Glycerol to dairy calves – effects on intestinal health and fluid balance

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by

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Institutionen för husdjurens utfodring och vård

Examensarbete 346
30 hp E-nivå

Swedish University of Agricultural Science
Department of Animal Nutrition and Management

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1. ABSTRACT

The bacterium *Lactobacillus reuteri*, which natural habitat is in the intestine of mammals and birds, uses glycerol as a substrate for production of the antimicrobial compound reuterin. Glycerol has been shown to decrease the number of *Escherichia coli* in human feces and it is believed to be due to *in situ* production of reuterin. *E. coli* belongs to the large family of *Enterobacteriaceae,* naturally occurring in the intestine, and pathogenic strains of *E. coli* have been shown to be one of several bacteria causing diarrhoea in calves. Consequences of diarrhoea are dehydration and energy deficiency and without treatment, i.e. addition of oral rehydration solution (ORS), the situation can be life threatening for the calf. Besides the nutritional value of glycerol, it has been shown that glycerol can maintain the fluid balance in humans, rats and steers. There seems to be no studies done concerning ORS containing glycerol given to calves.

One aim of the present study was to investigate if glycerol, by stimulating the formation of the substance reuterin and thus decreasing the amount of *Enterobacteriaceae* in calf feces, has a positive effect on the intestinal flora in healthy young calves compared to glucose and water. Secondly, the aim was to evaluate if glycerol can contribute to maintenance of the fluid balance. Furthermore the aim was to investigate if glycerol supplementation may ameliorate effects on metabolism of 24 h feed and fluid deprivation.

Twenty dairy calves of the Swedish Red Breed, one to three weeks old at the start of the experiment were used. The calves were randomly divided to one of three oral supplementations: glycerol (0.67 g glycerol/kg BW and 0.33 g glucose/kg BW) (n=8), glucose (1g glucose/kg BW) (n=8) and control (without addition of ORS) (n=4). The calves were provided with 400 ml of ORS twice a day during the adaptation period, day 1 to 11. Day 11 to 12, the calves were deprived of feed, milk and water for 24 h accepts for 800 ml ORS at 16.00 hr day 11 and 08.00 hr day 12. Blood and feces samples were collected 2 to 3 days before the start of the treatment (day 0) and after 11 days of treatment (day 11). Further, blood samples were collected after the 24 h long deprivation period (day 12). The blood was analyzed for packed cell volume (PCV), total plasma protein (TPP), plasma osmolality and plasma concentration of glycerol, glucose and insulin. The feces were analyzed for the concentration of short-chain fatty acids, lactate and 1,3-propandediol using a HPLC assay, and bacterial quantification of lactobacilli and enterobacteria was determined by plate counts. Detection of reuterin producing lactobacilli and *E. coli* and determination of 16S rRNA gene sequences of *Lactobacillus* isolates were also performed.

The PCV, concentration of TPP in plasma and osmolality increased from day 11 to 12 for all treatments, however, no differences among treatments were found within or between days. The effect of treatment showed that calves provided with ORS containing glycerol had higher values for plasma osmolality and a tendency to lower amount of PCV in blood, compared to the other treatments. The concentration of glycerol in plasma was shown to be higher for calves provided ORS with glycerol day 11 and 12 compared to calves treated with glucose and control calves. The concentration of glucose in plasma increased from day 11 to 12 in calves provided ORS with either glycerol or glucose whereas the glucose concentration in plasma tended to decrease in control calves. The insulin concentration in plasma increased from day 11 to 12 in calves treated with glucose. The concentration of short-chain fatty acids, lactate and 1,3-propandediol in feces remained unchanged irrespective treatment and day. The number of *Lactobacillus* and *Enterobacteriaceae* in feces was not affected by treatment. The number of *Enterobacteriaceae* decreased from day 0 to 11, whereas the number of

Lactobacillus remained unchanged. *L. reuteri* and *E. coli* was detected in feces regardless of treatment both day 0 and 11.

It can be concluded that ORS containing a mixture of glycerol/glucose can maintain the fluid balance in calves in greater extent as ORS containing glucose. Calves receiving pure glucose ORS were markedly hyperglycemic and hyperinsulinemic, whereas calves receiving ORS containing a mixture of glycerol/glucose did not develop hyperglycemia and insulin fluctuated less. ORS containing a mixture of glycerol/glucose was shown to not change the amount of lactobacilli and *E. coli* in these healthy young calves. Probably glycerol was efficiently absorbed in the small intestine and thereby not available for the intestinal microbiota. Further studies are needed to determine whether glycerol, alone and not in a mixture with glucose, possesses health promoting properties in calves. It is also suggested that further studies focus on the effect of glycerol on either fluid balance or intestinal flora in calves.

2. INTRODUCTION

Glycerol can be derived from the production of biodiesel and due to the growth of the biodiesel industry the availability of glycerol as a feed supplementation for cattle has increased (Bernesson, 2007). Glycerol is an energy-rich, glucogenic substance which can improve the energy supply to dairy cows both before and after calving (Schröder and Südekum, 1999). One of the aims of the study is to investigate if glycerol besides its nutritional value possesses properties beneficial for the health in calves.

Functional feed can be defined as feed supplement which besides its nutritional value also contains components which may improve health (Roberfroid, 2000). The bacterium *Lactobacillus reuteri* has its natural habitat in the gut in mammals and birds and produce a substance called reuterin in the presence of glycerol (Axelsson *et al*., 1989). Reuterin has broad-spectrum antimicrobial properties and can inhibit growth of e.g. *Escherichia coli* (Spinler *et al*., 2008). A low concentration of glycerol (10mM) has been shown to be enough to decrease the number of *E. coli* in human feces and the result was assumed to be due to *in situ* production of reuterin (Cleusix *et al*., 2008).

The most common disease that affects young calves is diarrhoea (Radostits *et al*., 2007). Diarrhoea among calves results in great economic losses due to treatment costs, reduced growth rate and in worst case death (Millemann, 2009). Infectious agents that can be found in feces samples from diarrheic calves are e.g. *Cryptosporidium* (Silverlås *et al*., 2009), *E. coli* and rotavirus (de la Fuente *et al*., 1999; Gulliksen *et al*., 2009). Among the consequences of diarrhoea are dehydration and energy deficiency and it is therefore of great importance that the calf acquires both milk and oral rehydration solution (ORS) in order to maintain fluid and electrolyte balance as well as the energy balance (Radostits *et al*., 2007). Without treatment the situation can be life threatening for the calf. ORS found on the Swedish market often contains of glucose as the main energy source (DeLaval, 2011; Lantmännen, 2011). There seems to be no studies investigating ORS containing glycerol given to calves even though glycerol has been shown to support the fluid balance in humans (Freund *et al*., 1995), rats (Allen *et al*., 1999) and steers (Parker *et al*., 2007).

An aim of the present study was to investigate if glycerol has a positive effect on the intestinal flora in healthy young calves. The hypothesis was that glycerol stimulates the formation of reuterin and thus decreasing the amount of *Enterobacteriaceae* in calf feces compared to glucose and control. Furthermore, the aim was to evaluate if glycerol can contribute to maintenance of the fluid balance and to inverstigate if glycerol supplementation may ameliorate effects on metabolism of 24 hours feed and fluid deprivation. The hypothesis was that feed and fluid deprived calves receiving glycerol have lower packed cell volume (PCV) and total plasma protein concentration (TPP) than calves receiving only glucose which would indicate that they were better off in maintaining the plasma volume during deprivation.

3. LITERATURE REVIEW

3.1. Functional feed

The primary role of the diet is to provide nutrients to meet the nutritional need (Roberfroid, 2000). Further, it is important that the appearance, texture and taste of the diet meet the preferences. During the last years more attention has been focused on the diet´s beneficial health effects especially concerning human diets and the concept functional food has been developed. Functional food should besides meeting nutritional needs also promote well-being and reduce the risk for diseases. The concept has gained increased interest in the animal feed industry. Garlic flakes aimed for horses is stated to have beneficial effects on e.g. the immune defense and digestion (Timotej and Andersen, 2011) and oligosaccharides aimed for birds is claimed to stimulate the growth of *Bifidobacterium* and *Lactobacillus* in the intestine (JH Biotech, 2011) are examples of products found on the feed market. In the American feedlot system where concentrate stand for the main part of the diet, forage are considered as functional feed since it helps to avoid digestive dysfunctions (Ware and Zinn, 2004). At the Swedish feed market concentrate and milk replacer with vitamin E and betaine for calves can be found. Vitamin E is claimed to support the immune system and betaine is claimed to reduce the risk of diarrhoea (Svenska Foder, 2011). Functional feed with glycerol aimed for calves seems not yet be on the market.

