

Replacing lactose from calf milk replacers

Effects on digestion and post-absorptive metabolism



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Thesis

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Propositions

1. Starch entering the small intestine of ruminants is largely fermented rather than digested.
(this thesis)
2. Health status of individual calves in later life explains more variation in feed efficiency than characterization of digestion, metabolism, behavior and immunology in early life.
(this thesis)
3. The increase of fructose in the human diet (Marriott et al., 2009) is not the cause for the increase in the prevalence of obesity.
4. Altruism in recycling behavior (Hopper and Nielsen, 1991) limits efficient waste recycling.
5. Gut feeling depends on gut filling.
6. Stress is caused more by high demands imposed by yourself than by others.

Propositions belonging to the thesis, entitled

‘Replacing lactose from calf milk replacers - Effects on digestion and post-absorptive metabolism’.

Myrthe S. Gilbert

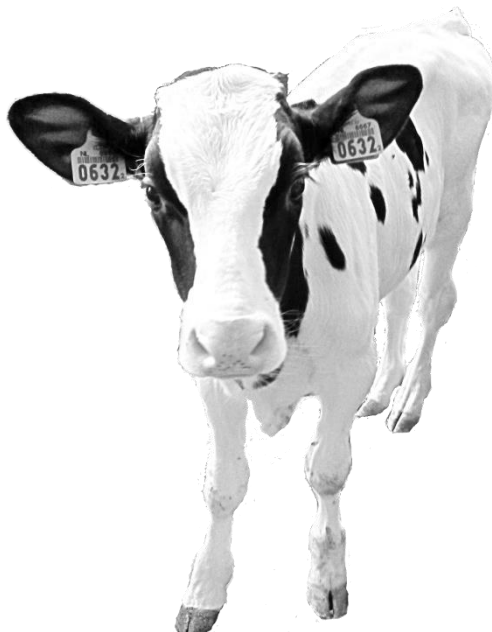
Wageningen, 18 December, 2015.

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Chapter 1

General introduction



PROJECT OUTLINE

Calves raised for white veal production are fed milk replacer (MR) and solid feed. The largest part of the energy provided to veal calves originates from the MR. Calf MR contains 40 to 50% lactose. Lactose originates from whey, which is a by-product from cheese production. Lactose is highly digestible (Coombe and Smith, 1974, Van den Borne et al., 2006), however, high and strongly fluctuating dairy prices are a major economic incentive to replace lactose from the calf MR by alternative energy sources. Furthermore, heavy veal calves often develop problems with glucose homeostasis and insulin sensitivity, which can result in urinary excretion of glucose (Vicari et al., 2008, Labussière et al., 2009) and hepatic steatosis (Gerrits et al., 1998). There are indications that high lactose intake of veal calves contributes to the problems with glucose homeostasis (Hugi et al., 1997). Replacing lactose with other energy sources could alter or alleviate these problems. Therefore, the project entitled “Replacing lactose by processed plant polysaccharides for calf nutrition” was designed. The main aim of the project was to successfully replace lactose from the calf MR. Replacement of lactose should not have detrimental effects on nutrient digestion and glucose homeostasis. Therefore, the objectives were to study the effects of replacing lactose by other carbohydrate or energy sources 1) on nutrient digestion and fermentation and 2) on glucose homeostasis and insulin sensitivity. This PhD thesis focusses mainly on the first objective.

VEAL PRODUCTION

White veal originates from calves that are slaughtered before 8 months of age. The major veal producing countries in Europe are France, the Netherlands, Italy, Germany and Belgium. In the EU-28, over 4.4 million veal calves were slaughtered in 2013, of which 1.2 million in the Netherlands (source: Eurostat). Over 90% of the veal that is produced in the Netherlands is exported, mainly to Italy, Germany and France.

In practice, male dairy calves and female calves not kept for replacement are purchased from dairy farms at approximately 14 days of age and are transported to specialized gathering facilities. At these facilities, calves are mixed and re-grouped and transported to specialized veal farms, where they are usually housed in individual pens during the first 4 to 6 weeks after arrival, in order to allow individual monitoring. Thereafter, the calves are group-housed until slaughter age. In the Netherlands, white veal calves are usually slaughtered at an age of approximately 29 weeks.

VEAL CALF NUTRITION

During the first 3 days after birth, it is advised to provide calves with colostrum, however, colostrum supply to male dairy calves and female calves not kept for replacement can vary considerably between dairy farms. Thereafter, calves are usually fed MR. For the production of white veal, calves are fed low-iron MR. This results in a pale meat color, which is valued by consumers. In 1997, the EU Council Directive 97/2/EC was introduced, stipulating the minimum provision of 50 to 250 g of fibrous feeds per day to veal calves. The amount and composition of this fibrous feed (i.e. concentrates and roughages) provided in practice differs, however, the largest part of the gross energy provided to white veal calves still originates from the milk replacer (50 to 75%).

When a calf ingests MR, the esophageal groove closes, allowing the MR to by-pass the reticulorumen (Abe et al., 1979). Therefore, the MR flows directly into the abomasum, and from there into the small intestine (Figure 1.1). The nutrients from the MR, therefore, need to be enzymatically hydrolyzed and absorbed in the small intestine, like in monogastric animals. Solid feed, on the other hand, enters the reticulorumen, where it is fermented by the microbial population, resulting in production of microbial protein and volatile fatty acids.

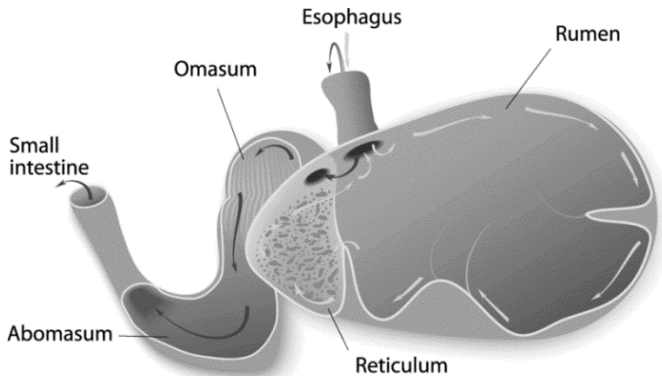


Figure 1.1 Schematic overview of the stomach compartments of ruminants. In milk-fed calves, the rumen is smaller and the abomasum is larger compared to adult ruminants and the functioning of the esophageal groove reflex allows milk replacer to by-pass the reticulorumen and to flow directly into the abomasum.

Calf MR typically contains 20% crude fat, 20% crude protein and up to 50% lactose. Traditionally, MR is based on skim milk powder, which is a by-product of the production of butter. Casein (i.e. the main protein in skim milk powder) and fat form a clot in the

abomasum, which increases the retention of dry matter, crude protein and fat in the abomasum (Cruywagen et al., 1990). Since the 1970s, whey powder, a by-product from cheese production, has been used as the main ingredient in calf MR, which results in a non-clotting MR. Also, products resulting from further processing of whey powder, such as delactosed whey-powder, lactose and whey protein concentrates are used in calf MR. However, whey is also used in foods (Kosikowski, 1979), and the availability and prices of whey fluctuate strongly. Whey prices are generally higher compared to for instance corn starch (as demonstrated by the annual means of whey powder prices in central USA and corn starch prices in Midwest USA; Figure 1.2). This provides a strong incentive to (partially) replace these whey-derived ingredients from the calf MR. Dairy proteins can already be replaced from the MR by vegetable proteins (i.e. from soy and wheat; Akinyele and Harshbarger, 1983, Lalles et al., 1995, Toullec and Formal, 1998). However, successful replacement of lactose from the calf MR has not yet been achieved.

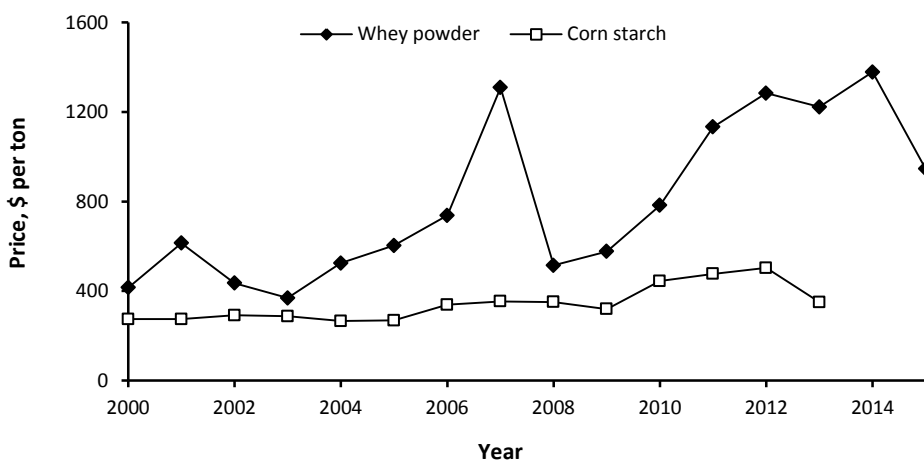


Figure 1.2 Annual means of whey powder prices in Central USA from 2000 to July 2015 and corn starch prices in Midwest USA from 2000 to 2013. Source: USDA Agricultural Marketing Service, 2015.

Lactose properties

The calf MR is produced as a dry powder and is then dissolved in warm water and supplied to the calf as a liquid MR. Therefore, all MR ingredients have to be dispersible in warm water. The physical-chemical properties of lactose allow the production of a dry MR powder that is highly soluble in warm water.

Lactose is a disaccharide composed of glucose and galactose (Figure 1.3) and in the small intestine of calves, lactose is hydrolyzed into glucose and galactose by the brush-border

enzyme lactase. Lactase activity is high in milk-fed calves (up to 180 U/g mucosal protein; Siddons, 1968, Le Huerou et al., 1992) and remains high as a result of the continuous feeding of lactose (Huber et al., 1964, Toofanian et al., 1973). This high lactase activity results in a high apparent ileal (97% of intake; Coombe and Smith, 1974) and total tract disappearance of lactose (100% of intake; Van den Borne et al., 2006) in milk-fed calves.

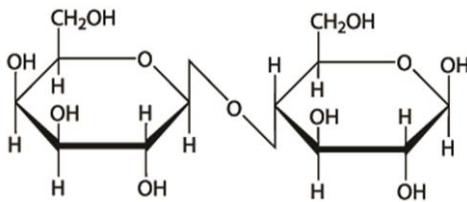


Figure 1.3 Structural overview of lactose.

Glucose is preferentially absorbed from the calf small intestine over galactose. Galactose is metabolized to glucose (Coombe and Smith, 1973) in the liver and slowly released to the blood. Galactose feeding, therefore, results in a lower insulin response compared to glucose feeding as shown in humans (Coss-Bu et al., 2009, Mohammad et al., 2011). Despite this, persistently high intakes of lactose in veal calves contribute to the development of problems with glucose homeostasis and insulin sensitivity (Hugi et al., 1997), which could lead to urinary glucose excretion (Vicari et al., 2008, Labussière et al., 2009) and even hepatic steatosis (Gerrits et al., 1998). Replacing lactose with other energy sources will likely result in different (ratios of) monosaccharides, which could possibly affect glucose homeostasis and insulin sensitivity.

In summary, a successful replacer of lactose should be dispersible in warm water, not cause digestive problems and reduce problems with glucose homeostasis.

Potential lactose replacers

Replacing lactose from the calf MR should not cause digestive problems. Therefore, lactose replacers require enzymatic hydrolysis and subsequent absorption of their end-products from the small intestine. In addition, lactose could be replaced by monomeric sources which do not require prior enzymatic hydrolysis. Absorption from the small intestinal lumen is then required in order to prevent digestive problems such as osmotic diarrhea. An overview of carbohydrate-degrading enzyme activities measured in milk-fed calves, their corresponding substrates and end-products of enzymatic hydrolysis and modes of transport of monomeric substrates from the small intestinal lumen is shown in Table 1.1.

Table 1.1 Overview of carbohydrate-degrading enzyme activities measured in milk-fed calves, their corresponding substrates and end-products of enzymatic hydrolysis and modes of transport of monomeric substrates from the small intestinal lumen.

Enzyme	Enzyme activity in vivo ¹	Substrate	End-product(s)	Transport from the small intestinal lumen	
				active	passive
α -amylase	19 ²	starch	maltodextrins		
		maltodextrins	maltose, isomaltose		
maltase	2.0 - 17.6 ³	maltose	glucose		
isomaltase	0.3 - 3.7 ³	isomaltose	glucose		
sucrase	0 ³	sucrose	glucose, fructose		
-	-	glucose	-	SGLT1 ⁴	paracellular ⁵
-	-	fructose	-	-	GLUT5 ⁶
-	-	glycerol	-	Na ⁺ -dependent ⁷	paracellular ⁸

¹ Enzyme activities are expressed in U/g protein measured in mucosal scrapings of the small intestine, except for α -amylase, which is expressed in mg glucose equivalent/ml measured in pancreatic juice.

² Value obtained from Morrill et al. (1970) and is expressed in mg glucose equivalent/ml measured in pancreatic juice.

³ Values obtained from Siddons (1968), Coombe and Siddons (1973), Toofanian et al. (1973) and Le Huerou et al. (1992).

⁴ Sodium-dependent glucose transporter (Hediger and Rhoads, 1994).

⁵ As reviewed by Harmon et al. (2004).

⁶ Facilitative transporter of fructose (Burant et al., 1992, Zhao et al., 1998).

⁷ There are indications that Na⁺-dependent transport is involved in glycerol absorption from the small intestinal lumen (Kato et al., 2005).

⁸ Höber and Höber (1937).

Starch. Starch is one of the most important carbohydrates used in animal feed and is composed of amylose and amylopectin (Figure 1.4). Amylose is a polymer of glucose molecules linked by α -1,4-linkages, whereas amylopectin also contains α -1,6-branching linkages (reviewed by French, 1973). Accessible α -1,4-linkages in starch are cleaved by pancreatic α -amylase, mainly resulting in maltodextrins, maltose and isomaltose. Thereafter, the brush-border enzyme maltase is required to further hydrolyze these maltodextrins and maltose to glucose, whereas the brush-border enzyme isomaltase cleaves the α -1,6-linkages.

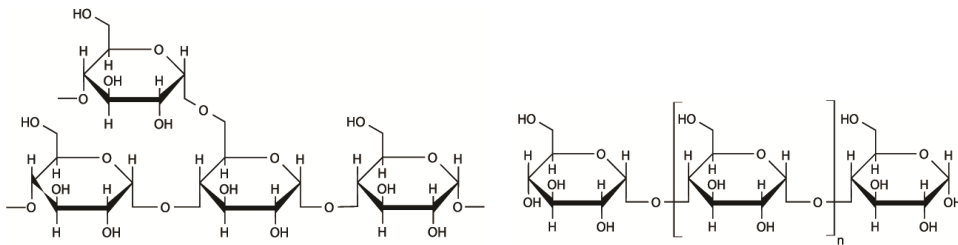


Figure 1.4 Structural overview of amylopectin (left) and amylose (right).

Starch has been studied as an ingredient in calf MR. Burt and Irvine (1970) showed a decrease from 93.7 to 87.5% in apparent total tract dry matter disappearance in milk-fed calves when replacing 37% of the lactose with cooked corn starch. Apparent ileal disappearance of partially acid-hydrolyzed starch (60% of intake) and maltose (43% of intake; Coombe and Smith, 1974) is lower than total tract disappearance of amylopectin (89% of intake) and amylose (83% of intake; Huber et al., 1961b). In addition, apparent ileal starch disappearance is substantially higher in pigs (98% of intake; Knudsen et al., 1993) than in calves. This indicates a limitation to enzymatic hydrolysis of starch in the small intestine of calves, resulting in starch fermentation in the large intestine. Total tract disappearance of starch can, therefore, not be used to assess the amount starch enzymatically hydrolyzed in the small intestine. Ileal disappearance would be more appropriate, however, there are indications that fermentation contributes to ileal disappearance of starch as well. For instance, in steers, the pH of ileal digesta decreased linearly with increasing infusion amount of raw corn starch or partially hydrolyzed corn starch in the abomasum (Kreikemeier et al., 1991, Branco et al., 1999). In addition, only 57% of the corn starch that disappeared in the small intestine of steers after infusion in the abomasum (at 66 g/h) could be recovered as portal glucose appearance (Kreikemeier and Harmon, 1995). Which enzyme is rate-limiting in the enzymatic hydrolysis of starch in calves is unknown. In ruminants, there are indications that brush-border enzyme activity (i.e. maltase or isomaltase; Kreikemeier and Harmon, 1995) or α -amylase is rate-limiting in the enzymatic hydrolysis of starch (as reviewed by Huntington, 1997). However, infusing amylase into the jejunum of steers fed a diet containing 60% unmodified corn did not increase the small intestinal disappearance of starch (Remillard et al., 1990).

Enzyme activities can be measured as well to assess the capacity for enzymatic starch hydrolysis. In milk-fed calves, starch-degrading enzyme activities are most often measured when fed little or no starch (Table 1.1). Under these conditions, maltase and isomaltase activity are much lower than lactase activity (Siddons, 1968, Toofanian et al., 1973, Le Huerou et al., 1992). Morrill et al. (1970) determined α -amylase activity in pancreatic juice

in male calves up to 8 weeks of age fed milk replacer, calf starter and hay. Pancreatic α -amylase activity varied considerably between calves, but was overall considered low. The absence of including starch in the MR makes interpretation of such enzyme activities difficult, because starch-degrading enzyme activities might change in the presence of its substrate. This hampers the identification of the rate-limiting enzyme in starch hydrolysis in milk-fed calves. In monogastric animals, enzyme activities required for starch hydrolysis adapt to starch supply. For instance, in adult rats, pancreatic α -amylase activity increased rapidly (within two days) when changing from a high corn oil diet to a high starch diet (Deschodt-Lanckman et al., 1971). In a review by Brannon (1990), it was concluded that α -amylase content and synthesis increase with 50-500% and 200-800%, respectively, when providing a high starch diet to rats. In pigs, feeding a high starch diet resulted in a higher mucosal maltase activity and a higher α -amylase activity in pancreatic tissue compared to feeding a high fat diet (Flores et al., 1988). This adaptive response of starch-degrading enzymes results in a high apparent ileal starch disappearance in pigs (98% of intake; Knudsen et al., 1993). Whether such adaptation of starch-degrading enzymes to substrate occurs in milk-fed calves as well is unknown. In ruminating Holstein steers, infusion of partially hydrolyzed starch in the abomasum resulted in a lower pancreatic α -amylase activity compared to infusion of water or casein (Walker and Harmon, 1995, Swanson et al., 2002). This indicates that α -amylase, at least in ruminants, is not up-regulated in the presence of starch as occurs in rats and pigs (discussed above). Similarly, maltase activity measured in brush-border membrane vesicles prepared from the jejunum of steers was not increased after infusion of starch hydrolysate in the abomasum compared to infusion in the rumen (Bauer et al., 2001). In these infusion studies, starch is infused for 7 to 10 days and the possibility that starch-degrading enzymes in ruminants and/or calves require longer adaptation periods to adapt to starch cannot be excluded.

Starch or starch hydrolysates used in studies to assess apparent starch disappearance in calves or ruminants are not always characterized. Sometimes, native or unmodified starches are used, but usually, starches are partially hydrolyzed, either by using acid treatment or incubation with heat-stable α -amylase, in order to make these starches soluble in water and more accessible for enzymes. The resulting starch hydrolysate, however, can differ in properties such as chain length and degree of polymerization and, therefore, require different ratios of starch-degrading enzyme for complete hydrolysis to glucose. The lack of starch (hydrolysate) characterization hampers the identification of the rate-limiting enzyme in starch hydrolysis by comparison of studies.

In conclusion, it remains unknown whether starch-degrading enzyme activities can adapt to substrate supply and which enzyme is rate-limiting in the hydrolysis of starch in the

small intestine of veal calves. There are indications that starch fermentation occurs in the small intestine, but the quantitative contribution to small intestinal starch disappearance is unknown. This knowledge is required in order to evaluate the potential of starch (hydrolysate) as a replacer of lactose in calf MR.

Sucrose. Sucrose is a disaccharide of glucose and fructose and could be a potential lactose replacer. However, sucrase activity is not present in the brush-border of milk-fed calves (Siddons, 1968, Toofanian et al., 1973, Le Huerou et al., 1992). In a study by Huber et al. (1961a), calves were fed whole milk supplemented with 2.5% sucrose from 3 days until 6 weeks of age and sucrase activity was not detected in the small intestine (Huber et al., 1961a). This is in agreement with a low apparent total tract disappearance of sucrose in calves fed whole milk supplemented with 10% sucrose (57% of intake; Huber et al., 1961b). These findings indicate that sucrose was most likely degraded by microbes and emphasize that total tract disappearance of carbohydrates does not reflect enzymatic hydrolysis.

In conclusion, sucrase activity is not inducible in milk-fed calves and replacing lactose with sucrose in the calf MR is not recommended.

Glucose. Monosaccharides, such as glucose and fructose, can be absorbed directly from the small intestinal lumen without prior enzymatic hydrolysis. Glucose is transported from the small intestinal lumen into the enterocyte by the Na⁺-dependent transporter SGLT1 (Hediger and Rhoads, 1994). Passive transport might also contribute to glucose absorption from the small intestinal lumen in ruminants, however, this probably only occurs at high (non-physiological) glucose concentrations (as reviewed by Harmon et al., 2004).

In milk-fed calves, the absorption rate of glucose from the small intestine (in g/h per m of small intestinal loop) increased with increasing amounts of glucose infused in the small intestinal loop (Coombe and Smith, 1973). However, glucose absorption from the small intestinal loop decreased from complete absorption to 50% absorption when increasing glucose infusion from 0.9 to 25.5 g/h (Coombe and Smith, 1973), suggesting a limit to glucose absorption in milk-fed calves. This is in agreement with studies in ruminants, where absolute amounts of glucose that disappeared from the small intestine increased, but glucose that disappeared from the small intestine as a percentage of glucose infused decreased from 97 to 71% when increasing abomasal glucose infusion from 20 to 60 g/h (Kreikemeier et al., 1991). In the latter study, fermentation could have contributed to this glucose disappearance as well, as indicated by a decrease in pH of ileal digesta (Kreikemeier et al., 1991).

Despite this limit to glucose absorption, sodium-dependent glucose transport from the small intestine appears to be induced by the luminal presence of glucose in ruminants. For instance in sheep, sodium-dependent glucose transport from the proximal small intestine decreased rapidly in lambs, as a consequence of weaning, but maintaining lambs on milk replacer or infusing adult sheep in the duodenum with glucose, increased glucose transporter activity in brush-border membrane vesicles (Shirazi-Beechey et al., 1991). Similar findings were reported for steers, where infusion of starch hydrolysate in the abomasum compared to infusion in the rumen resulted in a two-fold increase in Na⁺-dependent glucose uptake in brush-border membrane vesicles prepared from the jejunum (Bauer et al., 2001). This indicates that SGLT1 activity in ruminants is dependent on luminal presence of its substrate.

In conclusion, absolute amounts of glucose absorption increase with an increase in supply to the small intestine of calves. However, glucose absorption relative to glucose supply decreases with an increase in supply, indicating a saturation in glucose absorption from the small intestine at higher glucose concentrations.

Fructose. Fructose is absorbed from the small intestinal lumen by GLUT5 (Burant et al., 1992) and GLUT5 mRNA has been detected throughout the small intestine of lactating dairy cows and in the jejunum of a calf using Northern blotting (Zhao et al., 1998). A low reducing sugar response is measured in jugular blood after feeding a fructose solution to milk-fed calves (Velu et al., 1960, Siddons et al., 1969). This suggests either a rapid uptake of fructose by the liver from hepatic portal blood as seen in rat and human (as reviewed by Mayes, 1993) and subsequent post-absorptive conversion of fructose into products other than reducing sugars (i.e. glycogen, lactate, CO₂) or a low absorption of fructose from the intestinal lumen. A low absorption capacity results in incomplete disappearance of fructose from the small intestine, resulting in fructose fermentation. This is confirmed by the occurrence of diarrhea after feeding these fructose solutions to milk-fed calves (Velu et al., 1960, Siddons et al., 1969).

In conclusion, the transporter for absorbing fructose from the intestinal lumen into the enterocyte appears to be present in calves, but the absorptive capacity for fructose might be limited.

Glycerol. Glycerol is a by-product from biodiesel production (Thompson and He, 2006) and has been evaluated as a dietary ingredient for cattle and pigs (Duttlinger et al., 2012, Parsons et al., 2009). Glycerol is easily absorbed from the small intestinal lumen, with 78% of the glycerol absorbed within 25 minutes after infusion in isolated loops of the small intestine of rats (Höber and Höber, 1937). In situ measurements showed that glycerol

disappearance from the rat intestinal lumen was reduced when Na^+ was absent, indicating that Na^+ -dependent carriers are also involved in the absorption of glycerol (Kato et al., 2005). Feeding an oral rehydration solution containing glycerol to 3 week old Swedish Red calves increased plasma glycerol levels from 0 to approximately 7.7 mmol/L at 60 min after intake (Werner-Omazic et al., 2013), indicating that calves are able to absorb glycerol from the small intestine. Replacing 37.5% of the lactose by glycerol in the MR did not affect growth in calves from 3 to 35 days of age (Ebert et al., 2008). In addition, glycerol could affect water absorption in the small intestine. Infusing a rehydration solution containing glycerol into the jejunum of rats resulted in greater water absorption from the intestinal lumen compared to infusing a rehydration solution containing glucose (Wapnir et al., 1996).

In conclusion, calves are able to absorb glycerol from the small intestine. Partially replacing lactose with glycerol did not have adverse effects on growth performance in young milk-fed calves and glycerol could have positive effects on water absorption, suggesting that glycerol is a high-potential replacer of lactose. However, it is unknown whether absorption of glycerol is limited at higher inclusion levels of glycerol in the MR.

GLUCOSE METABOLISM AND INSULIN SENSITIVITY

Heavy veal calves often develop problems with glucose homeostasis, characterized by prolonged elevated postprandial glucose and insulin concentrations in jugular blood and high postprandial insulin to glucose ratios (Hugi et al., 1997). This can develop into a reduced insulin-dependent uptake of glucose in the tissues (i.e. insulin resistance), which can lead to urinary glucose excretion (Vicari et al., 2008, Labussière et al., 2009) and hepatic steatosis (Gerrits et al., 1998). Feeding MR containing 423 g lactose/kg DM resulted in greater postprandial hyperglycemia compared to feeding MR containing 290 g lactose/kg DM (Hugi et al., 1997), indicating that high lactose intakes contribute to these problems with glucose homeostasis.

Replacing lactose from the MR by monosaccharides results in the luminal presence of different (ratios of) monosaccharides. Lactose is split into equimolar amounts of glucose and galactose by lactase and glucose is preferentially absorbed from the small intestine over galactose (Coombe and Smith, 1973). Galactose can be metabolized to glucose in the human liver (as reviewed by Rippe and Angelopoulos, 2013), however, the amount of galactose converted into glucose depends on the specific conditions (i.e. whether galactose is ingested/infused separately or in combination with glucose). When galactose was ingested alone in milk-fed calves, approximately 33% of the reducing sugar response in the jugular blood was attributed to glucose (Coombe and Smith, 1973), suggesting that

not all the galactose is released as glucose in the jugular blood. Urinary galactose excretion in milk-fed calves has been reported as well (Hof, 1980). Replacing lactose with glucose might result in a greater postprandial glucose response. Replacing lactose with starch might have a similar effect if starch would be completely hydrolyzed to glucose. A greater postprandial glucose concentration could result in greater glucose excretion with the urine when the renal threshold for glucose reabsorption (8.3 mmol/L; Hostettler-Allen et al., 1994) is exceeded.

Whether fructose is absorbed from the small intestine of calves remains questionable because of the occurrence of diarrhea after fructose ingestion, however, possible post-absorptive effects of fructose are discussed below. The fractional uptake of fructose by the rat liver is 54.9 and 71.5% in the fed and fasting state, respectively (Topping and Mayes, 1971). Hepatic uptake of glucose was only 24% of that of fructose from 30 to 90 min after infusion of glucose or fructose in the isolated rat liver (Topping and Mayes, 1976). Therefore, the postprandial increase in fructose in jugular blood is low (from 5 to 10 mg/dl at 60 min postprandial) compared to the postprandial increase in glucose (from 90 to 160 mg/dl at 60 min postprandial) after equal intakes of fructose or glucose, respectively, in humans (Tappy et al., 1986). As reviewed by Rippe and Angelopoulos (2013), 50% of the fructose which is metabolized by the liver is converted into glucose, 25% into lactate and 15 to 18% into glycogen. Only 1 to 5% is converted into free fatty acids. This indicates that only 2 to 9% of the fructose is oxidized directly. However, fructose oxidation is greater than glucose oxidation (Tappy et al., 1986). This suggests that the majority of the fructose is oxidized after conversion into other substrates. Fructose does not elicit insulin release as demonstrated in rats (Bruckdorfer et al., 1973, Curry, 1989). In humans, the increase in insulin is 33 ± 4 μ U/ml after ingestion of 75g of glucose compared to 8 ± 2 μ U/ml after ingestion of equal amounts of fructose (Tappy et al., 1986). The insulin to glucose ratio in humans was greater when consuming a high glucose beverage with the meal compared to consuming a high fructose beverage (Teff et al., 2004). If similar post-absorptive responses occur in calves, replacing lactose with fructose might alter glucose metabolism.

Feeding glycerol with an oral rehydration solution to 3 week old Swedish Red calves increased plasma glycerol levels, but almost no increase in plasma glucose was found. Insulin response did not differ significantly between glucose and glycerol feeding with the oral rehydration solution (Werner-Omazic et al., 2013), suggesting that the insulin to glucose ratio is different when feeding glycerol compared to glucose. However, when feeding oral rehydration solutions containing either 100% glucose or 33% glucose and 67% glycerol at 1 g/kg of BW, the insulin to glucose ratio was similar between the two oral

rehydration treatments (Werner-Omazic et al., 2013). It is, therefore, not completely clear what the effects of glycerol are on glucose and insulin metabolism in calves.

OBJECTIVES AND OUTLINE OF THIS THESIS

This research was part of a multidisciplinary collaboration between the VanDrie Group, Tereos Syral, the University Medical Center Groningen and Wageningen University and was performed within the framework of the Dutch Carbohydrate Competence Center (CCC).

The objective of this thesis was to study the effects of replacing lactose from the MR by alternative carbohydrate or energy sources. To this end, the rate-limiting enzyme in starch hydrolysis was studied and the effects of replacing lactose with starch on digestion, fermentation and enzyme activity were determined. Secondly, the effects of replacing lactose with glucose, fructose or glycerol on energy and protein metabolism and glucose and insulin metabolism were studied. Thirdly, the effects of replacing lactose with a combination of glucose, fructose and glycerol on growth performance and feed efficiency were evaluated.

In Chapter 2, a titration approach with four starch products differing in size and branching was used to identify the capacity and the rate-limiting enzyme in starch digestion in milk-fed calves. In Chapter 3, the effects of replacing lactose with these four starch products on nutrient disappearance, enzyme activity and starch fermentation are presented. The effects of replacing lactose with glucose, fructose or glycerol on energy and protein metabolism are described in Chapter 4 and on glucose and insulin metabolism in Chapter 5. Chapter 6 presents the relation between physiological measurements in early life and growth performance and feed efficiency in later life and examines whether the ability of calves to cope with MR in which lactose is partially replaced by alternative energy sources can be predicted in early life. A general discussion is included in Chapter 7, with emphasis on the evaluation of lactose replacers and the question whether ruminants are equipped to deal with large amounts of starch. Finally, general conclusions are provided.

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Chapter 2

A titration approach to identify the capacity for starch digestion in milk-fed calves



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ABSTRACT

Calf milk replacers (MR) commonly contain 40 to 50% lactose. For economic reasons, starch is of interest as a lactose replacer. Compared with lactose, starch digestion is generally low in calves. It is, however, unknown which enzyme limits the rate of starch digestion. The objectives were to determine which enzyme limits starch digestion and to assess the maximum capacity for starch digestion in milk-fed calves. A within-animal titration study was performed, where lactose was exchanged stepwise for one of four starch products (SP). The four corn-based SP differed in size and branching, therefore, requiring different ratios of starch-degrading enzymes for their complete hydrolysis to glucose: gelatinized starch (α -amylase and (iso)maltase); maltodextrin ((iso)maltase and α -amylase); maltodextrin with α -1,6-branching (isomaltase, maltase and α -amylase) and maltose (maltase). When exceeding the animal's capacity to enzymatically hydrolyze starch, fermentation occurs, leading to a reduced fecal dry matter (DM) content and pH. Forty calves (13 weeks of age) were assigned to either a lactose control diet or one of four titration strategies ($n = 8$ per treatment), each testing the stepwise exchange of lactose for one SP. Dietary inclusion of each SP was increased weekly by 3% at the expense of lactose and fecal samples were collected from the rectum weekly to determine DM content and pH. The increase in SP inclusion was stopped when fecal DM content dropped below 10.6% (i.e. 75% of the average initial fecal DM content) for 3 consecutive weeks. For control calves, fecal DM content and pH did not change over time. For 87% of the SP-fed calves, fecal DM and pH decreased already at low inclusion levels, and linear regression provided a better fit of the data (fecal DM content or pH v. time) than non-linear regression. For all SP treatments, fecal DM content and pH decreased in time ($P < 0.001$) and slopes for fecal DM content and pH in time differed from CON ($P < 0.001$ for all SP), but did not differ between SP treatments. Fecal DM content of SP-fed calves decreased by 0.57% and fecal pH by 0.32 per week. In conclusion, fecal DM content and pH sensitively respond to incremental inclusion of SP in calf MR, independently of SP characteristics. All SP require maltase to achieve complete hydrolysis to glucose. We, therefore, suggest that maltase activity limits starch digestion and that fermentation may contribute substantially to total tract starch disappearance in milk-fed calves.

Keywords: milk-fed calf, starch digestion, starch fermentation, maltose, maltodextrin

INTRODUCTION

Calf milk replacers (MR) contain 40 to 50% lactose. Lactose is highly digestible (Burt and Irvine, 1970, Coombe and Smith, 1974, Van den Borne et al., 2006), and in a typical MR, lactose accounts for 40% of the digestible energy available to the calf. However, the volatility of lactose prices stimulates MR producers to replace lactose by other sources, particularly starch or products originating from starch. Starch digestion has been studied in calves, and total tract digestibility varies from 63 to 95% (Huber et al., 1968, Burt and Irvine, 1970, Nitsan et al., 1990). Ileal disappearance of lactose averaged 97% of intake, whereas ileal disappearance of starch averaged 60% of intake in calves (Coombe and Smith, 1974). Similar ileal disappearance (66%) was found in steers after infusion of starch in the abomasum (Kreikemeier and Harmon, 1995). The low ileal disappearance of starch compared with lactose indicates that enzyme activity required for the hydrolysis of starch to glucose is limited in calves. Which enzyme limits starch digestion in milk-fed calves is, however, unknown.

Starch requires different enzymes for complete hydrolysis to glucose. Pancreatic α -amylase cleaves accessible α -1,4-glycosidic bonds (Dona et al., 2010), resulting mainly in maltose, maltotriose and dextrans, although glucose can appear as well. Thereafter, maltase is required for the further hydrolysis of maltose and maltodextrins to glucose, whereas isomaltase is required to remove α -1,6-branches present in isomaltodextrins. Unlike in calves with a developed rumen, where little starch escapes rumen fermentation, starch inflow in the small intestine in milk-fed calves is directly related to inclusion of starch in the MR. The measurement of activities of luminal or brush border enzymes is usually conducted in calves fed little or no starch. Under these conditions, lactase activity is usually high, up to 180 U/g mucosal protein, maltase activity does not exceed 27 U/g protein and isomaltase activity is even lower than maltase activity (Siddons, 1968, Toofanian et al., 1973, Le Huerou et al., 1992). α -Amylase activity measured in pancreatic juice varied considerably between individual milk-fed calves, but was overall considered low (19 mg glucose equivalent/mL pancreatic juice), because no significant amount of starch would be cleaved at this level of α -amylase activity (Morrill et al., 1970). Adaptation of enzyme activities to substrate supply has been shown in rats (glucose/sucrose, Howard and Yudkin, 1963, starch, Deschodt-Lanckman et al., 1971); pigs (protein, Corring and Saucier, 1972, starch/pectin, Mosenthin et al., 1994); and calves (lactose, Huber et al., 1964, Toofanian et al., 1973). Enzyme activity measured in absence of its specific substrate might, therefore, be misleading. This hampers the identification of the rate-limiting enzyme in starch hydrolysis in milk-fed calves. Therefore, the objective was to identify the rate-limiting enzyme in the hydrolysis of starch in milk-fed calves.

In addition, time required for enzymes to adapt to specific available substrates is an important factor when assessing enzyme activity. In pigs, chymotrypsin and trypsin activity increased 2 days after switching from a protein-free diet to a 30% protein diet (Corring and Saucier, 1972). In rats, α -amylase activity tripled in 5 days after switching from a 0% starch diet to a 67% starch diet (Deschodt-Lanckman et al., 1971). It seems that enzymes adapt to a specific diet in 2 to 5 days, at least in omnivores. In ruminating calves and steers, α -amylase responses to starch infusion in the abomasum are absent or even negative (Walker and Harmon, 1995, Swanson et al., 2002). However, often only short-term effects are investigated. It is unknown how enzyme activity responds to an increase in starch availability in milk-fed calves.

