archaeal structure *in vivo* were *Methanobrevibacter olleyae*. Currently, studies have indicated a correlation between the community structure within the genus *Methanobrevibacter* and enteric methane production (Danielsson *et al.*, 2012; King *et al.*, 2011). Depending on the phylogenetic relationship, it was suggested that *Methanobrevibacter* be divided into two groups: *Methanobrevibacter ruminatium* and *Methanobrevibacter olleyae* in one group (RO) and *Methanobrevibacter smithii*, *Methanobrevibacter gottschalkii, Methanobrevibacter millerae* and *Methanobrevibacter thaueri* in the other (SGMT). King *et al*. (2011) and Danielsson *et al*. (2012) indicated that SGMT correlated positively with increasing methane production. However, the addition of glycerol to the *in vitro* system was similar with the *in vitro* control, and thus there is doubt as to whether the increased methane production shown in **Paper III** was due to differences in archaeal structure. It is more likely that the increased methane formation was a result of changes in the VFA profile.

8.1 Cellulolytic bacteria

Findings by Roger *et al*. (1992) reported that the growth and cellulolytic activity of the rumen bacterial species *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* were inhibited when glycerol was supplemented at a

concentration of 5% *in vitro*. Also, the growth of an anaerobic fungal species, *Neocallimastix frontalis*, was inhibited along with impaired cellulolytic activity. One suggestion is that glycerol may render the substrate less available to the bacterial cell (Roger *et al.*, 1992). However, these results must be viewed with caution, as the inhibition of growth and cellulolytic activity could be an effect of both the glycerol supplementation and the specific *in vitro* conditions used in the study, or both. In general, the growth of ruminal cellulolytic bacteria is inhibited at pH values below 6.0, and results in reduced cellulolytic activity in the rumen (Russell & Wilson, 1996; Russell & Dombrowski, 1980). No differences in pH were observed among treatments after 48 h of incubation (**Paper III**). The pH value was 6.84 at the start and 6.01 at the end of the incubation with glycerol, and thus within the optimum range for cellulolytic bacteria activity. This finding is consistent with other studies (Shin *et al.*, 2012; Carvalho *et al.*, 2011; DeFrain *et al.*, 2004; Schröder & Südekum, 1999). Wang *et al*. (2009a) reported a linear decrease in ruminal pH with increasing amounts of glycerol (100, 200 and 300 g glycerol/day) in rumen-cannulated steers. However, pH values did not drop below 6.0. In contrast, Kijora *et al*. (1998) showed a decrease in pH from 6.32 to 5.42 during a seven-day period when young bulls were provided with 200 g glycerol twice daily into the rumen.

9 Oral rehydration solution

Dehydration and energy deficiency often follow when calves are affected by diarrhoea, the most common disease in young calves (Svensson *et al.*, 2003) and an important cause of mortality (Barrington *et al.*, 2002). Oral rehydration solutions can be used in order to maintain the fluid and electrolyte balance, otherwise the situation could be life threatening for the calf (Constable *et al.*, 2001). The hypothesis was that absorption of fluid and electrolytes from the intestine would be facilitated by ORS containing glycerol compared with ORS containing glucose (**Paper IV**). Calves were subjected to fluid and feed deprivation for 24 h in order to mimic the energy deficiency and dehydration that often take place when calves suffer from infections. Results showed that calves responded with reduced plasma glucose levels (**Paper IV**). These findings support previous studies and underline the recommendations of continued milk feeding to calves with diarrhoea (Constable *et al.*, 2001). Calves provided with ORS containing a glycerol/glucose mix or glucose responded with elevated plasma glucose concentrations (**Paper IV**). This was expected since both solutions contained glucose and calves can readily absorb glucose from the small intestine. The increase in glucose was most pronounced in calves provided ORS with glucose only (Table 4 in **Paper IV**). The insulin concentration in plasma followed the same pattern, as expected, and obtained values in agreement with Katoh *et al*. (2004). The insulin to glucose ratio in calves provided with different ORS was similar, suggesting that calves responded similarly to the glucose load. However, calves receiving an oral load of ORS with glycerol alone did not respond with increased plasma glucose or plasma insulin, suggesting that ORS containing glycerol might have positive effects on the metabolism of dehydrated calves, in the sense of reduced risk of hyperglycaemia and hyperinsulinaemia.