Addition of strains of lactobacilli to feed stuff to enhance the utilization of feed, performance and/or the health of the calf has been studied. *Lactobacillus acidophilus* added to milk replacer has been shown to maintain initial body weight compared to control calves fed milk replacer without *L. acidophilus*, who lost approximately 112 g/day up to 2 weeks of age (Cruywagen *et al*., 1996). *Lactobacillus plantarum* in milk replacer given to Holstein calves has been shown to increase the daily weight gain and feed efficiency (Kawakami *et al*., 2010). The bacterium *Lactobacillus reuteri* has been shown to be an effective therapeutic agent against acute rotavirus diarrhoea in children (Shornikova *et al*., 1997) but it seems not yet been studied as feed additive for calves.

3.1.1. Lactobacillus reuteri

L. reuteri is a heterofermentative bacterium that can be found in the gastrointestinal tract of mammals and birds (Axelsson *et al*., 1989; Talarico *et al*., 1989). In the presence of glycerol, resting cells of *L. reuteri* produce a substance called 3-hydroxypropionaldehyde (3-HPA) also known as reuterin (Chung *et al*., 1989). Reuterin is a broad- spectrum antimicrobial substance with ability to inhibit growth of Gram negative and Gram positive bacteria, yeasts, moulds and protozoa. *L. reuteri´*s production of reuterin occurs during anaerobe conditions at 37℃ and pH ranging from 5 to 9, whereas reuterin has been shown to be active during both anaerobic and aerobic conditions (Axelsson, 1990). Axelsson (1990) showed that not all strains of *L. reuteri* from pigs have the ability to produce reuterin.

When *L. reuteri* ferment carbohydrates, glycerol works as a hydrogen acceptor, resulting in higher growth rates and cell yield of *L. reuteri* compared to when no glycerol is available (Talarico *et al*., 1990). Reuterin is produced as an intermediate step in the conversion of glycerol to 1,3- propanediol where NAD^+ is generated from NADH (Figure 1) (Luthi-Peng *et al*., 2002).

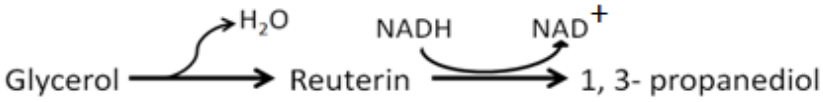


Figure 1. *Conversion of glycerol to 1,3- propanediol with reuterin as an intermediate step.*

Chung *et al*. (1989) showed that *L. reuteri* was able to produce a basal amount of reuterin by itself but the production was significantly increased in the presence of heterologous cells e.g. *E. coli, Salmonella typhimurium, Clostridium sporogenes and Streptococcus cremoris.* Reuterin is a bioactive substance that undergoes rapid structural changes and is therefore difficult to quantify in vivo (Talarico *et al*., 1990). Two markers can be used to estimate the production of reuterin; the concentration of 1,3- propanediol and the amount of *E. coli* which is highly sensitive to 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 affected at concentrations of 4 to 5 units per ml (Chung *et al*., 1989). Schaefer *et al*. (2010) suggested that reuterin induce oxidative stress in cells by modifying thiol groups in proteins. The mechanism of action of reuterin is still unclear despite many years of investigation.

3.1.2. Glycerol

The interest of biofuels made from renewable resources has increased, because of its environmental benefits (Fangrui and Milford, 1999). Biodiesel, a renewable fuel, is produced by transesterification of natural oils and fats and glycerol is a by-product from the biodiesel production. The interest of glycerol as a feed supplement to cattle has increased during the last years due to decreased prices of glycerol (DLBR LandbrugsInfo, 2011). The structure and chemical properties of glycerol have similarities with propylene glycol and propionic acid (Figure 2). Propylene glycol is used as a feed supplement to cattle because it is assumed to increase milk production and prevent ketosis (Nielsen and Ingvartsen, 2000). Propionic acid is produced when the rumen microorganisms ferment carbohydrates and the only acid that can be converted to glucose in animal cells (Sjaastad *et al*., 2003).

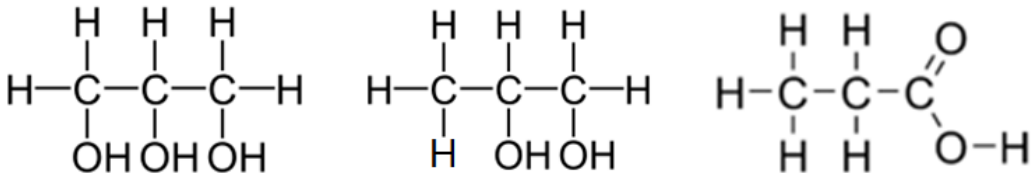


Figure 2*. Chemical composition of glycerol, propylene glycol and propionic acid.*

Glycerol is a water-soluble, colorless, odorless, syrupy liquid with a distinct sweet taste (Hart *et al*., 2003). Further, glycerol forms the backbone of triglycerides and is essential for the synthesis of fat in animals and plants. When the plasma concentration of glucose decreases glycerol can be used in the process of gluconeogenesis to form more glucose (Sjaastad *et al*., 2003).

3.1.3. The roll of glucose and insulin

Glucose is a common and important energy source for the body e.g. used by red blood cells and the brain, moreover the only energy source used by the brain in nonstarvation situations (Berg *et al*., 2003). Glucose is the major monosaccharide absorbed from the intestine in monogastric and pre-ruminating animals and is transported to the liver via the portal blood system (Sjaastad *et al*., 2003). The liver keep the concentration of glucose in the blood stable

either by absorbing excess glucose and store it as glycogen or by exporting glucose to the blood by mobilizing glycogen stores or produce glucose from other sources. The concentration of glucose in plasma can differ markedly between newborn and adult animals (Radostits *et al*., 2007). The concentration of glucose in plasma is for a 24 h old calf approximately 7.2 mM/l (Radostits *et al*., 2007) and for a three week old calf it ranges from 2.9 to 4.7 mM/l (Katoh *et al*., 2004; Radostits *et al*., 2007).

Insulin is a peptide hormone which responds to the concentration of glucose and amino acids in the blood (Sjaastad *et al*., 2003). The secretion of insulin is stimulated by hormones in the gastrointestinal tract which are released when nutrients are absorbed from the small intestine. The most important function of insulin is to regulate the plasma glucose concentration but also to increase triglyceride synthesis and regulate plasma concentration of amino acids. Katoh *et al*. (2004) concluded that the insulin concentration in calves is affected by age and also the quality of the diet may be involved in insulin alterations.

3.2. The gastrointestinal tract of the calf

3.2.1. Anatomy and development

The forestomach in the young calf is not fully developed and the digestion and absorption of nutrients is therefore comparable to monogastric animals (Phillips, 2010). The development of the forestomach takes part during the first months of the calf´s life. Tamate *et al*. (1962) compared the impact of diet on the development of the stomach in calf. The diets consisted of milk or milk, hay and grain or milk plus various substances such as fatty acids or plastic sponges administered into the rumen. Calves fed milk, hay and grain had a faster development of the forestomach compared to calves fed solely milk. Butyric and propionic acid stimulated the papillary development in the rumen if administered in sufficient amount and the plastic sponges promoted an increase in the muscular development of the rumen.