Previous studies have shown that fecal dry matter (DM) content decreased with decreased starch utilization (i.e. fecal starch concentration, blood reducing sugar response) in ruminants (Huber et al., 1961, Ørskov et al., 1970). Furthermore, a linear decrease was found in ileal pH when infusing starch or dextrin in the abomasum of steers (Kreikemeier et al., 1991, Branco et al., 1999). This decreased pH may indicate that starch is fermented rather than enzymatically hydrolyzed. In the current study, it is proposed that gradually exchanging lactose for a selected starch product (SP), allowing ample time for the adaptation of starch-degrading enzymes, and measuring changes in fecal DM content and pH can be used to identify the maximum capacity for the enzymatic digestion of that particular SP. It is assumed that the rate-limiting enzyme for starch hydrolysis can be identified by comparison of the response in fecal DM and pH to SP, selected based on the requirement for different (ratios of) digestive enzymes for their complete degradation to glucose.

The objectives were to determine which enzyme limits starch digestion and to assess the maximum capacity for starch digestion in milk-fed calves.

MATERIAL AND METHODS

Experimental design, animals and housing

Forty male Holstein-Friesian calves of 13 weeks of age (103.6 ± 1.1 kg) were used. Calves were assigned to one of five MR treatments varying in carbohydrate source. The control treatment (CON) contained lactose as the only carbohydrate source. In the other MR treatments, one of four SP was increased stepwise at the expense of lactose. The selected industrial SP originated from corn and included gelatinized starch (GS; Tate&Lyle Europe, Boleraz, Slovakia), maltodextrin (MD; Tereos Syral, Marckolsheim, France; dextrose equivalent (DE) ≈ 13), maltodextrin with a high level of α -1,6-branching (MDB; Tereos Syral, Marckolsheim, France; DE ≈ 9) and maltose (MT; Tereos Syral, Marckolsheim,

France). These SP (see the 'Diets and feeding' section for details) differ in size and branching and consequently require different ratios of starch-degrading enzymes for their complete hydrolysis to glucose. GS requires α -amylase and (iso)maltase; MD requires (iso)maltase and α -amylase; MDB requires isomaltase, maltase and α -amylase; and MT requires maltase only. The inclusion level of SP increased every week by 3% at the expense of lactose, to a maximum of 36%. For CON, the lactose content was maintained at 52.7%. Different fecal responses to increasing exposure of these SP can be used to identify the rate-limiting enzyme in starch digestion and the maximum capacity for starch digestion (Figure 2.1). The hypothetical maximum capacity for the digestion of a SP for an individual calf is defined as the inflection point in the relation between inclusion level of SP and the fecal DM content or pH, estimated using non-linear regression. Differences in inflection points can then be used to identify the rate-limiting enzyme in starch digestion.

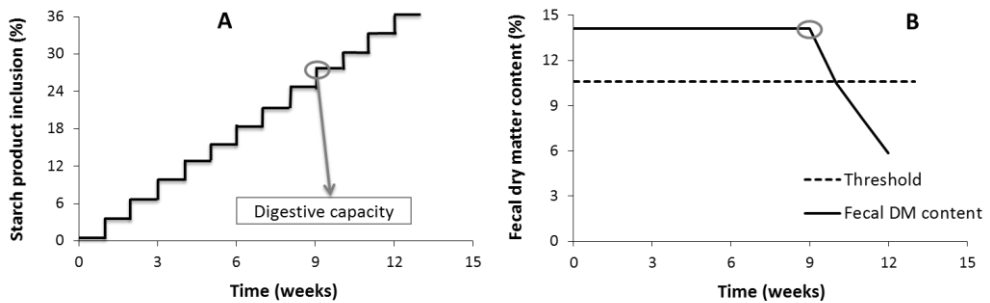


Figure 2.1 Approach to identify the maximum digestive capacity for each of the four starch products (SP) tested. (A): The solid line represents the inclusion level of a SP, which was increased every week by 3% points. (B): The dotted line represents the lower reference threshold for fecal dry matter (DM) content. When the fecal DM content was below the threshold for 3 consecutive weeks, the titration was stopped. The solid line represents the hypothetical response of the fecal DM content, decreasing when the calf's capacity to enzymatically hydrolyze SP is exceeded. The capacity for SP digestion is defined as the estimated inclusion level after which this decrease occurs.

As fecal DM content and pH tend to fluctuate over time, criteria were developed in order to determine when the increase in SP inclusion should be stopped. A fecal DM content of 10.6% was set as the lower reference threshold (see the 'Results' section for details). For each calf, SP inclusion increased until the fecal DM content was below this reference threshold for 3 consecutive weeks. Calves may also respond to high SP intakes by increasing MR refusals. In calves with a fecal DM content below 10.6%, the increase in SP inclusion was, therefore, stopped when MR refusals exceeded 10% for 3 consecutive days. In calves with a fecal DM content above 10.6%, the increase in SP inclusion was stopped

when MR refusals exceeded 25% per week, with MR refusals occurring during at least seven feedings.

Calves were housed in pairs on wooden-slatted floors. Per calf, 2.7 m² were available. Lights were on from 0600 to 1800 h. The stable was mechanically ventilated and the average temperature was 11 ± 2.9°C and the average humidity was 94 ± 8.8% (both mean ± SD). The experiment was approved by the Animal Care and Use Committee of Wageningen University.

Diets and feeding

Until 13 weeks of age, calves were fed a commercial MR and crushed barley from 3 to 8 weeks of age. At 13 weeks of age, all calves were adapted to the CON diet in 4 days (Table 2.1), which all calves received thereafter for a week. After this week, calves were assigned to the dietary treatments.

All calves were fed individually according to their metabolic BW (kg^{0.75}) at twice the metabolizable energy requirements for maintenance (ME_m) with estimated ME content based on the CON treatment. ME_m was set at 460 kJ/kg^{0.75} per day (Van Es et al., 1967). For SP treatments, lactose was exchanged for SP on a weight basis. MR was provided in buckets. Solid feed was not provided, as this would lead to difficulties in estimating starch entry into the intestinal tract.

To allow the SP inclusion level to increase up to 36%, a basal MR was formulated, which was included at 625 g/kg for each experimental MR. The remaining 375 g/kg consisted of lactose for the CON treatment and a combination of lactose and SP for the SP treatments. The ingredient and nutrient composition of MR is shown in Table 2.1. Each calf received 8.9 g crude protein/kg^{0.75} per day, 9.5 g crude fat/kg^{0.75} per day and 27 g carbohydrate/kg^{0.75} per day.

MR was mixed with water at 66°C to obtain a concentration of 143 g/kg and was supplied to the calves at a temperature of ~42°C at 0600 and 1600 h in two equal portions. Water was available ad libitum.

Measurements

Each week, measurements were performed in order to determine whether the titration of SP for individual calves should be pursued or stopped according to the criteria described above. Feces were collected directly from the rectum 4 days after exposure to the SP inclusion level and at the same day for the CON calves. Collection started directly after the morning feeding. Throughout the experiment, feces were collected successfully during the first attempt for ~75% of the calves. For the other calves, feces were collected after the next feeding. A maximum of three attempts was required in order to collect fecal samples

from all calves. Fecal samples were analyzed directly after sampling for DM content and pH. Calves were weighed weekly in order to adapt the feeding level to their metabolic BW.

Table 2.1 Ingredient and analyzed nutrient composition of the experimental milk replacer.

Ingredient	g/kg	Nutrient	g/kg DM ³
Lactose or SP ¹	375.0	Dry matter (g/kg)	979
Basal diet		Crude ash	65
Delactosed whey powder	285.4	Crude protein ($N \times 6.25$)	177
Whey protein concentrate	123.2	Crude fat	182
Fat		Lactose ^{1,4}	527
Lecithin	11.4	Lysine ⁴	19
Palmstearin	46.2	DL-Methionine ⁴	8.7
Palm oil	30.0	L-Threonine ⁴	12.3
Soy oil	45.0	Fe (mg/kg DM)	47.1
Palm-kernel oil	37.5		
Emulsifier	2.4		
Calcium formate	14.8		
Premix ²	8.0		
Mono ammonium phosphate	6.2		
L-lysine HCl	7.2		
Methionine	5.7		
Threonine	1.4		
Citric acid	0.6		

SP = starch product; DM = dry matter.

¹ In the control treatment, lactose was the only source of carbohydrate. In the four SP treatments (i.e. gelatinized starch, maltodextrin, maltodextrin with a high degree of α -1,6-branching and maltose), lactose was stepwise replaced by a specific SP as presented in Figure 2.1.

² Provided (per kg of experimental diet): crude protein, 1.2 g; starch, 0.1 g; lactose, 3.8 g; crude ash, 0.3 g; Ca, 2.5 mg; P, 335 mg; Na, 0.5 mg as sodium selenite; K, 1.0 mg as potassium iodide; Cl, 0.6 mg as choline chloride; Mg, 129 mg as magnesium oxide; Fe, 13 mg as ferrous sulphate; Cu, 2.0 mg as copper sulphate; Zn, 26 mg as zinc sulphate; Mn, 10 mg as manganese sulphate; Se, 0.1 mg as sodium selenite; I, 0.3 mg as potassium iodide; retinol, 2.2 mg; cholecalciferol, 26 μ g; DL- α -tocopherol acetate, 26 mg; menadione, 0.5 mg; L-ascorbic acid, 25 mg; thiamine, 1.3 mg; riboflavin, 2.6 mg; niacin, 8.9 mg; D-pantothenic acid, 4.6 mg; pyridoxine, 1.6 mg; cobalamin, 26 μ g; biotin, 25 μ g; choline, 52 mg; folic acid, 186 μ g.

³ Values are given in g/kg DM unless stated otherwise.

⁴ Calculated content.

Analytical procedures

Fecal samples were analyzed for DM content by drying at 70°C overnight followed by drying at 103°C for 4 h (ISO, 1999). Fecal pH was determined within 60 min after sampling using a pH meter (Hanna instruments, type: HI 9024, Woonsocket, Rhode Island, USA). The SP were characterized by high-performance size exclusion chromatography (HPSEC) and by high-performance anion exchange chromatography (HPAEC; Zhao et al., 2012). The molecular weight distribution of the SP samples was analyzed by HPSEC using an Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA, USA), equipped with a RI72 refractive index detector (Showa Denko K.K., Tokyo, Japan). The SP samples were solubilized in water (2.5 mg/mL) and 25 µL of the solution was injected. Separation of molecular weight fractions was performed on three TSK gel superAW columns in series (AW4000-AW3000-AW2500, each 6 × 150 mm; Tosoh Bioscience, Tokyo, Japan) in combination with a guard column (3.5 × 46 mm; Tosoh). For elution, 0.2 M sodium nitrate was used at a flow rate of 0.6 mL/min at 55°C.

The oligosaccharide profile of the SP samples was analyzed by HPAEC (Dionex ISC 3000; Dionex), equipped with a DionexCarboPac PA-1 column (2 × 250 mm) in combination with a CarboPac PA-1 guard column (2 × 50 mm). The SP samples were solubilized in water (0.01 to 0.1 mg/mL) and 10µL of the solution was injected using a Dionex ISC3000 autosampler and eluted (0.3 mL/min) using a gradient of 5 to 400 mM NaOAc in 100 mM NaOH during 50 min. Detection was performed using a Dionex ED40 detector in the pulsed amperometric detection mode.

The degree of branching of SP was analyzed by determining the molecular weight distribution before and after de-branching the SP samples with pullanase. Solubilized SP samples were analyzed by SEC using Multi Angle Laser Light Scattering for detection and dimethyl sulfoxide as eluent. The degree of polymerization (DP) of the de-branched part was determined using Pullulan standard calibration. From the increase in the chromatogram after de-branching and the DP of this de-branched part, the percentage of branches in the SP was calculated.

Statistical analyses

Initial BW, fecal DM content and pH were analyzed for treatment effects by ANOVA using the GLM procedure in SAS 9.2. To analyze the effect of SP inclusion, fecal DM content and pH was regressed against time. In this way, it was analyzed whether fecal DM content or pH changed over time for CON calves. For SP-fed calves, time coincided with increasing inclusion level of SP. One week corresponded to a 3% increase in SP. Fecal DM content and pH decreased already at very low SP inclusion levels. Therefore, linear regression provided a better fit of the data (fecal DM content or pH v. time) than non-linear

regression [linear-plateau model, Koops and Grossman, 1993; $Y = a + b \times \text{TIME} - b \times \log(1 + \exp(\text{TIME} - c))$ or quadratic model; $Y = a + b \times \text{TIME} + c \times \text{TIME}^2$], based on the F-test and correction for the number of model parameters. Inflection points were, therefore, not estimated. The mixed procedure in SAS 9.2 was used to investigate the effect of time (corresponding to SP inclusion for SP-fed calves) on fecal DM content, pH and BW. The following model was used:

$$Y_{ij} = \mu + \text{TREAT}_i + \text{TIME}_j + (\text{TREAT} \times \text{TIME})_{ij} + \text{TIME}_0 + \varepsilon_{ij}$$

where, Y is the dependent variable (fecal DM content, fecal pH or BW) during the titration, μ the mean intercept, TREAT_i the treatment ($i = 1, 2, 3, 4, 5$), TIME_j the time in weeks ($j = 1, 2, \dots, 13$, where 1 week corresponds to an increase of 3% in inclusion level of SP for the SP-fed calves), TIME_0 represents the fecal DM content, fecal pH or BW during the first experimental week, which was taken as a covariate in the model and ε_{ij} the error term. Calf was included in the repeated statement to account for repeated measurements per calf. Based on fit statistics (AIC and BIC), the first-order autoregressive covariance structure was used for fecal DM content and pH and the Toeplitz covariance structure was used for BW. Model residuals were checked on homogeneity of variance. Data transformations were used for pH (square) and for BW (transformed $\text{BW} = (\text{BW}^c - 1)/c$, with $c = 4$) to obtain homogeneity of variance. Differences were considered significant when $P < 0.05$. When main effects were significant, pairwise comparisons were made using the estimate statement. Pearson correlation coefficient was estimated between fecal DM content and fecal pH using the CORR procedure. Results are expressed as non-transformed estimates for parameters analyzed with the mixed procedure and as means for the initial parameters analyzed with the GLM procedure.

RESULTS

SP characteristics

The characteristics of the industrial SP are shown in Table 2.2 and the molecular weight distributions of the SP are shown in Figure 2.2. Figure 2.2 shows that GS contained only polymers and was, therefore, a suitable product for evaluating α -amylase activity when comparing results of the GS treatment with the MT treatment. Table 2.2 shows that MT contained 609 g/kg DM maltose and was, therefore, a suitable product for evaluating maltase activity. MD and MDB were selected so that the main difference between these SP would be the degree of branching in order to evaluate isomaltase activity. The degree of branching was higher for MDB than for MD, although the difference in degree of branching was only 1%. This branching is located in the higher molecular weight fraction,

because no iso-linkages were detected in the oligomers with HPAEC (data not shown). The dextrose equivalent was slightly higher for MD compared with MDB, which was (partly) caused by higher concentrations of glucose, maltose and maltotriose in MD.

Table 2.2 Characteristics of the four starch products used in the experimental milk replacers fed to calves.

	Gelatinized starch	Maltodextrin	Branched maltodextrin ¹	Maltose
Dextrose equivalent ² (minimum - maximum)	0	11 - 15	8 - 10	43 - 47
Glucose ³ (g/kg DM)	0	4	3	31
Maltose ³ (g/kg DM)	0	47	21	609
Maltotriose ³ (g/kg DM)	0	63	37	165
Degree of polymerization > 3 (g/kg DM) ⁴	1000	886	939	195
Branching ⁵				
Branching (% of total product)	3.6	3.4	4.4	0.0
Degree of polymerization of branched part	21.1	11.6	11.6	- ⁶

¹ Maltodextrin with a high degree of α -1,6-branching.

² Adapted from product specifications for maltodextrin, maltodextrin with a high degree of α -1,6-branching and maltose.

³ Analyzed by high-performance anion exchange chromatography.

⁴ Calculated as 1000-glucose-maltose-maltotriose.

⁵ Analyzed by size exclusion chromatography and enzymatic de-branching.

⁶ Maltose has no branched part and, therefore, no degree of polymerization is given.

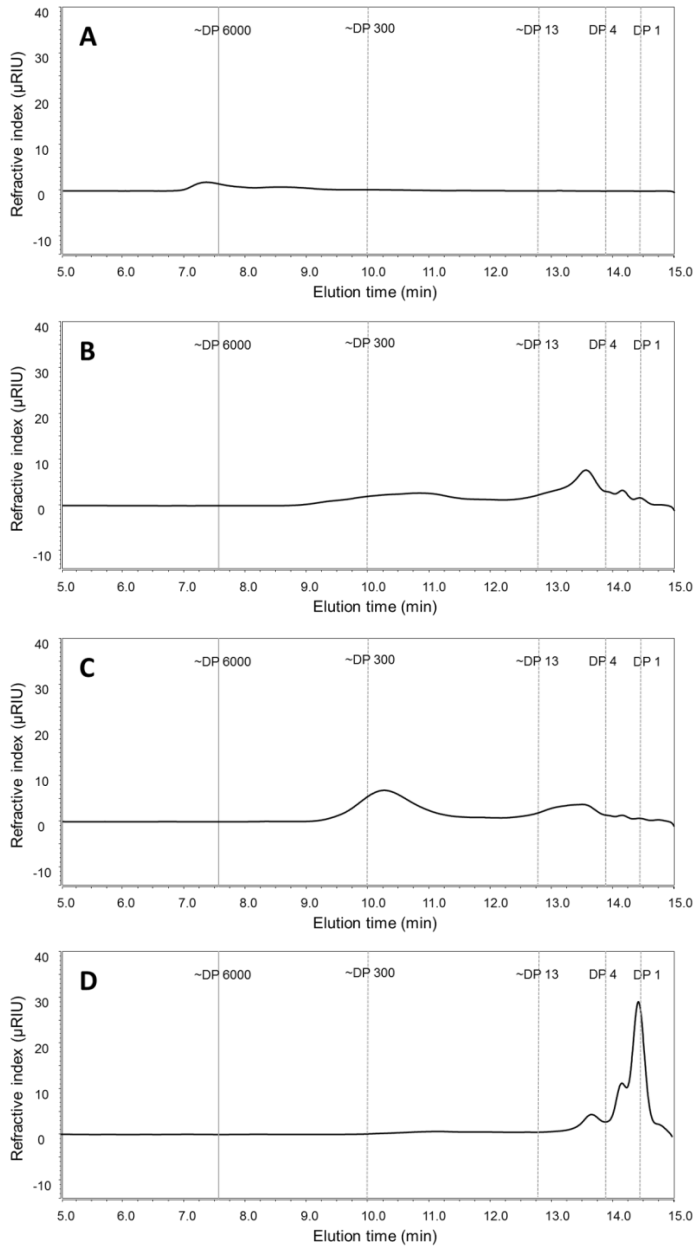


Figure 2.2 The molecular weight distribution of the four starch products fed to milk-fed calves. (A): Gelatinized starch; (B): maltodextrin; (C): maltodextrin with a high degree of α -1,6-branching; (D): maltose.

General

At the start of the first experimental week, when all calves received CON, calves weighed 103.6 ± 1.1 kg. Fecal DM content averaged $14.1 \pm 1.3\%$ and fecal pH averaged 7.6 ± 0.1 (Table 2.3). The fecal DM content, rather than fecal pH, was taken as the main criterion in the titration, because it appeared from our initial observations that fecal pH responded less sensitively to SP inclusion than fecal DM content. Therefore, a fecal DM content of 10.6%, being 75% of the initial DM content, was set as the lower reference threshold. The increase in SP inclusion of one calf was stopped according to MR refusal criteria as described previously. For all other calves, the increase in SP inclusion was stopped according to the fecal DM content criterion.

One calf from the CON treatment was excluded from the experiment based on persistent MR refusals and ruminal drinking, which resulted in bloating. One calf from the MT treatment died during the titration, but the cause of death could not be established. At an inclusion level of 18%, DM intake equaled $50.3 \text{ g/kg}^{0.75}$ for GS, $50.4 \text{ g/kg}^{0.75}$ for MD, $50.2 \text{ g/kg}^{0.75}$ for MDB and $50.5 \text{ g/kg}^{0.75}$ for the MT treatment. At this time point in the titration, DM intake equaled $51.0 \text{ g/kg}^{0.75}$ for the CON treatment. An interaction between treatment and time was detected for BW ($P < 0.05$; Table 2.3). The estimated BW gain of the calves was 1131 ± 31 g/day, but BW gain of GS and MT calves was lower compared with CON calves.

Digestive capacity

For CON calves, fecal DM content and pH did not change over time. Only for 8 out of 38 calves, the linear-plateau model converged. However, for none of these eight calves, the linear-plateau model provided a better fit than the linear model. For 87% of the SP-fed calves, fecal DM content and pH decreased already at low SP inclusion levels. For these calves, linear regression provided a better fit of the data (fecal DM content or pH v. time) than quadratic regression. Hence, inflection points could not be estimated. For all SP treatments, the fecal DM content and fecal pH decreased in time ($P < 0.001$), where time corresponded to an increasing inclusion level of SP. Slopes of fecal DM content and pH in time are shown in Table 2.3. The slopes for fecal DM content and fecal pH of all SP treatments differed from CON ($P < 0.001$ for all SP treatments). Slopes did not differ among SP treatments. The mean squared error for the time related slope in fecal DM content decreased from 6.9 to 6.1 when the covariate was included. The mean squared error for the slope in untransformed fecal pH was 0.5, both with and without inclusion of the covariate. Furthermore, fecal DM content and pH were positively correlated ($r = 0.62$, $P < 0.001$).

Table 2.3 Initial BW, fecal DM content and fecal pH and estimated changes in fecal DM content, fecal pH and BW in time of calves fed a milk replacer containing only lactose (control) or increasing inclusion levels of gelatinized starch, maltodextrin, maltodextrin with a high degree of α -1,6-branching or maltose.

Treatment	Control	Gelatinized starch	Maltodextrin	Branched maltodextrin ¹	Maltose	Pooled SEM	<i>P</i>			
							Treatment	Time	Treatment × Time	Time ₀
Initial (Time ₀) ²										
Number of calves	8 ³	8	8	8	8 ³					
BW (kg)	102.9	103.5	105.8	103.1	102.4	1.1	0.14	-	-	-
Fecal DM (%)	14.1	13.2	12.9	14.9	15.2	1.3	0.65	-	-	-
Fecal pH	7.6	7.6	7.4	7.7	7.8	0.1	0.23	-	-	-
Change of parameters in time ⁴										
Fecal DM (%/week)	0.14 ^A	-0.47 ^B	-0.51 ^B	-0.55 ^B	-0.77 ^B	0.11	0.35	< 0.001	< 0.001	< 0.001
Fecal pH (per week)	-0.02 ^A	-0.32 ^B	-0.33 ^B	-0.34 ^B	-0.29 ^B	0.03	0.28	< 0.001	< 0.001	0.69
BW (g/day)	1169 ^A	1130 ^B	1166 ^{AB}	1135 ^{AB}	1056 ^B	31	0.29	< 0.001	0.019	< 0.001

DM = dry matter.

¹ Maltodextrin with a high degree of α -1,6-branching.

² Initial values were determined at the start of the experiment when all calves received the control diet. Initial values are expressed as means.

³ n = 8 for the initial BW, fecal DM and fecal pH, n = 7 for the change of parameters in time.

⁴ Changes of parameters in time were estimated as described in the text. One week corresponds to an increase in starch product inclusion of 3%. Changes of parameters in time are expressed as non-transformed estimates.

^{AB} Means in the same row with different superscripts are significantly ($P < 0.01$) different.

DISCUSSION

SP characteristics

The SP were selected based on their size and branching in order to assess enzyme activity. GS contained only polymers and MT contained only low molecular weight fractions and mainly maltose. Therefore, these treatments were used to assess α -amylase activity. Although MDB was more branched compared to MD (29% increase), only 4.4% of the MDB was branched. This contrast was smaller than anticipated and might have been too small to detect differences in isomaltase activity. However, both MD and MDB contained α -1,6 linkages, whereas no α -1,6 linkages were detected in MT. Therefore, the fecal responses of calves fed MD and MDB can be compared with calves fed MT in order to assess isomaltase activity indirectly.

General

All SP treatments had a numerically lower daily BW gain compared with the CON treatment, although this could (partly) be ascribed to the lower DM intake for SP-fed calves because of the lower DM content in the SP than in the lactose. However, the reduction in fecal DM content with increasing intake of SP did only lead to a significant reduction in daily gain for the GS and MT treatment. Although the study was not designed to measure a response in daily gain, a reduction in growth (Huber et al., 1968, Natrajan et al., 1972), as a result of fermentation instead of digestion, was expected. The lower daily gain for the MT treatment is associated with a greater numerical decrease in fecal DM content in time, but not with fecal pH. Probably, the MT treatment resulted in a higher osmotic pressure and subsequent greater passage rate and, therefore, resulted in a decreased daily gain. There was a strong positive correlation between fecal DM content and pH, which corresponds with previous findings in milk-fed calves (Roy, 1969). The decrease in fecal pH with increasing inclusion level indicates the increase in fermentative degradation of SP.

We used a within-animal approach to investigate the effect of inclusion level of SP on fecal DM content and pH in milk-fed calves. This approach allowed us to incorporate the initial fecal DM content and pH as a covariate. Including this covariate in the model for fecal DM content improved the estimation of the slopes (mean squared error decreased from 6.9 to 6.1 when including the covariate). Another advantage of this approach is that it requires less animals compared with the conventional between-animal titration approach. Testing four SP treatments at for instance five inclusion levels and five animals per treatment would already require 100 calves, whereas our approach required only 40 calves. However, in our approach inclusion level of SP was confounded with time and, therefore,

age. Therefore, the control treatment was included. For the CON treatment, no change over time was found in fecal DM content and pH. The slopes for fecal DM content and pH of the SP treatments differed from CON, indicating that these negative slopes found for the SP treatments were caused by inclusion of SP and not by other factors such as age.

Digestive capacity

Fecal DM content and pH did not change in time for CON calves. A linear relation between time and fecal DM content and pH was found for most SP calves, indicating that the digestive capacity was already exceeded at low levels of SP intake. The linear relation shows that every per cent increase of SP in the MR, results in a constant decline in fecal DM content and pH, regardless the inclusion level. This indicates that fermentation contributes to starch disappearance already at low inclusion level. Apparent ileal digestibility of lactose is high in calves (97% of intake; Coombe and Smith, 1974), and including SP in the MR instead of lactose is, therefore, likely to reduce feed efficiency. Nonetheless, it may still be attractive to include starch in MR when starch is relatively cheap compared with lactose.

Roughly 50% of the calves suffered from diarrhea (fecal DM content < 10.6%) at a SP inclusion level of 18% (9 g of SP/kg^{0.75} per day). This is in contrast with data from Nitsan et al. (1990), where diarrhea was not observed (DM content 16 to 20%) in calves fed 9 g of corn starch/kg^{0.75} per day. However, starch concentration in the MR was not equal between the morning and afternoon meal in the study of Nitsan et al. (1990) and the calves used were younger compared with the calves used in the current study. The fecal pH after the afternoon meal was lower for the corn starch-fed calves (6.0 ± 0.13) compared with glucose-fed calves (7.3 ± 0.28; Nitsan et al., 1990), which is in agreement with our findings for SP-fed calves. The occurrence of diarrhea when providing starch has been documented before in milk-fed calves (Flipse et al., 1950, Huber et al., 1961) and sheep (Ørskov et al., 1970). We found a linear decrease in fecal DM content with increasing inclusion level of SP, indicating that, despite the slow increase in SP inclusion level, adaptation of enzymes is limited in milk-fed calves. This corresponds with findings by Natrajan et al. (1972) who did not find differences in total tract starch digestibility between milk-fed calves that were adapted for either 4 days or 12 weeks to starch inclusion. At the ileal level, however, differences in starch digestion may have occurred in that study.

The decline in fecal pH with increasing SP inclusion is in agreement with studies in ruminants. An inverse linear relation was found between abomasal starch or dextrin infusion and ileal digesta pH in steers (Kreikemeier et al., 1991) and between ileal glucoside content and ileal pH in sheep (Mayes and Ørskov, 1974). This linear decline

indicates that the enzymatic capacity for starch digestion is already low at low inclusion levels of starch and suggests that fermentation may contribute substantially to SP disappearance. Small intestinal starch disappearance has been studied in ruminants. In steers, small intestinal disappearance was 66% for corn starch after abomasal infusion of 66 g/h. Of this corn starch disappearance, only 23% was recovered as glucose in the portal vein (Kreikemeier and Harmon, 1995). The unaccounted part of the starch disappearance can be explained by intestinal fermentation or glucose metabolism in gastro-intestinal tissues. The latter was accounted for by correcting for the negative portal glucose uptake when infusing water, leading to a corrected portal glucose flux of 57% of the small intestinal disappearance. This indicates that 43% of the corn starch that disappeared in the small intestine could have been fermented. The fermentation hypothesis is supported by a higher concentration of short-chain fatty acids in the ileal digesta of starch- and dextrin-infused steers (Kreikemeier and Harmon, 1995).

The relationship between fecal DM content and SP inclusion level appeared linear and no inflection points could be estimated. We assumed that differences in slopes, instead of differences in inflection points, between SP treatments would enable us to deduce the rate-limiting enzyme. As the slopes of the GS and MT treatment did not differ, α -amylase can be considered non-limiting in starch hydrolysis. If isomaltase would be the limiting enzyme in starch hydrolysis, the drop in fecal DM content and pH for MD and MDB calves would be expected to be larger than the drop for MT calves. The lack of differences between SP treatments implies that maltase activity limits starch digestion in milk-fed calves, because all SP require maltase to achieve complete hydrolysis to glucose.

Alternatively, glucose absorption from the intestinal lumen could limit the uptake of starch-derived glucose and result in a reduced fecal DM content. To further investigate this, maltose feeding should be compared with glucose feeding. However, a high oral glucose load could lead to osmotic diarrhea, not necessarily coinciding with a decrease in fecal pH. Indeed, infusion of glucose in the abomasum of steers at 80 g/h (Kreikemeier et al., 1991) and feeding a glucose solution of 4.8 g/kg BW to calves by nipple bottle (Sen et al., 2006) resulted frequently in diarrhea. Comparing glucose to maltose feeding in a titration approach, as in the current study, would therefore be difficult because osmotic diarrhea is likely to occur. Even in our study, osmotic pressure could have contributed to the decrease in fecal DM content, especially in the MT treatment. Providing a maltose solution to cattle resulted in diarrhea in all age groups (varying from 22 to 600 days), whereas diarrhea was not observed after providing a starch solution (Huber et al., 1961). Results from other studies suggest that glucose absorption is not rate-limiting in starch uptake from the small intestine. When maltose was fed to calves, apparent ileal maltose disappearance was 43% of intake and the remaining sugars in the ileum did not contain

any free glucose (Coombe and Smith, 1974). Abomasal infusion of corn starch in steers resulted in an ileal disappearance of starch of 66% and the remaining sugars in the ileum contained only 4% free glucose (Kreikemeier and Harmon, 1995). Abomasal infusion of glucose in steers resulted in a higher portal glucose uptake compared with starch or dextrin infusion (Kreikemeier et al., 1991, Kreikemeier and Harmon, 1995). We suggest that maltase activity probably limits starch digestion in milk-fed calves.

In conclusion, fecal DM content and pH sensitively respond to incremental inclusion of SP. This suggests that the maximum capacity for starch digestion is already exceeded at low levels of SP intake. The linear decrease is independent of the SP size and degree of branching. This indicates that maltase limits starch digestion in milk-fed calves and that fermentation may contribute substantially to total tract starch disappearance in milk-fed calves.

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Chapter 3

Fermentation in the small intestine contributes substantially to intestinal starch disappearance in calves



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ABSTRACT

The proportion of starch disappearing from the small intestinal lumen is generally lower in ruminants than in monogastric animals, and there are indications that the starch digestion capacity in ruminants is limited. Milk-fed calves were used to study the rate-limiting enzyme in starch hydrolysis and to quantify starch fermentation in ruminants. Forty male Holstein-Friesian calves were fed milk replacer containing either lactose (control) or one of four corn starch products. The following starch products differed in the enzyme ratios required for their complete hydrolysis to glucose: gelatinized starch [α -amylase and (iso)maltase], maltodextrin [(iso)maltase and α -amylase], maltodextrin with α -1,6-branching (isomaltase, maltase, and α -amylase), and maltose (maltase). In the adaptation period, calves were stepwise exposed to an increasing dose of the starch product for 14 weeks to allow maximal adaptation of all enzyme systems involved. In the experimental period, apparent total tract and ileal starch product disappearance, total tract starch product fermentation, and α -amylase, maltase, and isomaltase activities were determined at 18% inclusion of the starch product. Maltase and isomaltase activities in the brush border did not increase for any of the starch product treatments. Luminal α -amylase activity was lower in the proximal (3.9 ± 3.2 and 2.7 ± 1.7 U/mg Co for control and starch product calves, respectively) but greater in the distal small intestine of starch-fed calves than in control calves (0.0 ± 0.0 and 6.4 ± 1.5 U/mg Co for control and starch product calves, respectively; means \pm SE for control and means \pm pooled SEMs for starch product treatments). Apparent ileal ($61.6 \pm 6.3\%$) and total tract ($99.1 \pm 0.4\%$) starch product disappearance did not differ between starch product treatments, suggesting that maltase activity limits starch digestion in ruminants. Total tract starch product fermentation averaged 414 ± 43 g/d, corresponding to 89% of intake, of which half was fermented before the terminal ileum, regardless of starch product treatment. Fermentation, rather than enzymatic digestion, is the main reason for small intestinal starch disappearance in milk-fed calves.

Keywords: starch digestion, starch fermentation, milk-fed calf, ruminant, maltase, α -amylase, isomaltase

INTRODUCTION

Adult ruminants usually absorb only small amounts of glucose from starch, because microbes in the rumen readily degrade starch, resulting in the production of volatile fatty acids (VFA). Required glucose is mainly synthesized *de novo* from propionic acid or amino acid precursors (Danfær et al., 1995). Mammalian neonates depend for their energy supply mainly on lactose and fat provided by the milk of the mother. After weaning of monogastric animals, the carbohydrate supply shifts from lactose to starch, resulting in a decreased lactase activity and an increased maltase activity (Reddy and Wostmann, 1966, Marion et al., 2005). In ruminants, weaning causes a shift from lactose to VFA as the main energy supply. When glucose demand is high (e.g., in lactating cows), nutritional strategies are applied to maximize the duodenal flow of starch, escaping ruminal degradation. It is assumed that this starch is enzymatically hydrolyzed, resulting in the absorption of glucose from the small intestine. It is, however, questionable whether a large portion of this starch is absorbed as glucose in ruminants, because the portal glucose appearance was substantially lower than small intestinal starch disappearance in steers (Kreikemeier and Harmon, 1995).

To assess small intestinal starch disappearance in ruminants, the flux of starch into the small intestine needs to be quantified. Available methods include measuring the duodenal flux of starch or infusing starch directly into the abomasum, both of which require the use of cannulas. Alternatively, milk-fed calves could be used to administer starch directly into the small intestine. Closure of the esophageal groove of calves allows the milk replacer to flow directly into the abomasum and subsequently into the small intestine (Abe et al., 1979). By adding starch to the milk replacer fed to pre-ruminant calves, their capacity to digest starch can be assessed. Previous studies in milk-fed calves indicated that ileal disappearance of partially acid-hydrolyzed starch was low (60% of intake) compared with that of lactose (97% of intake; Coombe and Smith, 1974). Similarly, the ileal disappearance of corn starch in steers (66% of abomasal infusion; Kreikemeier and Harmon, 1995) was low compared with that in pigs (99% of intake; Lin et al., 1987). In addition, there are indications that fermentation contributes to starch disappearance from the small intestinal lumen of ruminants. For instance, ileal pH decreased linearly with increasing infusion amount of raw corn starch or partially hydrolyzed corn starch in the abomasum of steers (Kreikemeier et al., 1991, Branco et al., 1999). Hence, the capacity to enzymatically hydrolyze starch may be limited in ruminants.

Pancreatic α -amylase activity appears to be greater in omnivores (Deren et al., 1967, Kidder and Manners, 1980) than in ruminant steers (Russell et al., 1981), although the methods for determining α -amylase activity often vary, which complicates comparisons

between studies. Maltase activity was 6.5 times greater (Deren et al., 1967, Kidder and Manners, 1980) and isomaltase activity was 44 times greater (Kidder and Manners, 1980) in omnivores than in pre-ruminant calves fed a milk replacer containing 3% starch (Le Huerou et al., 1992). There are indications that pancreatic α -amylase activity (as reviewed by Huntington, 1997) or brush border enzyme activities (Kreikemeier and Harmon, 1995) are limiting factors for starch hydrolysis in ruminants.

The objectives of the current study were as follows: 1) to determine the rate-limiting enzyme in starch hydrolysis and 2) to quantify starch fermentation in milk-fed calves. Milk-fed calves were also considered as a model for ruminants. Four products derived from starch were selected to be included in the milk replacer. These starch products (SP) differed in degree of polymerization and branching and consequently required different ratios of starch-degrading enzymes for their complete hydrolysis to glucose. The combination of apparent ileal SP disappearance, intestinal SP fermentation, and activities of starch-degrading enzymes in calves fed these selected SP was expected to provide insight into the rate-limiting enzyme, after allowing ample time for enzyme systems to adapt to the different SP.