Changes in plasma concentrations of TPP were used as an indirect marker of plasma volume changes in **Paper IV**, as described by Constable *et al*.

(1998). Calves receiving ORS with glucose, as well as control calves, responded with an increased plasma concentration of TPP, indicating dehydration. In contrast, calves provided with ORS containing a glycerol/glucose mix did not show increased plasma concentrations of TPP, suggesting that this solution may have ameliorated the negative effects of fluid and feed deprivation. This result is in line with numerous studies, reviewed by Goulet (2009), in humans and rats, showing that solutions containing glycerol can maintain fluid volume. Furthermore, Wapnir *et al*. (1996) and Allen *et al*. (1999) reported that ORS containing glycerol can improve the rate of water and electrolyte absorption in rats.

Estimations of dehydration in calves based on physical examination have been discussed for almost 50 years (Watt, 1965). Skin tent duration in the neck region and eyeball recession are useful clinical tools for identifying dehydrated calves (Constable *et al.*, 1998). Skin tent duration and eyeball recession were similar among treatments in **Paper IV**, and calves showed a modest dehydration after 24 h without feed and fluid. The result is based on a four-point scale used before and after the deprivation period as a measure of the degree of dehydration (Smith, 2009). Monitoring changes in BW is an alternative method for detecting acute dehydration in calves (Bywater, 1983). However, the gastrointestinal content is reduced in calves when deprived of feed and fluid, and this variation has probably more influence on BW loss than dehydration does. In **Paper IV**, all calves lost weight corresponding to approximately 9% of initial BW during the deprivation period (24 h), most likely explained by both reduced gastrointestinal contents and fluid deficit.

In **Paper IV**, the plasma concentration of glycerol increased markedly following oral glycerol supplementation. Both glucose and glycerol concentrations in plasma peaked 60 min after ORS intake, indicating that glycerol was absorbed from the gastrointestinal tract as rapidly as glucose (Figure 1 in **Paper IV**). The same pattern has been shown in humans where ingested glycerol was rapidly absorbed into blood (Massicotte *et al.*, 2006; Murray *et al.*, 1991). One interesting observation was that calves receiving ORS with glycerol or glucose via an oesophageal probe had a considerably delayed increase (approximately 90 to 180 min) in plasma concentrations of both glycerol and glucose. This is in line with Kaske *et al*. (2005) who reported that calves fed colostrum via drench exhibited a delayed increase of serum immunoglobulin concentrations with approximately 3 h compared to calves fed colostrum via nipple bottle. When calves ingest fluid by drench the oesophageal groove reflex does not progress sufficiently, thus the fluid initially enters into the reticulorumen at a far smaller volume than that of the abomasum of young calves. The delayed uptake of glycerol and glucose observed in the

drenched calves in **Paper IV** was probably due to the passage of ORS from the reticulorumen to the abomasum. These calves were omitted from the statistical analysis in **Paper IV**.

9.1 Reuterin

Glycerol can act as a hydrogen acceptor when *Lactobacillus reuteri* ferments carbohydrates and results in higher growth rates and cell yield of *L. reuteri* than fermentation without the presence of glycerol (Talarico *et al.*, 1990). Also, Chung *et al*. (2011) have shown that *L. reuteri* can produce a basal amount of reuterin by itself and production was enhanced by the presence of heterologous cells *e.g. E. coli*, *Salmonella typhimurium*, *Clostridium sporogenes* and *Streptococcus cremoris*.