3.2.2. Microbiota

The ecosystem in the gastrointestinal tract (GI-tract) consists of a highly complex structure of bacteria and its function and composition is of high importance when it comes to the individual health and nutrition (Axelsson *et al*., 1989; Conway, 1997). The bacterial colonization of the GI-tract in the offspring starts both during and after birth (Conway, 1997). Factors that influence the bacterial structure of the GI-tract is e.g. contact with adults, environment at birth, diet and antibiotics. The microbiota in calves consists almost entirely of coliforms and streptococci after a few days of life, shortly after clostridia and lactobacilli are present. The microbiota in the calf´s intestine shows high diversity and differs between locations in the GI-tract (Contrepois and Gouet, 1973; Jonsson, 1985; Kawakami *et al*., 2010), varies with age (Hartman *et al*., 1966; Contrepois and Gouet, 1973; Jenny *et al*., 1991; Rada *et al*., 2006) and is affected by diet (Hartman *et al*., 1966; Lukas *et al*., 2007).

3.3. Diarrhoea in the neonatal calf

Diarrhoea can be defined as major loss of water in the feces due to an increase in the fecal water content, an increase in the volume of feces excreted or a combination of the two (Radostits *et al*., 2007). The cause of diarrhoea can be inflammation of the intestine, malabsorption, indigestible diet, carbohydrate engorgement etc.

Diarrhoea is commonly occurring among calves (Radostits *et al*., 2007) and is an important cause of neonatal calf mortality (Barrington *et al*., 2002). In a Swedish study the morbidity in 3081 dairy heifer calves from birth to 90 days of age was investigated (Svensson *et al*., 2003),

where 317 calves developed diarrhoea and made diarrhoea to the most commonly occurring disease in the study with an incidence risk of 9.8%. Further, 75% of the calves developed diarrhoea in a Danish/Swedish study with 68 preweaned dairy calves (Svensson and Jensen, 2006). In a Dutch study with 424 calves, three weeks old or younger, from 108 farms, found that 43% of the calves had non-normal feces (Bartels *et al*., 2010). The texture of the feces was graded according a three-graded scale, where normal feces, scored 0, was defined as firm consistency, brown color and the perineum and tail was clean and dry and score 1 represented feces with a paste-like consistency, yellow color and feces scored 2 had a watery consistency. The perineum and/or tail was smeared with feces at both score 1 and 2.

The etiology of calf diarrhoea is multifactorial and diarrhoea occurs when the balance between the immunity of the calf, the exposure of infectious agents and the calf´s environment is disturbed (Lundborg *et al*., 2005; Gulliksen *et al*., 2009). Svensson *et al*. (2003) showed that diarrhoea is most prevalent in calves at a young age and is decreasing with increasing age (Figure 3).

The occurrence of different enteropathogens differ with age (Björkman *et al*., 2003; Smith, 2009a), enterotoxigenic *E. coli* is the most common enteropathogen in the age of 1 to 3 days and rotavirus in the age of 7 to 9 days (Smith, 2009a). Bartels *et al*. (2010) investigated the occurrence of *E. coli*, *Cryptosporidium parvum*, coronavirus, rotavirus and *Clostridium perifringes* in feces from 424 calves, in the age of 1 to 21 days. The study showed that *E. coli* was the least detected enteropathogen whereas *C. perifringes* was the most detected. In a Norwegian study with feces from 759 dairy calves it was shown that rotavirus and *Cryptosporidium* was the most detected enteropathogen (Gulliksen *et al.*, 2009). Björkman *et al*. (2003) conclude that rotavirus was the major pathogen causing diarrhoea whereas coronavirus and *E. coli* was of less importance in calves in the age up to 90 days.

Common methods to treat calves with diarrhoea are usage of oral rehydration solution (ORS), reduction or withdrawal of the milk/milk replacer or the concentration ratio, usage of antibiotics and homeopathic drugs (Svensson *et al*., 2003).

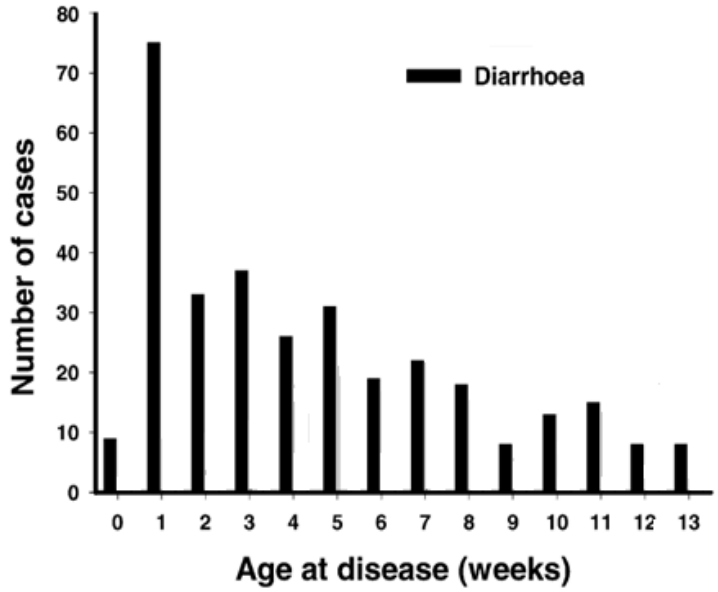


Figure 3. *Age distribution of 317 diarrheic dairy calves from 0 to 90 days (Svensson et al., 2003).*

3.3.1. Dehydration

Dehydration and deficit of energy are the most common consequences of diarrhoea (Radostits *et al*., 2007). The lack of water in a dehydrated calf also causes an imbalance of the electrolytes in the body and the situation can be life-threatening. The first response to dehydration is withdrawal of fluid from the tissues to maintain a normal level of blood volume. The second response is reduction of the blood volume, causing an increased packed cell volume (PCV). The parameters PCV, total plasma protein (TPP) and osmolality generally increase in a dehydrated animal. However, Dalton (1967b) investigated the effect of starvation on plasma composition in calves and it was shown that the plasma osmolality did not differ between pre-starvation and the fourth day of starvation. Möllerberg *et al*. (1989) found that the PCV in healthy, one and two week old, dairy calves was 32±3 and 30±2, respectively. Dalton (1967a) investigated variations in calf plasma composition in relation to age and found that the PCV and plasma osmolality did not vary from birth to the age of 22 days, with means of 43% and 284 mOsmol/l, respectively. The same study also showed that TPP varied with age, newborn calves and calves at the age of 3-5 days had an average TPP concentration of 4.9 g/100 ml plasma and 6.0 g/100 ml plasma, respectively.

Skin tent duration is a useful clinical tool to identify dehydrated calves. It is estimated by picking up the skin on the neck into a fold and count the seconds it takes for the skin to go down to its start position (Radostits *et al*., 2007; Smith, 2009b) (Table 1). The longer the skin remains folded the more severe is the dehydration. Eyeball recession is also an indicator of the degree of dehydration but may not be as accurate as skin tent duration since the position of the eyeballs depends on the body fat stores and can vary between individuals (Smith, 2009b).

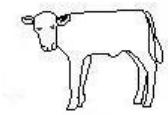
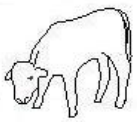
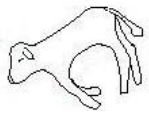
3.3.2. Oral rehydration solution (ORS)

The most important and widespread method to compensate losses due to diarrhoea and dehydration is the usage of ORS (Svensson *et al*., 2003; Wapnir *et al*., 1996). ORS has to fulfill four requirements; supply sodium to normalize the extracellular fluid volume; provide agents that improves the absorption of sodium and water from the intestine; provide an alkalinizing agent to correct the metabolic acidosis that often occur in diarrheic calves and to provide energy (Smith, 2009b). It has previously been suggested to withdraw the milk from diarrheic calves to reduce the severity of diarrhoea and only provide ORS (Smith, 2009a) but it can also cause negative effects (Heath *et al*., 1989). Nowadays, it is recommended to keep feeding the diarrheic calves milk to prevent energy deficiency (Svenska djurhälsovården, 2011).