MATERIAL AND METHODS

Experimental design and animals

Four industrial products derived from starch (hereafter referred to as SP) were selected; gelatinized starch (GS; Tate & Lyle Europe), maltodextrin (MD; Tereos Syral; dextrose equivalent = \sim 13), maltodextrin with a high level of α -1,6-branching (MDB; Tereos Syral; dextrose equivalent = \sim 9), and maltose (MT; Tereos Syral). The SP characteristics have been described in detail (Chapter 2). These SP differed in degree of polymerization and branching and consequently required different ratios of starch-degrading enzymes for their complete hydrolysis to glucose. GS requires α -amylase and (iso)maltase; MD requires (iso)maltase and α -amylase; MDB requires isomaltase, maltase, and α -amylase; and MT requires maltase only. A proportional increase in α -amylase requirement is expected with an increase in degree of polymerization.

The experiment was submitted to and approved by the Animal Care and Use Committee of Wageningen University. Forty male Holstein-Friesian calves of 13 weeks of age (104 ± 1.1 kg) received one of five milk replacer treatments varying in carbohydrate source as their only source of nutrients. The control (CON) treatment contained lactose as the only carbohydrate source. In the other treatments, one of four SP was included at 18% in the milk replacer at the expense of lactose.

The experiment consisted of an adaptation and an experimental period. In the adaptation period, calves were exposed to the dietary treatments for 14 weeks. Within these 14 weeks, the amount of SP inclusion gradually increased (until a maximum of 36%) and fecal dry matter (DM) content and pH were measured to assess the maximum SP inclusion amount without digestive problems for each individual calf (Chapter 2). The median of this maximum SP inclusion amount was 18% and was applied in the experimental period. The experimental period started when calves were 27 weeks of age (217 ± 3.9 kg) and lasted 3 weeks. Measurements on total tract and ileal nutrient disappearance, total tract SP fermentation, blood glucose response to a meal, and enzyme activities were performed during the experimental period. It was hypothesized that differences in these measurements between SP treatments could be used to identify the rate-limiting enzyme system in starch digestion.

Diets and housing

Calves were fed individually according to their metabolic weight ($\text{kg}^{0.75}$) at twice the metabolizable energy requirements for maintenance, with estimated metabolizable energy content based on the CON treatment. The metabolizable energy requirement for maintenance was set at $460 \text{ kJ/kg}^{0.75}$ per day (Van Es et al., 1967). Milk replacer was mixed with water to obtain a concentration of 154 g/kg and was supplied to the calves at a temperature of $\sim 42^\circ\text{C}$ in a bucket at 0600 and 1600 h in 2 equal portions. Ingredient and nutrient compositions have been described (Chapter 2). In short, milk replacer contained 171 g/kg crude protein, 176 g/kg crude fat, 63 g/kg crude ash, 21 g/kg moisture, and 522 g/kg lactose for the CON treatment. For the SP treatments, 180 g lactose/kg was exchanged for SP on a wt:wt basis. All SP originated from corn and differed in natural ^{13}C enrichment [1.0931 atom percentage (atom%) for GS, 1.0936 atom% for MD, 1.0919 atom% for MDB, and 1.0935 atom% for MT] from lactose (1.0727 atom%) and the remaining part of the diet (1.0767 atom%). All diets included cobalt-EDTA as an indigestible marker (1.3 g Co-EDTA/kg milk replacer). Chromium chloride (7.69 g CrCl_3 hexahydrate) was added to the last milk replacer feeding before anesthesia. Solid feed was not provided, because this would lead to difficulties in estimating starch flow into the intestinal tract. Water was provided ad libitum.

Calves were housed in pairs ($2.7 \text{ m}^2/\text{calf}$) on wooden, slatted floors. During the collection of feces for the measurement of total tract nutrient disappearance, calves were housed individually for 8 consecutive days. During this period, calves could change posture freely but could not turn around, facilitating the collection of feces. Lights were on from 0600 to 1800 h. The stable was mechanically ventilated, and the temperature and humidity

averaged $5.2 \pm 2.63^{\circ}\text{C}$ and $94 \pm 9.3\%$, respectively (both mean \pm SD), during the experimental period.

Sample collection and measurements

Calves were weighed weekly and were habituated to individual housing for 4 d. Feces were collected semi-quantitatively during 4 consecutive days to measure total tract nutrient disappearance and to calculate total tract SP fermentation on the basis of fecal ^{13}C excretion (see calculations; Gerrits et al., 2012). Feces were collected from the buckets underneath the slatted floor twice a day and were weighed, homogenized, sampled, and stored at -20°C until analysis.

To assess the appearance of glucose from lactose, GS, or MT in peripheral blood, a pulse dose of naturally ^{13}C -enriched lactose (1.0917 atom%, included at 375 g/kg milk replacer) or naturally ^{13}C -enriched SP (1.0930 atom%, included at 180 g/kg milk powder) was provided with the regular morning meal to CON, GS, and MT calves, respectively. Blood samples were taken from the jugular vein by venipuncture at -30, 30, 60, 120, 180, 240, and 360 min after feeding and collected in heparin tubes. Plasma was harvested after centrifugation. Plasma samples were stored at -20°C until analysis of ^{13}C enrichment in plasma glucose. In addition to the CON treatment, the GS and MT treatments were selected for the measurement of ^{13}C enrichment of plasma glucose because these treatments differed most in degree of polymerization.

Postmortem, digestus, and mucosal scrapings were collected from gastro-intestinal sections to assess ileal nutrient disappearance, passage of milk replacer, and enzyme activity. Calves received chromium chloride at 4 h before anesthesia by pentobarbital injection. Calves were lifted by the forelegs to prevent reflux of milk replacer from the abomasum into the rumen, and subsequently killed by exsanguination. After opening the abdominal cavity, the digestive tract was ligated at 5 positions to prevent digesta from flowing between gastro-intestinal segments. Clamps were placed before the rumen, between the rumen and abomasum, after the abomasum, ~ 100 cm before the ileocecal valve, and just before the ileocecal valve; the gastro-intestinal tract was then removed from the calf. The last ~ 100 cm of the small intestine was taken as the ileum. The reticulorumen, abomasum, ileum, and large intestine (colon and cecum) were separated from the small intestine. The length of the small intestine and ileum was recorded. The small intestine without ileum was spread out and divided into 2 equal segments, i.e., small intestine 1 and small intestine 2. The contents of each of the 3 small intestinal segments and of the reticulorumen, abomasum, and large intestine were collected quantitatively, weighed, homogenized, sampled, and stored at -20°C . Then, each small intestinal segment was cut open longitudinally at 50% of its length and rinsed with PBS. Mucosal scrapings

were collected from ~80 cm² of each small intestinal segment by using a glass slide and were stored at -20°C. This complete process was finished within 20 min for each calf. The slaughter of all calves was performed in 3 d, and treatments were balanced over days.

Analytical procedures

Fecal and ileal digestus samples were thawed, pooled per calf, and homogenized. DM content in fresh feces was determined according to ISO 6496 (ISO, 1999b). DM content of feed samples was determined by vacuum drying to a constant weight at 80°C.

For the determination of nutrient disappearance, homogenized feces were subdivided and either oven-dried (for crude fat, ash, and cobalt analysis) or freeze-dried (for total glucose analysis). Ileal digesta were freeze-dried. Dry feces and ileal digesta were ground to pass a 1-mm screen and subsequently analyzed for DM content (ISO, 1999b). Nitrogen content was determined in fresh feces and feed samples (Kjeldahl method; ISO, 2005) and in freeze-dried ileal digesta (Dumas combustion method, AOAC 990.03 by using a Thermo Quest NA 2100 Nitrogen and Protein analyzer from Interscience; AOAC, 2005). Crude fat content was determined after acid hydrolysis (ISO, 1999a) in oven-dried feces and in feed samples. Crude ash content was determined in fecal samples according to ISO 5984 (ISO, 2002). For the determination of SP disappearance, total glucose content (Total Starch kit from Megazyme; AOAC 996.11; AOAC, 2005) was determined in SP samples, freeze-dried feces, and freeze-dried ileal digesta. Total glucose was determined instead of starch because SP were composed of starch-derived poly- and oligosaccharides and glucose (Chapter 2). Analyzing starch content only could, therefore, underestimate the total amount of glucose present in the fecal or ileal samples originating from the SP. However, in these fecal and ileal samples, glucose originating from lactose could be present as well. Therefore, galactose was analyzed and it was assumed that the glucose originating from lactose could be calculated from the galactose content, assuming equimolar quantities of glucose and galactose.

Galactose and lactose concentrations were determined in fresh feces and fresh ileal digesta by using high-performance anion-exchange chromatography (Dionex ICS 3000; Dionex) according to Zhao et al. (2012). First, 200 mg sample was suspended in 4 mL Milli-Q water (Merck Millipore), and samples were equilibrated overnight at 4°C. Enzymes were inactivated in a water bath at 100°C for 10 min. Samples were centrifuged for 10 min at 5000 × g. Subsequently, the supernatant was filtered through a 0.2-µm filter, diluted as required, and injected (10 mL). For galactose, samples were eluted (0.3 mL/min) by using water during 35 min with the addition of 0.5 mol/L NaOH post-column (0.1 mL/min). For lactose, samples were eluted (0.3 mL/min) by using a gradient of 0.1 mol/L NaOH to 0.4

mol/L sodium acetate in 0.1 mol/L NaOH during 15 min followed by 1 mol/L sodium acetate in 0.1 mol/L NaOH for 5 min.

Total carbon and carbon with an atomic mass of 13 g (^{13}C) were determined in the SP and oven-dried feces after ball milling by using the isotope ratio MS combustion technique (Finnigan Delta V Advantage isotope ratio mass spectrometer; Finnigan MAT). Cobalt concentration in oven-dried feces and in freeze-dried ileal digesta and chromium concentration in the digesta of each gastrointestinal segment was analyzed by atomic absorption spectrophotometry (by using a SpectrAA 300 atomic absorption spectrophotometer; Varian B.V.). The recovery of chromium in each gastrointestinal segment was determined as an indicator of the passage rate of digesta and calculated from the quantity of digesta collected from each segment and its chromium concentration. Total chromium recovery was calculated as the sum of chromium recoveries in all segments.

Digestus samples of small intestine 1 and small intestine 2 were thawed and analyzed for α -amylase activity (Amylase Activity Assay Kit; Biovision). One unit of α -amylase represents the amount of α -amylase that hydrolyzes the substrate of the kit (ethylidene-para-nitrophenol-G7), resulting in 1 μmol nitrophenol/min at 25°C and pH 7.2. Luminal α -amylase activity was expressed as units per milligram of cobalt. Mucosal scrapings of 4 randomly selected calves per treatment were analyzed for lactase, maltase, and isomaltase activity by using the method of Dahlqvist (1964). One unit of brush border enzyme represents the hydrolysis of 1 μmol disaccharide/min at 37°C. Protein concentration was analyzed in the mucosal scraping samples by using the bicinchoninic acid procedure (Smith et al., 1985). Brush border enzyme activity was expressed as units per gram of protein.

Blood plasma samples of CON, GS, and MT calves were analyzed for ^{13}C enrichment in glucose. Samples were derivatized by adding 1 mL ethanol to 100 mL of plasma followed by centrifugation (10 min at 400 \times g at 4°C). The supernatant was evaporated under a constant stream of nitrogen at 60°C for 20 min. Subsequently, 50 mL pyridine and 100 mL acetic anhydride were added and mixed for 1 min by using a vortex, followed by acetylation overnight. Samples were solubilized with 1250 mL acetone and analyzed for ^{13}C enrichment in glucose by using GC combustion isotope ratio MS (GC Combustion III ThermoQuest Finnigan).

Calculations

Apparent total tract and ileal nutrient disappearance was calculated by using the following equation:

$$(1) \text{ Disappearance (\% of intake)} = [1 - (\text{Co}_{\text{feed}} / \text{Co}_{\text{digesta}}) \times (\text{Nutrient}_{\text{digesta}} / \text{Nutrient}_{\text{feed}})] \times 100$$

where Co_{feed} is the cobalt concentration in the milk replacer (mg/kg DM), $\text{Co}_{\text{digesta}}$ is the cobalt concentration in the feces or digesta (mg/kg DM), $\text{Nutrient}_{\text{digesta}}$ is the nutrient concentration in the feces or digesta (g/kg DM), and $\text{Nutrient}_{\text{feed}}$ is the nutrient concentration in the milk replacer (g/kg DM). Apparent total tract and ileal DM disappearance was calculated by using the following equation:

$$(2) \text{ Dry matter disappearance (\% of intake)} = [1 - (\text{Co}_{\text{feed}} / \text{Co}_{\text{digesta}})] \times 100$$

Total tract SP fermentation was calculated by the method of Gerrits et al. (2012) on the basis of the measured ^{13}C enrichment in basal milk replacer, lactose, SP, and feces. In short, in all SP treatments, the SP were derived from corn, resulting in higher natural ^{13}C enrichment in the SP than in the basal milk replacer and lactose (see the section ‘Diets and housing’ for details). An increase in fecal ^{13}C enrichment would result from fecal SP excretion or from microbial biomass that has incorporated the ^{13}C from SP. The SP excretion in feces was analyzed (see ‘Analytical procedures’), and microbial biomass from SP fermentation could be calculated by the difference. In this calculation, it was assumed that 1 g carbohydrate fermented results in 0.2 g microbial biomass in the feces [derived from 0.3 kJ fecal biomass/kJ carbohydrate (Livesey, 1991) and assuming 15.56 kJ/g carbohydrate (Blaxter, 1989) and 23.13 kJ/g biomass (Cordier et al., 1987)].

The ^{13}C enrichment in glucose was determined in the peripheral blood plasma of the CON, GS, and MT calves. Basal plasma samples (30 min before feeding) were taken as background values. In the postprandial samples, the ^{13}C enrichment in excess of background was calculated for plasma glucose and was expressed per gram of naturally ^{13}C -enriched carbohydrate provided.

Statistical analyses

All statistical analyses were performed with SAS 9.2 (SAS Institute). Mean daily body weight gain, length of the small intestine, intestinal disappearance of nutrients, and SP fermentation variables were analyzed for treatment effects by ANOVA with the use of the general linear model procedure. Day of slaughter did not affect ileal nutrient disappearance and was, therefore, excluded from the model. Studentized residuals of all

models were checked for homogeneity of variance. Apparent total tract crude fat disappearance (square root), apparent ileal nitrogen disappearance [transformed data = (original data^c - 1)/c, with c = 8], atom percentage of the feces (log), and fecal SP excretion (log) were transformed to obtain homogeneity of variance. Visual inspection of studentized residuals revealed 1 outlier for fecal DM output, 1 outlier for fecal nitrogen output, and 1 outlier for SP fermentation variables. There was no biological reason for excluding the data of these calves, and conducting the model with or without these calves did not affect the main treatment effect. Therefore, all calves were included in the data analysis. Enzyme activities and recovery of chromium were analyzed for treatment, segment, and treatment × segment effects by using the MIXED procedure. Segment was included as a repeated statement, with calf as the subject. On the basis of fit statistics (Akaike and Bayesian information criterion), the heterogeneous first-order autoregressive covariance structure was used for all enzyme activities and for recovery of chromium. Isomaltase and α-amylase activities and recovery of chromium were log-transformed, and lactase activity was square root-transformed to obtain homogeneity of variance. When interaction effects were found, treatment effects were analyzed for each segment separately. Total chromium recovery was analyzed for treatment effects by ANOVA with the use of the general linear model procedure.

The ¹³C enrichment of plasma glucose in excess of background was analyzed for treatment, time, and treatment × time effects by using the MIXED procedure. Time was included as a repeated statement, with calf as the subject. On the basis of fit statistics (Akaike and Bayesian information criterion), the first-order autoregressive covariance structure was used. When an interaction effect was found, treatment effects were analyzed for each time point separately. Visual inspection of studentized residuals revealed 3 outliers in the ¹³C enrichment of plasma glucose data. There was no biological reason for excluding the data of these calves, and conducting the model with or without these calves did not affect main effects. Therefore, these calves were included in the data analysis. Differences were considered significant when $P < 0.05$. When main effects were significant, pairwise comparisons were made by using Tukey's method. Results are expressed as non-transformed means and their pooled SEM.

RESULTS

One calf from the CON treatment was excluded from the experiment because of persistent milk replacer refusals and ruminal drinking, which resulted in bloat. One calf from the MT treatment died before the end of the experiment, with the cause of death remaining unknown upon autopsy. Mean daily body weight gain of the calves during the experimental period was 1.05 ± 0.05 kg/d. A greater mean daily body weight gain ($P = 0.018$) was found for the GS treatment (1.14 ± 0.05 kg/d) than for the CON (0.96 ± 0.06 kg/d) and MT (0.91 ± 0.03 kg/d) treatments, which was related to the slightly higher DM intake in the GS treatment in the period of feces collection ($P < 0.001$; Table 3.1).

Nutrient disappearance

Apparent total tract and ileal nutrient disappearance values are presented in Table 3.2. Six calves had no or insufficient ileal digesta to perform all nutrient analyses, resulting in incomplete data for these calves. The apparent DM disappearance at the ileum ($P < 0.001$) and total tract ($P = 0.029$) was greater for the CON treatment than for all SP treatments. Apparent total tract disappearance of crude fat was numerically greater for the SP calves than for CON calves ($P = 0.072$). Apparent total tract disappearance of lactose was complete for all calves. Small amounts of lactose were measured in the ileal digesta of the SP calves but not in the ileal digesta of CON calves, resulting in a treatment effect for the apparent ileal disappearance of lactose ($P < 0.001$). Apparent total tract disappearance of nitrogen was greater for CON calves than for GS, MDB, and MT calves ($P = 0.003$). At the ileum, however, nitrogen disappearance was not affected by treatment. Apparent total tract and ileal disappearance of SP did not differ between SP treatments. The length of the small intestine was not affected by treatment ($P = 0.88$) and averaged 29 ± 1.2 m.

SP fermentation

Fecal DM output was greater in SP calves than in CON calves ($P = 0.004$; Table 3.1). Only small amounts of SP were excreted in the feces of SP calves. The SP calves had a greater ^{13}C enrichment of the feces than did the CON calves ($P < 0.001$; Table 3.1). Total tract SP fermentation was not affected by SP treatment and averaged 414 ± 43.0 g/d, corresponding to 89% of SP intake (Table 3.1).

Table 3.1 Body weight, feed intake, and fecal output characteristics of, and total tract starch product fermentation in, calves fed a milk replacer containing lactose as the only carbohydrate source or 18% gelatinized starch, maltodextrin, maltodextrin with a high degree of α -1,6-branching, or maltose at the expense of lactose.¹

	Treatment					Pooled SEM	<i>P</i> ²
	CON	GS	MD	MDB	MT		
n	7	8	8	8	7		
Body weight (kg)	224	218	235	227	217	4	-
Dry matter intake (g/d)	2711	2913	2786	2662	2527	43	-
Starch product intake (g/d)	-	507	501	447	416	9	-
Fecal output characteristics							
Fecal dry matter output (g/d)	168 ^b	259 ^a	246 ^a	222 ^{ab}	226 ^{ab}	16	0.004
Fecal starch product ³ output (g/d)	-	5.3	5.4	2.9	5.3	2.2	0.87
Fecal ¹³ C enrichment (atom%)	1.0776 ^b	1.0829 ^a	1.0832 ^a	1.0825 ^a	1.0828 ^a	0.0004	< 0.001
Fecal biomass from starch product fermentation ⁴ (g/d)	-	89	90	77	75	8.6	0.46
Starch product fermentation ⁴ (g/d)	-	447	451	384	373	43	0.46
Starch product fermentation ⁴ (% of intake)	-	89	90	86	90	8.8	0.99

¹ Values are means. Labeled means without a common letter in a row differ, $P < 0.05$. Atom%, atom percentage; CON, control calves fed milk replacer containing lactose as the only carbohydrate source; GS, calves fed milk replacer containing 18% of gelatinized starch at the expense of lactose; MD, calves fed milk replacer containing 18% of maltodextrin at the expense of lactose; MDB, calves fed milk replacer containing 18% of maltodextrin with a high degree of α -1,6-branching at the expense of lactose; MT, calves fed milk replacer containing 18% of maltose at the expense of lactose.

² *P*-values apply to the treatment effect. When no mean for the CON treatment is shown, the *P*-value applies to differences between the starch product treatments only.

³ Starch product was calculated by measuring total anhydrous glucose excretion and was corrected for glucose originating from lactose by assuming that the glucose originating from lactose equaled galactose excretion.

⁴ Fermentation of starch products estimated from fecal ¹³C excretion and assuming that 0.2 g fecal microbial biomass requires 1 g starch product to be fermented. For details, see text.

Table 3.2 Apparent total tract and ileal nutrient disappearance in calves fed a milk replacer containing lactose as the only carbohydrate source or 18% of gelatinized starch, maltodextrin, maltodextrin with a high degree of α -1,6-branching, or maltose at the expense of lactose.¹

	Treatment					Pooled SEM	<i>P</i> ²
	CON	GS	MD	MDB	MT		
Apparent total tract disappearance (% of intake)							
n	7	8	8	8	7		
Dry matter	93.7 ^a	91.1 ^b	91.2 ^{ab}	91.7 ^{ab}	91.0 ^b	0.6	0.029
Nitrogen	86.1 ^a	79.8 ^b	81.9 ^{ab}	79.3 ^b	79.9 ^b	1.2	0.003
Crude fat	91.0	93.6	95.6	94.9	94.4	1.1	0.072
Lactose	100.0	100.0	100.0	100.0	100.0	0.0	-
Starch product ³	-	99.0	99.0	99.4	98.8	0.4	0.79
Apparent ileal disappearance (% of intake)							
n	6	6 ⁴	8	7	7 ⁵		
Dry matter	88.0 ^a	81.9 ^{ab}	78.7 ^b	79.4 ^b	78.6 ^b	1.5	< 0.001
Nitrogen	83.1	83.6	81.5	84.1	82.9	1.8	0.74
Lactose	100.0 ^a	99.7 ^a	99.5 ^{ab}	99.5 ^{ab}	99.0 ^b	0.1	< 0.001
Starch product ³	-	64.0	60.5	63.6	58.5	6.3	0.91

¹ Values are means. Labeled means without a common letter in a row differ, $P < 0.05$. CON, control calves fed milk replacer containing lactose as the only carbohydrate source; GS, calves fed milk replacer containing 18% of gelatinized starch at the expense of lactose; MD, calves fed milk replacer containing 18% of maltodextrin at the expense of lactose; MDB, calves fed milk replacer containing 18% of maltodextrin with a high degree of α -1,6-branching at the expense of lactose; MT, calves fed milk replacer containing 18% of maltose at the expense of lactose.

² *P*-values apply to the treatment effect. When no mean for the CON treatment is shown, the *P*-value applies to differences between the starch product treatments only.

³ Starch product disappearance was calculated by measuring total anhydrous glucose intake and excretion and was corrected for glucose originating from lactose by assuming that the glucose originating from lactose equaled galactose excretion.

⁴ For the GS treatment, the number of calves for apparent ileal dry matter disappearance was 7; for the other nutrients the number of calves was 6.

⁵ For the MT treatment, the number of calves for apparent ileal nitrogen disappearance was 6; for the other nutrients the number of calves was 7.

Enzyme activity

Brush border enzyme activity for lactase, maltase, and isomaltase and luminal activity for α -amylase are presented in Table 3.3. Lactase activity did not differ between dietary treatments and was greater in small intestine 1 compared with small intestine 2 ($P < 0.001$) and the ileum ($P < 0.001$). Maltase and isomaltase activity was not significantly greater for any of the SP treatments compared with the CON treatment. Luminal α -amylase activity was measured in small intestine 1 but was not detected in small intestine 2 for CON calves, whereas this activity was higher in small intestine 2 than in small intestine 1 in all SP treatments (treatment \times segment, $P = 0.015$).

Passage

Three calves were excluded from the analysis on chromium recovery because of missing samples. Two calves were identified as ruminal drinkers (chromium recovery in the rumen $>50\%$ of the pulse dose) and were excluded from the analysis. Total chromium recovery did not differ between treatments ($P = 0.24$) and averaged $85 \pm 3.1\%$ of intake. Recovery of chromium per gastro-intestinal segment is shown in Figure 3.1. In the CON treatment, almost half of the provided chromium was recovered in small intestine 2, and $4.7 \pm 1.92\%$ of the chromium had reached the large intestine. A treatment \times segment interaction was found ($P = 0.008$), which was caused by a treatment effect in small intestine 2 ($P = 0.033$) and in the large intestine ($P = 0.033$). The MD and MT treatments had a lower chromium recovery in small intestine 2 ($P = 0.029$ and $P = 0.074$, respectively) and a greater chromium recovery in the large intestine ($P = 0.056$ and $P = 0.033$, respectively) compared with the CON treatment, indicating a higher passage rate of the milk replacer in the second part of the small intestine for these calves.

^{13}C in plasma glucose

The response of ^{13}C in excess of background in peripheral plasma glucose of the CON, GS, and MT calves is shown in Figure 3.2. The response of ^{13}C in excess of background in time differed between treatments (treatment \times time interaction, $P < 0.001$). At all postprandial time points, the enrichment of plasma glucose was substantially greater ($P < 0.001$) after intake of ^{13}C -enriched lactose (CON) than after intake of ^{13}C -enriched GS or MT. The latter 2 treatments did not differ at any point in time.

Table 3.3 Brush border enzyme activity and luminal α -amylase activity in small intestinal segments of calves fed a milk replacer containing lactose as the only carbohydrate source or 18% of gelatinized starch, maltodextrin, maltodextrin with a high degree of α -1,6-branching, or maltose at the expense of lactose.¹

	Treatment					Pooled SEM	<i>P</i>		
	CON	GS	MD	MDB	MT		TRT	SEG	TRT × SEG
Brush border activity (U/g protein)									
n	5	4	4	4	4				
Lactase							0.53	< 0.001	0.47
Small intestine 1	193	182	163	176	197	12.6			
Small intestine 2	14	5	4	6	4	4.4			
Ileum	0.8	0.7	0.7	0.9	1.1	0.3			
Maltase							0.046	< 0.001	0.58
Small intestine 1	9	6	4	3	4	1			
Small intestine 2	46	41	19	21	18	12			
Ileum	32	17	22	18	17	8			
Isomaltase							0.032	< 0.001	0.97
Small intestine 1	3	2	1	2	2	0.4			
Small intestine 2	13	13	5	8	10	4.0			
Ileum	11	7	7	8	7	2.6			
Luminal α -amylase activity (U/mg Co)									
n	7	8	8	8	7		0.074	0.002	0.015
Small intestine 1	3.9	1.9	3.9	3.2	1.8	2.0			
Small intestine 2	0.0	5.2	8.9	5.7	5.9	1.4			

¹ Values are means. One unit (U) of brush border enzyme represents the hydrolysis of 1 μ mol disaccharide/min at 37°C and 1 U of α -amylase represents the amount of α -amylase that hydrolyzes the substrate of the kit (ethylidene-para-nitrophenol-G7), resulting in 1 μ mol nitrophenol/min at 25°C and pH 7.2. Co, indigestible marker cobalt, provided continuously as Co-EDTA with the milk replacer; CON, control calves fed milk replacer containing lactose as the only carbohydrate source; GS, calves fed milk replacer containing 18% of gelatinized starch at the expense of lactose; MD, calves fed milk replacer containing 18% of maltodextrin at the expense of lactose; MDB, calves fed milk replacer containing 18% of maltodextrin with a high degree of α -1,6-branching at the expense of lactose; MT, calves fed milk replacer containing 18% of maltose at the expense of lactose; SEG, segment, the small intestine without the ileum (i.e., last ~100 cm of the small intestine) was divided equally into small intestine 1 and small intestine 2; TRT, dietary treatment.

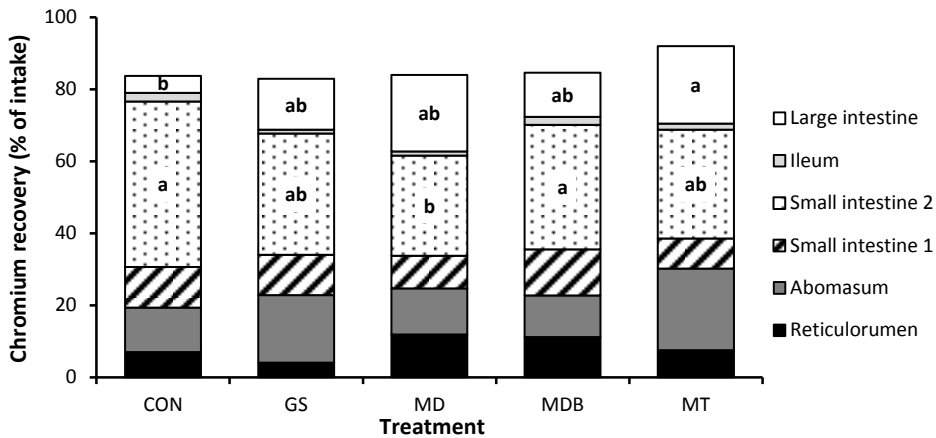


Figure 3.1 Recovery of chromium (provided as chromium chloride hexahydrate) per gastrointestinal segment of calves at 4 h after feeding a control milk replacer or a milk replacer containing 18% of one of four starch products. Labeled means without a common letter within the same gastrointestinal segment differ, $P < 0.05$; $n = 6 - 7$. The pooled SEM equaled 3.1% for the reticulorumen, 3.3% for the abomasum, 1.3% for small intestine 1, 3.9% for small intestine 2, 0.5% for the ileum, and 4.3% for the large intestine. CON, control calves fed milk replacer containing lactose as the only carbohydrate source; GS, calves fed milk replacer containing 18% of gelatinized starch at the expense of lactose; MD, calves fed milk replacer containing 18% of maltodextrin at the expense of lactose; MDB, calves fed milk replacer containing 18% of maltodextrin with a high degree of α -1,6-branching at the expense of lactose; MT, calves fed milk replacer containing 18% of maltose at the expense of lactose.

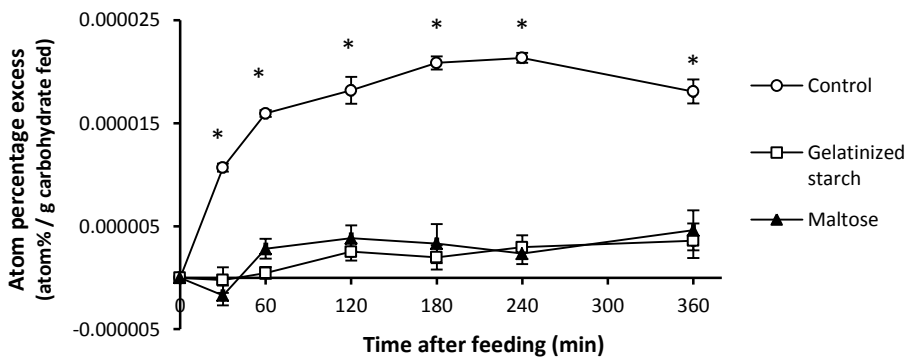


Figure 3.2 The ^{13}C enrichment in excess of background in peripheral plasma glucose of calves fed a milk replacer containing 37.5% naturally ^{13}C -enriched lactose (control) or 18% naturally ^{13}C -enriched gelatinized starch or maltose. The atom percentage in excess of background is corrected for the dose of the naturally ^{13}C -enriched carbohydrate fed. Values are means \pm SEM, $n = 7 - 8$. The response of ^{13}C in excess of background in time differed between treatments (treatment \times time interaction, $P < 0.001$). * Control is different from other means at that time, $P < 0.05$.

DISCUSSION

This experiment was conducted to determine the rate-limiting enzyme in starch hydrolysis and to quantify starch fermentation in milk-fed calves. The greatest maltase activity was 46 U/g protein in small intestine 2 of CON calves. This is very low when compared with other, non-herbivorous species, for example, weaned pigs (180 U/g protein halfway along the small intestine; Kidder and Manners, 1980), neonatal pigs of ~22 d of age fed a milk replacer (600 U/g protein in the jejunum; Oliver et al., 2002) and rats fed a high-maltose diet (178 U/g protein; Deren et al., 1967). Similarly, the greatest isomaltase activity in our study was 13 U/g protein in small intestine 2 of CON calves, whereas this amount was ~80 U/g protein halfway the small intestine of weaned pigs (Kidder and Manners, 1980). Lactase activity averaged 182 ± 12.6 U/g protein in small intestine 1 across all treatments. This high lactase activity was expected, because lactase activity remains high in calves through the continuous feeding of lactose (Huber et al., 1964, Toofanian et al., 1973). We measured luminal α -amylase activity in small intestinal digesta. Often, α -amylase activity is measured in pancreatic tissue samples or in pancreatic juice, making comparisons with our study difficult. Abomasal infusion of partially hydrolyzed starch in steers decreased pancreatic α -amylase activity (Walker and Harmon, 1995, Swanson et al., 2002) and pancreatic juice α -amylase activity and secretion (Walker and Harmon, 1995). In steers, α -amylase is probably not the limiting step in small intestinal starch digestion, because infusing amylase into the jejunum of steers fed a diet containing 60% cracked maize did not increase the small intestinal digestibility of starch (Remillard et al., 1990).

The selected SP used in this study require different ratios of starch-degrading enzymes for their complete hydrolysis to glucose. Differences between treatments in activities of starch-degrading enzymes, and ileal SP disappearance, allow the rate-limiting enzyme for starch hydrolysis in milk-fed calves to be determined. The activity of α -amylase was greater for SP calves than for CON calves in small intestine 2 and is probably not limiting in starch digestion. This is in agreement with studies in steers (Remillard et al., 1990, Kreikemeier and Harmon, 1995). Maltase and isomaltase activities were not increased in any of the small intestinal segments after providing their specific substrate for 14 weeks in the MT and MDB treatments, respectively. In addition, ileal SP disappearance did not differ between treatments. Because all SP require maltase activity to achieve complete hydrolysis to glucose, our data indicate that maltase limits starch digestion in milk-fed calves.

The possibility that nutrients from the milk replacer (i.e., fat or lactose) inhibit brush border enzyme activity cannot be completely excluded in this study. For example, Lee et al. (2012) showed an inhibitory effect of sucrose on maltase-glucoamylase activity in vitro.

In rabbits, however, replacing dietary starch for lactose did not affect apparent ileal starch disappearance (Gutiérrez et al., 2002). To the best of our knowledge, such studies have not been performed in ruminants. Surprisingly, the maltase activity was greatest for the CON calves. Similar negative effects of partially hydrolyzed starch on pancreatic α -amylase activity have been shown before in steers (Walker and Harmon, 1995, Swanson et al., 2002).

In piglets, greater luminal α -amylase activity (U/g DM digesta) was found in the duodenum than in the jejunum and ileum (Feng et al., 2007). This is in contrast with our results, in which greater α -amylase activity was found in small intestine 2 than in small intestine 1 for SP calves, whereas no α -amylase activity was found in small intestine 2 for CON calves. This may indicate that a substantial portion of the amylase found in the digesta in small intestine 2 of SP calves is of microbial origin or that the amylase moves with the undigested substrate more rapidly from small intestine 1 to small intestine 2, as confirmed by the chromium recovery data, which indicate an increased rate of passage in the SP treatments compared with the CON treatment. The chromium recovery in the reticulorumen averaged $10 \pm 4.7\%$, indicating that the esophageal groove reflex was still functioning.

The lack of sufficient starch-degrading enzyme activity in the brush border resulted in a decreased apparent ileal disappearance of DM in the MD, MDB, and MT treatments compared with the CON treatment. Therefore, greater amounts of substrate were available for colonic fermentation in calves fed SP. On the basis of apparent ileal (62%) and apparent total tract (99%) disappearance, 37% of the ingested SP was fermented in the large intestine. Total tract SP fermentation, estimated from ^{13}C excretion in feces, averaged 89% of SP intake, regardless of the SP source, indicating that 52% of the SP intake was fermented before the terminal ileum. Milk replacer that leaked into the reticulorumen averaged $11 \pm 5.1\%$ for SP-fed calves. This indicates that 41% of the SP intake was fermented in the small intestine. This 41% is calculated by the difference and is based on an indirect method for quantifying microbial biomass in the small intestine.

As an alternative approach, total tract SP fermentation can be estimated from the extra DM output of SP calves compared with CON calves, assuming that the extra DM output in SP calves, corrected for differences in undigested fecal SP, fat, or ash, would be largely microbial biomass. SP calves produced more feces than did CON calves (+70 g DM/d), but fecal fat excretion was lower in SP calves than in CON calves (-16 g fat/d). This results in an increase of 86 g nonfat fecal DM/d when feeding SP to calves. The difference in fecal DM output between SP and CON calves was partly caused by SP excretion directly (5 g SP/d) but could not be explained by increased ash excretion in SP calves. This leaves 81 g DM/d that is unaccounted for, which is hypothesized to be undigested microbial mass resulting

from SP fermentation. Assuming 1 g SP fermented per 0.2 g fecal microbial biomass (Livesey, 1991), this would require 405 g SP to be fermented, corresponding to 87% of SP intake. Both methods for quantifying total tract SP fermentation indicate that, regardless of the SP source, the majority of SP intake is fermented in calves. This is in agreement with the greater apparent ileal compared with apparent total tract nitrogen disappearance in SP calves, indicating a net influx of nitrogen into the large intestinal lumen. A lower apparent ileal nitrogen disappearance for SP calves could be expected after substantial small intestinal starch fermentation as well; however, apparent ileal nitrogen disappearance did not differ between SP treatments and the CON treatment. Most probably, a considerable portion of the microbial biomass produced in the small intestine is digested before the terminal ileum. We quantified SP fermentation at a SP inclusion amount of 18%. A linear decrease in fecal DM content and pH was observed with increasing SP inclusion amount during the adaptation period in these calves, irrespective of SP source (Chapter 2), showing that fermentation contributes to SP disappearance at lower inclusion amounts as well.