Reuterin is a antimicrobial substance produced as an intermediate step when glycerol is converted to 1,3-propanediol where NAD^+ is generated from NADH (Luthi-Peng *et al.*, 2002) (Figure 7). Reuterin undergoes rapid structural degradation and is therefore difficult to quantify in vivo (Luthi-Peng *et al.*, 2002; Talarico *et al.*, 1990). However, Cleusix *et al*. (2008) reported that glycerol was metabolised to 1,3-propanediol in an *in vitro* system with intestinal microbiota from humans. Consequently, this metabolite may act as an indirect marker of intestinal reuterin synthesis. The number of *E. coli*, which is highly sensitive to reuterin, can also be used to estimate the production of reuterin (Cleusix *et al.*, 2008). The viability of *L. reuteri* is affected by reuterin concentrations exceeding 20 to 30 units/ml, whereas *E. coli* is already affected at concentrations of 4 to 5 units/ml (Chung *et al.*, 2011).

One hypothesis was that calves provided with ORS containing a glycerol/glucose mix would have an increased formation of reuterin, and thus decreased faecal shedding of *Enterobacteriaceae* compared with calves provided with ORS containing glucose and control calves during the 10-day experimental period (**Paper IV**). However, results showed that calves receiving ORS with the glycerol/glucose mixture did not respond with an increased concentration of 1,3-propanediol, suggesting that synthesis of the antimicrobial substance reuterin was not amplified in calves provided with glycerol. This is supported by the 16S rRNA sequencing of bacteria, which confirmed that *L. reuteri* was present in faeces, and the proportion of reuterin-positive *Lactobacillus* was similar among treatments during the adaptation period. Furthermore, no differences in faecal shedding of *Lactobacillus* and *Enterobacteriaceae* among treatments were observed. Taken together, these results imply that glycerol was absorbed from the small intestine and was virtually not available for the microbiota in the lower gut. The implication is

supported by the fact that glycerol was not detected in faecal samples. The number of *Lactobacillus* in calf faeces are in agreement with findings by Karney *et al*. (1986). The VFA pattern in faeces was not influenced by ORS treatment, supporting the conclusion that ORS containing a glycerol/glucose mix had no effect on the intestinal microbiota of calves.

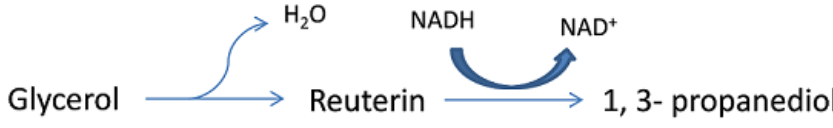

Figure 7. Conversion of glycerol to 1,3-propanediol with reuterin as an intermediate step.

10 Main conclusions

Taken together, the results presented in this thesis show that supplementation of glycerol may be a potential nutrient source for early lactating cows on grass silage based diets, and in ORS to young calves. Calculations based on results from **Paper II**, showed that approximately 70% of the glycerol entering the rumen escaped rumen microbial digestion and was absorbed from the gastro-intestinal tract. This finding is favourable especially for high-yielding dairy cows, as the absorbed glycerol can be efficiently converted to glucose via gluconeogenesis in the liver. Furthermore, results from **Paper IV** showed that glycerol was rapidly absorbed from the gastrointestinal tract in young calves. Based on results from the studies performed in **Paper I-IV**, the following conclusion was drawn:

- There were no significant differences between the two glycerol purities, >99% and 88.1% glycerol respectively, on milk yield or composition, total intake of dry matter or metabolic parameters in dairy cows in early lactation.

- Inclusion at levels of 500 g glycerol/day irrespective of purities used in **Paper I** in diets to lactating cows in early lactation is well tolerated, and may increase the milk yield and enhance the feed conversion rate in dairy cows in early lactation.

- Glycerol can be used as a beneficial component of ORS for young calves in doses of at least 1.0 g/kg BW glycerol (>99% purity) mixed with water (800 ml).

- Oral rehydration solutions containing glycerol may ameliorate the dehydration effect in young calves under feed and fluid deprived

conditions. Hyperglycaemia and hyperinsulinaemia was evaded in calves receiving ORS containing glycerol and suggests a favourable metabolic response to glycerol.