Examples of ORS found on the Swedish market are Feedtech® electrolyte (DeLaval, 2011), Effydral® (Effydral, 2011) and Protect Diakur®Super (Lantmännen, 2011), with glucose or lactose as energy source. There seems to be few if any studies where ORS containing glycerol given to calves has been used even though glycerol has been shown to improve the fluid balance of several other species (Freund *et al*., 1995; Allen *et al*., 1999; Parker *et al*., 2007). Parker *et al.* (2007) exposed 2.5-year-old *Bos indicus* steers to stress during 48 h of transportation and showed that steers given 2 g glycerol/kg BW before transportation, maintained the body water better than steers without addition of glycerol. Humans, in rest, who ingested a solution containing glycerol, increased the fluid retention compared to when water alone was ingested and it was due to lower urine flow rates associated with lower free water clearance (Freund *et al*., 1995). Allen *et al*. (1999) showed that if ORS with glucose is replaced by two thirds with glycerol, the water and sodium absorption is improved in rats.

Further, Wapnir *et al*. (1996) showed that the net water absorption/secretion was greater for ORS with glycerol compared to glucose in rats.

Table 1. *Degree of dehydration and associated symptoms (Radostits et al., 2007; Smith, 2009b; Extension, 2011)*

Body water loss (%)	Symptoms	
0-4	No clinical signs, skin tent duration < 1 s	
4-6	Decreased urine output by calf, PCV 40-45 %	
6-8	Mild depression, dry mouth and nose, further reduction in urine output, skin tenting 1-2 seconds, PCV 50 %, 2-4 mm eyeball recession	
8-10	Calf depressed, laying down, dry gums, skin tenting 2-5 seconds, PCV 55%, 4-6 mm eyeball recession	
10-12	Calf remains down, legs and ears cold, skin tenting 5-10 seconds, PCV 60%, 6-8 mm eyeball recession	
12-14	Shock and death, skin tenting > 10 s, 8-12 mm eyeball recession	

4. MATERIAL AND METHODS

The study was carried out at Kungsängen Research Centre at the Swedish University of Agricultural Sciences in Uppsala, Sweden, and the experimental design and all handling of animals were approved by Uppsala Ethical Committee, Sweden.

4.1. Animals, Management and Experimental Design

Twenty dairy calves, nine heifers and eleven bulls, of the Swedish Red Breed, in average 14 days old (range 7 to 21 days), during a period of twelve days, were used in the study. The experimental period was divided into two parts, adaptation period (day 1-11) and deprivation period (day 11-12).

The calves were separated from their dams within 24 h after birth, and moved to straw-bedded single pens (1.0×1.2 m^2) in a separate indoor calf barn during three days (Figure 4.). The calves were fed approximately 2.5 l of colostrum from their dams twice a day from a nipple bottle. At day 4 they were moved to group pens, with maximum 14 calves/pen, (5.9×5.0 m^2) with slatted concrete floor and a straw-bedded area. A commercial milk replacer (Elitekalv Xtra, Kvarnbyfoder, Sweden) was provided by a transponder controlled automatic milk feeder (CF300A, DeLaval, Tumba, Sweden). The ration of milk replacer was 5 l/day between 4 to7 days of age and then increased with 0.5 l/day until the final ration of 10 l/day was reached at approximately 15 days of age. Commercial concentrate (Talang, Lantmännen, Sweden) was provided by a transponder controlled automatic feeder (CF300A, DeLaval, Tumba, Sweden). The ration of concentrate was 0.25 kg/day at start and then gradually increased to a maximum of 1.5 kg/day. Round bale silage, first cut, was provided ad libitum in troughs and drinking water was available in automatic water bowls.

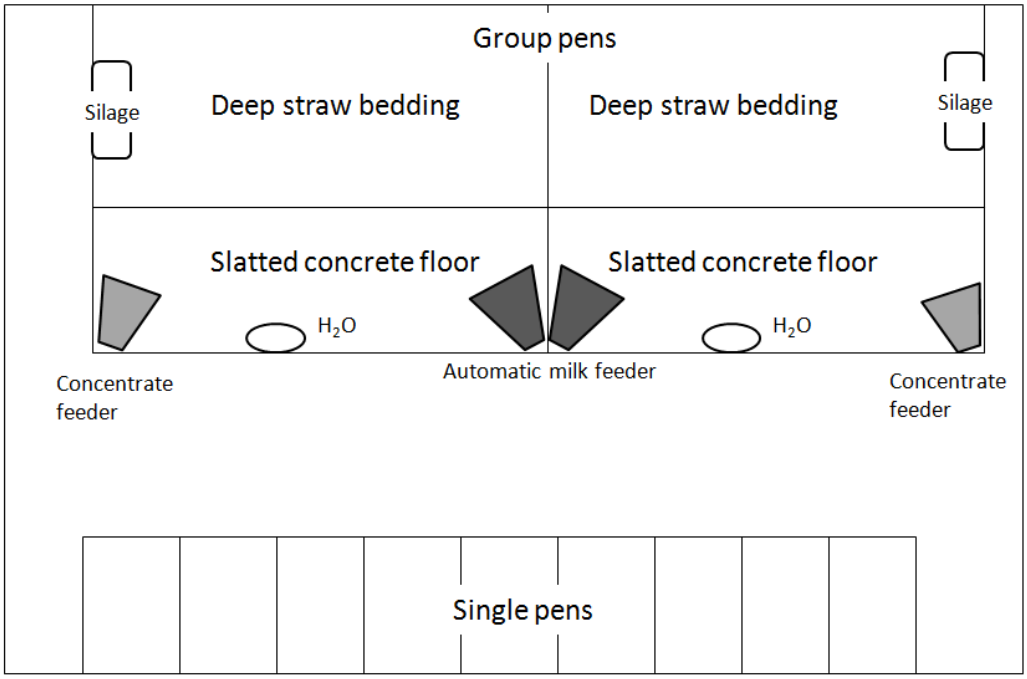


Figure 4. Schematic outline of indoor calf barn.

The calves were divided according to expected day of birth into four groups. Calves within the same group were kept in the same pen and were given the treatment simultaneously. Calves in each group were randomly assigned to one of three oral supplementations: 0.67 g

glycerol/kg BW and 0.33 g glucose/kg BW (glycerol) (n=2), 1g glucose/kg BW (glucose) (n=2) and control (n=1) without addition of ORS during d 1 to 11.

During the adaptation period, day 1 to 11, the ORS (400 ml) was adjusted to body temperature and offered twice a day (08.00 and 16.00 h) from nipple bottles in excess of ordinary feeding ratio and water. The automatic milk feeder was blocked for two h before feeding to increase the calves' willingness to ingest the oral supplementation. During the deprivation period, day 11-12, the calves were kept in straw-bedded single pens, without access to feed, milk and water. Body tempered ORS (800 ml) was offered at 16.00 h at day 11 and 08.00 h at day 12 from nipple bottles. The control group was offered 800 ml of body tempered water at the same occasion day 11 and 12

4.2. Sample collection

Control samples of feces and blood were collected 2 to 3 days before the start of the experiment (day 0). Further samples were collected during the morning on day 11 (blood and feces) and 12 (blood), approximately one h after the ORS had been provided the calves. Blood was collected from the jugular vein into heparinised 10 ml vacuum tubes with lithium heparin as anticoagulant (Venoject, Terumo Europe N.V., Leuven, Belgium). Approximately 40 g of feces were obtained fresh from the calves upon rectal stimulation and transferred into 50 ml sterile tubes. The texture of the feces were scored and recorded according to a five-graded scale, ranged from firm, dry feces to watery blood mixed feces (Silverlås, pers. comm.). Approximately 1 g of feces from each sample were transferred into sterile 1.8 ml tubes and stored in -70°C until bacterial quantification. The rest of the feces samples were stored in sterile 50 ml tubes at -20 °C until high performance liquid chromatography (HPLC) analysis.

At each sampling occasion the calves were weighed and body temperature was measured to control the health status of the calves. The calves' weight, skin tent duration in the neck region and eyeball recession was performed on a four-grade scale, from normal to severed dehydrated calf, before and after the deprivation period as a measure of the degree of dehydration (Radostits *et al*., 2007; Smith, 2009b).