A clear increase in ^{13}C enrichment of plasma glucose was found after feeding naturally ^{13}C -enriched lactose to CON calves. In contrast, only a marginal increase was found after feeding naturally ^{13}C -enriched GS or maltose, indicating that only small amounts of glucose in peripheral blood originate from GS or maltose. This corresponds with the result that 66% of the small intestinal SP disappearance is attributed to fermentation, which results in the production of VFA, and leaves only 34% of the small intestinal SP disappearance for enzymatic digestion, which results in the release of glucose; however, propionate could also have been used as a precursor for gluconeogenesis and could have contributed to the low ^{13}C enrichment in plasma glucose in GS and MT calves.

The low brush border enzyme activity, the large amount of total tract and small intestinal SP fermentation, the reduced retention time of digesta in the small intestine, and the small increase in ^{13}C enrichment in plasma glucose after SP feeding show that calves are not able to enzymatically hydrolyze starch in substantial amounts. Portal glucose flux studies (Kreikemeier et al., 1991, Kreikemeier and Harmon, 1995) have evaluated starch digestion in the small intestine of ruminants as well but are often inconclusive. Fifty-five percent of the infused starch (60 g/h) into the abomasum of steers disappeared before the ileocecal junction. However, only 43% of this small intestinal disappearance could be accounted for as net portal glucose uptake, after correction for the negative portal glucose uptake found with water infusion to account for glucose use by the portal-drained viscera (Kreikemeier et al., 1991). This leaves 57% of the small intestinal starch disappearance unaccounted for. The arterial use of glucose by the portal-drained viscera was increased after infusion of partially hydrolyzed starch in the abomasum compared

with infusion in the rumen (Harmon et al., 2001). This unexpected increase indicates that the arterial glucose use by the portal-drained viscera is greater when luminal starch/glucose is present, suggesting that correction after water infusion in portal glucose flux studies is insufficient. However, infusing this partially hydrolyzed starch (800 g/d) into the rumen instead of into the abomasum did not alter the flux of VFA across the portal-drained viscera (Harmon et al., 2001). This confuses the message of these studies and corresponds to our observations on small intestinal fermentation of starch. Direct quantification of enzymatic starch hydrolysis in ruminants remains to be studied and could be studied by measuring a response in ^{13}C glucose in the portal circulation after abomasal infusion of ^{13}C -enriched starch.

In translating our results obtained with milk-fed calves to functional ruminants, we assume that a functioning rumen and the resulting type of digesta does not affect the capacity of the ruminant small intestine to enzymatically hydrolyze starch and absorb its end products. Possible matrix effects of the milk replacer were discussed above. The possibility that rumen microbes, flowing through the small intestinal tract, interfere with the non-microbial enzymatic hydrolysis of starch cannot be excluded, but this is considered unlikely.

Our data lead to the hypothesis that maltase is the rate-limiting enzyme in starch digestion in milk-fed calves, and that the largest part of small intestinal starch disappearance is due to fermentation rather than enzymatic hydrolysis to glucose. Such extensive fermentation in the small intestine has not been demonstrated before and could occur in ruminants as well.

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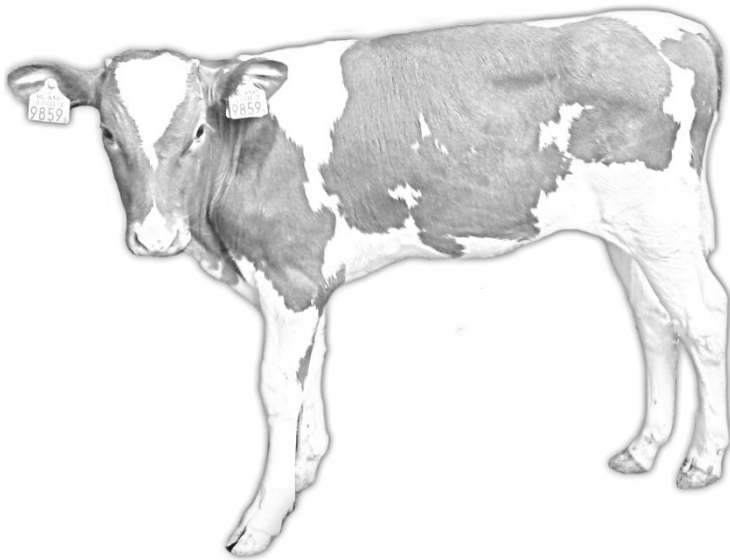
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Chapter 4

Effects of replacing lactose from milk replacer by glucose, fructose or glycerol on energy partitioning in veal calves



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ABSTRACT

Calf milk replacers contain 40 to 50% lactose. Fluctuating dairy prices are a major economic incentive to replace lactose from milk replacers by alternative energy sources. The objective was, therefore, to determine the effects of replacement of lactose with glucose, fructose or glycerol on energy and protein metabolism in veal calves. Forty male Holstein-Friesian calves (114 ± 2.4 kg) were fed milk replacer containing 46% lactose (CON) or 31% lactose and 15% of glucose (GLUC), fructose (FRUC) or glycerol (GLYC). Solid feed was provided at 10 g dry matter (DM)/kg of body weight^{0.75} (BW^{0.75}) per day. After an adaptation of 48 d, individual calves were harnessed, placed in metabolic cages and housed in pairs in respiration chambers. Apparent total tract disappearance of DM, energy and nitrogen (N) and complete energy and N balances were measured. GLUC, FRUC and GLYC calves received a single dose of 1.5 g of [U-¹³C]glucose, [U-¹³C]fructose or [U-¹³C]glycerol, respectively, with their milk replacer at 0630 h and exhaled ¹³CO₂ and ¹³C excretion with feces was measured. Apparent total tract disappearance was decreased by 2.2% for DM, 3.2% for energy and 4.2% for N in FRUC compared to CON calves. Energy and N retention did not differ between treatments, and averaged 299 ± 16 kJ/kg of BW^{0.75} per day and 0.79 ± 0.04 g/kg of BW^{0.75} per day, respectively, although FRUC calves retained numerically less N (13%) than other calves. Recovery of ¹³C isotopes as ¹³CO₂ did not differ between treatments and averaged $72 \pm 1.6\%$. The time at which the maximum rate of ¹³CO₂ production was reached was more than 3 h delayed for FRUC calves, which may be explained by a conversion of fructose before being oxidized. Recovery of ¹³C in feces was greater for FRUC calves ($7.7 \pm 0.59\%$) than for GLUC ($1.0 \pm 0.27\%$) and GLYC calves ($0.5 \pm 0.04\%$), indicating incomplete absorption of fructose from the small intestine resulting in fructose excretion or fermentation. In conclusion, energy and N retention was not affected when replacing >30% of the lactose with glucose, fructose or glycerol. Increased fecal losses of DM, energy and N were found in FRUC calves compared to CON, GLUC and GLYC calves. Post-absorptive losses occurred with the urine for glucose and glycerol, which caused a lower respiratory quotient for GLUC calves during the night. Fructose was oxidized more slowly than glucose and glycerol, probably as a result of conversion into other substrates before oxidation.

Key words: veal calf, glucose, fructose, glycerol, energy retention

INTRODUCTION

Calf milk replacers (MR) contain 40 to 50% lactose. Apparent ileal lactose disappearance is high in milk-fed calves, up to or exceeding 97% of intake (Coombe and Smith, 1974, Chapter 3). High and fluctuating dairy prices are a major economic incentive to replace lactose from MR by alternative energy sources. It has been recently demonstrated that starch products are not suitable for replacing lactose as maltase activity likely limits starch digestion and the vast majority of ingested starch products from MR disappears from the intestinal tract by fermentation rather than after hydrolysis by animal enzymes and subsequent absorption of glucose (Chapter 2 and 3). Therefore, energy sources which do not require enzymatic hydrolysis prior to absorption from the intestinal lumen might be more suitable lactose replacers. Glucose, fructose and glycerol are potential lactose replacers. Glucose and fructose are absorbed from the small intestinal lumen by specific carriers, i.e. glucose by the Na⁺-dependent transporter SGLT1 (Hediger and Rhoads, 1994, Zhao et al., 1998) and fructose by GLUT5 (Burant et al., 1992, Zhao et al., 1998). Glycerol is easily absorbed from the small intestinal lumen, with 78% of the glycerol absorbed within 25 minutes after infusion in isolated loops of the small intestine of rats (Höber and Höber, 1937). There are indications that Na⁺-dependent carriers are also involved in the absorption of glycerol (Kato et al., 2005).

Ingestion of glucose, fructose or glycerol by milk-fed calves results in the luminal presence of different (ratios of) monosaccharides and glycerol compared with the ingestion of lactose. Lactose is split into equimolar amounts of glucose and galactose by the brush-border enzyme lactase (Coombe and Smith, 1973). Lactase activity is high in milk-fed calves (Toofanian et al., 1973, Le Huerou et al., 1992, Chapter 3). Glucose is preferentially absorbed from the small intestine over galactose (Coombe and Smith, 1973) and replacing lactose with glucose might, therefore, result in a greater postprandial blood glucose concentration which could result in an increased excretion of glucose via urine when the renal threshold is exceeded (Hostettler-Allen et al., 1994, Vicari et al., 2008a). Feeding a fructose solution to milk-fed calves results in a low reducing sugar response in blood sampled from the jugular vein (Velu et al., 1960, Siddons et al., 1969), suggesting either a rapid uptake of fructose by the liver from hepatic portal blood as seen in rat and human (as reviewed by Mayes, 1993) and subsequent post-absorptive conversion of fructose into products other than reducing sugars (i.e. glycogen, lactate, CO₂) or a low absorption of fructose from the intestinal lumen. The latter might result in incomplete disappearance of fructose from the small intestine, resulting in substrate fermentation. Almost no increase in plasma glucose was found after feeding glycerol with an oral rehydration solution to 3 week old Swedish Red calves (Werner-Omazic et al., 2013). Feeding fructose or glycerol,

therefore, probably results in a lower postprandial increase in glucose compared to lactose feeding. Also, fructose does not elicit insulin release (Curry, 1989). Feeding glycerol or glucose to 3 week old Swedish Red calves resulted in similar increases in plasma insulin, whereas plasma glucose levels were lower after glycerol feeding (Werner-Omazic et al., 2013). Glycerol or fructose feeding might, therefore, affect the insulin to glucose ratio and, consequently alter glucose metabolism compared with lactose feeding. Also, heavier milk-fed calves often develop insulin resistance (Hostettler-Allen et al., 1994, Hugi et al., 1997) and replacing lactose might affect the development of insulin resistance.

Replacing lactose with glucose, fructose or glycerol not only results in the luminal presence of different (ratios of) monosaccharides and glycerol, but could potentially alter glucose homeostasis and, thereby, energy partitioning. The objective of this study was to determine the effects of replacement of lactose with glucose, fructose or glycerol on energy and protein metabolism in veal calves. The effects on glucose homeostasis and insulin sensitivity are presented in Chapter 5.

MATERIAL AND METHODS

This experiment was approved by the Animal Care and Use Committee and conducted at the research facilities of Wageningen University.

Animals and experimental design

Forty male Holstein-Friesian calves were selected from one farm based on body weight, age and clinical health. Calves arrived at the research facilities in 3 batches and were equally divided over 5 blocks of 8 calves based on body weight. Within each block, pairs of calves were assigned to one of four milk replacer treatments ($n = 10$ per treatment, $n = 2$ per treatment per block). Each block was separated by one week, which was required because of the capacity of the respiration chambers. At the start of the experiment, mean \pm SE weight and age of the calves were 114 ± 2.4 kg and 97 ± 1.4 d, respectively.

After arrival, calves received the control milk replacer for 11 d, and, thereafter, the adaptation period started and calves were assigned to one of four MR. The control treatment (CON) contained 462 g lactose per kg MR as the only source of carbohydrate. In the other MR treatments, one of three lactose replacers [glucose (GLUC), fructose (FRUC) or glycerol (GLYC)] was included, replacing 150 g lactose per kg MR. Calves were fed these MR for 48 d before the experimental period started in which measurements on energy and protein metabolism were performed. This long adaptation period was applied in order to determine the long-term effects of these lactose replacers on energy and protein metabolism.

Housing

During the first 42 d of the adaptation period, calves were housed in groups of 5 on wooden, slatted floors without bedding material. Per calf, 2.0 m² was available. Each group received the same MR, but the MR was provided individually. Within one group, calves were assigned to multiple blocks. Lights were on from 0630 to 1730 h. The stable was mechanically ventilated and the mean \pm SE minimum and maximum temperature and humidity were 17.1 \pm 0.10 and 19.7 \pm 0.13°C and 60 \pm 0.60 and 77 \pm 0.50% during the entire experiment.

During the last 6 d of the adaptation period, calves were housed in metabolic cages (0.8 \times 1.8 m) and harnessed for habituation. During the experimental period, calves were housed in pairs in one of the four identical respiration chambers for 6.5 d. Calves were housed in similar metabolic cages in the respiration chamber as during habituation. During housing in the chambers, audiovisual contact between calves was possible. Temperature and relative humidity were kept constant in the respiration chambers at 18°C and 65%, respectively.

Diets and feeding

Calves were fed individually according to their metabolic bodyweight (BW^{0.75}) at twice the metabolizable energy requirement for maintenance. The metabolizable energy requirement for maintenance was set at 460 kJ/kg of BW^{0.75} per day (Van Es et al., 1967). Milk replacer was provided in buckets and calves were locked in the headlock of the fence during MR feeding (maximum of 60 min) to allow individual MR intake. The ingredient and nutrient composition of the experimental MR are shown in Table 4.1. The CON MR was composed of 850 g/kg basal MR and an additional 150 g/kg of lactose. For the GLUC, FRUC and GLYC treatment, the 150 g of lactose was exchanged for glucose (Tereos Syral, Marckolsheim, France), fructose (Tate&Lyle Europe, Boleraz, Slovakia) or glycerol (Triconor Distribution BV, Soest, The Netherlands) on a gross energy (GE) basis. The MR was mixed with water to obtain a concentration of 140 g/L. When the milk volume reached 9.0 L, the concentration was increased to 160 g/L. Milk replacer was supplied to the calves at a temperature of ~42°C at 0630 and 1530 h in two equal portions. Milk replacer refusals were weighed and recorded after each feeding. Water was available ad libitum.

Solid feed supply equaled 10 g DM/kg of BW^{0.75} per day and was composed of 20% wheat straw and 80% concentrates on DM basis. Concentrates were composed of 458 g/kg corn, 279 g/kg barley, 205 g/kg lupines, 34 g/kg premix and 24.4 g/kg palm oil. Analyzed DM, crude protein and GE content equaled 907 g/kg, 157 g/kg DM and 18.0 kJ/g DM for concentrates and 921 g/kg, 31.7 g/kg DM and 18.2 kJ/g DM for wheat straw, respectively.

Solid feed was provided per pen (i.e. 5 calves) during group housing and per calf during individual housing in metabolic cages. Solid feed was provided in two portions per day, directly after MR feeding. Solid feed refusals were weighed and recorded once a day, before feeding MR in the morning.

Table 4.1 Ingredient and nutrient composition of the experimental milk replacer.

Ingredient	g/kg	Nutrient	g/kg DM ³
Lactose or lactose replacer ¹	150.0	Dry matter ⁴ (g/kg)	966
Basal diet		Crude ash	74
Whey protein concentrate	391.1	Crude protein ($N \times 6.25$)	205
Delactosed whey	244.5	Crude fat	209
Fat		Lactose ^{1,5}	478
Lard	72.4	Lysine ⁵	16.5
Tallow	72.4	Methionine ⁵	4.6
Coconut oil	39.2	Threonine ⁵	13.2
Lecithin	5.9	Fe (mg/kg DM)	49.9
Emulsifier	5.9	Gross energy (MJ/kg)	19.8
Calcium formate	7.5		
Premix ²	10.0		
Methionine	1.1		

¹ The control treatment contained 462 g lactose per kg MR as the only source of carbohydrate. In the other MR treatments, one of three lactose replacers was included, replacing 150 g lactose per kg MR by glucose, fructose or glycerol on a gross energy basis. Gross energy content equaled 15.14 kJ/g of lactose, 13.96 kJ/g of glucose, 15.38 kJ/g of fructose and 17.65 kJ/g of glycerol.

² Provided (per kg of experimental diet): crude protein, 0.7 g; starch, 5.0 g; crude ash, 1.5 g; Ca, 17 mg; P, 7.5 mg; Na, 0.7 mg; K, 7.3 mg; Cl, 13 mg; Mg, 0.5 g; Fe, 45 mg; Cu, 8.0 mg; Zn, 0.1 mg; Mn, 43 mg; Se, 0.3 mg; I, 1.0 mg; Vitamin A, 25,013 IU; Vitamin D₃, 4,002 IU; Vitamin E, 150 IU; Vitamin K₃, 2.1 mg; Vitamin C, 0.3 g; Vitamin B₁, 8.2 mg; Vitamin B₂, 10 mg; Vitamin B₃, 35 mg; Vitamin B₅, 18 mg; Vitamin B₆, 10 mg; Vitamin B₁₂, 0.1 mg; biotin, 0.2 mg; choline, 0.4 g; folic acid, 0.7 mg.

³ Values are provided in g per kg of dry matter unless stated otherwise.

⁴ Dry matter content of the basal milk replacer.

⁵ Calculated content.

Measurements

Calves were weighed weekly to adapt the feeding level to their metabolic BW. In addition, calves were weighed before and after energy balance measurements. A complete energy and nitrogen (N) balance was performed in the respiration chamber during 5 d. The balance started after 0.5 d. On day 6 in the respiration chamber, calves were subjected to an intravenous glucose tolerance test to measure insulin sensitivity (see Chapter 5) and, therefore, this day was not taken into account for the energy and N balance measurements. Gas exchange (O_2 , CO_2 , CH_4) was measured in 12-min intervals (Verstegen and Hoogerbrugge, 1987). Feces were collected quantitatively per calf using plastic bags attached to the harnesses of the calves and were changed 2 or 3 times a day. Feces were stored at $-20^\circ C$ pending further processing. Feces were pooled over 3 periods within the 5 d balance period. Feces were pooled for the 48 h before isotope feeding (see below) for the determination of background ^{13}C enrichment, for the 48 h after isotope feeding for the determination of ^{13}C enrichment and for the last 24 h. For the determination of total tract nutrient disappearance, part of the feces of all 3 periods were pooled. Urine was collected quantitatively per calf using buckets under the metabolic cages containing 1500 mL of sulfuric acid to prevent microbial activity and volatilization of N through NH_3 . Acidified urine was weighed and specific gravity was determined. Acidified urine was sub-sampled and stored at $-20^\circ C$ until analyses. Outgoing air was sub-sampled and ammonia was captured in sulfuric acid, collected quantitatively and sampled. Water that condensed on the heat exchanger was collected quantitatively and sampled. Milk replacer refusals were weighed, sampled and stored at $-20^\circ C$. Solid feed refusals were collected quantitatively and stored at $-20^\circ C$. Basal MR, lactose, glucose, fructose, glycerol, concentrates and wheat straw were sampled weekly.

On the third day in the respiration chamber, GLUC, FRUC and GLYC calves received 1.5 g of [$U-^{13}C$]glucose (99 atom%, Cambridge Isotope Laboratories), [$U-^{13}C$]fructose (99%, Cambridge Isotope Laboratories) or [$U-^{13}C$]glycerol (99 atom%, Sigma Aldrich), respectively, with their morning MR meal. The [$U-^{13}C$]fructose was not fed during the first block and 0.75 g of [$U-^{13}C$]fructose (99 atom%, Sigma Aldrich) was provided to the FRUC calves in the second block. The ^{13}C enrichment in expired CO_2 was measured every 12 min using non-dispersive infrared spectrometry (Alferink et al., 2003) in order to calculate oxidation of the ^{13}C -enriched lactose replacers. Recovery of ^{13}C in feces was measured as an indicator of absorbance of these lactose replacers. When a lactose replacer is not absorbed from the intestinal lumen, it can either be excreted with the feces directly, or, more likely, be fermented by microbes. Both result in the excretion of ^{13}C in feces, the latter through the excretion of microbes which have incorporated ^{13}C of the lactose replacers into their own biomass. On this day, the responses in blood glucose and insulin

to MR feeding were measured by collecting 9 blood samples using central venous catheters (Careflow, Becton Dickinson, Alphen aan den Rijn, The Netherlands), which were inserted into the jugular vein before calves were moved to the respiration chambers (see Chapter 5).

Chemical analyses

Basal MR samples were pooled and analyzed for DM, N and GE content. Concentrates and wheat straw samples were pooled, ground to pass a 1 mm screen and analyzed for DM, N and GE content. Solid feed refusals were pooled per calf, ground to pass a 1 mm screen and analyzed for DM, N and GE content. Milk replacer refusals were thawed, homogenized and analyzed for DM content after freeze drying, and the N and GE contents were assumed equal to the N and GE contents of the MR dry matter. Urine was thawed and analyzed for N and glucose content and freeze-dried before GE analysis. Feces were freeze-dried and ground to pass a 1 mm screen. Feces collected before isotope feeding were analyzed on ^{13}C enrichment. Feces collected after isotope feeding were analyzed for DM content, ^{13}C enrichment and total carbon (C) content. Feces collected over the total balance period were analyzed for DM, N and GE content. Last, N was analyzed in condensed water containing ammonium and in sulfuric acid solution containing aerial ammonia.

Dry matter content was determined according to ISO standard 6496 (ISO, 1999b). Kjeldahl N and crude fat content were analyzed according to ISO standard 5983-2 (ISO, 2005) and ISO standard 6492 (ISO, 1999a), respectively. Gross energy content was analyzed with bomb calorimetry according to ISO standard 9831 (ISO, 1998). Crude ash content was analyzed according to ISO standard 5984 (ISO, 2002). Total C content and ^{13}C enrichment were analyzed after ball milling, using combustion isotope ratio mass spectrometry (IRMS; Finnigan Delta V advantage isotope ratio mass spectrometer, Finnigan MAT, Bremen, Germany). Urinary glucose was analyzed with an enzymatic colorimetric method using the Roche/Hitachi Modular P800 automatic analyzer (Roche Diagnostics, Mannheim, Germany). Analyses were performed in duplicate, except urinary glucose which was analyzed once.

Calculations

Apparent total tract disappearance of DM, N and energy were calculated as the difference between intake and excretion with the feces, expressed as a percentage of intake. Gross energy intake was calculated by subtracting the feed refusals from feed provided and multiplying with the GE content. Digestible energy (DE) intake was calculated by subtracting the energy excreted with feces from GE intake. Metabolizable energy (ME)

intake was calculated by subtracting energy excreted with urine and CH₄ from DE intake. Energy retention was calculated by subtracting heat production (calculated using the equation of Brouwer, 1965) from ME intake. The metabolizability of DE was calculated as ME : DE × 100. The respiratory quotient (RQ) was calculated as the ratio between CO₂ production and O₂ consumption. The diurnal fluctuation in RQ was calculated as the maximum minus the minimum RQ.

Nitrogen retention was calculated by subtracting N losses with feces, urine, condensed water and sulfuric acid solution from N intake. Protein retention was calculated as N retention × 6.25. Energy retained as fat was calculated by subtracting energy retained as protein (protein retention in g × 23.7 kJ/g) from energy retention.

The ¹³CO₂ production from [U-¹³C]glucose, [U-¹³C]fructose and [U-¹³C]glycerol was calculated by multiplying CO₂ production with ¹³C enrichment in expired CO₂ and corrected for background enrichment. The area under the curve was integrated for calculating ¹³CO₂ production over a period for 30 h after isotope feeding. Oxidation of [U-¹³C]glucose, [U-¹³C]fructose and [U-¹³C]glycerol was expressed as a percentage of the dose provided, after correction for the number of C-atoms, chemical purity and isotopic enrichment. Oxidation was corrected for an incomplete bicarbonate recovery of 72%, which was observed in milk-fed calves by Van den Borne et al. (2007). For the pattern of ¹³CO₂ production in time, ¹³CO₂ production was averaged per h and expressed as a percentage of the dose provided.

The excretion of ¹³C in feces was calculated. Total C output with the feces after isotope feeding (g/48 h) was calculated by multiplying the amount of feces produced (g DM/48 h) with the carbon content. Total C output in mol/48 h was calculated by multiplying total C output (g DM/48 h) with the mean molecular weight of carbon in the feces after isotope feeding, which was calculated from the ¹³C enrichment of the feces. Finally, ¹³C excretion with feces after isotope feeding was calculated by multiplying the total C output (mol/48 h) with the atom percentage excess (i.e. the atom percentage of feces after isotope feeding corrected for background enrichment). Recovery of ¹³C isotopes in feces was expressed as a percentage of the dose provided, after correction for the number of C-atoms, chemical purity and isotopic enrichment.

Statistical analyses

All statistical analyses were performed with SAS 9.3 (SAS Institute, Inc., Cary, NC). Studentized residuals of all models were checked for homogeneity of variance based on the Shapiro-Wilk test. Observations were classified as outlier when the studentized residual was greater than 3 or lower than -3. Apparent total tract disappearance, fecal pH and DM content, average daily gain, urinary glucose excretion and N and energy balance

traits were analyzed for treatment and block effects by ANOVA using the GLM procedure. Pairs of calves (housed in one respiration chamber) were considered as the experimental unit for the N and energy balance traits and the individual calf was the experimental unit for the other variables. The CORR procedure was used to estimate the Pearson correlation coefficients.

Circadian patterns of RQ and heat production were averaged per hour and analyzed on treatment, time, treatment \times time interaction and block effects using the GLIMMIX procedure. Time was included as a random statement with calf as subject. Based on fit statistics (AIC and BIC), the Toeplitz covariance structure was used. The Kenward and Roger method was used for computing the denominator degrees of freedom (Kenward and Roger, 1997). The diurnal fluctuation in RQ and the 24h RQ were analyzed for treatment effects by ANOVA using the GLM procedure. One outlier was identified for the diurnal fluctuation in RQ, which was a calf which was housed individually in the respiration chamber in block 3. This observation was deleted for the analysis on the diurnal fluctuation in RQ.

Excretion of ^{13}C in feces (experimental unit is calf) and as $^{13}\text{CO}_2$ in expired breath (experimental unit is pair of calves) were analyzed on treatment and block effects by ANOVA using the GLM procedure. Recovery of ^{13}C in the feces was square root-transformed to obtain homogeneity of variance.

Differences were considered significant when $P < 0.05$ and considered a trend when $0.05 < P < 0.10$. When main effects were significant, pairwise comparisons were made using the Tukey method. Results are expressed as non-transformed means and their pooled SEM.

RESULTS

Average daily gain in the adaptation period did not differ between treatments and averaged $1,292 \pm 40.5$ g. One GLUC calf did not enter the respiration chamber due to ruminal drinking (i.e. MR leaking into the rumen due to malfunction of the esophageal groove reflex) resulting in bloat. Another GLUC calf was, therefore, housed individually in a respiration chamber. This calf was allowed to have visual contact with calves in the adjacent respiration chamber to minimize stress. This individually housed calf did not have any feed refusals. Two experimental units (FRUC in block 1 and GLUC in block 5) were excluded from statistical analyses due to development of diarrhea, and incomplete feces collection, in a FRUC calf, and development of a high body temperature ($> 40^\circ\text{C}$ for two d) in a GLUC calf. For measurements where calf was the experimental unit (i.e. apparent total tract disappearance and fecal ^{13}C excretion), only these individual calves were excluded from statistical analyses.

Digestibility

In 4 calves (2 GLUC and 2 FRUC calves), part of the feces had leaked from the fecal bags and was collected from the funnel underneath the metabolic cage. These feces were collected and analyzed and added to fecal excretion. Feces production of FRUC calves (309 ± 22.4 g DM per day) was greater compared with the other treatments (232 ± 12.6 g DM per day on average; $P = 0.002$). This resulted in a decreased apparent total tract disappearance of DM ($P = 0.002$) and energy ($P = 0.003$) for the FRUC treatment compared with the other treatments (Table 4.2). Apparent total tract N disappearance was also decreased for FRUC calves ($P < 0.001$). Fecal pH was lower ($P < 0.001$) for FRUC calves (6.1 ± 0.12) than for other calves (7.2 ± 0.14). Fecal DM content was affected by treatment ($P = 0.029$) and was lowest for FRUC calves (133 ± 7.4 g/kg) and highest for GLYC calves (167 ± 6.9 g/kg), with intermediate values for CON (149 ± 5.6 g/kg) and GLUC calves (153 ± 9.1 g/kg).

Table 4.2 Apparent total tract disappearance of dry matter, energy and nitrogen in calves fed a milk replacer containing 462 g lactose per kg (CON; $n = 10$) or replacing 150 g lactose per kg by iso-energetic amounts of glucose (GLUC; $n = 8$), fructose (FRUC; $n = 9$) or glycerol (GLYC; $n = 10$) and recovery of ^{13}C in feces after feeding a single dose of ^{13}C -labeled glucose, fructose or glycerol.

Parameter	Treatment				Pooled SEM	P
	CON	GLUC	FRUC	GLYC		
Apparent total tract disappearance (% of intake)						
Dry matter	91.6 ^a	91.8 ^a	89.5 ^b	92.6 ^a	0.52	0.002
Energy	90.5 ^a	90.6 ^a	87.6 ^b	92.0 ^a	0.77	0.003
Nitrogen	86.8 ^a	87.5 ^a	82.9 ^b	88.5 ^a	0.69	< 0.001
^{13}C recovery with feces (% of dose ¹)	-	1.0 ^b	7.7 ^a	0.5 ^b	0.36	< 0.001

¹ $n = 8$ for FRUC.

^{ab} Means without a common letter in a row differ ($P < 0.05$).

Energy and N balance

The effects of dietary treatments on energy and N balances are shown in Table 4.3. Energy retention did not differ between treatments and averaged 299 ± 16 kJ/kg of $\text{BW}^{0.75}$ per day. Urinary energy losses were greater ($P = 0.034$) for GLUC calves than for FRUC calves. Urinary glucose excretion was affected by treatment ($P < 0.001$), with greater urinary glucose losses for CON (8.3 ± 1.11 kJ/kg of $\text{BW}^{0.75}$ per day) and GLUC (13.2 ± 3.71 kJ/kg of $\text{BW}^{0.75}$ per day) calves than for FRUC (1.3 ± 0.34 kJ/kg of $\text{BW}^{0.75}$ per day) and GLYC calves

(1.9 ± 0.52 kJ/kg of $BW^{0.75}$ per day). Methane production was greatest for GLYC calves ($P = 0.008$) and equaled $8.7 \pm 0.37\%$ when expressed as percentage of GE intake from solid feed, but only accounted for 1.7% of total energy losses. Methane production was not related to solid feed refusals ($r = -0.15$, $P = 0.55$). There was a trend for metabolizability of DE ($P = 0.05$), which was caused by a greater metabolizability of DE for FRUC calves ($95.2 \pm 0.09\%$) than for GLUC calves ($93.4 \pm 0.99\%$). The efficiency by which ME was retained did not differ between treatments and averaged $30 \pm 1.4\%$.

Nitrogen retention (0.79 ± 0.04 g N/kg of $BW^{0.75}$ per day) and the efficiency by which digested N was retained ($52 \pm 2.5\%$) did not differ between treatments. However, fecal N losses were higher ($P < 0.001$) for FRUC calves (0.30 ± 0.01 g N/kg of $BW^{0.75}$ per day) than for other calves (0.22 ± 0.01 g N/kg of $BW^{0.75}$ per day). Nitrogen retention ($P = 0.044$) and the efficiency by which digested N was retained ($P = 0.046$) decreased with blocks later in time.

Circadian patterns of heat production and RQ are shown in Figure 4.1 and 4.2, respectively. Both patterns followed the ingestion of meals (time effect $P < 0.001$ for both). A treatment \times time interaction ($P = 0.002$) was found for RQ. In general, the RQ for GLYC calves was lower than for other calves at 2 to 6 h after feeding. During the night, the RQ was lower for GLUC calves than for CON calves at 13 ($P = 0.08$) and 15 hours ($P = 0.018$) after the afternoon feeding. The diurnal fluctuation in RQ was affected by treatment ($P = 0.032$), with a greater diurnal fluctuation for GLUC calves (0.14 ± 0.014) compared to GLYC calves (0.12 ± 0.006), and intermediate values for CON (0.12 ± 0.009) and FRUC (0.13 ± 0.007) calves. The 24h RQ was lower ($P = 0.010$) for GLYC calves (0.89 ± 0.001) than for other calves (0.91 ± 0.004). A treatment \times time interaction was not found for heat production.

Isotope recovery

Recovery of ^{13}C in feces and as $^{13}\text{CO}_2$ in expired breath after feeding [$\text{U-}^{13}\text{C}$]glucose, [$\text{U-}^{13}\text{C}$]fructose or [$\text{U-}^{13}\text{C}$]glycerol are presented in Table 4.2 and Figure 4.3. Recovery of isotopes with feces was greater ($P < 0.001$) for FRUC calves than for GLUC and GLYC calves. Recovery of isotopes as $^{13}\text{CO}_2$ did not differ between treatments ($P = 0.10$) and averaged $72 \pm 1.6\%$. The time at which the maximum rate of $^{13}\text{CO}_2$ production was reached was more than 3 h delayed ($P = 0.008$) for FRUC calves ($T_{\text{max}} = 515 \pm 39$ min after feeding) compared with GLUC and GLYC calves ($T_{\text{max}} = 294 \pm 30$ min after feeding on average; Figure 4.3).

Table 4.3 Nitrogen and energy balances in calves fed a milk replacer containing 462 g lactose per kg (CON) or replacing 150 g lactose per kg by iso-energetic amounts of glucose (GLUC), fructose (FRUC) or glycerol (GLYC).

Parameter	Treatment				Pooled SEM	P
	CON	GLUC	FRUC	GLYC		
n, pairs of calves	5	4 ¹	4	5		
BW (kg)	185	181	188	186	7.6	-
N balance						
(g N/kg of BW ^{0.75} per day)						
Total N intake	1.78	1.78	1.78	1.76	0.015	-
Fecal N excretion	0.23 ^b	0.21 ^b	0.30 ^a	0.20 ^b	0.012	0.003
Urinary N excretion	0.70	0.66	0.73	0.71	0.039	0.96
N losses with air and condensed water	0.05	0.04	0.04	0.04	0.007	0.83
N retention ²	0.79	0.86	0.71	0.81	0.036	0.16
Efficiency ² (% of digested N)	51.4	55.1	47.8	52.0	2.45	0.59
Energy balance						
(kJ/kg of BW ^{0.75} per day)						
GE intake from MR	992	988	991	976	6.0	-
GE intake from SF	166	168	167	176	10.2	-
Fecal energy excretion	110 ^{ab}	106 ^{ab}	136 ^a	92 ^b	7.8	0.027
Urinary energy excretion	43.8 ^{ab}	56.4 ^a	35.6 ^b	48.8 ^{ab}	5.0	0.034
Methane production ²	15.0 ^a	12.7 ^b	13.7 ^{ab}	16.1 ^a	0.8	0.008
Heat production	683	680	696	686	11.8	0.91
Energy retention	306	302	277	310	16.2	0.70
As protein ²	118	128	105	120	5.4	0.16
As fat	188	174	172	190	15.7	0.85
Efficiency (% of metabolizable energy)	30.9	30.6	28.5	31.1	1.39	0.72
Respiratory quotient ³	0.912 ^a	0.908 ^{ab}	0.910 ^a	0.893 ^b	0.003	0.010

¹ In block 3, only one calf was housed in the respiration chamber.

² N retention ($P = 0.044$), N retention efficiency ($P = 0.046$), methane production ($P = 0.014$) and energy retention as protein ($P = 0.044$) were affected by block.

³ There is a trend for a lower respiratory quotient for GLYC compared to GLUC ($P = 0.085$).

^{ab} Means without a common letter in a row differ ($P < 0.05$).

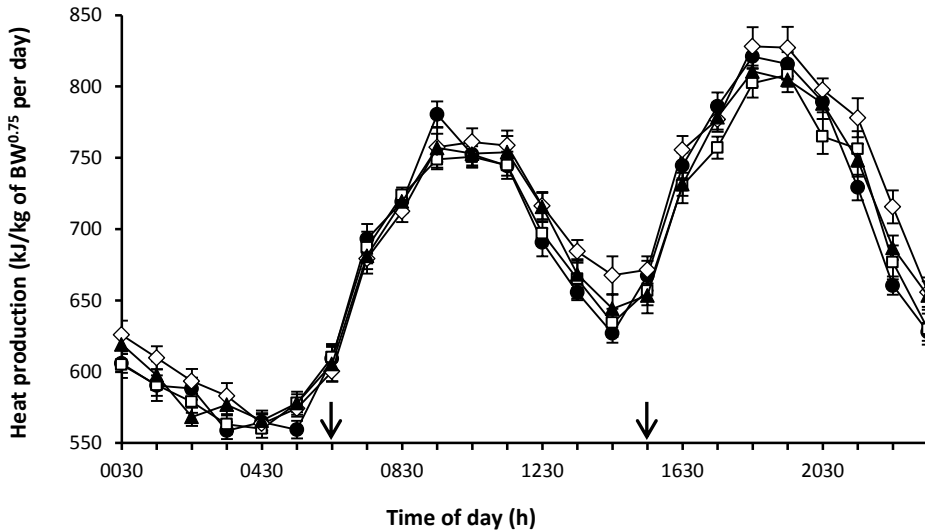


Figure 4.1 Circadian pattern of heat production of calves fed a milk replacer containing 462 g lactose per kg (control; ●) or replacing 150 g lactose per kg by iso-energetic amounts of glucose (□), fructose (◇) or glycerol (▲). Arrows indicate feeding times. No treatment × time interaction was found ($P = 0.82$).