- Synthesis of the antimicrobial substance reuterin was not elevated in young calves receiving ORS containing glycerol.

- The main fraction of glycerol was absorbed across the rumen epithelium (~60%), largely by passive diffusion and probably not facilitated by carriers. Glycerol also disappeared from the rumen compartment via outflow through the omasal orifice (~10%) and was thus available for absorption in the small intestine. Glycerol also disappeared from the rumen compartment by microbial digestion (~30%).

- Glycerol supplementation did not decrease enteric gas production in the gas *in vitro* system used in **Paper III**.

- Evaluation of bacterial and archaeal community structures indicated that the addition of glycerol *in vitro* generated differences at genus level within the bacterial structure with an increased relative abundance of unclassified *Ruminococcaceae*, and *Anaerovibrio*. The dominating sequences within the archaeal structure were related to *Methanobrevibacter thaueri*.

11 Future perspectives

Below, some suggestions for future research in relation to the findings of the present study are noted:

- Currently in Sweden, crude glycerol is hardly used at all in farm animals. However, there is a growing demand for biodiesel worldwide and the capacity to produce biodiesel in Europe today is far higher than demand for it (Biodiesel 2020, 2008). Crude glycerol is generally a more profitable by-product than glycerol (>99%), with approximately half the price of glycerol (>99%). An increased market for crude glycerol might shed a new light on this promising nutrient source in the diet of dairy cows. Furthermore, over-consumption of nitrogen and phosphorus is an issue in numerous feeding strategies. Thus, crude glycerol with negligible amounts of nitrogen and phosphorus could be a favourable nutrient source in feed rations with high proportion of grass silage.

- Presently available results of glycerol inclusion in the diets are inconsistent. Studies elucidating the upper limit of crude glycerol supplementation to dairy cows are further advocated. Furthermore, most of the studies have been conducted on a relatively low number of animals and for short periods. Increased knowledge of the value of crude glycerol as a nutrient source in high-yielding dairy cow diets could be determined by measuring milk production, feed intake and metabolic parameters in a large-scale experiment with different levels of crude glycerol included in the rations and an increased number of animals and days of lactation.

- It is still not investigated how ORS with glycerol affect young calves with diarrhea. To fully explore the potential of ORS with glycerol further studies of young calves affected by diarrhea would be of interest.

- The knowledge and understanding of the interactions, mechanisms and process performance of the microbial population in the rumen when glycerol is added to the rumen of dairy cows could be extended. In the present thesis glycerol was only added to the gas *in vitro* system. *In vivo* studies of glycerol supplementation on the microbial population with focus on adaptive mechanisms diurnal variation *etc.* are therefore advocated. Analysis of the rumen samples by use of modern molecular sequencing techniques could then produce new knowledge in this field.

12 Svensk sammanfattning

Under de senaste decennierna har intresset för biobränslen som framställs av förnybara resurser ökat. Biodieselindustrin genererar stora mängder av restprodukten glycerol (synonym: glycerin, 1,2,3 - propantriol). Expansionen av biodieselindustrin har gett förutsättningar för användning av glycerol som fodertillsats till nötkreatur. Idag är utfodringen av rå glycerol till nötkreatur ovanligt i Sverige. Det finns dock olika tillskottsfoder som innehåller glycerol med renhet >99% på den svenska fodermarknaden. I dagsläget är priset på raffinerad glycerol (>99%) omkring 5-6 SEK/kg, i Sverige.

Glycerol ingår i ämnesomsättningen som en funktionell grupp i triglycerider och i flera andra essentiella lipider, och gruppen behövs för att växter och djur ska kunna lagra energi i form av fett. Glycerol är energirikt och innehåller omkring 16 MJ omsättbar energi/kg torrsubstans för idisslare, och kan omvandlas till glukos i levern via glukoneogenesen.