4.3. Analysis

4.3.1. Blood analysis

The blood was analysed for PCV and TPP within one h after sampling. For analysis of PCV a subsample was transferred into micro haematocrit tubes and centrifuged at 3000 x *g* for 10 min at room temperature. The blood samples were centrifuged at 1800 × *g* for 25 min at 20°C. The plasma was harvested and TPP was determined by refractometry (Master Refractometer, Atago, Tokyo, Japan). The residual plasma samples were stored at -20°C until further analysis of osmolality, insulin, glucose and glycerol.

After thawing, all plasma samples were centrifuged at 3000 x *g* for 5 min at room temperature. Plasma osmolality was measured by freezing point depression (Model 3250 Osmometer, Advanced Instruments, Inc., Massachusetts, USA). Plasma was analysed for glycerol and glucose using an enzymatic colorimetric test (Glycerol, R-BIOPHARM AG, Darmstadt, Germany and GLUCOSE liquicolor, Human, Wiesbaden, Germany),by spectrophotometry (Shimadzu UV-1800, Shimadzu Suzhou Instruments Wfg. Co., Jiangsu, China). The concentration of plasma insulin was analyzed with a commercial enzyme

immunoassay method evaluated for bovine (Mercodia Ultrasensitive Bovine Insulin ELISA, Uppsala, Sweden).

4.3.2. Feces analysis

4.3.2.1. HPLC

The concentrations of acetate, propionate, *n*-butyrate, lactate and 1, 3-propanediol, were quantified using a HPLC assay described by Andersson and Hedlund, (1983). Five g of feces were mixed with 5 mM sulfuric acid to a total volume of 25 ml. The samples were placed in an ultrasound bath for 5 min at 25°C and then centrifuged at 2100 × *g* for 10 min at 20°C. The extract was filtered through a 0.2 µm nylon membrane and 200 µl of the clear solution was mixed with 800 µl of the internal standard solution (0.1 % of pivalic acid in 5 mM sulphuric acid) before injection into a HPLC-system that consisted of Alliance 2795 Separations Module together with a Temperature control Module II and 2414 RI Detector (Waters Corporation, Massachusetts, USA). Column packet ReproGel H 9 µL 300x8 mm (Dr. Maisch HPLC GmbH, Ammerbush-Entringen, Germany) was used for the separations and maintained at 60°C, using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.8 ml/min.

4.3.2.2. Microbial analysis

Bacterial quantification

Bacterial quantifications of lactobacilli and enterobacteria in feces sample from day 0 and 11 for each calf were performed. The concentration of lactobacilli was determined by plate counts on Rogosa agar (Merck, Darmstadt, Germany), incubated in anaerobic jars at 37°C for 48 h and the concentration of enterobacteria was determined by plate counts on Violet Red Bile Glucose (VRBG) agar (Oxoid, Basingstoke, UK), incubated at 37°C for 24 h.

Reuterin assay

Detection of reuterin producing *Lactobacillus* bacteria were done on 51 fresh plates with growth of approximately 20 colony forming units (CFU) of *Lactobacillus* per plate, both samples from day 0 and 11 and all treatments were tested. Briefly, 10 ml of glycerol agar (500 mM, 1% agar) were poured on fresh plates with colonies of lactobacilli and incubated at 37°C for 30 to 45 min. Further, 5 ml of 0.1 % 2,4-dinitrophenyl hydrazine in 2 M HCl were added and then poured of after 3 min and additionally 5 ml of 5 M KOH were dispensed. Positive bacterial colonies were surrounded by red-brown zones.

Determination of 16S rRNA gene sequence of bacteria

Bacteria of *Lactobacillus* were cultured on MRS plates, i.e. 6 CFU positive for reuterin and 4 CFU negative for reuterin were picked and suspended in 10 µL sterile water in PCR tubes. The Ready-To- Go PCR beads (GE Healthcare, Buckinghamshire, UK)) were used for the PCR reaction. Adding 0.5 µl DNA, 1 µl 8f primer (5´-AGAGTTTGATCCTGGCTCAG-3´; 10 pmol/ µl), 1 µl 926r primer (5´-CCGTCAATTCCTTTRAGTTT-3´; 10 pmol/µl) and 22.5 µL sterile water and the PCR program 30 x (94°C for 40 s, 55°C for 40 s, 72°C for 60 s), 72°C for 7 min was used. The PCR products were analysed by agarose gel electrophoresis and purified with QIAquick PCR Purification kit (Qiagen, Hilden, Germany). The amplified gene was sequenced at Uppsala Genome Center, Rudbeck laboratory, Uppsala, Sweden using the primer 8f. The sequences were compared to the GenBank database using standard nucleotide BLAST at NCBI (http://www.ncbi.nih.gov) as well as with the Ribosomal Database Project 10 (http://rdp.cme.msu.edu) analysis tool Sequence Match.

Confirmation of E. coli

Enterobacteriaceae isolated from eleven fecal samples i.e. 4 glycerol, 4 glucose and 3 controls, from day 11 were tested. Five colonies per sample were inoculated to a pre-warmed tube containing Lactose-Tryptone-Lauryl Sulphate Broth (Oxoid, Basingstoke, UK) and a Durham tube, for detection of gas production, and incubated at 44°C for 24 h. A positive result was indicated by gas production. Indole production was detected by addition of 0.5 ml of *Kovac's reagent*. A red color appeared in tubes positive for indole. Bacteria producing gas and indole were regarded as *E. coli*.

4.4. Statistical analysis

Nineteenth calves completed the experiment. One calf treated with glycerol was excluded from the study due to general in appetence during the experiment. Blood samples from five calves were excluded from the statistical analyses day 11, due to a mistake in the daily management routines. Statistical analyses of the difference among treatments were made using procedure MIXED in SAS (SAS 9.2, SAS Institute, 2008). The model contained fixed effects of treatment (3 levels), within day (3 levels) and between days (2 levels), group (4 levels) and all 2 way interactions. The effects of treatment on plasma concentrations of glycerol, glucose, insulin and TPP, and concentration of PCV in blood, and amount of *Enterobacteriaceae* and *Lactobacillus* in feces were analyzed using repeated measures by day. Unstructured covariances were used in all models for samples within calf. The fixed effects were considered significant at $P < 0.05$, and pair wise comparisons were made to evaluate significant differences. Non-significant interactions ($P>0.10$) were excluded from the model for each variable tested, except for the interaction between day and treatment. Effect of sex was tested in the initial model, but the effect was not significant and therefore dropped from the model. Results from the statistical analyses are presented as least squares means and associated standard error of the mean.

5. RESULTS

5.1. Blood and plasma composition

The PCV and concentration of TPP in blood did not differ among treatments, within (P=0.57) and (P=0.17) or between days (P=0.39) and (P=0.32), respectively (Table 2 and 3). However, the effect of treatment tends to differ with higher PCV (P=0.11) and concentration of TPP (P=0.14) in control calves. The PCV in blood decreased from day 0 to 11 (P=0.005) while it increased from day 11 to 12 (P=0.02) for all treatments. Calves in group 2 had higher amount of PCV in blood and plasma osmolality compared to calves in group 1 and 4 (P=0.02), and group 3 and 4 (P=0.005), respectively, probably due to a rotavirus infection in the barn. Day was significant both within and between days, (P=0.003) and (P=0.002), respectively, with increased concentration of TPP in plasma from day 11 to 12 (P=0.002) for all treatments. The plasma osmolality did not differ among treatments, within (P=0.51) or between days (P=0.40). However, the plasma osmolality increased from day 11 to 12 (P=0.005) for all treatments. The effect of treatment showed that calves provided with ORS containing glycerol had higher values for plasma osmolality compared to the other treatments (P=0.03).