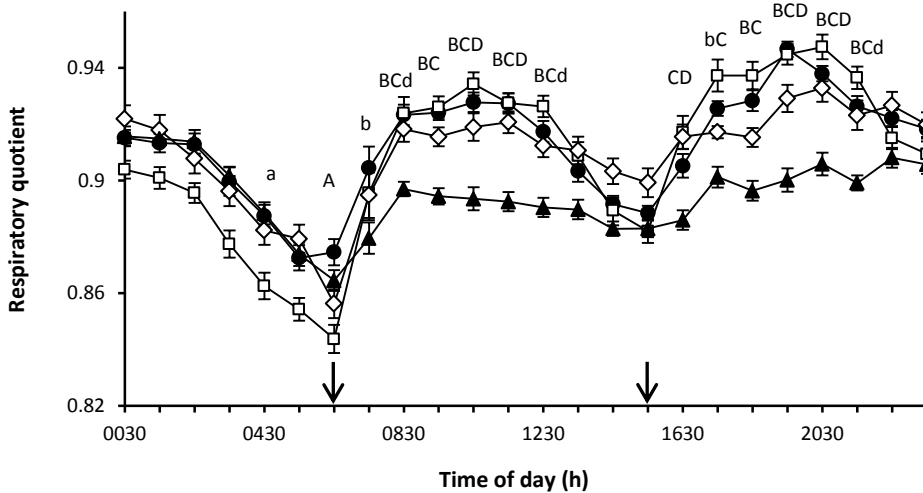


Figure 4.2 Circadian pattern of the respiratory quotient of calves fed a milk replacer containing 462 g lactose per kg (control; ●) or replacing 150 g lactose per kg by iso-energetic amounts of glucose (□), fructose (◇) or glycerol (▲). Arrows indicate feeding times. A treatment × time interaction found ($P = 0.002$) was found. A, control differs from glucose; B, control differs from glycerol; C, glucose differs from glycerol; D, fructose differs from glycerol ($P < 0.05$) and a, control differs from glucose; b, control differs from glycerol; d, fructose differs from glycerol ($P < 0.10$).

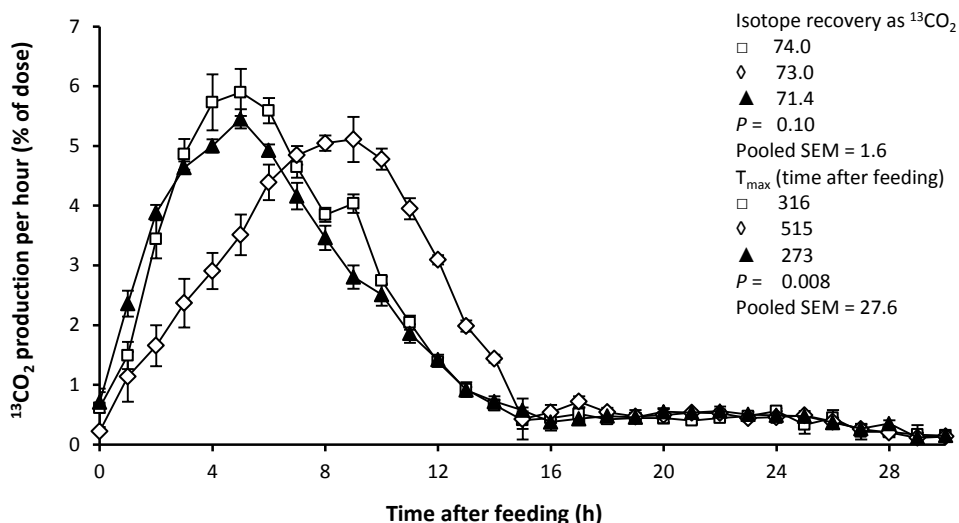


Figure 4.3 Production of $^{13}\text{CO}_2$, isotope recovery as $^{13}\text{CO}_2$ and time of maximum rate of production of $^{13}\text{CO}_2$ (T_{\max}) after feeding a single dose of ^{13}C -labeled glucose (\square), fructose (\diamond) or glycerol (\blacktriangle) to calves with the milk replacer containing iso-energetic amounts of glucose, fructose or glycerol compared to 150 g of lactose per kg in the control milk replacer.

DISCUSSION

The objective of this study was to determine the effects of replacing lactose with glucose, fructose or glycerol on energy and protein metabolism in veal calves. Apparent total tract DM, N and energy disappearance of the calves in our study were lower compared with those of calves fed exclusively MR (Van den Borne et al., 2006b, Labussière et al., 2009), which can be ascribed to the lower apparent total tract disappearance of the solid feed (Berends et al., 2012). The FRUC calves consistently had a lower apparent total tract disappearance of DM, energy and N than other calves. The increased fecal N losses in FRUC calves could result from increased N losses at the end of the ileum, as a result of a higher passage rate resulting in increased dietary or endogenous N losses. Alternatively, the increased fecal N losses could result from increased N losses in the large intestine, as a result of an influx of urea into the large intestine which is used for microbial protein synthesis. The FRUC calves also had the greatest fecal ^{13}C excretion (i.e. $7.7 \pm 0.59\%$). This fecal ^{13}C originates from the ^{13}C -labeled fructose, but the form in which this ^{13}C is excreted is unknown. The ^{13}C could be excreted as fructose with the feces, although this seems unlikely because fructose can be fermented by microbes in the large intestine. Fermentation of fructose, resulting in microbial biomass and volatile fatty acids, could

result in this increase in fecal ^{13}C excretion. This is supported by the low fecal pH and increased fecal N losses for FRUC calves. Assuming that all ^{13}C is excreted as microbial biomass and that only 20% of the fermented carbohydrate is excreted as microbial biomass with the feces [derived from 0.3 kJ fecal biomass/kJ carbohydrate (Livesey, 1991) and using 15.56 kJ/g carbohydrate (Blaxter, 1989) and 23.13 kJ/g biomass (Cordier et al., 1987)], then 38.5% of the ^{13}C -labeled fructose was fermented. The true disappearance of fructose, therefore, ranged between 61.5 and 92.3%. Either way, it indicates that ileal disappearance of fructose is lower than that of glucose and glycerol, which could result in osmotic or fermentative diarrhea. Diarrhea has been reported after feeding a fructose solution to milk-fed calves (Velu et al., 1960, Siddons et al., 1969).

Despite the reduced fructose absorption, energy retention did not differ between the dietary treatments. Energy retention averaged 299 ± 16 kJ/kg of $\text{BW}^{0.75}$ per day, which is in a similar range as in a study by Berends et al. (2012), who found an energy retention of 295 kJ/kg of $\text{BW}^{0.75}$ per day for milk-fed calves of 164 kg of BW fed 9 g DM of solid feed/kg of $\text{BW}^{0.75}$ per day and a MR with a GE content of 21.7 MJ/kg DM. Energy losses with urine were greatest for GLUC calves, which was caused by greater urinary glucose excretion for GLUC calves. Urinary glucose excretion has been shown in milk-fed calves, and increased with age and feeding level (Vicari et al., 2008a, Labussière et al., 2009), with protein-free energy intake (Gerrits et al., 2008) and with nutrient asynchrony (Vicari et al., 2008b). To assess whether glycerol is excreted with urine as well, the urinary energy excretion which was not explained by glucose (assuming 15.56 kJ/g glucose; Blaxter, 1989) and urea N (assuming 22.6 kJ/g urea N; Baldwin, 1995) was calculated. This unexplained urinary energy excretion was greater ($P = 0.009$) for GLYC calves (30.8 ± 1.01 kJ/kg of $\text{BW}^{0.75}$ per day) than for other calves (20.5 ± 3.40 kJ/kg of $\text{BW}^{0.75}$ per day), indicating that these calves excreted glycerol with the urine. Energy retention was not decreased for GLYC calves, which is in agreement with studies where no effect on growth performance was observed when replacing dietary corn with glycerol for growing-finishing pigs (Duttlinger et al., 2012) or when replacing 37.5% of the lactose with glycerol in the MR for calves from 3 to 35 d of age (Ebert et al., 2008).

Nitrogen retention did not differ between treatments when using a pair of calves as experimental unit (Table 4.3). Feed intake and fecal and urinary N losses were measured individually and N retention was lower ($P = 0.027$) in FRUC calves than in GLUC calves when calf was used as the experimental unit. This decreased N retention for FRUC calves was mainly caused by increased fecal N losses. Nitrogen retention and the efficiency by which digested N was retained decreased with blocks later in time, which is in agreement with the review of Van den Borne (2006a) and with Labussière et al. (2009) who reported a decrease in N retention efficiency as calves got older and heavier.

Circadian patterns of RQ and heat production followed the ingestion of meals. The 24h RQ of GLYC calves was lower than for other calves due to the lower RQ for the complete oxidation of glycerol (0.857; Elia and Cummings, 2007). The RQ of GLUC calves was lower when approaching the morning meal, which was also reflected in the greater diurnal fluctuation in GLUC calves. This indicates that lipid oxidation was greater for GLUC calves during the night, as a response to low carbohydrate availability for oxidation. This is probably related to the greater amount of time that the renal threshold for glucose (8.3 mmol/L; Hostettler-Allen et al., 1994, Vicari et al., 2008a) is exceeded in jugular blood in the GLUC calves after feeding (Chapter 5), resulting in greater excretion of urinary glucose after feeding and, therefore, less glucose is available for oxidation during the night. Indeed, urinary glucose excretion and the RQ during the night (i.e. the average RQ of the last 6 h before morning feeding) were negatively related ($r = -0.50$, $P = 0.033$), and this relation was mainly caused by a high relation in the GLUC calves ($r = -0.90$, $P < 0.10$).

Before the 1530 h feeding, the RQ of FRUC calves appeared to be greater than that of other calves, although only numerically. Probably, fructose is converted to other metabolites, before being oxidized. This is in agreement with the delay in $^{13}\text{CO}_2$ production after feeding ^{13}C -labeled fructose compared with glucose and glycerol. Fructose could be converted into glucose (calves; Siddons et al., 1969, Kurz and Willett, 1992), lactate (human and rat; Pereira and Jangaard, 1971, Swanson et al., 1992, Chong et al., 2007, Teff et al., 2009) and glycogen (rat; Pereira and Jangaard, 1971). In humans, 50% of the fructose which is metabolized by the liver is converted into glucose, 25% into lactate, 15-18% into glycogen and 1-5% into free fatty acids (as reviewed by Rippe and Angelopoulos, 2013). This indicates that only 2-9% of the fructose is oxidized directly, which is in agreement with the delay in $^{13}\text{CO}_2$ production that we found after feeding. The conversion of fructose into VFA before oxidation is possible as well in our study. Despite the delay in the maximum rate of $^{13}\text{CO}_2$ for FRUC, the recovery of the isotopes as $^{13}\text{CO}_2$ was equal between lactose replacers and averaged 72%. This is slightly lower than recovery of $^{13}\text{CO}_2$ after feeding ^{13}C -labeled glucose in a previous study with heavy milk-fed calves (80% on average; Van den Borne et al., 2007). The recovery of isotopes as $^{13}\text{CO}_2$ expressed as a percentage of the amount of ^{13}C not excreted with feces results in a recovery of 74.8 ± 2.36 , 79.1 ± 1.31 and $70.8 \pm 0.91\%$ for GLUC, FRUC and GLYC calves. This suggests that oxidation of digested FRUC is greater than that of digested GLYC and GLUC ($P = 0.005$). This corresponds with the urinary energy losses, which are lowest for FRUC calves, and is in agreement with humans, where ingested fructose is oxidized to a greater extent than ingested glucose (Tappy et al., 1986). The amount of isotopes not lost with feces or breath was greatest for GLYC calves ($P = 0.002$) and equaled 29.0 ± 0.90 , 24.9 ± 2.31 and $19.3 \pm$

1.25 % for GLYC , GLUC and FRUC calves, respectively. This is in agreement with our results on energy retention (GLYC > GLUC > FRUC).

In conclusion, energy and N retention were not affected when replacing >30% of the lactose from the calf milk replacer with glucose, fructose or glycerol. Apparent total tract disappearance of dry matter, energy and nitrogen of GLUC and GLYC calves were comparable with CON calves, but fecal losses of dry matter, energy and nitrogen were increased for FRUC calves. Post-absorptive losses occurred with the urine for glucose and glycerol, which caused a lower RQ for GLUC calves during the night. Furthermore, fructose was oxidized more slowly than glucose and glycerol, probably as a result of conversion into other substrates before oxidation.

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Chapter 4

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Chapter 5

Lactose in milk replacer can partly be replaced by glucose, fructose or glycerol without affecting insulin sensitivity in veal calves



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ABSTRACT

Calf milk replacer (MR) contains 40 to 50% lactose. Lactose strongly fluctuates in price and alternatives are desired. Also, problems with glucose homeostasis and insulin sensitivity (i.e. high incidence of hyperglycemia and hyperinsulinemia) have been described for heavy veal calves (BW > 100 kg). Replacement of lactose by other dietary substrates can be economically attractive, and may also positively (or negatively) impact the risk of developing problems with glucose metabolism. An experiment was designed to study the effects of replacing one third of the dietary lactose by glucose, fructose or glycerol on glucose homeostasis, insulin sensitivity and growth performance in veal calves. Forty male Holstein-Friesian calves (114 ± 2.4 kg) were fed a MR containing 462 g lactose per kg (CON), or a MR in which 150 g of lactose per kg MR was replaced by glucose (GLUC), fructose (FRUC) or glycerol (GLYC). During the first 10 days of the trial, all calves received CON. The CON group remained on this diet, and the other groups received their experimental diets for a period of 8 weeks. Measurements were conducted during the first (baseline) and last week of the trial. A frequently sampled intravenous glucose tolerance test was performed to assess insulin sensitivity and 24h urine was collected to measure glucose excretion. During the last week of the trial, a bolus of 1.5 g of [U- 13 C] substrates was added to their respective meals and plasma glucose, insulin and 13 C-glucose responses were measured. Growth was not affected by treatment (daily gain 1.19 ± 0.03 kg). Insulin sensitivity was low at the start of the trial and remained low (1.2 ± 0.1 and 1.0 ± 0.1 [mU/L] $^{-1}$ ×min $^{-1}$), and there was no treatment effect. Glucose excretion was low at the start of the trial (3.4 ± 1.0 g/d), but increased ($P < 0.01$) in CON and GLUC calves (26.9 ± 3.9 and 43.0 ± 10.6 g/d), but not in FRUC and GLYC calves. Postprandial glucose was higher ($P < 0.05$) in GLUC, lower ($P < 0.05$) in FRUC and similar in GLYC, compared CON calves. Postprandial insulin was lower ($P < 0.05$) in FRUC and GLYC, similar in GLUC, compared to CON calves. Postprandial 13 C-glucose increased substantially in FRUC and GLYC calves, indicating that calves are able to partially convert these substrates to glucose. We conclude that replacing one third of lactose in MR by glucose, fructose or glycerol differentially influences postprandial glucose homeostasis, but does not affect insulin sensitivity and growth performance in veal calves.

Key words: veal calves, lactose, milk replacer, fructose, glycerol, insulin sensitivity, glucose homeostasis

INTRODUCTION

Veal calves are fed with milk replacer (MR), roughage and concentrates. Despite the tendency to increase the amounts of roughage and concentrates in the diet, the vast majority (60 to 70%) of the digestible nutrient intake originates from MR. Upon closure of the esophageal groove, MR bypasses the rumen and flows directly into the abomasum. Lactose is the predominant, if not the only, carbohydrate source in MR. Calf MR commonly contains approximately 45% lactose, which is efficiently digested and absorbed from the calf's intestinal lumen (Burt and Irvine, 1970, Coombe and Smith, 1974).

However, the commercial availability of lactose (or whey) for feed applications is limited and not constant, resulting in large fluctuations in raw material prices. Replacement of lactose by other ingredients would increase the flexibility for composing MR recipes, reduce costs, and possibly, depending on the alternative, also reduce the carbon footprint of veal production. Importantly, a prolonged high intake of lactose, combined with substantial amounts of fat, has been associated with impaired glucose homeostasis. Hyperglycemia, hyperinsulinemia and insulin resistance have been observed in veal calves in the second phase of the fattening period (Hostettler-Allen et al., 1994, Hugi et al., 1997). Such metabolic problems may eventually result in diabetes and (pro)inflammatory stress, as demonstrated in humans (Hotamisligil, 2006, Shoelson et al., 2006) and in hepatic steatosis (Gerrits et al., 2008).

Starch or starch-based products, such as maltodextrins, are the most obvious alternatives for lactose. These products are widely available and are also attractive from an economic perspective. However, we recently demonstrated that calves have difficulties digesting starch-based products from MR diets, probably due to low activities of α -amylase and maltase in the small intestine. Nonetheless, the vast majority of starch does not reach the end of the small intestine, which can probably be explained by fermentation (Chapter 2 and 3).

Apart from starch-based products, glucose, fructose and glycerol may also replace lactose in MR. Partly replacing lactose by fructose and glycerol may beneficially affect postprandial glucose homeostasis. These substrates have lower glycemetic (and insulinemic) responses than lactose (Foster-Powell et al., 2002). It is believed that a lower glycemetic (and insulinemic) response is beneficial for health, especially in subjects with impaired glucose metabolism (Howlett and Ashwell, 2008). In humans, fructose and glycerol are almost completely absorbed and metabolized by the liver (Grunnet and Lundquist, 1967, Schaefer et al., 2009, Sun and Empie, 2012). Therefore, the effects of these substrates on postprandial glucose homeostasis will likely depend on the rate and extent of conversion of these substrates to glucose by the liver. In human, fructose is only partly (29-51%)

converted to glucose by the liver (Sun and Empie, 2012). Whether this is also the case for veal calves is not clear. Compared to fructose and glycerol, glucose may lead to higher glycemic (and insulinemic) responses than lactose (Foster-Powell et al., 2002), and thus may negatively affect postprandial glucose homeostasis. Whether prolonged exposure to these substrates (as partial replacer of lactose in veal calves) also affects insulin sensitivity and growth performance is not known.

The objective of the current study was, therefore, to study the effects of partial replacement of dietary lactose by glucose, fructose and glycerol on growth performance, glucose homeostasis and insulin sensitivity in veal calves. The effects on energy and protein utilization are described in Chapter 4.

MATERIAL AND METHODS

Animals and housing

Forty male Holstein-Friesian calves were housed at the research facility of the Department of Animal Sciences at Wageningen University, The Netherlands. At the start of the trial calves were 97 ± 1.4 d of age and weighed 114 ± 2.4 kg (both mean \pm SEM).

Calves were housed in groups, except for 6 d during the pre-experimental period (i.e. first 10 d) and the last 14 d of the trial. During these periods calves were housed individually in metabolic cages (dimensions: 0.80×1.8 m). During group housing, calves were housed in pens (5 calves/pen), which were fitted with wooden slatted floors and galvanized fencings. Per calf, 2 m^2 was available. Ventilation occurred by ceiling fans, and illumination by natural light and artificial (fluorescent lamps) light between 0630 and 1730 h. The average temperature and humidity were $18.5 \pm 0.4^\circ\text{C}$ and $69.5 \pm 1.2 \%$, respectively (both mean \pm SEM).

Experimental procedures complied with the Dutch Law on Experimental Animals, and the ETS123 (Council of Europe 1985 and the 86/609/EEC Directive) and were approved by the Animal Care and Use Committee of Wageningen University.

Experimental design, diets and feeding

Calves were fed twice the metabolizable energy requirements for maintenance, which was set at 460 kJ/kg metabolic body weight ($\text{kg}^{0.75}$) per day (Van Es et al., 1967). Individual body weights were measured weekly and the feeding rate was adjusted accordingly.

The trial consisted of a pre-experimental period (first 10 days) and an experimental period of 55 days. During the pre-experimental period, all calves received the control MR diet, which contained 462 g of lactose per kg MR. The composition of the MR is given in Table 5.1. Thereafter, calves were assigned to one of four dietary treatments. The control group

(CON) remained on the control MR diet. In the other groups, 150 g of lactose (per kg MR) was replaced by iso-energetic amounts of either glucose (GLUC; Tereos Syral, Marckolsheim, France), fructose (FRUC; Tate & Lyle Europe, Boleraz, Slovakia) or glycerol (GLYC; Triconor Distribution BV, Soest, The Netherlands). All calves remained on their respective diets for a period of 55 days. The introduction of the lactose replacers occurred gradually; by increasing the lactose replacement by 50 g per kg MR every 3 days.

Table 5.1 Composition of the control milk replacer.

Ingredient	g/kg	Nutrient	g/kg DM ³
Basal milk replacer			
Delactosed whey powder	244.5	Dry matter ⁴ (g/kg)	966
Calcium formate	7.5	Crude protein ($N \times 6.25$)	205
Coconut oil	39.2	Crude fat	209
Lard	72.4	Crude ash	74
Tallow	72.4	Lactose ⁵	478
Lecithin	5.9	Fe (mg/kg DM)	49.9
Ricinoleate emulsion	5.9	Gross energy (MJ/kg)	19.8
Premix ¹	10.0		
Whey protein concentrate	391.1		
Methionine	1.1		
Additional			
Lactose ²	150		

¹ Premix (per kg of milk replacer): crude protein, 0.7 g; starch, 5.0 g; crude ash, 1.5 g; Ca, 17 mg; P, 7.5 mg; Na, 0.7 mg; K, 7.3 mg; Cl, 13 mg; Mg, 0.5 g; Fe, 45 mg; Cu, 8.0 mg; Zn, 0.1 g; Mn, 43 mg; Se, 0.3 mg; I, 1.0 mg; Vitamin A, 25,013 IU; Vitamin D₃, 4,002 IU; Vitamin E, 150 IU; Vitamin K₃, 2.1 mg; Vitamin C, 0.3 g; Vitamin B₁, 8.2 mg; Vitamin B₂, 10 mg; Vitamin B₃, 35 mg; Vitamin B₅, 18 mg; Vitamin B₆, 10 mg; Vitamin B₁₂, 0.1 mg; biotin, 0.2 mg; folic acid, 0.7 mg; choline 0.4 g.

² For the GLUC, FRUC and GLYC groups, 150 g lactose per kg was replaced iso-energetically by glucose, fructose and glycerol, respectively.

³ Values in g per kg dry matter unless stated otherwise.

⁴ Dry matter of the basal milk replacer.

⁵ Calculated content.

In addition to MR, each calf received 10 g DM of solid feed per kg^{0.75} per day. The solid feed consisted of 80% concentrates and 20% wheat straw (based on DM). The concentrates were composed of 279 g/kg barley, 458 g/kg corn, 205 g/kg lupines, 24 g/kg palm oil, and 34 g/kg premix.

MR was fed on individual basis, at a concentration of 140 g/L and supplied at a temperature of ~42°C. The concentration increased to 160 g/L when the MR volume ≥ 9.0 L. Solid feed was provided per pen (5 calves/ pen) during group housing, and per individual calf when the calves were housed in metabolic cages. Calves were allowed ad libitum access to water. Feeding took place twice a day, at 0630 and 1530 h, via buckets. MR was supplied first, followed by solid feed. MR refusals (max 60 min after feeding) were weighed and recorded twice a day, prior to solid feed supply, whereas solid feed refusals were weighed and recorded once a day, prior to the morning MR feeding.

Experimental procedures

The measurements were concentrated during the pre-experimental period (first 10 days of trial; measurement period 1) and last 7 days of the trial (measurement period 2).

Frequently sampled intravenous glucose tolerance test (FSIGTT). A FSIGTT was performed during both measurement periods (on day 9 and 65 of the trial). Five (measurement period 1) and 12 days (measurement period 2) prior to the test, calves were moved to metabolic cages and were prepared with a central venous catheter (Careflow, Becton Dickinson, Alphen aan den Rijn, The Netherlands) in the jugular vein for glucose and insulin infusion, and blood sampling.

All calves were fasted overnight for 16 to 19 h prior to the FSIGTT. At $t = 0$ min, an intravenous glucose bolus of 0.3 g/kg BW (20% glucose solution, B. Braun, Oss, The Netherlands) was administered within 2 min, followed by an intravenous insulin bolus of 0.03 IU/kg BW (100 IU/mL solution, Insuman[®] Rapid, Sanofi-Aventis, Gouda, The Netherlands) at $t = 20$ min (administered within 1 min). Blood samples were collected from the jugular catheter at $t = -8, -4, 2, 4, 6, 8, 10, 12, 14, 16, 19, 22, 25, 30, 35, 40, 50, 60, 75, 90, 120, 150, 180$ min relative to the intravenous glucose bolus. Blood samples were transferred immediately into 6 mL lithium heparin vacutainer tubes (Becton Dickinson, Breda, The Netherlands) and stored on ice. Plasma was collected, after centrifugation, within 1.5 h after blood sampling and stored at -80°C until analysis of plasma glucose and insulin concentrations. In addition, plasma triglycerides, HDL and LDL cholesterol concentrations were analyzed in the fasting plasma samples.

The insulin sensitivity index was calculated according to Bergman's minimal model approach, using MinMod Millennium (MinMod Inc., Los Angeles, CA, USA; version 6.0.2), a computer-based software for the calculation of insulin sensitivity from FSIGTT data (Pacini and Bergman, 1986, Boston et al., 2003). Insulin sensitivity derived by this method (IS_{minmod}) encompasses both peripheral and hepatic IS (Bergman et al., 1979). Therefore, another index of insulin sensitivity, the Quantitative Insulin Sensitivity Check Index

(QUICKI), was calculated from the fasting plasma glucose and insulin levels collected during the FSIGTT. QUICKI primarily reflects hepatic IS (Chen et al., 2003, Muniyappa et al., 2008). This index was calculated using the following formula:

$$\text{QUICKI} = 1 / [\log [\text{glucose (mg / dL)}] + \log [\text{insulin (mU / L)}]]$$

Urine collection. Prior to catheterization (for the FSIGTT), calves were shaved (especially the tail region) and harnessed. Plastic bags were connected to the harness to collect feces. Clean urine was quantitatively collected for 3 days (measurement period 1) and 5 days (measurement period 2). The urine was collected via funnels in buckets, which were placed under the cages. Each bucket contained 1500 mL of a 25% sulfuric acid solution (BOOM, Meppel, The Netherlands). At the end of the collection period, the amount of urine was weighed, and an aliquot of 100 mL was transferred into a urine collection cup and frozen at -20°C until analysis of glucose, catecholamines and cortisol.

Postprandial blood glucose, ¹³C-glucose and insulin concentrations. On day 61 of the trial (measurement period 2), a bolus of 1.5 g of [U-¹³C]-enriched substrates (99% isotopic enrichment; Cambridge Isotope Laboratories, Tewksbury, MA, USA) was added to the morning MR meal. Hence, GLUC calves received [U-¹³C] glucose, FRUC calves received [U-¹³C] fructose, and GLYC calves received [U-¹³C] glycerol, whereas CON calves did not receive ¹³C-enriched substrates. Solid feed was not provided during this (enriched) test meal.

Blood samples were collected from the jugular vein at t = -10, 15, 30, 60, 120, 180, 240, 300 and 360 min relative to MR feeding. Blood samples were transferred into 6 mL lithium heparin vacutainer tubes (Becton Dickinson, Breda, The Netherlands) and stored on ice. Plasma was collected after centrifugation and was stored at -80°C until analysis. Plasma glucose and insulin concentrations, and ¹³C enrichment in plasma glucose were measured. For glucose and insulin, the ΔC_{max} (= maximum concentration – fasting concentration), time to maximum concentration and the incremental area under the curve (iAUC_{0-6h}) were calculated. For ¹³C enrichment in plasma glucose, only the iAUC_{0-6h} was calculated. The iAUC_{0-6h} was calculated using the trapezoid method (Le Floch et al., 1990).

Analytical procedures

Plasma glucose, triglycerides, HDL and LDL cholesterol were measured on a Roche-Hitachi Modular automatic analyzer (Roche Diagnostics) using enzymatic colorimetric methods. Insulin was measured using a bovine ELISA kit (Merckodia, Uppsala, Sweden).

Catecholamines in urine were measured using an online SPE-LC/MS/MS method previously described by de Jong et al. (2010). Cortisol was measured using the online SPE-LC/MS/MS method described by Jones et al. (2012). This method was originally developed for saliva samples but was successfully applied on urine samples. The $^{13}\text{C}:^{12}\text{C}$ ratio in plasma glucose was measured using a modified version of the GC/C/IRMS method previously described by Vonk et al. (2000a, 2000b). The modifications are described in detail elsewhere (Eelderink et al., 2012). A calibration curve was used to calculate the molar percentage excess (7 standards; range 0.01-2.00 molar percent excess) of the measured samples. Then, the molar percentage excess was multiplied by the plasma glucose concentration to obtain $\Delta[^{13}\text{C}]$ -glucose concentrations.

Statistical analyses

The SPSS (IBM, Version 22) statistical software was used for all statistical analyses. Data are presented as means \pm SEM. Insulin sensitivity, urinary glucose excretion and fasting blood levels of glucose, insulin, cholesterol, triglycerides, catecholamines and cortisol were tested for treatment effects by ANCOVA using GLM. Calf was the experimental unit and initial values (measured during pre-experimental period) were used as covariates. Growth and data derived from postprandial blood concentrations (i.e. $\Delta\text{C}_{\text{max}}$, time to maximum, and $\text{iAUC}_{0-6\text{h}}$) were tested for treatment effects by ANOVA using GLM. Normality of the studentized residuals was assessed by visual inspection. Non-normal distributed data were (log) transformed to obtain normality. P -values < 0.05 were considered significant and P -values < 0.10 were considered a trend toward significance. When treatment effects were significant, the Šidák method (Šidák, 1967) was used to correct for pairwise comparisons.

RESULTS

Growth performance

There was no difference in feed refusals between groups. One calf in the GLUC group was excluded from the trial due to ruminal drinking. The average daily gain, measured over the 65 d trial period, was on average 1.19 ± 0.03 kg/d and did not differ between treatments (Figure 5.1).

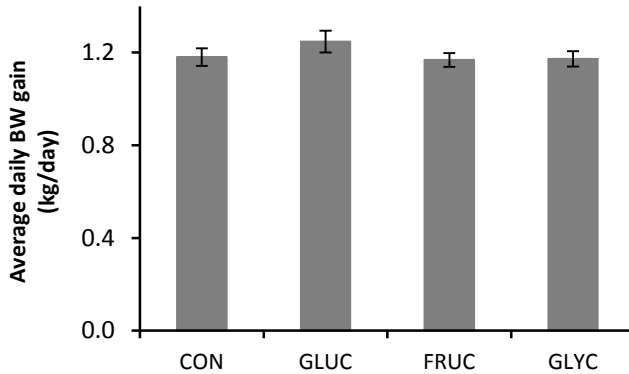


Figure 5.1 Growth performance of veal calves fed a control milk replacer (CON, 462 g lactose/kg milk replacer; $n = 10$) or a milk replacer in which 150 g of lactose per kg milk replacer was replaced by iso-energetic amounts of glucose (GLUC; $n = 9$), fructose (FRUC; $n = 10$) or glycerol (GLYC; $n = 10$). Error bars represent the SEM. There was no significant difference between treatment groups ($P = 0.77$).

Insulin sensitivity

Initial values for IS_{minmod} and QUICKI (measured during the pre-experimental period) were $1.2 \pm 0.1 \times 10^{-4} \text{ ((mU/L)}^{-1} \times \text{min}^{-1})$ and 0.371 ± 0.001 , respectively. These values decreased by 20 and 10%, respectively, during the trial, but these changes were not affected by dietary treatment (Figure 5.2).

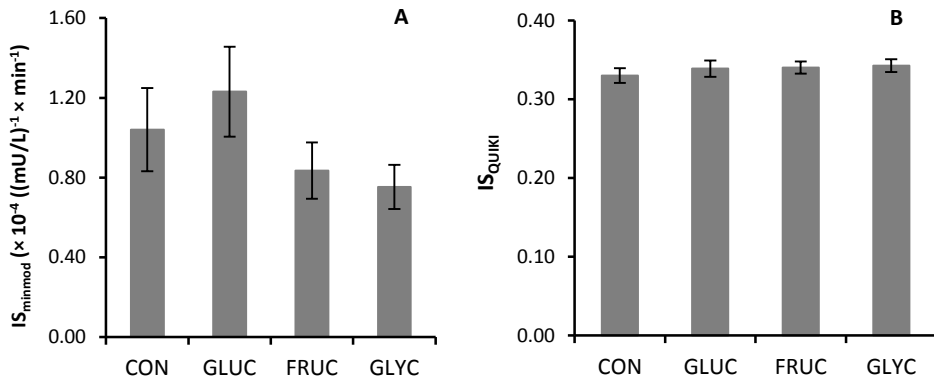


Figure 5.2 Insulin sensitivity (IS) in veal calves fed a control milk replacer (CON, 462 g lactose/kg milk replacer; $n = 10$) or a milk replacer in which 150 g of lactose per kg milk replacer was replaced by iso-energetic amounts of glucose (GLUC; $n = 9$), fructose (FRUC; $n = 10$) or glycerol (GLYC; $n = 10$). Insulin sensitivity was calculated using the FSIGTT minimal model (A) and QUICKI (B). Error bars represent SEM. There was no significant difference between treatment groups ($P = 0.70$ and 0.20 , respectively).

Fasting blood metabolites and hormones

Fasting plasma glucose concentrations (collected during the FSIGTT) were greater ($P < 0.05$) for FRUC calves (5.8 mmol/L) than for GLUC calves (5.3 mmol/L; Table 5.2). Fasting plasma insulin concentrations, however, were not affected by treatment but increased by 78% during the trial. Fasting triglyceride concentrations were not affected by treatment but decreased by 64% during the trial. Fasting HDL- and LDL-cholesterol concentrations were not affected by treatment.

Table 5.2 Fasting plasma metabolite and hormone concentrations and urinary excretion of stress-related markers (means \pm SEM) in veal calves fed a control milk replacer (CON, 462 g lactose/kg milk replacer; $n = 10$) or a milk replacer in which 150 g of lactose per kg milk replacer was replaced by iso-energetic amounts of glucose (GLUC; $n = 9$), fructose (FRUC; $n = 10$) or glycerol (GLYC; $n = 10$), for a period of 55 days.

	Treatment				<i>P</i>
	CON	GLUC	FRUC	GLYC	
Fasting plasma concentration					
Glucose (mmol/L)	5.7 \pm 0.1 ^{ab}	5.3 \pm 0.1 ^a	5.8 \pm 0.1 ^b	5.6 \pm 0.1 ^{ab}	0.016
Insulin (mU/L)	12.7 \pm 2.2	10.8 \pm 1.3	9.8 \pm 1.8	11.7 \pm 2.1	0.79
Triglycerides (mmol/L)	0.15 \pm 0.01	0.15 \pm 0.01	0.13 \pm 0.01	0.14 \pm 0.01	0.94
HDL-cholesterol (mmol/L)	2.3 \pm 0.2	2.6 \pm 0.2	3.1 \pm 0.2	2.7 \pm 0.1	0.078
LDL-cholesterol (mmol/L)	0.41 \pm 0.06	0.54 \pm 0.07	0.68 \pm 0.10	0.56 \pm 0.07	0.14
Urinary excretion					
Adrenaline (μ g/kg BW per day)	0.07 \pm 0.01	0.05 \pm 0.01	0.06 \pm 0.01	0.05 \pm 0.01	0.25
Noradrenaline (μ g/kg BW per day)	0.68 \pm 0.08	0.68 \pm 0.10	0.77 \pm 0.07	0.65 \pm 0.10	0.24
Dopamine (μ g/kg BW per day)	0.71 \pm 0.11	0.77 \pm 0.09	0.83 \pm 0.05	0.68 \pm 0.10	0.14
Cortisol (μ g/kg BW per day)	0.07 \pm 0.01	0.08 \pm 0.02	0.08 \pm 0.01	0.06 \pm 0.01	0.50

^{ab} Different superscripts indicate pairwise differences ($P < 0.05$).

Postprandial glucose, insulin and ¹³C enrichment in plasma glucose

Postprandial blood glucose and insulin concentrations were measured after 51 days of adaptation to the experimental diets. On this day, fasting glucose and insulin concentrations were measured again for calculation of the postprandial parameters (Table 5.3). The treatment effects did not differ from the fasting measurements conducted during the FSIGTT (Table 5.2). Directly after feeding, plasma glucose and insulin concentrations increased (Figure 5.3), but the time to the maximum plasma concentrations of glucose and insulin did not differ between treatments. The postprandial increase in plasma glucose concentration (ΔC_{\max}) differed between treatments, with the levels being higher ($P < 0.05$) in GLUC calves than in FRUC and GLYC calves. The ΔC_{\max} for insulin also differed between treatments, with the levels being higher ($P < 0.05$) in GLUC and CON calves than in FRUC calves. The $iAUC_{0-6h}$ for glucose was higher ($P < 0.05$) for GLUC calves than the other calves, and higher ($P < 0.05$) for CON calves than for FRUC calves. The $iAUC_{0-6h}$ for insulin was higher ($P < 0.05$) for GLUC and CON calves than for FRUC and GLYC calves. The $iAUC_{0-6h}$ for the $\Delta[^{13}\text{C}]$ -glucose did differ between treatments, with $\text{GLUC} > \text{GLYC} > \text{FRUC}$ (Table 5.3 and Figure 5.4).

Urinary glucose excretion

During the pre-experimental period, urinary glucose excretion was low (3.4 ± 1.0 g/d). At the end of the trial (i.e. after 55 days of adaptation to the experimental diets), the glucose excretion was substantially higher ($P < 0.01$) for CON and GLUC calves (26.9 ± 3.9 and 43.0 ± 10.6 g/d, respectively; Figure 5.5).

Urinary excretion of catecholamines and cortisol

The urinary excretion of four stress-related markers (adrenaline, nor-adrenaline, dopamine, and cortisol) was measured during this trial. Urinary excretion of these stress-related markers was not affected by dietary treatment (Table 5.2).