Glycerol utvärderades redan under 1960 och 1970-talet som ett preparat för behandling av ämnesomsättningssjukdomar hos mjölkkor under tidig laktation. Den höga kostnaden för glycerol hämmade efterfrågan av produkten, men i slutet av 1990-talet när biobränsleindustrin utökades och därigenom genererade stora mängder glycerol som restprodukt sjönk priset. Således har den ökade tillgången på glycerol skapat ett förnyat intresse för användning av glycerol som ett fodertillskott till mjölkkor, speciellt i tidig laktation. Kostnaden för rå glycerol är ungefär halva priset jämfört med raffinerad glycerol och därmed är den mer attraktiv som fodertillsats. Under 2000-talet har olika utfodringsstrategier liksom olika nivåer och renheter av glycerolprodukter använts i utfodringen av mjölkkor i tidig laktation.

Målsättningen med studierna som presenteras i avhandlingen var att ta fram grundläggande information om tillskott av glycerol till mjölkkor och kalvar. Informationen kan sedan utnyttjas av rådgivare och mjölkproducenter, och inom foderindustrin.

12.1 Sammanfattning av studierna och resultat

Utfodring av rå respektive raffinerad glycerol till mjölkkor

Fyrtiotvå kor i tidig laktation användes i försöket för att undersöka effekterna av utfodring med rå respektive raffinerad glycerol. Korna tilldelades en av tre behandlingar: tillskott av 500 g rå glycerol/dag, tillskott av 500 g raffinerad glycerol/dag och en kontroll utan tillskott av extra energi i fodret. Försöksperioden startade vid kalvning och fortsatte under de fyra första laktationsveckorna. Mjölkproduktion, foderintag och ämnesomsättnings-parametrar utvärderades.

Resultaten från studien visade ingen skillnad i mjölkavkastning mellan de två glycerolkvaliteterna. Däremot tenderade mjölkavkastningen att vara högre hos kor som fick raffinerad glycerol jämfört med kontrollkorna utan extra energitillskott i fodret. Varken ensilageintaget eller det totala torrsubstansintaget påverkades av de olika glycerolkvaliteterna, vilket tyder på att metanol och andra föreningar som rå glycerol innehåller inte minskade foderintaget. Kornas energistatus studerades indirekt med markörer i blodplasma, fria fettsyror och ketonkroppar (NEFA och β – hydroxybutyrat). Dessa påverkades dock inte av behandlingarna, och inga skillnader i hullförändring påvisades under den fyra veckor långa försöksperioden, vilket tyder på att kornas energibalans inte påverkades av behandlingen.

Omsättning av glycerol i våmmen

Fyra olika försök genomfördes för att undersöka via vilka vägar glycerol försvinner från våmmen hos idisslare. Det första försöket syftade till att undersöka hur glycerol absorberas över våmepitelet. Från ett slakteri erhölls vävnader från får som användes för isolering av våmepitel. I det andra försöket gavs en engångsdos av 500 g glycerol, tillsammans med en markör till tre våmfistulerade kor för att mäta hur stor andel av glycerolen som passerade ut från våmmen via bladmagsöppningen. Mikrobernas nedbrytning av glycerol studerades *in vitro* i det tredje försöket. Det fjärde försöket syftade till att kvantifiera absorption av glycerol från våmmen genom att ersätta våminnehållet hos tre våmfistulerade kor med buffertlösning som innehöll olika koncentrationer av glycerol (15, 30 och 45 mmol/l glycerol).

Resultaten från studierna visade att den största delen glycerol absorberades över våmepitelet (~60%), med passiv diffusion. Glycerol försvann även från våmmen genom utflöde via bladmagsmagsöppningen (~10%) och blev därmed

tillgänglig för absorption i tunntarmen. Ungefär 30% av glycerolen försvann genom mikrobiell nedbrytning i våmmen.

Metanproduktion och våmmikrobiologi

Den tredje studien syftade till att utvärdera effekten av olika fodermedelstillsatser: glycerol (15 och 30 mmol/l) och extrakt från cashewnötskal (5 och 10 mg), på metanproduktion samt bakterie- och arkéepopulationer i våmmen hos mjölkkor. Våmvätska som samlats från tre våmfistulerade lakterande kor inkuberades i ett gas *in vitro* system under 48 timmar. Vid olika tidpunkter (8, 24 och 48 timmar) togs prover för analys av bildningen av metan och flyktiga fettsyror och för studier av hur mikrobpopulationen i våmmen påverkades.