Calves provided with ORS containing glycerol had higher concentration of glycerol in plasma day 11 and 12 (P<0.001) compared to ORS with glucose or control and also increased glycerol concentration in plasma day 12 compared to day 11 (P<0.001). Calves provided with ORS containing glucose had the highest concentration of glucose in plasma whereas the control group had the lowest at day 12 (P<0.001). The concentration of glucose in plasma increased, from day 11 to 12, for calves provided with ORS containing glucose and glycerol, (P=0.002) and (P<0.001), respectively. Further, the glucose concentration in plasma tended to decrease (P= 0.06) in control calves from day 11 to 12. Calves in group 4 had higher concentration of glucose in plasma compared to group 1, 2 and 3, (P=0.02). The concentration of insulin in plasma increased from day 11 to 12 for calves provided with ORS containing glucose (P=0.001) and there was a tendency for calves provided with ORS containing glycerol (P=0.08). The concentration of insulin in plasma did not differ among treatments within days (P=0.07). Day was significant within day (P=0.03), with increased concentration of insulin in plasma from day 11 to 12 for all treatments. There was a difference in treatment with higher values for glucose compared to the other treatments (P=0.03). Calves in group 1 and 4 had higher concentration of insulin in plasma compared to calves in group 2 and 3 (P=0.02).

Table 2. *The effect of oral rehydration solution containing glucose or glycerol on packed cell volume, total plasma protein, plasma osmolality, plasma concentration of glycerol, glucose and insulin in dairy calves, within day. Data presented as least squares means ± SEM*

Treatment[1]	Glycerol	Glucose	Control	Glycerol	Glucose	Control	Glycerol	Glucose	Control
Day		0			11			12	
PCV[3], %	40±1.5	40±1.4	42±2.3	36±0.8	37±0.7	38±1.0	36±1.4	39±1.3	42±1.7
TPP[4], g/L	52±2.3	54±2.1	50±3.0	51±1.7	49±1.5	54±2.1	53±2.3	54±2.1	58±2.9
Osmolality, m*Osm*/kg	285±3.3	285±3.1	281±4.3	284±1.6	279±1.5	278±1.9	292±2.1	287±2.0	281±2.6
Glycerol, m*M*/L	0.2±0.16	0.4±0.15	0.2±0.21	3.3±0.44^{a}	0.1±0.40^{b}	0.2±0.56^{b}	8.1±0.27^{a}	0.1±0.25^{b}	0.2±033^{b}
Glucose, m*M*/L	5.3±0.22	5.3±0.21	5.6±0.29	5.9±0.59	7.0±0.53	5.5±0.74	8.2±0.70^{a}	13.5±0.63^{b}	4.0±0.86^{c}
Insulin, µg/L	0.3±0.03	0.3±0.03	0.4±0.04	0.3±0.16	0.5±0.15	0.4±0.21	1.2±0.44	2.4±0.41	0.1±0.54

[a-c] Values within a row with different superscripts differ (P<0.05) within day, [1] Oral rehydration solution; Glucose (1g glucose/kg BW), Glycerol (0.67 g glycerol/kg BW and 0.33 g glucose/kg BW), Control (water) [3] Packed cell volume [4] Total plasma protein

Table 3. *The effect of oral rehydration solution containing glucose or glycerol on packed cell volume, total plasma protein, plasma osmolality, plasma concentration of glycerol, glucose and insulin in dairy calves, between days. Data presented as least squares means ± SEM*

Treatment[1]	Glycerol		Glucose		Control		
Day	11	12	11	12	11	12	P-value[4]
PCV[2], %	35±1.0	36±1.5	37±0.8	39±1.3	38±1.0	42±1.8	0.39
TPP[3], g/L	53±1.4	55±1.7	49±1.4	53±1.5	54±1.6	58±2.0	0.32
Osmolality, m*Osm*/kg	284±1.7	293±2.1	279±1.5	286±2.0	278±2.0	281±2.5	0.40
Glycerol, m*M*/L	3.3±0.43^{a}	8.1±0.29^{b}	0.1±0.39	0.2±0.27	0.2±0.54	0.2±0.35	<0.001
Glucose, m*M*/L	5.5±0.55^{a}	8.0±0.72^{b}	7.0±0.49^{a}	13.3±0.65^{b}	5.7±0.67	4.0±0.87	<0.001
Insulin, µg/L	0.4±0.23	1.3±0.47	0.6±0.21^{a}	2.4±0.42^{b}	0.3±0.25	0.1±0.55	0.032

[a,b] Values within a row with different superscripts differ between days, (P<0.05) [1] Oral rehydration solution; Glucose (1g glucose/kg BW), Glycerol (0.67 g glycerol/kg BW and 0.33 g glucose/kg BW), Control (water) [2] Packed cell volume [3] Total plasma protein [4] Level of significance, P < 0.05

5.2. Body weight, Skin tent duration and Eyeball recession

The calves' body temperature was normal at all sampling occasions throughout the experiment. The initial average weight was 44 kg (44±1.2 kg). Effect of treatment on weight was not influenced within day (P=0.34) or between days (P=0.60) (Table 4). However, there was a difference between day 0 to 11, due to the calves daily weight gain and day 11 to 12, due to the deprivation period (P<0.001). The weight loss, day 11 to 12, was approximately 6% and defined as mild dehydration (Smith, 2009b). Weight differences between the groups were found (P=0.04), due to the calves´ age and sex distribution in respective group. The measured skin tent duration and eyeball recession were not significant among treatments, P=0.30 and P=0.19, respectively (Table 4). However, there were significant differences between day 11 and 12, skin tent duration increased from 2 to 4 s (P=0.001), moderate dehydration (Smith, 2009b), and eyeball recession increased from 0 to 2 mm (P=0.001).

Table 4*. The effect of oral rehydration solution containing glucose or glycerol on body weight, skin tenting and eyeball recession in dehydrated calves, between days. Data presented as least squares means ± SEM*

Treatment[1]	Glycerol		Glucose		Control	
Day	11	12	11	12	11	12
Body weight, kg	51±2.5	48±2.1	50±2.3	47±2.0	53±3.3	51±2.8
Skin tent duration, s	2±0.3	4±0.5	3±0.3	4±0.5	2±0.4	5±0.7
Eyeball recession, mm	0±0.3	2±0.6	0±0.3	1±0.5	1±0.4	2±0.7

[1] Oral rehydration solution; Glucose (1g glucose/kg BW), Glycerol (0.67 g glycerol/kg BW and 0.33 g glucose/kg BW), Control (water)

5.3. Short-chain fatty acids, lactate and 1,3-propanediol

Acetate (P=0.20), propionate (P=0.65), *n*-butyrate (P=0.29), lactate (P=0.24) and 1,3-propanediol (P=0.33) concentrations in fecal material were not influenced by treatments between day 0 and 11(Table 5). The concentration of lactic acid in calf feces decreased from day 0 to 11 (P=0.017) and the concentration of *n*-butyric acid tended to decrease from day 0 to 11 (P=0.07).

Table 5*. The effect of oral rehydration solution containing glucose or glycerol on the concentration of acetic-, propionic-, n-butyric- and lactic acid as well as 1,3-propanediol in calf feces. Data presented as least squares means ± SEM*

Treatment[1]	Glycerol		Glucose		Control	
Day	0	11	0	11	0	11
Acetic acid, m*M*/L	63.2±11.29	61.1±5.90	49.1±10.53	62.9±5.46	79.1±14.89	56.4±7.72
Propionic acid, m*M*/L	22.6±5.99	27.5±2.67	24.3±5.59	27.6±2.46	29.8±7.91	26.3±3.48
n-Butyric acid, m*M*/L	32.5±4.65	21.2±1.83	23.1±4.34	21.9±1.69	25.0±6.13	20.4±2.38
Lactic acid, m*M*/L	7.4±5.58	3.6±1.74	13.0±5.21	6.6±1.61	22.9±7.37	2.0±2.27
1,3 - Propanediol, m*M*/L	0.5±0.11	0.7±0.13	0.7±0.10	0.6±0.12	0.7±0.14	0.5±0.17

[1] Oral rehydration solution; Glucose (1g glucose/kg BW), Glycerol (0.67 g glycerol/kg BW and 0.33 g glucose/kg BW), Control (water)

5.4. Fecal scoring and microbial analysis

The texture of the feces did not differ among treatments during the experiment (P=0.06) (Table 6). Throughout the experiment the average score was 2 (2±0.9), referred as pasty on the five-grade scale (Silverlås, 2010). Effect of treatment on bacterial quantification *Enterobacteriaceae* of and *Lactobacillus* were not significant between day 0 and 11, (P=0.16) and (P=0.25), respectively (Table 6). The amount of *Enterobacteriaceae* decreased from day 0 to 11 (P=0.01) while the amount of *Lactobacillus* was unchanged (P=0.88). Results from the reuterin assay show 19 plates (of 51 fresh plates) with reuterin activity, proved by cfu surrounded by a red-brown zone (Figure 5). However, there were no consistent differences among treatments within or between days. The 16S rRNA gene sequencing showed that all bacteria positive for reuterin was *Lactobacillus reuteri* and the reuterin negative bacteria were different species of *Lactobacillus* (*L. vaginalis, L. salivarius, L. rhamnosus* and *L. reuteri*). The confirmation of *E. coli* showed detection of gas and indole production in all samples tested.