Table 5.3 Postprandial responses of plasma glucose and insulin, and ^{13}C enrichment in plasma glucose (means \pm SEM) in veal calves fed a control milk replacer (CON, 462 g lactose/kg milk replacer; $n = 10$) or a milk replacer in which 150 g of lactose per kg milk replacer was replaced by iso-energetic amounts of glucose (GLUC; $n = 9$), fructose (FRUC; $n = 10$, except ^{13}C -glucose enrichment $n = 6$) or glycerol (GLYC; $n = 10$), for a period of 51 days.

	Treatment				<i>P</i>
	CON	GLUC	FRUC	GLYC	
Plasma glucose concentration					
Fasting values (mmol/L)	5.4 \pm 0.1 ^{ab}	4.9 \pm 0.1 ^a	5.5 \pm 0.2 ^b	5.4 \pm 0.2 ^{ab}	0.024
$\Delta\text{C}_{\text{Max}}$ (mmol/L)	4.7 \pm 0.5 ^{ab}	6.3 \pm 0.4 ^a	3.5 \pm 0.3 ^b	3.7 \pm 0.4 ^b	< 0.001
Time to maximum (min)	56 \pm 9	77 \pm 20	50 \pm 17	54 \pm 4	0.53
iAUC _{0-6h}	761 \pm 84 ^b	1233 \pm 110 ^a	383 \pm 77 ^c	560 \pm 91 ^{bc}	< 0.001
Plasma insulin concentration					
Fasting values (mU/L)	17.0 \pm 3.5	14.4 \pm 1.0	10.3 \pm 1.3	11.0 \pm 1.5	0.077
$\Delta\text{C}_{\text{Max}}$ (mU/L)	909 \pm 133 ^a	831 \pm 79 ^a	478 \pm 85 ^b	569 \pm 68 ^a	0.008
Time to maximum (min)	150 \pm 10	140 \pm 10	130 \pm 16	126 \pm 7	0.44
iAUC _{0-6h} $\times 10^3$	146 \pm 21 ^a	156 \pm 11 ^a	72 \pm 11 ^b	90 \pm 9 ^b	< 0.001
^{13}C -enriched plasma glucose ¹					
iAUC _{0-6h} $\times 10^{-2}$	4.8 \pm 1.0 ^d	887 \pm 55 ^a	151 \pm 14 ^b	250 \pm 18 ^c	< 0.001

¹ On top of the milk replacer diets (except for the control group) 1.5 g of the corresponding U- ^{13}C substrate (i.e.

^{13}C -glucose, fructose or glycerol) was added to their diets.

^{a-c} Different superscripts indicate pairwise differences ($P < 0.05$).

^d CON calves were not included in statistical analysis because they did not receive a ^{13}C -enriched substrate.

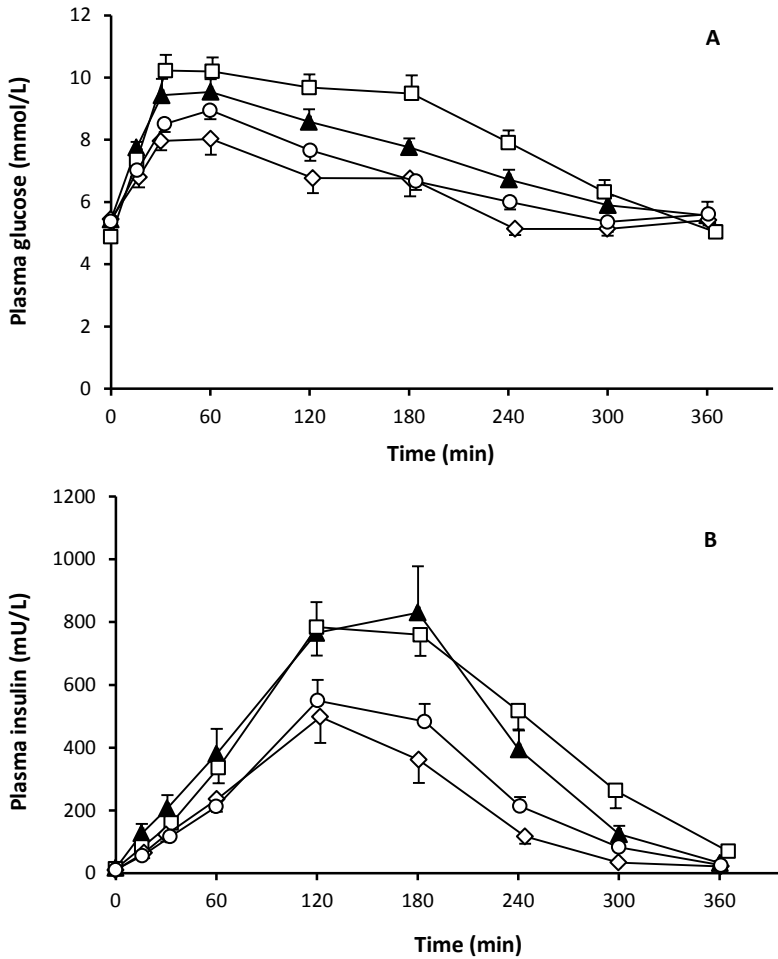


Figure 5.3 Plasma glucose (A) and insulin (B) responses in veal calves fed (at time = 0) a control milk replacer (▲, 462 g lactose/kg milk replacer; n = 10) or a milk replacer in which 150 g of lactose per kg milk replacer was replaced by iso-energetic amounts of glucose (□; n = 9), fructose (◇; n = 10) or glycerol (○; n = 10). Error bars represent SEM. Calculated parameters and statistics are given in Table 5.3.

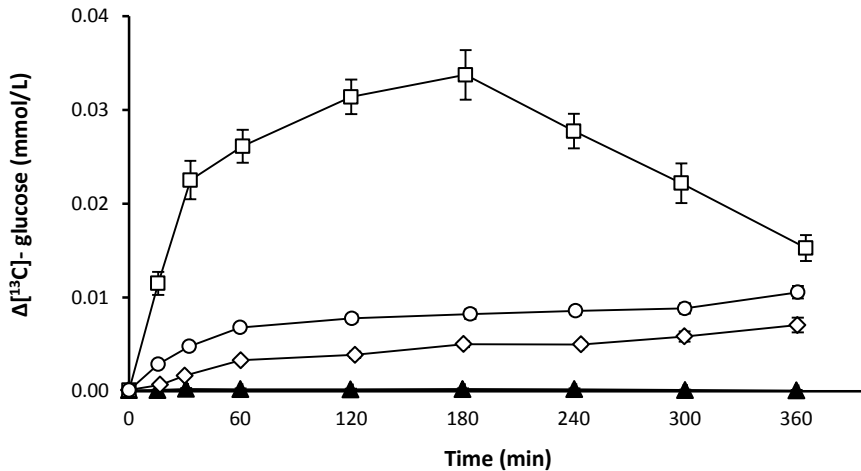


Figure 5.4 Changes in plasma $\Delta^{13}\text{C}$ -glucose concentration in veal calves fed (at time = 0) a control milk replacer (\blacktriangle , 462 g lactose/kg milk replacer; $n = 10$) or a milk replacer in which 150 g of lactose per kg milk replacer was replaced by iso-energetic amounts of glucose (\square ; $n = 9$), fructose (\diamond ; $n = 6$) or glycerol (\circ ; $n = 10$). On top of the milk replacer diets (except for the control group) 1.5 g of the corresponding U- ^{13}C substrate (i.e. ^{13}C -glucose, fructose or glycerol) was added to their diets. Error bars represent SEM. Calculated parameters and statistics are given in Table 5.3.

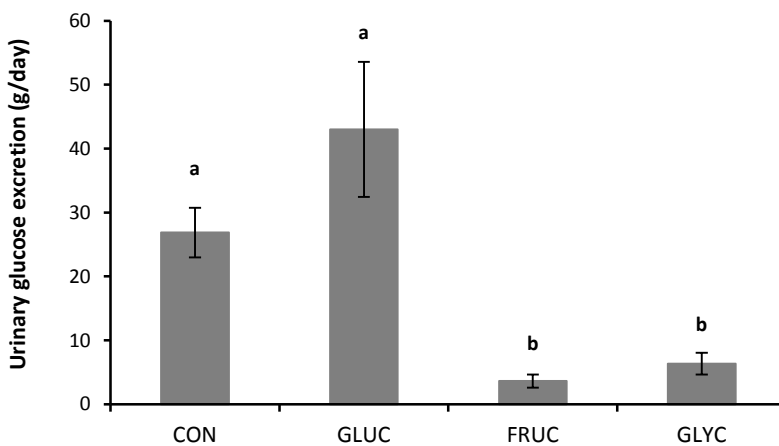


Figure 5.5 Urinary glucose excretion in veal calves fed a control milk replacer (CON, 462 g lactose/kg milk replacer; $n = 10$) or a milk replacer in which 150 g of lactose per kg milk replacer was replaced by iso-energetic amounts of glucose (GLUC; $n = 9$), fructose (FRUC; $n = 10$) or glycerol (GLYC; $n = 10$) Error bars represent SEM. ^{ab} Different letters indicate pairwise differences ($P < 0.05$).

DISCUSSION

Growth performance

The average growth rate during the trial was in agreement with what is normally achieved in practice at this feeding level. More importantly, there was no significant difference in growth performance between treatments. Data on energy partitioning (Chapter 4) showed that there was no difference in net energy retention between treatments. Thus, these results indicate that utilization of glucose, fructose and glycerol for growth does not differ from that of lactose in veal calves. These substrates might, therefore, be suitable lactose replacers in MR.

Fructose and glycerol gluconeogenesis

The total postprandial blood glucose response (iAUC_{0-6h}) was higher for GLUC calves than for the other calves, and higher for CON calves than for FRUC calves. In general, blood glucose levels reflect both the exogenous glucose influx, endogenous glucose production, as well as the glucose uptake into peripheral tissues (Priebe et al., 2010). The differences in iAUC_{0-6h} for glucose may, therefore, be due to a limited ability of the liver to convert fructose (and glycerol) into glucose. In order to assess appearance of exogenous substrates into the systemic circulation and to obtain indications on hepatic conversion into glucose, each calf (except for the CON calves) received 1.5 g of the corresponding U-¹³C substrate. The Δ [¹³C]-glucose levels increased in all groups (Figure 5.4) and the iAUC_{0-6h} differed between treatments, with GLUC calves being substantially higher than FRUC and GLYC calves, and GLYC being higher than FRUC. This indicates that fructose and glycerol are (partly) converted to glucose in calves, and may partly explain the differences observed in postprandial glucose concentrations. Nevertheless, the rates of conversion could not be calculated in our experiment, as the whole body glucose flux was not determined. Delarue et al. (1993) have studied the conversion of fructose to glucose in humans and have found that approximately 50% of the fructose was converted to plasma glucose within 6 hours after ingestion. Tran et al. (2010) have also studied gluconeogenesis from fructose in humans and reported a 37 and 29% conversion for men and women, respectively, within 6 hours after ingestion. Similar to our experiment, the plasma ¹³C-glucose enrichment increased consistently within the first 6 hours. This indicates that, for both humans and calves, there is an incomplete conversion of fructose to glucose within the first 6 hours after intake. Gluconeogenesis from glycerol has also been studied in humans, although less extensive than fructose. Massicotte et al. (2006) have assessed the metabolic fate of glycerol in humans during prolonged exercise and found that approximately 9% is converted to glucose within the first 2 hours after

ingestion. Glycerol is also a known glucogenic precursor in ruminants (i.e. cows and sheep), although conversion rates have not been quantified (Aschenbach et al., 2010, Werner-Omazic et al., 2015).

Glucosuria

Urinary glucose excretion was significantly higher in CON and GLUC calves than in FRUC and GLYC calves, where the urinary glucose excretion was negligible. Excretion of glucose via urine is a result of hyperglycemia (Chao, 2014, Wilding, 2014). This occurs when the renal threshold for glucose reabsorption is exceeded. In our experiment, the plasma glucose peak levels for the CON and GLUC calves exceeded the renal threshold of 8.3-11.1 mmol/L in calves (Wijayasinghe et al., 1984, Scholz and Hoppe, 1987, Hostettler-Allen et al., 1994, Stanley et al., 2002) and, therefore, urinary excretion was expected in these groups. Glucosuria is frequently observed in milk-fed calves (Hostettler-Allen et al., 1994, Hugi et al., 1997, Kaufhold et al., 2000). Although urinary glucose excretion differed between treatments, insulin sensitivity did not differ. This indicates that urinary glucose excretion in calves is not associated with insulin resistance, but rather relates to exceeding the glucose renal threshold.

Based on the significantly greater postprandial blood glucose levels and glycosuria for CON and GLUC than for FRUC and GLYC calves, it could be expected that calves assigned to the first two treatments would be more insulin resistant. Chronic postprandial hyperglycemia can lead to glucotoxicity, which can ultimately result in insulin resistance and diabetes (Campos, 2012). This was, however, not confirmed by the insulin sensitivity measurements.

Development of insulin sensitivity

The absence of effects on insulin sensitivity may be explained by the already low insulin sensitivity in calves before the dietary intervention. The average insulin sensitivity before the intervention was $1.2 \pm 0.1 \times 10^{-4} \text{ ((mU/L)}^{-1} \times \text{min}^{-1})$, which is $\sim 10\times$ lower than in neonatal calves at 3 and 6 weeks of age (Stanley et al., 2002) and ~ 3 to 6 times lower than in non-ruminants such as humans (Caumo et al., 2000, Vila et al., 2010) and dogs (Stefanovski et al., 2011). Instead, values were more comparable to those in lactating cows (Stanley et al., 2002). Several factors may contribute to the (decreased) insulin sensitivity observed in veal calves. One factor that may contribute is the high fat content in conventional calf MR ($\sim 200 \text{ g/kg MR}$). The composition of the digestible energy intake in veal calves MR (i.e. high fat and carbohydrate content) is similar to that of the adult western human diet (Schwarz et al., 2003, Cordain et al., 2005). In humans, a high dietary fat intake was found to be associated with insulin resistance (Storlien et al., 1996, Vessby,

2000, Marshall and Bessesen, 2002). However, despite their higher glucose (but lower fat) intake pigs and rats generally do not suffer from insulin resistance compared to veal calves, which indicates that interactions between dietary fat and glucose may be crucial for upsetting glucose metabolism (and insulin sensitivity) in veal calves. Another factor that may contribute to the (decreased) insulin sensitivity observed in veal calves is the discrepancy between their diet and ontogenetic background. Insulin resistance seems to be age-dependent in veal calves (Hugi et al., 1997, Hugi et al., 1998). In nature, calves between 4 and 6 months of age are grazing and plant fragments are fermented in the rumen along with the production of volatile fatty acids such as acetate, propionate and butyrate as the main sources of energy. Therefore, the veal calf, which is an ontogenetic ruminant, may not be equipped with the genetic capacity to deal with the large amounts of lactose, e.g. the capacity to use the glucose as precursor for de novo fatty acid synthesis (Roehrig et al., 1988). This may explain why a high lactose intake might induce insulin resistance in veal calves and why calves on conventional MR do not synthesize body fat from dietary carbohydrates (Van den Borne et al., 2007). Nevertheless, whether the decrease in insulin sensitivity is diet-related or part of the genetic programming of calves (transition from pre-ruminant to ruminant) is not clear and has to be investigated. Another factor that can modulate insulin sensitivity is environmental stress (Sato et al., 2011). Hugi et al. (1997) have measured the urinary excretion of noradrenaline and dopamine in veal calves and found a significant increase with age. They concluded that the enhanced activity of the nervous system (stress) might have contributed to the decrease in insulin sensitivity. In our study, however, none of the stress-related markers increased with age and there was no treatment effect. In addition, we performed Pearson bivariate correlation analysis between the measured insulin sensitivity values and the rates of urinary excretion of the different stress markers (data not shown). Significant correlations were not found between insulin sensitivity and any of the stress markers, suggesting that stress did not significantly affect insulin sensitivity in the current study.

Fasting blood glucose, insulin, triglycerides and cholesterol

The fasting glucose and insulin levels found in this trial were comparable to those reported by Hostettler-Allen et al. (1993, 1994) and Vicari et al. (2008). In our study, an increase in fasting plasma insulin levels was observed for all treatments, which suggests a decrease in hepatic insulin sensitivity (Muniyappa et al., 2008). The increase in plasma insulin is in agreement with findings by Breier et al. (1988) and Hugi et al. (1997), but in contrast with Hostettler-Allen et al. (1994) who did not detect an age-related increase in fasting plasma insulin levels.

In humans, insulin resistance is also often associated with dyslipidemia, characterized by a

high ratio between triglycerides and HDL-cholesterol (Li et al., 2008, Bitzur et al., 2009). However, whether this is also true for calves is unknown. Nevertheless, in our trial the triglyceride/HDL-cholesterol ratios were extremely low (ratios < 1) and decreased with age in all groups (lower triglyceride concentrations), suggesting that there was no evidence of dyslipidemia in these calves.

CONCLUSIONS

At least 150 g of lactose per kg MR can be replaced by glucose, fructose or glycerol without affecting growth performance in calves, indicating that these lactose replacers are used with a similar energetic efficiency. Lactose replacement by glucose, leads to increased postprandial blood glucose levels, and also increased losses of glucose in urine although not significant. Fructose and glycerol are (partly) converted into glucose, and lead to reduced postprandial glucose and insulin levels when lactose is replaced by fructose and reduced insulin levels when replaced by glycerol. Both substrates do not lead to significant urinary glucose losses. Despite differences in postprandial glucose homeostasis, lactose replacement did not affect insulin sensitivity, possibly because insulin sensitivity was already low at start of the trial.

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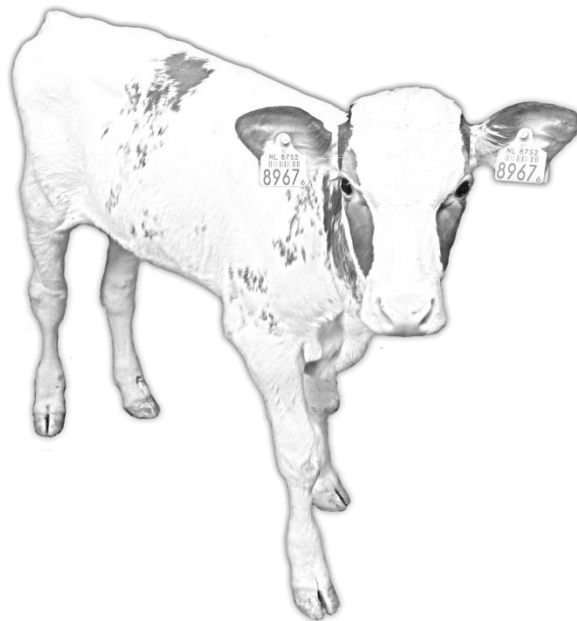
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Chapter 6

Predicting variation in feed efficiency in veal calves by characterizing feeding motivation, digestion, metabolism, behavior and immunology in early life



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ABSTRACT

High inter-individual variation in growth performance is reported in veal calves. High and fluctuating dairy prices are a major economic incentive to substitute lactose from calf milk replacer (MR) by alternative energy sources. Replacement of lactose, however, further increases the inter-individual variation in growth performance, indicating that calves differ in their capacity to deal with low-lactose MR. The objectives of the current study were to examine whether variation in growth performance in healthy veal calves can be explained by characterizing calves in early life and to determine whether their ability to cope with low-lactose MR can be predicted in early life. One hundred and eighty male Holstein-Friesian calves (17 ± 0.3 days of age) were characterized using targeted challenges in period 1 (2-11 weeks of age) related to feeding motivation, digestion, post-absorptive metabolism, behavior and stress and immunology. In period 2 (11-27 weeks of age), 130 calves were included and were equally divided over two MR treatments, i.e. a control MR which contained lactose as only carbohydrate source and a low-lactose MR in which 51% of the lactose was iso-calorically replaced by glucose, fructose and glycerol (2:1:2 ratio; GFG). Relations between early life characteristics and growth performance in later life were assessed in 117 clinically healthy calves. ADG in period 2 tended to be greater for control than for GFG calves. ADG in period 2 was explained for 12% by early life measurements. However, this was mainly related to variation in solid feed refusals. When ADG was adjusted to equal solid feed intake, only 4% of the variation in standardized ADG in period 2, reflecting feed efficiency, could be explained by early life measurements. This indicates that > 95% of the variation in feed efficiency in later life could not be explained by early life characterization. It is hypothesized that variation in health status explains substantial variation in feed efficiency in veal calves. Significant relations between fasting plasma glucose concentrations, fecal dry matter and fecal pH in early life and feed efficiency in later life depended on MR composition. These measurements are, therefore, potential tools for screening calves in early life on their ability to cope with low-lactose MR.

Key words: veal calves, feed efficiency, lactose, glycerol

INTRODUCTION

High inter-individual variation in growth performance is commonly reported in veal calves. Although animal health and feed intake affect daily gain, growth performance also differs substantially between individual clinically healthy calves at similar levels of nutrient intake. For instance, the coefficient of variation in ADG in milk-fed calves was 5.9% (13 to 26 weeks of age; Chapter 2), 12.3% (~6.5 to 9.5 weeks of age; Labussiere et al., 2008) and even 21.3% (3 to 6 weeks of age; Akinyele and Harshbarger, 1983). This variation is high compared to for instance growing-finishing pigs (coefficient of variation in ADG averaged 2.6% in pigs from 24-107 kg; Millet et al., 2012) and broilers (coefficient of variation in ADG was 2.0% in control-fed female broilers from 0 to 3 weeks of age; Lee et al., 2003).

Calf milk replacers (MR) contain 40 to 50% lactose. High and fluctuating dairy prices are a major economic incentive to replace lactose from MR by alternative energy sources. However, differences in growth rates between individual calves increased further when lactose was replaced from the MR. For instance, the coefficient of variation in ADG increased from 5.9 to 8.4% when lactose was partially replaced with maltose, although ADG decreased (Chapter 2), and increased from 3.6 to 80% when lactose was replaced with starch (Natrajan et al., 1972). This shows that variation in growth performance depends on feeding strategy and that not all calves are equally able to cope with lactose replacers (i.e. low-lactose MR). Digestive and metabolic processes may contribute to this increase in inter-individual variation in growth performance when feeding low-lactose MR. For example, the coefficient of variation in apparent ileal disappearance of dry matter increased from 5.0 to 6.9% when partly replacing lactose with maltose (Chapter 3) and the coefficient of variation in urinary glucose excretion increased from 42 to 82% when partly replacing lactose with glucose, fructose or glycerol (Chapter 5).

Under equal feeding and husbandry conditions in healthy calves, growth performance is largely determined by feed efficiency. Efficient utilization of nutrients depends on the calf's ability to cope with internal and external stressors, such as a meal, fasting, acute stress and infection pressure. This ability can be assessed by measuring the calf's responsiveness to specific stimuli. We designed targeted challenges related to digestion, metabolism, behavior and immunology and hypothesized that these can, under equal feeding and husbandry conditions in healthy calves, be used to characterize calves in early life. This characterization of calves in early life could be predictive of later life growth performance, potentially depending on MR composition.

Our objectives were 1) to examine whether variation in growth performance of veal calves can be explained by characterizing calves in early life and 2) to determine whether their ability to cope with low-lactose milk replacers can be predicted in early life.

MATERIAL AND METHODS

This experiment was conducted at the research facilities of the VanDrie Group (Scherpenzeel, The Netherlands) and was submitted to and approved by the Animal Care and Use Committee of Wageningen University.

Experimental design and animals

One-hundred and eighty male Holstein-Friesian calves of Dutch origin were used in this experiment. The experiment consisted of two periods. Period 1 (experimental week 1 to 10) started when calves arrived at the experimental facilities (average age and BW were 17 ± 0.3 d and 44 ± 0.2 kg, respectively) and lasted 9 weeks. In period 1, measurements (see 'Measurements') were performed on individual calves to characterize each calf. In period 2 (experimental week 10 to 28), the feeding trial was conducted in which a control, lactose-based MR was compared with a MR in which 51% of the lactose was replaced with glucose, fructose and glycerol. During period 2, general performance (see 'Measurements') was recorded. Average age and BW at the start of period 2 was 82 ± 0.3 d and 87 ± 0.4 kg, respectively.

Housing

Calves were housed on wooden, slatted floors. During period 1, calves were housed individually (1.2 m^2 per calf) in order to perform all measurements individually. During period 2, calves were housed in groups of 5 (1.8 m^2 per calf). Individual fixation of calves during feeding was achieved by locking calves in the headlock of the fence. Light was provided by daylight and artificial lights were on from 0600 to 1800 h. The stable was mechanically ventilated. The temperature and humidity averaged $19 \pm 3.9^\circ\text{C}$ and $77 \pm 9.3\%$ (mean \pm SD), respectively, during the experiment.

Diets and feeding

The ingredient and nutrient composition of the experimental MR are shown in Table 6.1. In period 1, all calves received MR with lactose as the only carbohydrate source. Solid feed was composed of concentrates, rapeseed straw and alfalfa and was supplied at a ratio of 70:15:15 on estimated dry matter (DM) basis. In period 2, each calf was assigned to one of two MR treatments, i.e. a control MR with lactose as only carbohydrate source (CON) or a MR in which 51% of the lactose was replaced with a mixture of glucose, fructose and glycerol (2:1:2; GFG) on gross energy basis. Solid feed was composed of concentrates and rapeseed straw and was supplied at a ratio of 80:20 on estimated DM basis. Concentrates were composed of 250 g/kg oats, 238 g/kg barley, 201 g/kg corn, 155 g/kg lupines, 111

g/kg corn gluten meal, 24 g/kg premix and 20 g/kg molasses. Analyzed crude protein content was 183 g/kg.

The feeding levels for MR and solid feed were based on a practical feeding scheme and were equal between calves. In period 1, MR allowance increased progressively from 400 to 1200 g/d and solid feed from 85 to 360 g/d. Solid feed was provided from one week after arrival onwards. In period 2, MR increased progressively from 1250 to 3000 g/d and solid feed from 630 to 1170 g/d.

Throughout the experiment, MR was mixed with warm water (66°C) and the concentration of reconstituted MR was 125 g/kg reconstituted MR in period 1 and increased from 125 to a maximum of 200 g/kg reconstituted MR in period 2. Reconstituted MR was supplied at a temperature of ~42°C in two equal meals, at 0600 and 1600 h, and calves were allowed access for 15 min, after which refusals were quantified. Solid feed was provided to each calf individually directly after the morning MR meal in period 1 and after each MR meal in period 2. In period 1, solid feed residuals were removed and quantified prior to the morning meal. In period 2, calves were group-housed, and were allowed to consume their solid feed portion for 60 min, after which solid feed refusals were removed and quantified.

Water was available through water nipples. In period 1, water was available ad libitum except for 2 h around MR feeding. In period 2, water was available ad libitum.

Table 6.1 Ingredient and nutrient composition of the experimental milk replacers.

Ingredient (g/kg)	Period 1	Period 2	
		Control	GFG ⁶
Lactose or lactose replacement ¹		250.0 ¹	
Whey	466.1	201.9	
Delactosed whey	149.7	150.0	
Whey protein concentrate	111.3	85.2	
Soy protein concentrate	10.0	62.5	
Soluble wheat protein	64.1	38.6	
Fat			
Lard	52.5	64.6	
Tallow	52.5	64.6	
Coconut oil	49.2	34.9	
Lecithin	5.0	5.2	
Emulsifier	5.0	5.2	
Premix	10.0 ²	10.0 ³	
Calcium formate	8.4	7.5	
Mono ammonium phosphate	-	5.7	
Lysine	10.0	6.7	
Methionine	2.1	3.1	
Threonine	2.0	1.6	
CaCO ₃	2.5	2.5	
Nutrient (g/kg DM ⁴)	Period 1	Period 2	
		Control	GFG ⁶
Dry matter (g/kg)	978	980	973
Crude ash	86	63	63
Crude protein (<i>N</i> × 6.25)	207	180	181
Crude fat	193	205	187
Lactose ^{1,5}	473	504	266
Fe (mg/kg DM)	45.9	12.2	12.9

¹ In period 2, two milk replacers were used. In the GFG milk replacer, lactose was replaced by 100 g/kg glucose, 50 g/kg fructose and 97.8 g/kg glycerol. The amount of glycerol was slightly adjusted to match the gross energy content of the combination of glucose, fructose and glycerol with that of the replaced lactose. Gross energy content was 15.6 kJ/g for lactose, 14.0 kJ/g for glucose, 15.4 kJ/g for fructose and 17.6 kJ/g for glycerol.

² The starter premix provided (per kg of experimental diet): crude protein, 0.7 g; starch, 5.0 g; crude ash, 1.5 g; calcium, 16.6 mg; phosphorus, 7.5 mg; sodium, 0.7 mg; potassium, 7.3 mg; chloride, 13.1 mg; magnesium, 0.5 g; Fe, 44 mg; copper, 8.0 mg; zinc, 110 mg; manganese, 43 mg; selenium, 0.3 mg; iodide, 1.0 mg; vitamin A, 25,013 IU; vitamin D₃, 4,002 IU; vitamin E, 135 mg; vitamin C, 0.3 g; vitamin K₃, 2.1 mg; vitamin B₁, 8.2 mg; vitamin B₂, 10.2 mg; vitamin B₃, 34.9 mg; vitamin B₅, 18.0 mg; vitamin B₆, 10.0 mg; vitamin B₁₂, 0.1 mg; biotin, 0.2 mg; choline, 0.4 g; folic acid, 0.7 mg.

³ The finisher premix provided (per kg of experimental diet): crude protein, 0.8 g; starch, 5.4 g; crude ash, 1.3 g; calcium, 17.0 mg; phosphorus, 8.1 mg; sodium, 0.8 mg; potassium, 7.8 mg; chloride, 13.2 mg; magnesium, 0.5 g; Fe, 0.3 mg; copper, 4.0 mg; zinc, 100 mg; manganese, 43 mg; selenium, 0.3 mg; iodide, 1.0 mg; vitamin A, 25,000 IU; vitamin D₃, 4,000 IU; vitamin E, 90 mg; vitamin C, 0.1 g; vitamin K₃, 2.1 mg; vitamin B₁, 8.2 mg; vitamin B₂, 10.2 mg; vitamin B₃, 34.8 mg; vitamin B₅, 18.0 mg; vitamin B₆, 6.2 mg; vitamin B₁₂, 0.1 mg; biotin, 0.2 mg; choline, 0.4 g; folic acid, 0.7 mg.

⁴ Values are given in g/kg dry matter (DM) unless stated otherwise.

⁵ Calculated content.

⁶ In the GFG milk replacer, glycerol was liquid and added separately when milk was prepared. The milk replacer powder without glycerol was analyzed on nutrient content. Glycerol was assumed to contain 995 g/kg glycerol and 5 g/kg moisture based on product specifications.

Measurements

Throughout the experiment, health and performance of the calves were regularly monitored. Calves were weighed once every four weeks. Rectal temperature was taken in 2-week intervals, and hemoglobin (Hb) concentration in jugular blood was determined in 4-week intervals. A minimum Hb concentration of 5.5 mmol/L was targeted and iron was injected i.m. when necessary. Medical treatment was applied when required based on clinical signs of illness and all treatments were recorded. In week 5, one fecal sample was taken directly from the rectum and analyzed for the presence of pathogens (Rotavirus, Coronavirus, E. Coli K99 and Cryptosporidium) using an immunoassay (BIO K 288 from Bio-X Diagnostics, Rochefort, Belgium). In period 1, measurements were performed to characterize the calves. Targeted challenges related to feeding motivation, digestion, post-absorptive metabolism, behavior, stress and immunology were performed. In addition, measurements were performed at arrival, before the first feeding, as an indicator for the individual conditions upon arrival. These categories are hereafter referred to as physiological platforms.

Feeding motivation. Feeding motivation for MR and concentrates was tested in week 8 and 9, respectively, during a single, ad libitum intake test, which replaced the normal feeding (i.e. 4.6 kg reconstituted MR and 330 g solid feed). For the test with MR, 14 kg of reconstituted MR was provided and unrestricted access was allowed for 10 min. For the test with concentrates, 500 g portions were provided hourly for three hours after the normal MR meal (4.8 kg of reconstituted MR), and calves were stimulated to stand up at each portion of concentrates provided. In addition, the drinking speed of MR was measured in week 8 during a morning and afternoon MR meal as the time required to finish the meal (in sec), divided by its size (4.6 kg of reconstituted MR).

Digestion. Feces were scored on consistency (firm, firm/normal, normal, normal/thin and thin) and color (black, grey, dark brown, brown, light brown, yellow, white) 9 times during period 1 (once or twice per week in week 1-6 and once in week 10). Mean fecal consistency and color score were calculated. In week 5, one fecal sample was taken directly from the rectum and fecal pH (Hanna Instruments, type: HI 9024, Woonsocket, Rhode Island, USA) and DM content were measured. In week 6, retention time of the MR meal (4.2 kg reconstituted MR) was determined by adding chromium chloride (53 mg CrCl₃ hexahydrate per g MR) as an indigestible colored marker (Benvenuti et al., 2014) to the MR meal and scoring first appearance of the green color in the feces by hourly scan-sampling for 28h.

Post-absorptive metabolism. In week 7, after overnight fasting, post-absorptive metabolism was assessed during a MR meal challenge followed by a fasting challenge. MR [506 ± 13 g MR (mean ± SD)] was provided with a movable MR mixer and dispenser and MR supply was calculated by the difference between the weighed amount of MR before feeding and the remaining MR in the dispenser after feeding, divided by the number of calves fed per feeding round. Each individual MR meal was supplemented with 41 mg of ¹³C urea (99 atom%, Sigma Aldrich). Jugular blood samples were collected by venipuncture at -1.6 (basal), 1, 2, 6, 15 and 24h after MR feeding in lithium heparin (for insulin, ¹³C urea and IGF-1) and sodium fluoride tubes (for glucose, L-lactate and NEFA). Plasma was harvested after centrifugation and stored at -20°C pending analysis for glucose (all samples), insulin (-1.6, 1, 2, 6, and 15h), L-lactate (all samples), NEFA (all samples), IGF-1 (-1.6h) and ¹³C urea enrichment (all postprandial time points for 10 randomly selected calves and for other calves at 1, 6 and 15h after feeding). As it can be expected that dilution of plasma ¹³C urea (after urea absorption) is caused by endogenous urea production by the calf, the rate of ¹³C urea dilution can be used as an indicator for urea production (as shown in pigs by Oosterveld et al., 2005). For the meal challenge, calculated variables included the increase in plasma glucose, L-lactate and insulin from basal to peak (i.e. peak was at 1h or 2h postprandial) and the decrease in plasma NEFA from basal to 1h postprandial. For the fasting challenge, variables included the decrease in plasma glucose and the increase in plasma NEFA concentration from 15 to 24h after feeding and the decrease in ¹³C urea enrichment from 6 to 15h after feeding.

Behavior and stress. In week 7, a human approach test was performed during the morning and afternoon feeding as described by Lensink et al. (2003). In short, in phase 1 (i.e. approach phase) one person approached a drinking calf and it was noted whether the calf stopped drinking or not. In phase 2 (i.e. touch phase; 10 min after phase 1), the withdrawal reaction of the calf to the outstretching arm of the approaching person was scored from 1 (no withdrawal) to 4 (strong withdrawal reaction). For both phases, the mean score of each calf was calculated.

In week 7, physical activity of calves was measured using scan sampling. During 3 d, 48 scans were performed per calf, the posture (standing or lying) of the calf was scored during 2.5 h after the morning feeding and during 2.5 h before the afternoon feeding. Activity was expressed as the percentage of the scans that the calf was standing.

In week 8, a restraint test was performed for each calf. After taking a basal blood sample, calves were restrained with a halter, which they had not been in contact with before, and the halter was connected to the feeding fence. After 12 min, a second blood sample was taken and the halter was removed. Blood samples were collected by venipuncture in

lithium heparin tubes, plasma was harvested and stored at -20 °C pending cortisol analysis. The increase in cortisol concentration to restraining was calculated as a proxy for acute stress.

In week 10, the calf's behavior during routine weighing was recorded during two phases. First, the animal caretaker opened the individual pen and the movement behavior of the calf towards the weighing scale was qualitatively assessed using a 5-point score (1: calf is resistant and does not move, the animal caretaker has to push the calf towards the weighing scale and 5: the calf runs towards the weighing scale). Second, the behavior of the calf during fixation on the weighing scale was scored from 1 (calf does not move) to 4 (calf is very restless and moves continuously).

Immunology. In week 9, a lipopolysaccharide (LPS) challenge was performed by injecting LPS (from *Escherichia coli* 055:B5, L6529, Sigma-Aldrich) in the jugular vein at 0.05 µg/kg of BW (Borderas et al., 2008) and rectal temperature and respiratory frequency were recorded before injection and every hour after injection up to 8 h.

In week 11 (in period 2), calves were subjected to a human serum albumin (HuSa) challenge. Calves with fever (rectal temperature > 40 °C) were excluded. HuSa (A8763, Sigma Aldrich) was injected i.m. at 50 µg/kg of BW dissolved in saline solution (at a concentration of 1 mg/mL) and blood samples were collected in lithium heparin tubes by venipuncture before HuSa injection and at 3, 7, 14 and 28 d after HuSa injection. Plasma was harvested after centrifugation and stored at -20°C pending specific immunoglobulin G (IgG) and M (IgM) titer analyses.