Metanproduktionen minskade inte när glycerol tillsattes till substratet i gas *in vitro* systemet. Däremot var koncentrationen av flyktiga fettsyror numeriskt högre när glycerol tillsattes jämfört med kontrollen utan fodermedelstillsats. Bakterie- (genusnivå) och arkée- (artnivå) populationerna för glycerolbehandlingen följde i stort samma mönster som *in vitro* kontrollen utan fodermedelstillsats. Det fanns dock några tydliga skillnader på släktnivå inom bakteriestrukturen med en ökning av oklassificerade *Ruminococcaceae* och *Anaerovibrio*. De dominerande sekvenserna inom arkéepopulationen var relaterade till *Methanobrevibacter thaueri*, och resultatet överenstämde med *in vitro* kontrollen utan fodermedelstillsats.

Vätskeersättningar till kalvar

I den fjärde studien undersöktes effekterna av vätskeersättningar med glycerol till kalvar med avseende på ämnesomsättningsparametrar och tarmmikrobiota. Studien var uppdelad i två försök. Under det första försöket gavs vätskeersättningar med glycerol eller glukos till fem kalvar. Försöket var upplagt med en change-over design, där varje behandling varade en dag följt av en sju-dagars återställningsperiod. I det andra tilldelades nitton kalvar en av tre behandlingar: vätskeersättning med glycerol/glukosmix, vätskeersättning med glukos och kontroll (utan vätskeersättning) under 10 dagar. Därefter blev kalvarna fråntagna vätska och foder under 24 timmar för att efterlikna den vätske- och energibrist som kan uppkomma hos sjuka kalvar.

Resultaten från studierna visade att glycerol absorberas snabbt från mag-tarmkanalen och var sannolikt inte tillgänglig för grovtarmens mikrobiota. Ingen ökad syntes av den antimikrobiella substansen reuterin kunde påvisas hos kalvar som gavs vätskeersättning innehållande glycerol jämfört med övriga

behandlingar. Kalvar som inte fick vätskeersättning under den 24 timmar långa perioden då kalvarna inte hade tillgång till foder och vatten hade en tendens till sänkta plasmaglukosnivåer. Plasmavolymen tenderade att minska mer hos kalvar som inte fick vätskeersättning samt hos de kalvar som fick vätskeersättning med glukos än hos kalvar som fick vätskeersättning med glycerol under den 24 timmar långa svältperioden. Detta resultat tyder på att vätskeersättningar innehållande glycerol skulle kunna ha en positiv effekt på kalvar som drabbas av vätske- och energibrist i samband med sjukdom.

12.2 Slutsatser

Sammanfattningsvis visar resultaten som presenteras i avhandling att tillskottsutfodring med glycerol kan fungera både som näringstillskott till mjölkkor under tidig laktation och i vätskeersättningar till unga kalvar. Minst 500 g glycerol/dag kan blandas i foderstaten till kor i tidig laktation, av rå- respektive raffinerad glycerol. Raffinerad glycerol kan öka mjölkavkastning och öka kornas foderutnyttjande i tidig laktation. Vidare kan glycerol (>99%) användas som en komponent i vätskeersättningar till unga kalvar i motsvarande doser som användes i denna studie. Beräkningar baserade på resultat från studierna i Paper II, tyder på att omkring 70% av glycerolen som tillfördes våmmen inte bröts ned av mikroorganismer i våmmen utan istället absorberas från mag-tarmkanalen. Resultaten tyder på att glycerol är särskilt positivt för högavkastande kor, eftersom den glycerol som absorberas effektivt kan omvandlas till glukos via glukoneogenesen i levern. Metanproduktion i våmmen minskade inte vid tillsats av glycerol och den mikrobiella populationen tycktes inte förändras nämnvärt vid tillsats av glycerol.

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