Table 6. *The effect of oral rehydration solution with glycerol or glucose on texture of calf feces, amount of Lactobacillus and Enterobacteriaceae in calf feces. Data presented as least squares means ± SEM*

Treatment[1]	Glycerol		Glucose		Control	
Day	0	11	0	11	0	11
Texture of feces	1.6±0.40	1.9±0.30	2.5±0.37	1.5±0.28	1.5±0.53	1.5±0.40
Lactobacillus, log CFU/ g feces	7.3±0.46	7.0±0.35	6.7±0.40	7.7±0.33	7.7±0.57	7.2±0.46
Enterobacteriaceae, log CFU/ g feces	5.5±0.60	5.0±0.27	5.8±0.56	5.2±0.25	6.1±0.80	3.7±0.35

[1] Oral rehydration solution; Glucose (1g glucose/kg BW), Glycerol (0.67 g glycerol/kg BW and 0.33 g glucose/kg BW), Control (water)

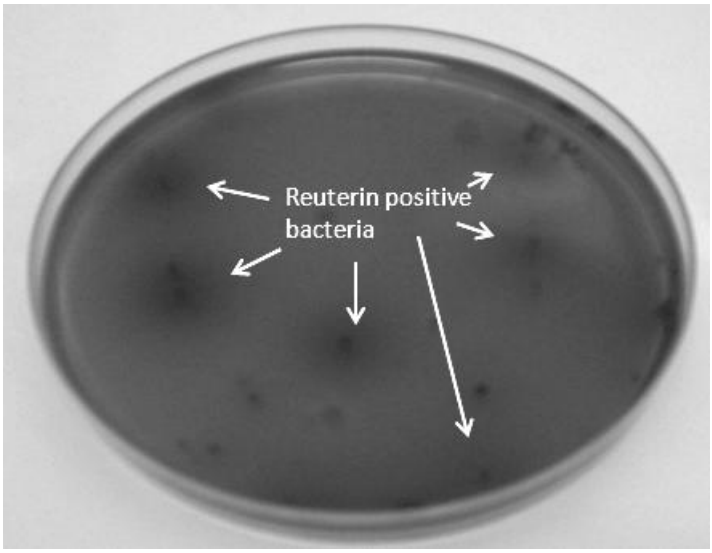


Figure 5. *Plate with growth of Lactobacillus, darker zones around colony forming units indicates reuterin positive bacteria.*

6. DISCUSSION

The PCV in blood, concentration of TPP in plasma and plasma osmolality in young calves increased after 24 h of deprivation and it is a normal reaction that occurs in the body due to dehydration (Dalton, 1967b; Radostits *et al*., 2007). According to the hypothesis should dehydrated calves treated with glycerol have lower concentrations of PCV in blood and TPP in plasma compared to dehydrated calves treated with glucose and control. No differences among treatments regarding the amount of PCV in blood, the concentration of TPP in plasma and plasma osmolality could be found within or between days. However, the effect of treatment show that calves provided with ORS containing glycerol had higher plasma osmolality and tended to have lower amount of PCV compared to glucose and control. The increased plasma osmolality shown in calves treated with ORS containing glycerol do not necessarily mean that those calves were dehydrated. The plasma osmolality will increase due to elevated concentration of low-molecular-weight substances (Smith, 2009a) and it might explain the higher plasma osmolality shown in glycerol treated calves. A more accurate measurement to use when analysing the degree of dehydration would be the PCV in blood. It would be of interest to extend the deprivation period to 3 to 4 days, as done by Dalton (1967b). Clear differences regarding amount of PCV in blood, concentration of TPP in plasma and plasma osmolality might appear and it would be possible to determine whether glycerol has the potential to keep up the fluid balance to a greater degree in a dehydrated calf compared to glucose. The results obtained for the PCV in blood in this study falls into the wide range of results shown in healthy calves, investigated in previous studies (Dalton, 1967a; Möllerberg *et al*., 1989; Radostits *et al*., 2007). The concentration of TPP in plasma in this study is slightly lower compared to values shown by Radostits *et al*. (2007) which may be due to insufficient amount and quality of colostrum. However, the concentration of TPP in plasma and plasma osmolality is in agreement with Dalton (1967a). It would be of interest to investigate the effect of different glucose: glycerol ratios in ORS given to calves. Allen *et al*. (1999) found that if glucose is replaced by two thirds glycerol, the net water absorption in rats' increase, however that might not be the optimal glucose: glycerol mix for calves.

Literature discussing the concentration of glycerol in plasma in calves is spare and it is therefore difficult to compare the results from this study with previous work. However, calves provided with ORS containing glycerol had higher plasma concentration of glycerol day 11 and 12, compared to calves treated with glucose and control calves. The values obtained for the concentration of glucose in plasma from the control calves, day 12, is in agreement with Radostits *et al.* (2007) whereas the values obtained from glycerol and glucose treated calves is higher compared to Radostits *et al.* (2007). When the glucose concentration in plasma increased, the insulin concentration followed the same pattern since the roll of insulin is to regulate the glucose concentration in plasma (Sjaastad *et al*., 2003). The values obtained for the concentration of insulin in plasma in this study is in agreement with Katoh *et al*. (2004). Differences in blood and plasma composition that occurred between groups are probably due to a rotavirus infection in the barn and the supply of colostrum.

The degree of dehydration differs depending on which parameter taken into account. Which parameter is the most reliable is questionable due to the accuracy of the equipment and the lack of previous experience regarding measurement of skin tent duration and eyeball recession. The eyeball recession may not be an accurate measurement of degree of dehydration since the position of the eyeballs depends on the body fat stores and can vary between individuals (Smith, 2009b). However, body weight loss, skin tent duration and eyeball recession are good clinical measurements of dehydration for the farmer to apply when calves are suffering of diarrhoea.

The formation of short-chain fatty acids by the intestinal bacteria is influenced by e.g. the bacterial composition of the microbiota (Macfarlane and Macfarlane, 2003) which is shown to vary with age (Hartman *et al*., 1966; Contrepois and Gouet, 1973; Jenny *et al*., 1991; Rada *et al*., 2006). The concentrations of acetic-, propionic-, *n*-butyric- and lactic acid in feces from this experiment are similar to results presented in previous studies (Sato and Koiwa, 2008; Sato 2010). The concentration of lactic acid decreased with increasing age, which is in agreement with Sato (2010). Sato (2010) showed that the concentration of *n*-butyric acid is lower in calves with diarrhoea compared to healthy calves. Impaired health status, due to a rotavirus infection in the barn, might explain the tendency of decreasing concentration of *n*-butyric found in this study.