Arrival. IgG concentration in plasma of neonatal calves is related to the quantity and quality of colostrum intake (Morin et al., 1997, Hammon and Blum, 1998). A jugular blood sample was collected by venipuncture in lithium heparin tubes to determine hematocrit and the titers of natural antibodies (IgG and IgM). Blood was transferred to non-heparinized capillaries and centrifuged at 9600 × g for 4 min using a microcentrifuge (Heraeus Pico 17, Thermo Scientific) and the percentage of red blood cells was calculated. Plasma was harvested from the remaining blood after centrifugation and stored at -20°C pending IgG and IgM titer analyses. In addition, a second blood sample was taken and analyzed on Hb concentration.

Calf shape. Calf shape was measured twice in period 1 (week 2 and 6). Heart girth and body length were measured in order to calculate the surface area of the body (assuming a cylinder and calculating surface area as $[(2\pi \times r^2) + (\pi \times d \times h)]$, with r = heart girth / 2π , d =

$2 \times r$ and $h =$ body length]. Surface area was divided by body weight to assess calf shape (cm^2 / kg of BW).

Analytical procedures

Milk replacers were sampled and analyzed for DM (ISO, 1999b), nitrogen (NEN, 1984), crude fat (ISO, 1999a) and crude ash (ISO, 2002). Fe was analyzed by atomic absorption spectroscopy. Fecal samples were thawed, homogenized and analyzed for DM according to ISO 6496 (ISO, 1999b). Concentrates were analyzed for nitrogen (ISO, 2005).

Plasma samples from the meal and fasting challenges were analyzed for glucose and L-lactate enzymatically using the Roche/Hitachi Modular P800 automatic analyzer (Roche Diagnostics, Mannheim, Germany). The concentration of NEFA was analyzed enzymatically using a kit (NEFA FS kit, DiaSys, Holzheim, Germany) and absorbance was measured with a spectrophotometer (Selectra ProM, ELITech Clinical Systems). Insulin concentration was analyzed with ELISA (Bovine Insulin ELISA kit, Mercodia, Uppsala, Sweden). For the determination of ^{13}C enrichment in urea, plasma samples were derivatized and analyzed using combustion isotope ratio mass spectrometry (IRMS; Finnigan Delta V advantage, Finnigan MAT, Bremen, Germany) as described by Dai et al. (2010). Cortisol and IGF-1 were analyzed by immunoassay (Siemens Medical Solutions Diagnostics) using the IMMULITE® 2000 analyzer (Siemens).

Plasma samples collected when calves arrived at the experimental facilities were analyzed for natural antibody titers [IgG and IgM, binding keyhole limpet hemocyanin (KLH)] and plasma samples from the HuSa challenge were analyzed for specific antibody titers (IgG and IgM, binding HuSa) by ELISA. In short, 96-well plates were coated with 100 μL per well of 4 $\mu\text{g}/\text{mL}$ of KLH (H8283, Sigma-Aldrich) or HuSA (A8763, Sigma-Aldrich), incubated overnight at 4 °C and washed with tap water containing 0.05% Tween20. Plasma samples were added to the plates in a 4-step serial dilution, starting with dilution 1:40, and plates were incubated for 1.5h at room temperature with 1:20,000 diluted sheep anti-bovine IgG heavy chain [(Sh α BolIgG(Fc)/PO), A10-118P, Bethyl, Montgomery, TX, US] and rabbit anti-bovine IgM [(R α BolIgM(Fc)/PO), A10-100P, Bethyl, Montgomery, TX, US], for IgG and IgM, respectively. After washing, tetramethylbenzidine and 0.05% H_2O_2 were added and the plates were incubated for 10 min at room temperature. Reactions were stopped with 1.25 M H_2SO_4 and extinctions were measured with a Multiscan instrument (Labsystems, Helsinki, Finland) at a wavelength of 450 nm. E_{max} was calculated as the highest mean extinction of a standard positive (pooled) plasma sample, which was added to each microtiter plate. Values of the dilutions that gave an extinction closest to 50% of E_{max} were used and titers were expressed as \log_2 values.

Statistical analyses

The objective was to relate the calves' capacity to cope with physiological and behavioral challenges in early life to growth performance in later life. In our statistical approach we first subjected the calculated variables (see 'Measurements') in early life to principal component analysis (PCA) per physiological platform (i.e. arrival, feeding motivation, digestion, post-absorptive metabolism and behavior and stress) to reduce and scale the data. Thereafter, factor scores were extracted for all calves for all principal components (PC) retained in the PCA of each platform. Finally, the relation between these PC plus their interaction with MR treatment in period 2 and growth performance in period 2 was determined with multiple regression analysis.

In this experiment we anticipated to include at least 120 clinically healthy calves in which the measurements were performed successfully. Therefore, we initially included 180 calves (period 1). The LPS challenge was performed in 32 calves, but because of the unexpected shock resulting in the death in 4 calves, this challenge was not performed for the other calves and the 28 calves remaining after the LPS challenge were excluded from the second part of the experiment (i.e. period 2). In addition, calves with extensive individual medical treatments in period 1 were excluded. As a consequence, 130 calves were included in period 2.

In order to successfully perform PCA on the measurements per physiological platform, missing values of individual calves were replaced with the mean of the other calves. Two calves were considered as missing values for the urea production data, because ^{13}C enrichment in plasma urea was too low at 6h after feeding (i.e. below the mean ^{13}C enrichment in plasma urea of 10 randomly selected calves at 24h postprandial, i.e. 1.071 atom%) to estimate a slope for ^{13}C urea dilution. Two calves were considered as missing values for all variables related to the meal and fasting challenges, as these calves appeared to have drunk their MR in the rumen (as indicated by a low increase in ^{13}C enrichment in plasma urea (< 0.005 atom%), from 1 to 6h postprandial, a postprandial increase in plasma glucose < 1 mmol/L and the absence of a postprandial decrease in plasma NEFA concentration). For fecal DM and pH, sixteen calves were considered as missing values as they tested positive on fecal pathogens. Additional analyses revealed that fecal pathogens were related to fecal DM ($P = 0.021$) and pH ($P = 0.023$). For the feeding motivation test with MR and for drinking speed of MR, eleven calves were considered as missing values, because they used a drinking nipple. A drinking nipple was used when calves showed difficulties with drinking MR directly from the bucket at arrival at the facilities. The mode of feeding MR affected intake during the feeding motivation test ($P = 0.011$) as well as drinking speed ($P < 0.001$). Furthermore, measurements performed within 3 d after individual medical treatment were considered as missing

values (4 calves). Measurements of retention time of MR were not included in the statistical analyses due a combination of factors. In 14 calves, the green color was not observed in the feces. In 20 calves, more than 20% of the MR was refused. Also, the presence of fecal pathogens tended to affect retention time ($P = 0.09$), which ultimately resulted in missing values for 35% of the calves.

Despite restricted feeding, not all calves consumed their solid feed allowance, which resulted in variation in solid feed intake. Solid feed intake ($P < 0.001$) affected ADG in period 2 (GLM procedure). Therefore, ADG in period 2 was adjusted for solid feed intake in period 2, by adding the residuals from the model $\text{ADG in period 2} = \text{solid feed intake in period 2}$ to the mean ADG in period 2. This adjusted ADG is the ADG at equal solid feed intake and, therefore, represents feed efficiency.

All statistical procedures were performed with SAS 9.3. PCA was performed with the principal axis method of the FACTOR procedure and prior communality estimates were set at one. Extracted PC were subjected to varimax rotation and PC with an eigenvalue greater than one were retained for further analysis. In the rotated factor pattern, variables with a loading ≥ 0.40 or ≤ -0.40 were considered to load on that PC. The effects of retained PC, MR treatment and the interaction between retained PC and MR treatment on adjusted ADG in period 2 were analyzed. Variables not included in the PCA (i.e. the specific IgG and IgM response to HuSa injection, IGF-1 concentration and calf shape) were also included in the models. First, the PC and above mentioned variables and the interaction with MR treatment were used in a univariate model. PC or interactions with the MR treatment with a P -value < 0.2 were selected. Secondly, these selected PC and interactions were included in a multivariate model. PC or interactions with a P -value > 0.1 were removed from the selection. Finally, the multivariate model with the greatest adjusted R-square and with all variables or interactions with $P < 0.1$ was selected. The same procedure as described above was used for selecting the model that explains most of the variation in unadjusted ADG in period 2. These models were performed with the MIXED procedure, including a random effect of pen. When an interaction effect was significant, the estimate statement was used to estimate the regression coefficient β and the significance of β . Model residuals were checked visually on homogeneity of variance.

To evaluate if the physiological platforms in period 1 had common underlying mechanisms, a secondary PCA was performed on all the retained PC per platform, IGF-1 concentration and calf shape.

Results are expressed as mean \pm SE, unless stated otherwise. Pearson correlation coefficients were estimated using the CORR procedure.

RESULTS

General

During the last two weeks of period 2, an outbreak of severe diarrhea occurred. These last two weeks were not taken into account and period 2, therefore, lasted 16 instead of 18 weeks. One calf was euthanized at the start of period 2 due to severe body weight loss and poor condition and three calves died in period 2 (two after bloating and one after severe diarrhea). Calves with MR refusals greater than 5% in period 2 ($n = 9$) were excluded from analyses, because these calves were assumed to refuse their MR due to health problems. Therefore, 117 calves were included in statistical analyses. MR intake, solid feed intake and ADG in period 1 averaged 53.6 ± 0.02 kg, 11 ± 0.2 kg and 701 ± 6 g, respectively.

Principal component analysis per physiological platform

Descriptive data of the measurements performed in period 1 are presented in Table 6.2. Loadings of all variables for each PC per platform are presented in Table 6.3. Within each platform, an attempt was made to interpret loading patterns of included variables on each PC, as described in the headings of Table 6.3. For the platform arrival, 2 PC, termed “Hb and hematocrit” and “natural antibodies” were retained, accounting for 90% of the total variance. For the platform feeding motivation, 2 PC, termed “feeding motivation” and “drinking speed” were retained, accounting for 76% of the total variance. For the platform post-absorptive metabolism, 5 PC, termed “postprandial response”, “fasting glucose and NEFA”, “fasting glucose”, “lactate and basal insulin” and “urea production” were retained, accounting for 76% of the total variance. For the platform behavior and stress, 4 PC, termed “reactivity”, “fearfulness”, “stress and activity” and “acute stress” were retained, accounting for 67% of the total variance.

Relation between early life measurements and growth performance in period 2

ADG in period 2 tended to be greater for CON (1292 ± 14 g/d) than for GFG calves (1267 ± 14 g/d; $P = 0.071$). Solid feed intake in period 2 was unaffected by MR treatment ($P = 0.89$). The effects of selected PC and separate variables from period 1 on ADG and adjusted ADG in period 2 are presented in Table 6.4. PC “drinking speed” ($P = 0.06$), PC “natural antibodies” ($P = 0.041$), PC “fearfulness” ($P = 0.030$), IGF-1 concentration ($P = 0.009$) and the specific IgM response to HuSa ($P = 0.044$) affected ADG in period 2. Interactions with MR treatment did not occur and were, therefore, excluded from the final model. Variance explained by the model and by pen (R^2) were 14.3 and 20.9%, respectively. Adjusted variance explained by the model (adjusted R^2) was 12.3%.

Effects of PC “fasting glucose” (interaction, $P = 0.012$), PC “fecal pH” (interaction, $P = 0.068$) and PC “fecal DM” (interaction, $P = 0.039$) on adjusted ADG in period 2 differed between the MR treatments. Variance explained by the model and by pen (R^2) was 7.9 and 30.6%, respectively. Adjusted variance explained by the model (adjusted R^2) was 3.5%. Results of the secondary PCA are presented in Table 6.5. Ten secondary PC (sPC) were retained, accounting for 74% of the total variance. For the first 6 sPC, at least 2 PC from different platforms loaded within a sPC.

Table 6.2 Descriptive data of measurements performed in early life of calves ($n = 117$).

Item	Mean	SD	Coefficient of variation
<u>Arrival</u>			
Hematocrit (%)	33.5	5.80	17.3
Hemoglobin (mmol/L)	6.2	1.09	17.6
IgG titer	3.2	1.38	42.7
IgM titer	4.3	1.53	35.1
<u>Feeding motivation</u>			
Milk replacer ¹ (kg)	10.4	1.16	11.2
Concentrates (kg)	0.32	0.24	74.0
Drinking speed ¹ (s/kg milk replacer)	15.8	7.17	45.3
<u>Digestion</u>			
Fecal dry matter content ¹ (g/kg)	179	26.8	15.0
Fecal pH ¹	7.3	0.32	4.3
Fecal consistency (score ₁₋₅)	3.1	0.36	11.5
Fecal color (score ₁₋₇)	3.8	0.30	7.9
Total tract retention time ¹ (h)	12.2	1.77	14.4
<u>Post-absorptive metabolism¹</u>			
Meal challenge			
Basal glucose (mmol/L)	5.3	0.50	9.4
Δ glucose _{basal-peak} (mmol/L)	2.8	1.37	48.4
Basal L-lactate, mmol/L	0.6	0.25	44.7
Δ L-lactate _{basal-peak} (mmol/L)	0.5	0.31	60.8
Basal insulin (μ g/L)	0.18	0.09	52.2
Δ insulin _{basal-peak} (μ g/L)	7.3	6.48	88.9
Basal NEFA concentration (mmol/L)	0.17	0.08	46.5
Δ NEFA _{basal-1h} (mmol/L)	0.07	0.07	89.0

Fasting challenge			
Δ glucose _{15-24h} (mmol/L)	1.2	0.28	23.3
Fasting glucose _{24h} (mmol/L)	3.8	0.46	12.2
Δ NEFA _{15-24h} (mmol/L)	0.11	0.13	112.5
Fasting NEFA _{24h} (mmol/L)	0.45	0.12	27.6
Δ ¹³ C urea _{6h-15h} (atom%)	0.027	0.009	34.2
Immunology			
IgG titer specific to HuSa ¹ , max. response	5.5	1.53	27.6
IgM titer specific to HuSa ¹ , max. response	1.3	0.93	69.0
Behavior and stress			
Human approach test¹			
Approach phase ² (score ₀₋₁)	0.1		
Touch phase ² (score ₁₋₅)	2.9		
Restraint test¹			
basal cortisol (nmol/L)	2.3	3.19	137.7
Δ cortisol (nmol/L)	2.1	4.63	221.4
Behavior during weighing¹			
Towards weighing scale ³ (score ₁₋₅)	3.7		
On weighing scale ³ (score ₁₋₄)	1.4		
Activity (% of time standing)	12.0	4.28	35.6
Other			
IGF-1 (μ g/L)	146.3	41.71	28.5
Calf shape (cm ² /kg of BW)	137.2	5.0	3.7

¹ For fecal DM content and pH, n = 100. For retention time, n = 76. For the feeding motivation test with milk replacer and for drinking speed, n = 106. For all variables regarding post-absorptive metabolism, n = 115 except for Δ ¹³C urea_{6h-15h}, where n = 113. For the human approach test, restraint test and behavior during weighing, n = 116. For the HuSa challenge, n = 105 for IgG and n = 106 for IgM.

² The human approach test was performed twice and the two scores were averaged per phase. For the approach phase, the number calves with score 0, 0.5 and 1 were 105, 9 and 3, respectively. For the touch phase, 11 calves scored 1; 8 calves scored 1.5; 8 calves scored 2; 16 calves scored 2.5; 25 calves scored 3; 19 calves scored 3.5 and 30 calves scored 4.

³ For the behavior towards the weighing scale, the number of calves with score 1, 2, 3, 4 and 5 were 0, 2, 63, 24 and 28, respectively. For the behavior on the weighing scale, the number of calves with score 1, 2, 3 and 4 were 84, 23, 8 and 2, respectively.

Table 6.3 Loadings per principal component (PC) extracted by principal component analysis performed separately per physiological platform in veal calves. The eigenvalue and percentage of variance explained per PC are provided and loadings > 0.4 and < -0.4 are in bold.

Item	PC1	PC2	PC3	PC4	PC5
<u>Arrival</u>	Hb and hematocrit	Natural antibodies			
Hematocrit (%)	0.99	-0.02			
Hemoglobin (mmol/L)	0.99	-0.04			
IgG titer	0.04	0.90			
IgM titer	-0.09	0.89			
Eigenvalue	1.98	1.61			
Variance explained (%)	49.5	40.2			
<u>Feeding motivation</u>	Feeding motivation	Drinking speed			
Feeding motivation milk replacer (kg)	0.80	-0.01			
Feeding motivation concentrates (kg)	0.80	0			
Drinking speed (s/kg milk replacer)	-0.01	1			
Eigenvalue	1.27	1.00			
Variance explained (%)	42.4	33.3			
<u>Digestion</u>	Fecal pH	Fecal DM			
Fecal dry matter (g/kg)	-0.06	0.95			
Fecal pH	0.61	-0.15			
Fecal consistency (score ₁₋₅)	0.74	0.41			
Fecal color (score ₁₋₇)	0.77	-0.04			
Eigenvalue	1.51	1.10			
Variance explained (%)	37.7	27.4			

<u>Post-absorptive metabolism</u>	Postprandial response	Fasting glucose and NEFA	Fasting glucose	Lactate and basal insulin	Urea production
Basal glucose (mmol/L)	-0.04	-0.14	0.90	0.07	0.04
Δ glucose _{0-peak} (mmol/L)	0.77	-0.29	-0.13	-0.07	-0.11
Basal L-lactate (mmol/L)	0.06	-0.09	0.10	0.87	-0.07
Δ L-lactate _{0-peak} (mmol/L)	0.43	0.01	0.07	-0.75	-0.19
Basal insulin (μ g /L)	0.01	0.30	0.34	0.47	-0.14
Δ insulin _{0-peak} (μ g/L)	0.74	-0.28	0.09	-0.12	-0.27
Basal NEFA (mmol/L)	0.91	0.08	-0.09	-0.02	0.20
Δ NEFA _{0-1h} (mmol/L)	0.88	0.07	-0.08	-0.05	0.27
Δ glucose _{15-24h} (mmol/L)	-0.35	0.21	0.62	0.11	-0.05
Fasting glucose _{24h} (mmol/L)	0.32	-0.56	0.56	0.01	0.12
Δ NEFA _{15-24h} (mmol/L)	-0.34	0.86	0.05	0.03	0.01
Fasting NEFA _{24h} (mmol/L)	0.13	0.91	-0.05	-0.02	-0.10
Δ ¹³ C urea _{6h-15h} (atom%)	0.09	-0.11	0.03	-0.02	0.90
Eigenvalue	3.28	2.23	1.68	1.59	1.10
Variance explained (%)	25.3	17.1	12.9	12.3	8.4
<u>Behavior and stress</u>	Reactivity	Fearfulness	Stress and activity	Acute stress	
Human approach test, approach phase (score ₀₋₁)	0.72	-0.05	0.11	-0.12	
Human approach test, touch phase (score ₁₋₅)	-0.15	0.80	0.10	-0.05	
Restraint test, basal cortisol (nmol/L)	0.00	-0.22	-0.74	0.22	
Restraint test, Δ cortisol (nmol/L)	-0.06	0.03	0.02	0.95	
Behavior towards weighing scale (score ₁₋₅)	0.79	0.12	-0.04	0.06	
Behavior on weighing scale (score ₁₋₄)	0.30	0.70	-0.06	0.09	
Activity (% of time standing)	0.08	-0.16	0.78	0.24	
Eigenvalue	1.27	1.22	1.18	1.03	
Variance explained (%)	18.1	17.4	16.8	14.8	

Table 6.4 Effects of principal components (PC) from period 1 (2-11 weeks of age) and interaction with milk replacer treatment on ADG and adjusted ADG in veal calves in period 2 (11-27 weeks of age) fed a milk replacer containing lactose as the only carbohydrate source (CON; n = 62) or a milk replacer in which 51% of the lactose was replaced by iso-energetic amounts of glucose, fructose and glycerol (GFG; n = 55).

Item	ADG period 2		
	<i>P</i>	β	
		estimate	SE
Treatment	0.071		
Feeding motivation PC2 (drinking speed)	0.060	18.7	9.8
Arrival PC2 (natural antibodies)	0.041	-20.1	9.7
Behavior and stress PC2 (fearfulness)	0.030	-21.2	9.6
IGF-1	0.009	0.62	0.23
IgM specific to HuSa	0.044	21.4	10.5

Item	Adjusted ADG ¹ period 2							
	<i>P</i>		β (CON treatment)			β (GFG treatment)		
	item	interaction ²	estimate	SE	<i>P</i>	estimate	SE	<i>P</i>
Treatment	0.134							
Post-absorptive metabolism PC3 (fasting glucose)	0.742	0.012	-19.2	9.0	0.035	14.8	9.9	0.138
Digestion PC1 (fecal pH)	0.808	0.068	10.9	8.7	0.215	-14.2	10.5	0.178
Digestion PC2 (fecal DM)	0.556	0.039	10.3	8.1	0.207	-18.5	11.1	0.100

¹ Adjusted ADG was calculated as the average daily gain adjusted for solid feed intake (see text for details).

² Interaction between item and MR treatment.

Table 6.5 Loadings per secondary principal component (sPC) extracted by secondary principal component analysis. The eigenvalue and percentage of variance explained per PC are provided and loadings > 0.4 and < -0.4 are in bold.

Item	sPC1	sPC2	sPC3	sPC4	sPC5	sPC6	sPC7	sPC8	sPC9	sPC10
Arrival PC1	0.03	0.69	0.32	-0.27	0.12	-0.25	-0.08	-0.02	0.14	0.07
Arrival PC2	0.01	-0.02	0.03	-0.02	0.02	-0.04	0.87	0.06	-0.03	0.00
Feeding motivation PC1	0.00	-0.04	0.02	-0.02	0.00	0.00	-0.04	-0.12	0.93	-0.01
Feeding motivation PC2	-0.58	0.37	-0.13	-0.01	0.33	0.14	0.08	0.21	0.07	0.31
Digestion PC1	0.28	-0.03	0.35	0.54	0.21	0.22	-0.01	-0.26	-0.15	0.04
Digestion PC2	0.09	-0.04	-0.20	-0.16	0.64	-0.03	-0.31	-0.09	-0.29	-0.01
Post-absorptive metabolism PC1	0.05	0.06	0.03	0.17	-0.12	0.67	-0.24	0.00	0.03	0.28
Post-absorptive metabolism PC2	-0.11	0.12	0.81	0.00	-0.10	-0.03	0.17	-0.03	-0.01	0.01
Post-absorptive metabolism PC3	0.72	0.01	0.05	-0.08	0.18	-0.05	0.07	0.36	-0.13	0.13
Post-absorptive metabolism PC4	-0.07	-0.05	-0.08	0.80	0.02	-0.15	-0.01	0.15	0.02	0.04
Post-absorptive metabolism PC5	0.08	0.78	-0.18	0.09	-0.22	0.10	0.04	0.00	-0.15	-0.19
Behavior and stress PC1	0.10	-0.08	0.04	0.26	0.71	-0.01	0.24	0.07	0.20	-0.08
Behavior and stress PC2	-0.05	-0.10	-0.05	-0.30	0.09	0.76	0.14	0.02	-0.02	-0.15
Behavior and stress PC3	0.13	-0.09	0.00	0.05	-0.05	0.04	0.01	-0.12	-0.02	0.89
Behavior and stress PC4	0.06	-0.32	0.62	0.00	-0.01	-0.01	-0.38	0.22	0.10	-0.05
IGF-1 concentration	0.80	0.17	-0.18	0.07	0.09	0.08	-0.03	-0.11	0.13	0.14
Calf shape	0.03	0.00	0.05	0.09	0.00	0.03	0.04	0.88	-0.11	-0.12
Eigenvalue	1.65	1.41	1.41	1.25	1.21	1.20	1.18	1.14	1.11	1.10
Variance explained, %	9.7	8.3	8.3	7.3	7.1	7.0	7.0	6.7	6.6	6.5

DISCUSSION

Principal component analysis per physiological platform

Calves were characterized using targeted challenges in early life. We subjected data from these measurements in early life to principal component analysis with the dual objective of data reduction and scaling of variables for subsequent multiple regression analysis. It should be noted that PCA is based on associations, and that resulting loading patterns do not necessarily reflect causal relations. However, within each platform, most loading patterns represented well-established relationships and could, therefore, be labeled with a brief description.

Arrival. High positive loadings for the Hb concentration and the hematocrit level measured at arrival (PC1) are in agreement with the positive relation between Hb and hematocrit found in calves (Rice et al., 1967). PC2 had high positive loadings for natural IgG and IgM antibody titers. These PC together indirectly provide information of the background and the first two weeks of life of the calves (i.e. prior to arrival). For instance, Hb and hematocrit values are lower for newborn calves from primiparous than from multiparous cows (Kume and Tanabe, 1993, 1996) and the IgG concentration in plasma of neonatal calves is related to colostrum intake (Hammon and Blum, 1998). However, serum IgG and IgM decrease in calves after birth and endogenous IgG and IgM production starts between 8-16 days of age (Husband et al., 1972), indicating that the IgG and IgM titers determined in our experiment are a combination of residual colostrum antibodies and antibodies endogenously produced by the calf.

Feeding motivation. PC1 had high positive loadings for intake during the ad libitum intake tests with MR and concentrates, indicating a positive association in feeding motivation for both MR and concentrates. This is in agreement with the positive relation between MR refusals and solid feed refusals in later life ($r = 0.37$, $P < 0.001$). The second PC was associated with drinking speed.

Digestion. PC1 had high positive loadings for fecal pH, fecal consistency score and fecal color score and PC2 had high positive loadings for fecal DM content and fecal consistency score. Fecal DM content and pH did not load on the same principal component, indicating that variation in fecal pH was not associated with variation in fecal DM content. A positive relation between fecal DM content and pH was found in calves in which intestinal fermentation was induced by the feeding of starch with the MR (Chapter 2 and 3).

Variation in fecal pH and fecal DM content in the current study was, therefore, probably not related to intestinal fermentation.

Post-absorptive metabolism. PC1 had high positive loadings on the postprandial increase of plasma glucose, insulin and lactate and the postprandial decrease in plasma NEFA. This postprandial response after ingestion of the MR meal is in agreement with the postprandial patterns found in milk-fed calves (Hugi et al., 1997, Vicari et al., 2008). PC2 suggests a negative relation between blood glucose and NEFA concentrations during fasting, which can be attributed to increased release of NEFA from adipose tissue at low insulin/glucagon ratios (as reviewed by Holtenius and Holtenius, 1996), which occurs during fasting when plasma glucose levels drop. PC3 is associated with fasting glucose concentrations, as the decrease in glucose concentration during fasting was highly related to the glucose concentration at $t = 15\text{h}$ ($r = 0.43$, $P < 0.001$). PC5 was primarily associated with the estimation of urea production.

Behavior and stress. The withdrawal reaction of calves to an outstretching arm and the movement score during confinement in a weighing scale were described as fearfulness (PC2) and the basal cortisol concentration and activity level were described as stress and activity (PC3). These two separate PC are in agreement with the suggested model of responsiveness in calves along the two independent axes fearfulness and activity, which combined could represent the coping style of a calf (Van Reenen et al., 2005).

Relation between early life measurements and growth performance in period 2

Our first objective was to explain variation in growth performance of healthy veal calves by variation in physiological responses to specific challenges in early life. Early life characteristics explained 12% of the variation in growth performance in later life. ADG in later life was positively related with early life drinking speed, IGF-1 concentration and specific IgM response to HuSA and negatively related with plasma natural antibodies at arrival and fearfulness in early life. The positive relation between IGF-1 concentration and growth performance in cattle has been reported before (Lund-Larsen et al., 1977). The negative relation between fearfulness and ADG is in agreement with the association found between fear-related behaviors in a social separation test and ADG in beef heifers (Müller and von Keyserlingk, 2006). These characteristics and growth performance are often measured at the same time, but our data indicate that these characteristics also have a predictive value for later life growth performance. We applied a restricted feeding schedule, in order to minimize variation in ADG related to variation in feed intake. However, refusals of solid feed occurred, resulting in variation in solid feed intake

between calves (coefficient of variation of solid feed intake in later life was 22%). Therefore, the relation between growth performance in later life and early life measurements were, at least in part, related to solid feed and total energy intake.

Variation in ADG, adjusted for solid feed refusals represents variation in feed efficiency. Early life characteristics explained only 4% of variation in feed efficiency in later life. Only interactions between early life characteristics and MR treatment affected feed efficiency significantly. Fasting plasma glucose was negatively related with feed efficiency in CON calves and positively, although numerically, related with feed efficiency in GFG calves. Similarly, the relation between feed efficiency and fecal pH and fecal DM differed between CON-fed calves and GFG-fed calves. The negative, although numerical, relation between early life fecal DM and later life feed efficiency in GFG-fed calves could potentially be caused by a positive effect of glycerol on water absorption (as demonstrated in the rat small intestine by Wapnir et al., 1996) in calves characterized with a low fecal DM content. However, this hypothesis requires further study. The characteristics fasting plasma glucose, fecal DM and fecal pH can potentially be used for screening calves in early life on their ability to cope with low-lactose MR.

A substantial part of the variation in feed efficiency (> 95%) remained unexplained. This could indicate that a large part of the variation cannot be predicted by early life characteristics and/or that a large part of the variation is due to factors not taken into account in this study. In our study, utmost care was taken to prevent observations taken from calves during periods of clinical disease. Yet, variation in health inevitably occurred and may have affected some observations in early life or feed efficiency in later life. Individual medical treatments in later life were registered for 23 calves. Days on medical treatment and adjusted ADG in later life were negatively related ($r = -0.19$, $P = 0.040$), indicating that clinical signs of illness or the medical treatment itself affected feed efficiency. The percentage of variance explained in feed efficiency increased from 3.5 to 7.9% when excluding the 23 calves that had received individual medical treatment, indicating that clinical disease contributed to inter-individual variation in feed efficiency.

The impact of variation in health status within our setting of minimized clinical disease is further illustrated by associations between calves housed together in one pen. Gastrointestinal and respiratory disorders often start localized within the stables. Usually, when > 10% of the calves have clinical signs of illness, metaphylactic treatments are applied to the complete batch of calves. This procedure is reflected in the substantially greater use of group antimicrobial treatment than individual antimicrobial treatment in veal systems (Pardon et al., 2012). Calves within close vicinity to an infected calf will be exposed to the pathogen and possibly subsequent disease, whereas (clinical) disease will likely be prevented in calves housed further away. Therefore, calves within a pen affect

each other more than calves between pens. This is also reflected in the large percentage (i.e. 30%) of variation in feed efficiency that was associated with the random pen effect in our study. This indicates the importance of health status in explaining variation in feed efficiency in veal calves.

Secondary principal component analysis

A secondary PCA was performed to evaluate if the physiological platforms in early life had common underlying mechanisms. The first 6 retained sPC had high loadings on PC from different platforms, indicating that these platforms do have common underlying mechanisms. For instance, drinking speed was negatively associated with fasting plasma glucose and IGF-1 concentration (sPC1). A low value for drinking speed (s/kg MR) corresponds to a high drinking speed, indicating that higher fasting glucose concentrations and IGF-1 concentration are associated with a high drinking speed. The positive association between fasting plasma glucose concentrations and IGF-1 concentration in the basal plasma sample is in agreement with data presented by Chelikani et al. (2004), where lowered plasma glucose concentrations coincided with lowered plasma IGF-1 concentrations in response to fasting in dairy cattle.

The associations found by secondary PCA suggest that the responses of calves to challenges related to feeding motivation, digestion, post-absorptive metabolism and behavior cannot be interpreted as separately functioning systems, but rather contribute together to the physiology of the animal.

CONCLUSIONS

Early life characterization of feeding motivation, digestion, post-absorptive metabolism, immunology and behavior in veal calves explained 12% of the variation in growth performance in later life. When growth performance was adjusted to equal solid feed intake, only 4% of the variation in standardized ADG, in fact reflecting variation in feed efficiency, could be explained by early life measurements. This indicates that > 95% of the variation in feed efficiency in later life could not be explained by early life characterization of calves. It is hypothesized that variation in health status explains substantial variation in feed efficiency in veal calves. Significant relations between fasting plasma glucose concentrations, fecal dry matter and fecal pH in early life and feed efficiency in later life depended on MR composition. These measurements are, therefore, potential tools for screening calves in early life on their ability to cope with low-lactose MR.

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Chapter 7

General discussion



INTRODUCTION

Lactose derived from whey is the main carbohydrate in the calf milk replacer (MR). The availability and prices of whey fluctuate strongly, providing a major incentive to (partially) replace lactose in the calf MR. The objective of this thesis was to evaluate the potential of alternative energy sources to replace lactose from calf MR. In the following paragraphs, the chapters of this thesis are discussed with emphasis on evaluation of the different lactose replacers used in this thesis and the capacity of ruminants to deal with large amounts of starch. New data are presented in text boxes. General conclusions are provided at the end of this chapter.

EVALUATION OF LACTOSE REPLACERS

In this thesis, eight (combinations of) lactose replacers were evaluated in three large scale in vivo experiments. In the first in vivo experiment, lactose was step-wise replaced by one of four starch products differing in size and branching (Chapter 2) and thereafter, a fixed inclusion level of the starch products was applied (Chapter 3). In the second in vivo experiment (Chapter 4 and 5), lactose was replaced by glucose, fructose or glycerol. In the third in vivo experiment (Chapter 6), lactose was replaced with a combination of glucose, fructose and glycerol. The percentage of lactose replaced in the studies reported in each chapter is summarized in Figure 7.1.

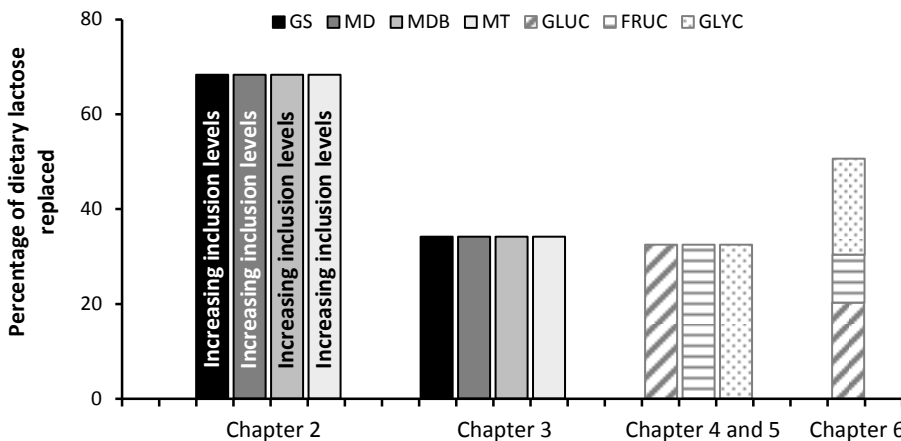


Figure 7.1 Percentage of lactose replaced in the calf milk replacer per chapter described in this thesis. GS, gelatinized starch; MD, maltodextrin MD; MDB, maltodextrin with a high degree of α -1,6-branching; MT, maltose; GLUC, glucose; FRUC, fructose; GLYC, glycerol.

Effects of lactose replacers on growth performance

To evaluate lactose replacers based on their effects on growth performance, experimental differences between studies should be considered. In Chapter 2 and 3, no solid feed was provided to the calves. In Chapter 4, solid feed was supplied at 10 g DM/kg^{0.75} per day, however, during group housing, solid feed was supplied per pen. In Chapter 6, both MR and solid feed were provided according to a feeding scheme used in practice and both MR and solid feed were provided individually. Total gross energy intake was lowest in Chapter 2 and 3 and greatest in Chapter 6. The effects of lactose replacers on growth performance can, therefore, be best evaluated relative to the lactose control treatment within each experiment (Figure 7.2).

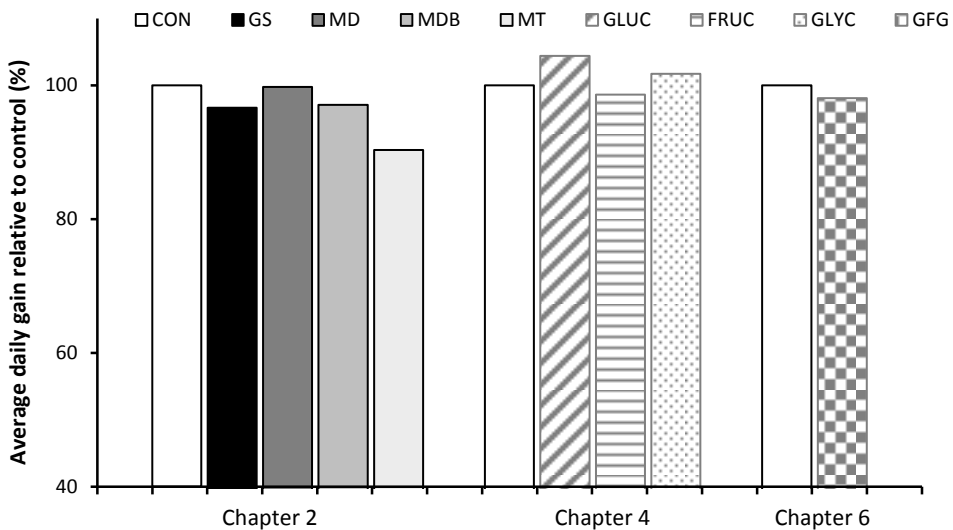


Figure 7.2 Average daily gain relative to control (%) within studies reported in each chapter. CON, milk replacer containing lactose as only carbohydrate source; GS, milk replacer containing increasing amounts of gelatinized starch; MD, milk replacer containing increasing amounts of maltodextrin; MDB, milk replacer containing increasing amounts of maltodextrin with a high degree of α -1,6-branching; MT, milk replacer containing increasing amounts of maltose; GLUC, milk replacer in which 32% of the lactose is replaced with glucose; FRUC, milk replacer in which 32% of the lactose is replaced with fructose; GLYC, milk replacer in which 32% of the lactose is replaced with glycerol; GFG, milk replacer in which 51% of the lactose is replaced with of glucose, fructose and glycerol.