Cleusix *et al*. (2008) who investigated the effect of *L. reuteri* and reuterin on intestinal microbiota from humans, measured the concentration of 1,3-propanediol as an indirect measurement of reuterin and showed that a glycerol concentration of 100 mM increased the concentration of 1,3-propanediol. It was not possible to distinguish differences of the concentration of 1,3-propanediol among treatments or between days. Probably glycerol was efficiently absorbed in the small intestine and thereby not available for *L. reuteri*. Treatment did not affect the texture of the feces. The data shows that the texture of the feces ranged from normal to watery and is in agreement with Bartels *et al*. (2010) who showed that the texture of calf feces can vary widely. ORS with glycerol fed to calves did not affect the concentration of *Lactobacillus* and *Enterobacteriaceae* in calf feces. It was believed that calves fed ORS with glycerol would have higher concentration of *Lactobacillus* and lower concentration of *Enterobacteriaceae* in feces compared to calves treated with glucose or control calves. However, the concentration of lactobacilli did not differ between days or among treatments. The concentration of lactobacilli in calf feces from this study falls into the wide range of lactobacilli in calf feces shown by Contrepois and Gouet (1973). *E. coli* belongs to the large family of *Enterobacteriaceae*. The results from this experiment showed that the concentration of *Enterobacteriaceae* decreased from day 0 to 11 and it might be explained by a natural decrease of *E. coli* as shown by Rada *et al*. (2006). *E. coli* was detected in all tested samples, an expected result since *E. coli* is naturally occurring in the intestine (Karaolis and Boedeker, 1997). Unfortunately, the concentration of *E. coli* was not determined in this experiment. However, glycerol has been shown to decrease the concentration of *E. coli* in human feces (Cleusix *et al*., 2008).

On the basis of the limited data from the reuterin test it is impossible to determine whether calves provided ORS with glycerol had a higher proportion of reuterin positive *Lactobacillus* compared to calves provided ORS with glucose or control calves. *L. reuteri* is naturally occurring in the intestine (Axelsson *et al*., 1989) therefore it is surprising that reuterin positive lactobacilli could not be detected in all reuterin tested plates. The result could be explained by lack of previous experience of performing reuterin tests and the ability to interpret the reactions that occurred on the plates. However, the result from the 16S rRNA sequencing of bacteria confirmed the data from the reuterin test and it was shown that *L. reuteri* was present in feces at day 0 and 11, regardless treatment. One of the reuterin negative bacteria turned out to be *L. reuteri*, indicating that strains of *L. reuteri* not producing reuterin might exist in the intestine of calves and not just in the intestine of pig as shown by Axelsson (1990).

7. CONCLUSION

- The results suggest that a mixture of glycerol/glucose was better ORS than the pure glucose solution. Glycerol may be a suitable alternative to use in fluid replacers for calves in the future.

- Calves receiving pure glucose ORS were clearly hyperglycemic and hyperinsulinemic, whereas calves receiving ORS containing a mixture of glycerol/glucose did not develop hyperglycemia and insulin fluctuated less.

- ORS containing a mixture of glycerol/glucose did not decrease the amount of *Enterobacteriaceae* or increase the amount of *Lactobacillus* in feces compared to ORS with glucose. Probably was glycerol efficiently absorbed in the small intestine and thereby not available for the microbiota in the large intestine.

- Further studies are needed to determine whether glycerol, alone and not in a mixture with glucose, possesses health promoting properties in calves. It is also suggested that further studies focus on the effect of glycerol on either fluid balance or intestinal flora in calves.

8. SAMMANFATTNING

Bakterien *Lactobacillus reuteri*, vars naturliga habitat är i mag-tarmkanalen hos människor, djur och fåglar, använder glycerol som substrat för framställning av den antimikrobiella substansen reuterin. Glycerol har visat sig minska mängden *Escherichia coli* i avföring hos människa och det tros bero en *in situ* produktion av reuterin. *E.coli* tillhör den stora familjen *Enterobacteriaceae* och är naturligt förekommande i tarmen. Patogena arter av *E.coli* har visat sig vara en av flera bakterier som orsakar diarré hos kalv. Diarré följs av dehydrering och energibrist och som utan behandling med vätskeersättning, kan vara livshotande. Förutom glycerols höga energivärde har glycerol egenskaper som visat sig upprätthålla vätskebalansen hos människor, råttor och stutar. Det verkar dock inte finnas några studier gjorda med fokus på vätskeersättningar innehållandes glycerol till kalvar.

Syftet med studien var att undersöka om glycerol, genom att stimulera produktionen av reuterin och därmed minska mängden *Enterobacteriaceae* i kalvträck, har en positiv effekt på tarmfloran hos friska kalvar jämfört med glukos och utan tillförsel av vätskeersättning (kontroll). Vidare var syftet att undersöka om glycerol kan upprätthålla plasmavolymen samt att undersöka skillnader i plasma metaboliter t.ex. glukos och insulin, hos kalvar vilka varit undanhållna mjölk, foder och vatten under 24 timmar.

Tjugo kalvar av rasen Svensk Rödbrokig Boskap i åldern 1 till 3 veckor vid försökets start användes i försöket. Kalvarna delades slumpmässigt till en av tre vätskebehandlingsgrupper: glukos (1 g glukos/kg kroppsvikt) (n=8), glycerol (0.67 g glycerol/kg kroppsvikt och 0.33 g glukos/kg kroppsvikt) (n=8), kontroll (ingen vätskeersättning) (n=4). Kalvarna tilldelades 400 ml kroppstempererad vätskelösning två gånger dagligen under tillvänjningsperioden, dag 1 till 11. Dag 11 till 12, undanhölls kalvarna mjölk, foder och vatten under 24 timmar och tilldelades endast 800 ml kroppstempererad vätskelösning, 16.00 dag 11 och 08.00 dag 12. Blod- och träckprover samlades in 2-3 dagar innan försökets start (dag 0) samt efter 11 dagars behandling (dag 11). Ytterligare blodprover samlades in efter ett dygns svält (dag 12). Blodet analyserades för hämatokrit, totalt plasmaprotein, osmolalitet, plasmakoncentration av glycerol, glukos och insulin. Avföringen analyserades för koncentrationen av kortkedjiga fettsyror, laktat och 1, 3-propandediol med hjälp av HPLC-analys, och mängden laktobaciller och enterobakterier bestämdes genom platträkning. Detektion av reuterin och *E.coli* samt fastställande av 16S rRNA gensekvensen av *Lactobacillus* genomfördes.

Resultaten visade att hämtokrit, koncentration av totala plasmaproteiner och plasma osmolaliteten ökade från dag 11 till 12. Hämatokrit, koncentrationen av totala plasmaproteiner och plasma osmolaliteten skiljde sig inte mellan behandlingsgrupperna inom eller mellan dagar. Plasmakoncentrationen av glycerol visade sig vara högre för kalvar behandlade med vätskeersättning innehållandes glycerol dag 11 och 12 jämfört med kalvar som behandlats med glukos eller kontroll kalvar. Koncentrationen av glukos i plasma ökade från dag 11 till 12 hos kalvar behandlade med vätskeersättning innehållandes glukos och glycerol medan glukoskoncentration i plasma tenderade att minska hos kontroll kalvar. Koncentrationen av insulin i plasma ökade från dag 11 till 12 hos kalvar som behandlats med glukos. Koncentrationen av kortkedjiga fettsyror, laktat och 1, 3-propandediol i träcken skiljde sig inte mellan behandlingarna varken inom eller mellan dagar. Mängden *Lactobacillus* och *Enterobacteriaceae* påverkades inte av behandling. Mängden av *Enterobacteriaceae* minskade från dag 0 till 11, medan mängden av *Lactobacillus* var

oförändrad. *Lactobacillus reuteri* och *E. coli* återfanns i träcken från alla behandlingsgrupper både dag 0 och 11.

Det kan konstateras att vätskeersättning innehållandes en blandning av glycerol och glukos kan upprätthålla vätskebalansen i större utsträckning än vätskeersättning innehållandes glukos. Kalvar som fick vätskeersättning innehållandes glukos var hyperglykemiska och uppvisade höga nivåer av insulin medan kalvar som fått vätskeersättning innehållandes en blandning av glycerol och glukos inte var hyperglykemiska och koncentrationen av insulin i plasma fluktuerade mindre. Vätskeersättning innehållandes en blandning av glycerol och glukos inte kan förbättra tarmfloran hos unga kalvar och det bero förmodligen på att glycerol effektivt absorberades i tunntarmen och därmed inte blev tillgängligt för tarmens mikroorganismer. Ytterligare studier behövs för att avgöra om glycerol, som enda komponent, har hälsofrämjande egenskaper hos kalvar. Framtida studier bör fokusera på effekten av glycerol på antingen vätskebalans eller tarmfloran hos kalvar.

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