Growth performance of calves fed gelatinized starch, maltose and a combination of glucose, fructose and glycerol was lower compared to control calves. However, no major growth depression was found for any of the lactose replacer treatments, when a minimum growth performance of 95% compared to the control treatment is set as a criterion. The

minor reduction in growth performance when replacing lactose by starch products, contradicts the large amount of starch fermentation observed in Chapter 3 (89% of intake in total tract and 41% of intake in the small intestine). A reduction in growth performance would be expected because the energetic utilization of fermented carbohydrate is ~30% lower than that of carbohydrate hydrolyzed and absorbed as glucose (as reviewed for pigs by Noblet and Le Goff, 2001). However, starch products had a positive effect on crude fat digestion (Chapter 3 and Textbox 7.1). In addition, urinary glucose excretion might have been reduced in starch-fed calves compared to control calves, due to lower postprandial glucose concentrations in peripheral plasma (Textbox 7.1). These positive effects of starch products could have contributed to the relatively low drop in growth performance of milk-fed calves when replacing lactose with starch products. However, this would only compensate ~45% of the expected decrease in growth performance due to fermented starch compared to digested lactose (see Textbox 7.1).

From Figure 7.2 it can be concluded that all lactose replacers can be used without causing a reduction in growth performance greater than 5%. However, in Chapter 2, a titration approach was applied. If a fixed percentage of lactose was replaced by starch products (for instance 34% as in Chapter 3) during the entire measurement period instead of a step-wise replacement, a greater reduction in growth performance can be expected due to the linear relation between starch fermentation and starch inclusion (Chapter 2).

Textbox 7.1 Effects of starch products on fat digestion and postprandial plasma glucose concentrations

Apparent total tract disappearance of crude fat tended to be greater for starch product-fed calves than for control calves. The effect of gelatinized starch on crude fat digestion was further investigated in the digesta samples collected from the small intestine (small intestine segment 1 and small intestine segment 2; Chapter 3) at slaughter from the control and gelatinized starch-fed calves. These samples were analyzed for lipase activity (lipase activity assay kit, BioVision 723-100, California, USA) and for residual fat (without acid hydrolysis; ISO, 1999). The data show that lipase activity was greater for the gelatinized starch-fed calves than for the control calves, both in small intestine 1 (95.7 ± 50.7 and 16.2 ± 10.2 U/mg Co, respectively) and small intestine 2 (53.2 ± 19.8 and 18.9 ± 8.6 U/mg Co, respectively). This resulted in a tendency for a greater apparent disappearance of crude fat in small intestine 1 and 2 for the gelatinized starch-fed calves compared to the control calves (26.3 ± 6.2 and $21.5 \pm 7.6\%$ of intake for small intestine 1 and 97.7 ± 0.3 and $94.7 \pm 0.8\%$ of intake for small intestine 2, respectively; Pluschke et al., submitted to Animal). Increased lipase activity in small intestinal digesta of rats (Schneeman et al., 1982) or in pancreatic juice of pigs (Langlois et al., 1987) has been reported when feeding a wheat bran diet compared to a control diet. In pigs, this was mainly due to an increased secretion of pancreatic juice (Langlois et al., 1987). In goats, a quadratic increase in lipase activity

in pancreatic juice is reported when increasing dietary starch concentration, however, this did not coincide with a significant increase in pancreatic juice secretion (Xu et al., 2009). The mechanisms responsible for the increased luminal lipase activity when feeding starch to milk-fed calves require further study. However, the data of Pluschke et al. show that feeding gelatinized starch increased the luminal activity of lipase, resulting in a greater crude fat digestion. Most likely, this effect was also present in the other starch product treatments, since the apparent total tract crude fat disappearance was numerically greater for all starch product treatments compared to the control treatment (Chapter 3).

The expected increase in growth as a result of a greater apparent total tract disappearance of crude fat can be estimated. Using a daily DM intake of 2700 g DM/d (Table 3.1, with a crude fat content of 182 g/kg DM; Table 2.1) and the apparent total tract crude fat disappearance reported in Table 3.2, the digestible energy intake from crude fat can be calculated. Assuming that 28% of the digestible energy is retained in a 61:39 ratio for fat and protein retention (derived from calves fed only MR at 164 kg of BW; Berends et al., 2012), the energy retained from extra digested crude fat can be calculated. This results in an increase in growth of 6 g/d for starch product-fed calves compared to control calves.

In the experiment described in Chapter 3, a meal challenge was performed at the end of the fattening period to evaluate the postprandial glucose response when replacing lactose with starch products (Figure 7.3). A lower postprandial increase in peripheral plasma glucose concentration was found for calves fed the starch products, due to the large amount of starch product disappearing after fermentation rather than after enzymatic hydrolysis to glucose (Chapter 3). The lower postprandial response in plasma glucose in starch-fed calves indicates that the renal threshold for glucose (8.3 mmol/L; Hostettler-Allen et al., 1994) was not exceeded or at least for a shorter period of time compared to control calves. The daily urinary glucose excretion for the control and starch product-fed calves can be estimated from the integrated AUC for plasma glucose (from Figure 7.3) and assuming 0.035 g urinary glucose per 1 mmol/L \times 6h in plasma glucose (derived from control calves in Table 5.3 and Figure 5.5). Assuming 15.56 kJ/g glucose (Blaxter, 1989) and that 29% of the metabolizable energy is retained in a 61:39 ratio for fat and protein retention (derived from calves fed only MR at 164 kg of BW; Berends et al., 2012), the decrease in growth related to urinary glucose excretion can be estimated. This results in a decrease in growth performance of 2.2 g/d for control calves compared to 1.2 g/d for starch product-fed calves.

Expected growth from feeding 18% starch product or 18% lactose in the MR can be estimated with a similar method. At a daily DM intake of 2700 g/d (with 18% dietary starch product or lactose) and assuming that 27% of the gross energy intake is retained in a 61:39 ratio for fat and protein retention (derived from calves fed only MR at 164 kg of BW; Berends et al., 2012) and assuming that the amount of starch products which is fermented (89% of intake, Chapter 3) is retained with a 30% lower efficiency as digested lactose, a decrease of 15 g/d can be expected when feeding 18% dietary starch compared to lactose to milk-fed calves. The greater crude fat disappearance and lowered urinary glucose excretion of starch product-fed calves compared to

control calves results in an estimated increase in growth performance of 7 g/d, which would compensate 45% of the expected drop in growth performance when replacing lactose from the MR by starch products.

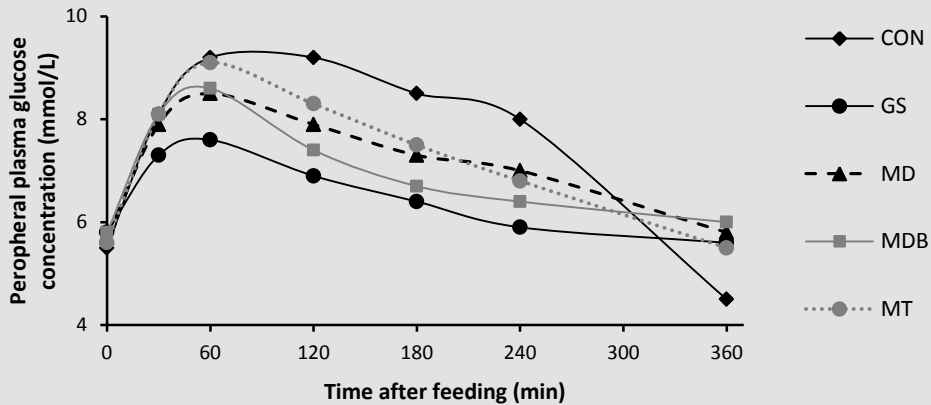


Figure 7.3 The postprandial response in peripheral plasma glucose in calves fed milk replacer containing lactose as only carbohydrate source (CON) or a milk replacer in which 34% of the lactose is replaced by gelatinized starch (GS), maltodextrin (MD), maltodextrin with a high degree of α -1,6-branching (MDB) or maltose (MT).

Small intestinal absorption of lactose replacers

Lactose replacers can be evaluated by their absorption from the small intestine as well. Fructose and glucose absorption were assessed indirectly in two pilot studies, conducted prior to the study described in Chapter 4 and 5. The results of these pilot studies are presented in Textbox 7.2. In one of the two calves in pilot study 1, fructose could be detected in peripheral plasma after feeding MR supplemented with fructose. At 60 min after feeding, peripheral fructose concentration was estimated at 44 $\mu\text{mol/L}$. This is low compared to humans at 60 min after ingestion of fructose, where plasma fructose concentration was 580 $\mu\text{mol/L}$ (after ingestion of fructose at 0.75 g/kg BW; Chong et al., 2007) and 555 $\mu\text{mol/L}$ (after ingestion of 75 g fructose; Tappy et al., 1986). However, a low peripheral fructose concentration does not exclude the possibility that fructose is absorbed from the small intestine, because fructose is rapidly cleared from the portal blood as demonstrated in rat liver (Topping and Mayes, 1971, 1976). The second calf in pilot study 1 developed osmotic diarrhea after feeding MR supplemented with fructose, suggesting that the absorption capacity for fructose was exceeded in this calf. The second pilot study was, therefore, performed to determine the inclusion level of fructose in the milk replacer without causing diarrhea. The incidence in diarrhea increased when increasing the inclusion of fructose and glucose in the MR (Figure 7.5). This indicates a

limit to the absorption capacity of veal calves and this limit is exceeded more quickly for fructose than for glucose.

Textbox 7.2 Absorption of fructose and glucose in calves

Two pilot studies were performed to assess absorption of fructose and glucose in veal calves. The first pilot study was performed to evaluate peripheral plasma fructose and glucose responses to a MR meal. Two male Holstein-Friesian calves (172 and 149 kg of BW) were used on three consecutive days. During morning MR feeding, calves received 60% of their normal MR meal (day 1), 60% of their normal MR meal supplemented with 300 g glucose (day 2) and 60% of their normal MR meal supplemented with 300 g fructose (day 3). Blood samples were collected from catheters inserted into their jugular vein at -15, 15, 30, 60, 120, 180, 240 and 360 min after MR feeding. Plasma samples were analyzed for glucose concentration using an enzymatic method and for fructose peak area using an in-house developed LC-MS/MS method at the University Medical Center Groningen.

Calf 2 only drank 0.5 L of the 4.9 L MR meal on day 1, the catheter was blocked during the first 4 postprandial sampling points on day 2, osmotic diarrhea developed on day 3 and fructose levels in peripheral plasma were below detection limit in this calf. Therefore, complete data were only obtained for calf 1 (presented in Figure 7.4). The peripheral plasma glucose response was clearly increased after glucose supplementation. The peak area for fructose in peripheral blood was greatest when feeding MR supplemented with fructose and fructose concentration at 60 min after feeding was estimated at 44 $\mu\text{mol/L}$.

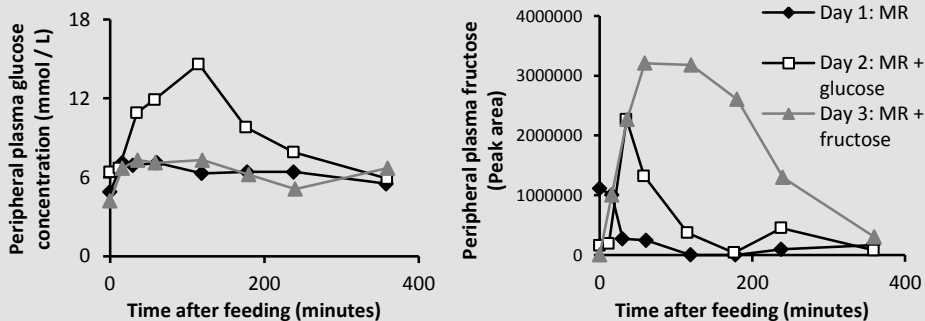


Figure 7.4 Response in peripheral plasma glucose and fructose in one calf fed only milk replacer (MR) or MR supplemented with 300 g of glucose or fructose.

One calf from the first pilot study developed osmotic diarrhea after feeding MR supplemented with fructose. The second pilot study was, therefore, performed to determine the inclusion level of fructose in the milk replacer without causing diarrhea. In addition, a combination of glucose and fructose was evaluated, because, in humans, fructose absorption is increased in the presence of glucose (Rumessen and Gudmand-Høyer, 1986).

Twenty pens with 5 calves (> 12 weeks of age) each were selected at a commercial veal farm and treatments were divided over pens and included fructose, glucose, fructose + glucose and a

control. During five consecutive days, increasing amounts of fructose, glucose or fructose + glucose (50:50 ratio on weight basis) were included in the MR on a weight basis at the expense of MR. In the control treatment, the normal MR was fed. Calves received MR twice a day and solid feed (concentrates and chopped wheat straw) once a day. Inclusion level 5 and 10% were both fed during two feedings and inclusion level 15 and 20% were both fed during 3 feedings. After each feeding, fresh feces in each pen were scored for consistency on a 5-point scale. Feces were categorized as firm, firm/normal, normal, normal/thin and thin.

Over 80% of the feces scored in the control pens was of normal consistency and no thin feces were observed in the control pens. The absorption of fructose was not increased in the presence of free glucose, because the percentage of feces scored as thin was greater at 20% inclusion of glucose + fructose (19% of feces scored as thin) than at 10% inclusion of fructose (0% of the feces scored as thin). The percentage of feces scored as thin increased from 0 to 44% for the fructose treatment and from 0 to 25% for the glucose treatment (Figure 7.5), when increasing the inclusion level from 5 to 20%. It should be noted that the monosaccharides were included at the expense of MR, resulting in a greater total carbohydrate content in the monosaccharide treatments compared to the control treatment.

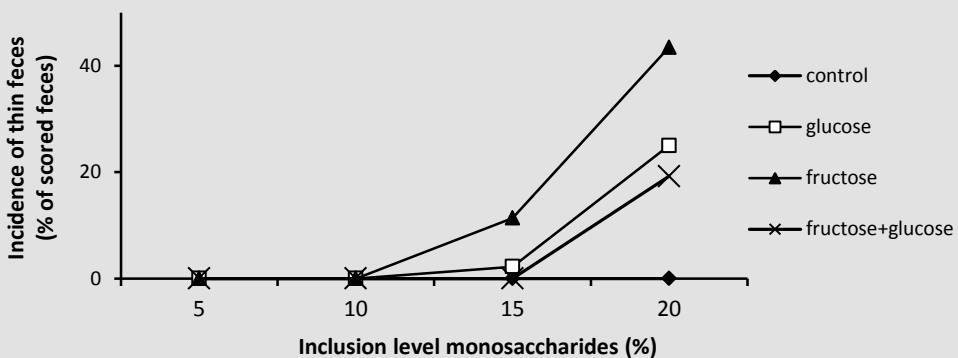


Figure 7.5 The incidence of thin feces in calves fed control milk replacer or milk replacer in which 5, 10, 15 or 20% of the milk replacer is replaced by fructose, glucose or a combination of fructose and glucose.

The absorption of lactose replacers from the small intestine was estimated in Chapter 3 and 4. Incomplete enzymatic hydrolysis and absorption of starch was assessed using ^{13}C enrichment of feces after feeding naturally ^{13}C -enriched corn starch products (Chapter 3) and incomplete absorption of glucose, fructose and glycerol was assessed using ^{13}C enrichment of feces after feeding ^{13}C stable isotopes (Chapter 4). These methods require low naturally ^{13}C enrichment of other dietary sources (Chapter 3) or measurement of background fecal ^{13}C enrichment before isotope feeding (Chapter 4). Incomplete (hydrolysis and) absorption of ^{13}C -enriched lactose replacers results in fecal ^{13}C excretion, either directly from fecal excretion of the ^{13}C -enriched lactose replacers or, more likely,

from ^{13}C -labeled microbial biomass as a result of fermentation of the ^{13}C -enriched lactose replacer.

In Chapter 3, 34% of the lactose was replaced by starch products and estimated enzymatic hydrolysis and absorption from the small intestine was 10% of intake for gelatinized starch, 9% of intake for maltodextrin, 13% of intake for maltodextrin with a high degree of α -1,6-branching and 9% of intake for maltose. Assuming a linear relation between starch product fermentation and inclusion of starch products (Chapter 2), the percentage of lactose which can be replaced while maintaining 90% enzymatic hydrolysis and absorption is estimated (Figure 7.6).

Similar estimates can be derived for glucose, fructose and glycerol. In Chapter 4, 32% of the lactose was replaced with glucose, fructose or glycerol. Recovery of ^{13}C in feces was 7.7% of the oral dose of ^{13}C provided as ^{13}C -labeled fructose. Assuming all the unabsorbed fructose was fermented and assuming 1 g of fecal microbial biomass per 5 g fructose fermented, this corresponds to 38% of the fructose fermented or 62% of the fructose absorbed. For glucose-fed calves, 1% of the ^{13}C -label originating from ^{13}C -labeled glucose was recovered with the feces, indicating a small intestinal absorption of 95%. A lower absorption of fructose than of glucose at equal inclusion level is in agreement with the greater incidence of diarrhea when feeding fructose compared to glucose to veal calves (Figure 7.5). For glycerol-fed calves, 0.5% of the ^{13}C -label originating from ^{13}C -labeled glycerol was recovered with the feces. In bulls infused with glycerol in the rumen for 7 days, glycerol was absorbed directly or fermented (mainly into propionate) in the rumen (Kijora et al., 1998). Assuming the intestinal microbial population of calves in the experiment described in Chapter 4 adapted to glycerol as well, this suggests a small contribution of fermentation to total tract glycerol disappearance. Assuming 1 g of fecal microbial biomass per 5 g glycerol fermented, small intestinal absorption was 97%. Assuming a linear relation between fermentation and inclusion level of glucose, fructose and glycerol, the percentage of lactose which can be replaced while maintaining 90% absorption is estimated (Figure 7.6).

Using 90% absorption from the small intestine as a criterion, lactose can be replaced completely by glycerol, for 62% by glucose and for 59% by a combination of glucose, fructose and glycerol (2:1:2 ratio). Less than 5% of the lactose can be replaced with starch products, when 90% enzymatic hydrolysis and absorption is required.

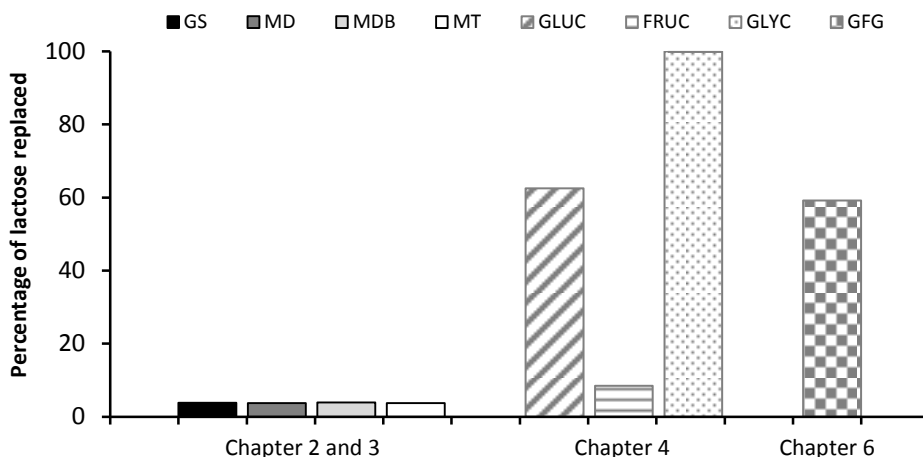


Figure 7.6 Estimated percentage of lactose which can be replaced by gelatinized starch (GS), maltodextrin (MD), maltodextrin with a high degree of α -1,6-branching (MDB), maltose (MT), glucose (GLUC), fructose (FRUC), glycerol (GLYC) or glucose, fructose and glycerol (2:1:2 ratio, GFG) when 90% of the lactose replacers should be (hydrolyzed and) absorbed from the small intestine.

Considerations and implications of lactose replacers

Glucose, glycerol and a combination of glucose, fructose and glycerol (2:1:2) are promising replacers of lactose from the calf MR. The data presented in this thesis can be used for feed evaluation for calves, but also for feed evaluation for dairy cattle regarding starch digestion in the small intestine.

In the studies described in this thesis, pure glycerol was used which is a viscous liquid. This liquid glycerol was added separately to the MR when preparing the reconstituted MR just before feeding. In practice, it would be desirable to include the glycerol in the calf MR powder. However, adding glycerol to the calf MR powder while maintaining a free-flowing powder is challenging and requires further study.

The abolition of the milk quota system in the EU as of April 2015 has resulted in an increase in milk production and a decrease in milk price. This might reduce the urgent requirement to replace lactose from calf MR formulations. However, as soon as non-EU countries increase the import of European dairy products, the demand for lactose replacers in calf MR will increase again. Whether a lactose replacer is used in commercial calf MR depends on the balance between digestibility and efficiency, resource availability and resource prices. With the data presented in this thesis, such trade-offs can be evaluated. Replacing lactose with energy sources of plant origin could have beneficial effects on the carbon footprint, but this requires further (life cycle assessment) study. Furthermore, the proportion of solid feeds in veal calf rations has increased over the last

years and might increase further in the coming years. Solid feeds are cheaper compared to MR and are, therefore, also promising ration ingredients to reduce feed costs for producing veal.

ARE RUMINANTS EQUIPPED TO DEAL WITH LARGE AMOUNTS OF STARCH?

“Digestive efficiency is related to the rumen in animals whose lower tract digestive capacity is limited, particularly for carbohydrates like starch, which the ruminant grazer gut has not seen in 10 million years!” (Van Soest, 1994). This puts current attempts to replace lactose with starch in the MR for veal calves and to increase starch supply to the small intestine in high-producing dairy cows into a different perspective.

Adaptability of starch-degrading enzymes

In Chapter 2, a long and gradual adaptation period was provided in order for calves to adapt to the selected starch products. Despite the length of this adaptation period, starch-degrading enzyme activities were not increased for any of the starch product treatments (Chapter 3). This is in agreement with a lower pancreatic α -amylase activity found after starch hydrolysate infusion in the abomasum of steers compared to infusion of water or casein (Walker and Harmon, 1995, Swanson et al., 2002) and with a lack of an increase in brush-border maltase activity after infusion of starch hydrolysate in the abomasum compared to infusion in the rumen (Bauer et al., 2001). This low capacity to enzymatically hydrolyze starch resulted in only 10% of the starch intake being enzymatically hydrolyzed and absorbed from the small intestine (Chapter 3). Calves, and adult cattle, apparently cannot increase the starch-degrading enzyme activity in response to substrate availability as occurs in pigs and rats (Flores et al., 1988, Brannon, 1990), where adaptive mechanisms to starch supply are highly active.

Glucose absorption

The possibility of glucose absorption being the rate-limiting step in complete starch disappearance from the small intestine cannot be excluded, but is unlikely. The final step to complete hydrolysis occurs in the brush-border and, therefore, little free glucose is expected in the small intestinal lumen after (iso)maltase action. Glucose could originate from α -amylase action directly, although the resulting release of glucose is minor (reviewed by Dona et al., 2010). Indeed, the contribution of free glucose to total glucoside flow at the ileum of steers after abomasal corn starch infusion was only 4% (Kreikemeier and Harmon, 1995).

Total glucose was analyzed in the ileal digesta samples described in Chapter 3. Calves were only fed MR, and total glucose originated from starch products or from free glucose released from lactose. The latter was corrected for by measuring ileal galactose and assuming that glucose originating from lactose equaled galactose quantity. In addition, free glucose concentration was analyzed together with galactose concentration in the ileal digesta samples using HPAEC. These results are summarized in Figure 7.7. Free glucose contributed one third to the total glucose present in the ileal digesta of calves fed starch products with the milk replacer (Figure 7.7). Free glucose concentrations in ileal digesta were not greater for calves fed starch products than for control calves, indicating that glucose absorption is not the (main) limiting factor in complete starch hydrolysis and absorption. The free glucose and galactose present in the ileal digesta can (partially) originate from mucosa sloughed into the ileal digesta when collecting the digesta. The numerically greater concentration of free than total glucose in control calves indicates a small discrepancy between analytical methods.

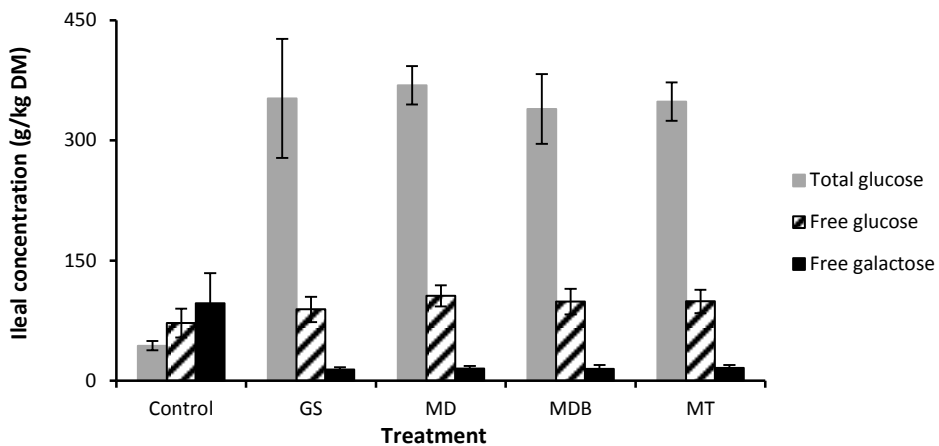


Figure 7.7 The concentration of total glucose (measured using a Total Starch kit of Megazyme), free glucose and galactose (measured by HPAEC) in ileal digesta of calves fed milk replacer containing lactose as only carbohydrate source (Control) or milk replacer in which 34% of the lactose was replaced with gelatinized starch (GS), maltodextrin (MD), maltodextrin with a high degree of α -1,6-branching (MDB) or maltose (MT).

Glucose absorption is likely not the main rate-limiting factor in small intestinal starch disappearance in ruminants either, illustrated by the observation by Kreikemeier and Harmon (1995) that the intestinal disappearance of abomasally infused glucose exceeds that of abomasally infused corn starch (85 vs. 66%). Furthermore, sodium-dependent

glucose transport from the small intestine of ruminants depends on small intestinal substrate. Lambs maintained on milk replacer had greater brush-border glucose transporter activity compared to weaned lambs (Shirazi-Beechey et al., 1991). The same authors reported an increase in brush-border glucose transporter activity when infusing adult sheep in the duodenum with glucose. Infusing starch hydrolysate in the abomasum of steers resulted in a two-fold increase in Na⁺-dependent glucose uptake compared to infusing starch hydrolysate in the rumen (Bauer et al., 2001). Throughout evolution, ruminants have apparently retained the ability to increase Na⁺-dependent glucose uptake in response to the small intestinal presence of substrate. This is in agreement with the results reported in Chapter 4, where glucose absorption from the small intestine of veal calves was estimated at 95% of glucose intake.

Insulin sensitivity

Heavy veal calves often develop problems with glucose homeostasis, characterized by prolonged elevated postprandial glucose and insulin concentrations in jugular blood and high postprandial insulin to glucose ratios (Hugi et al., 1997). This was confirmed by high and broad peaks in the postprandial pattern of plasma glucose and insulin concentrations after MR feeding reported in Chapter 5. However, dietary glucose supplied with the MR is mainly oxidized (74% of intake in Chapter 4 and $\geq 80\%$ of intake reported by Van den Borne et al., 2007). Therefore, absorption of glucose into tissues does occur, but slowly and it requires high insulin concentrations.

Insulin sensitivity was also evaluated in Chapter 5. Replacing 32% of the lactose with glucose, fructose or glycerol in the calf MR did not affect insulin sensitivity. Insulin sensitivity at the start of the experiment (15 weeks of age) was already low. In a study by Stanley (2005), insulin sensitivity dropped from $15.1 \times 10^{-4} \text{ min}^{-1} \text{ per } \mu\text{U/mL}$ at 3 weeks of age to $10.6 \times 10^{-4} \text{ min}^{-1} \text{ per } \mu\text{U/mL}$ at 6 weeks of age in 3 female Holstein calves. This suggests an age-dependent decrease in insulin sensitivity, although age was confounded with diet, because calves were already weaned at 6 weeks of age. However, insulin sensitivity decreased even further to $1.9 \times 10^{-4} \text{ min}^{-1} \text{ per } \mu\text{U/mL}$ as these 3 Holstein heifers got older and came into their first lactation (Stanley, 2005).

An age-dependent decrease in insulin sensitivity in calves is plausible. In ruminants, energy supply depends on the production of volatile fatty acids as a result of microbial degradation of solid feeds in the rumen and generally minor amounts of glucose are absorbed from the small intestine. Ruminants, therefore, depend on gluconeogenesis for their glucose supply. Acetic acid is absorbed by tissues for oxidation or lipogenesis, whereas propionic acid is a precursor for gluconeogenesis in the liver and the kidneys (Van Soest, 1994).

In summary, absorbed glucose is taken up by tissues and largely oxidized, but broad postprandial glucose and insulin peaks, combined with glucosuria, illustrate that glucose homeostasis is pushed to its limits in heavy veal calves. Calves and potentially also other animals with a fully functioning rumen lack the ability to increase starch-degrading enzyme activity in the presence of luminal substrate and show a strong decrease in insulin sensitivity with age independent of diet. This indicates an overall ontogenetic program that is aimed at volatile fatty acid production and an independency of small intestinal starch hydrolysis and glucose absorption. However, the ability to increase SGLT1 activity in response to luminal substrate presence appears to have been retained during ruminant evolution.

CONCLUSIONS

Lactose

Lactase activity is high in the proximal small intestine of milk-fed calves and results in complete apparent ileal disappearance of lactose. Postprandial plasma glucose concentrations are high in heavy veal calves fed a milk replacer with lactose as only carbohydrate source. This results in glucose excretion with the urine, but this urinary glucose excretion is low (i.e. 1%) when expressed to total energy losses in veal calves.

Starch

The activity of starch-degrading enzymes is not increased in response to substrate availability in the small intestinal lumen. In addition, brush-border maltase activity appears to be the rate-limiting enzyme in starch hydrolysis in calves. Intestinal starch fermentation in calves occurs already at low inclusion levels and when one third of the lactose is replaced by starch, 89% of the starch intake is fermented, of which half before the ileum. An increase in crude fat digestion and, possibly, a reduced urinary glucose excretion partially compensate the expected decrease in growth performance when feeding starch instead of lactose to milk-fed calves. However, less than 5% of the lactose can be replaced with starch, without deteriorating digestive performance by more than 10%.

Glucose, fructose and glycerol

Energy retention is not affected when replacing one third of the lactose from the milk replacer with glucose or glycerol. Nonetheless, some of the glucose and glycerol are excreted with the urine by veal calves. Glucose, fructose and glycerol supplied with the MR are largely oxidized (> 70% of intake), with fructose oxidation being slower than that

of glucose and glycerol. Glucose and glycerol are promising lactose replacers and the percentage of lactose that can be replaced without deteriorating digestive performance by more than 10% is estimated at 62% for glucose and at 100% for glycerol. The latter requires further (validation) study. Fructose absorption from the small intestine is incomplete when replacing one third of the lactose from the MR by fructose and less than 10% of the lactose can be replaced by fructose without deteriorating digestive performance by more than 10%. However, a combination of glucose, fructose and glycerol (in a 2:1:2 ratio) can replace half of the lactose from the MR without negatively affecting feed efficiency.

Insulin sensitivity in veal calves was very low at an age of 15 weeks. The further decrease in insulin sensitivity with age or body weight was not affected by the lactose replacers tested.

Early life prediction of feed efficiency and ability to cope with lactose replacers

Early life characterization of feeding motivation, digestion, post-absorptive metabolism, immunology and behavior in veal calves explained 12% of the variation in growth performance in later life. When growth performance was adjusted to equal solid feed intake, only 4% of the variation in standardized ADG, reflecting variation in feed efficiency, could be explained by early life measurements. Significant relations between fasting plasma glucose concentrations, fecal dry matter and fecal pH in early life and feed efficiency in later life depended on MR composition. These measurements are, therefore, potential tools for screening calves in early life on their ability to cope with a MR in which half of the lactose is replaced by glucose, fructose and glycerol (in a 2:1:2 ratio).

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List of abbreviations

^{13}C	carbon with an atomic mass of 13 gram
ADG	average daily gain
AIC	Akaike information criterion
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AOAC	Association of Analytical Communities
Atom%	atom percentage
AUC	area under the curve
BIC	Bayesian information criterion
BW	body weight
$\text{BW}^{0.75}$	metabolic body weight
$\Delta\text{C}_{\text{max}}$	maximum concentration – fasting concentration
C	carbon
Co	cobalt
CON	control
Cr	chromium
DE (Chapter 2 and 3)	dextrose equivalent
DE (Chapter 4)	digestible energy
DM	dry matter
DP	degree of polymerization
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FSIGTT	frequently sampled intravenous glucose tolerance test
FRUC	fructose
GC	gas chromatography
GE	gross energy
GFG	glucose, fructose, glycerol
GLM	general linear model
GLUC	glucose
GLYC	glycerol
GS	gelatinized starch
Hb	hemoglobin
HDL	high-density lipoprotein
HPAEC	high-performance anion exchange chromatography
HPLC	high-performance liquid chromatography
HPSEC	high-performance size exclusion chromatography
HuSa	human serum albumin
IGF-1	insulin-like growth factor 1
IgG	immunoglobulin G

IgM	immunoglobulin M
IS	insulin sensitivity
IRMS	isotope ratio mass spectrometry
ISO	International Organization for Standardization
kg ^{0.75}	metabolic body weight
KLH	keyhole limpet hemocyanin
LDL	low-density lipoprotein
LPS	lipopolysaccharide
MD	maltodextrin
MDB	maltodextrin with a high level of α -1,6-branching
ME	metabolizable energy
ME _m	metabolizable energy requirements for maintenance
MinMod	minimal model
MR	milk replacer(s)
MS	mass spectrometry
MT	maltose
N	nitrogen
NEFA	non-esterified fatty acids
PC	principal component(s)
PCA	principal component analysis
QUICKI	quantitative insulin sensitivity check index
r	Pearson correlation coefficient
R ²	percentage of variance explained
RQ	respiratory quotient
SD	standard deviation
SE	standard error
SEG	segment
SEM	standard error of the mean
SGLT1	Na ⁺ -dependent glucose transporter
SP	starch product(s)
sPC	secondary principal component(s)
SPE-LC/MS/MS	solid phase extraction liquid chromatography - tandem mass spectrometry
T _{max}	time of maximum rate of production
TREAT	dietary treatment
TRT	dietary treatment
U	unit
VFA	volatile fatty acids

Summary

Veal calves are fed milk replacer (MR) and solid feed. The largest part of the energy provided to veal calves originates from the MR. Calf MR contains 40 to 50% lactose, originating from whey, a by-product from cheese production. High and strongly fluctuating dairy prices are a major economic incentive to replace lactose from the calf MR by alternative energy sources. The objective of this thesis was to study the effects of replacing lactose from calf MR on nutrient digestion and fermentation and post-absorptive metabolism.

In Chapter 2 and 3, four starch products (SP) were evaluated for replacing lactose. The four SP differed in size and branching, and consequently required different ratios of starch-degrading enzymes for their complete hydrolysis to glucose. Gelatinized starch required α -amylase and (iso)maltase; maltodextrin required (iso)maltase and α -amylase; maltodextrin with α -1,6-branching required isomaltase, maltase and α -amylase and maltose required maltase. In [Chapter 2](#), adaptation to these SP was assessed during 14 weeks, using a within-animal titration study. Forty male Holstein-Friesian calves ($n = 8$ per treatment) were assigned to either a lactose control MR or one of four titration strategies, each testing the stepwise exchange of lactose for one of the SP. For control calves, fecal dry matter (DM) content and fecal pH did not change over time. The response in fecal DM content and fecal pH in time did not differ between SP treatments and decreased linearly with 0.57% and 0.32 per week, respectively, where one week corresponded to an increase in SP inclusion of 3%. This indicates that the capacity for starch digestion was already exceeded at low inclusion levels, resulting in SP fermentation. All SP required maltase to achieve complete hydrolysis to glucose and it was, therefore, suggested that maltase is the rate-limiting enzyme in starch digestion in milk-fed calves.

Following the titration, a fixed inclusion level of 18% of the SP in the MR was applied. Effects on starch-degrading enzyme activity, nutrient disappearance, SP fermentation and jugular glucose appearance were measured ([Chapter 3](#)). Lactase activity in the brush border was high in the proximal small intestine of all calves, resulting in a high apparent ileal disappearance of lactose ($\geq 99\%$ of intake). Maltase and isomaltase activities in the brush border were not increased for any of the SP treatments. Luminal α -amylase activity was lower in the proximal small intestine but greater in the distal small intestine of SP-fed calves compared to control calves. This amylase activity in the distal small intestine of SP-fed calves might have been of microbial origin. Apparent SP disappearance did not differ between SP treatments. The difference between apparent ileal (62%) and total tract (99%) SP disappearance indicated substantial SP fermentation in the large intestine (37% of intake). In addition, total tract SP fermentation was quantified using fecal ^{13}C excretion

which originated from the naturally ^{13}C -enriched corn SP. Total tract SP fermentation averaged 89% of intake, regardless of SP treatment. MR leaking into the reticulorumen was measured as the recovery of Cr in the reticulorumen at slaughter after feeding MR pulse-dosed with Cr 4h prior to slaughter. MR leaking into the reticulorumen averaged 11% for SP-fed calves. By difference, this leaves 41% of the SP intake fermented in the small intestine. This coincided with increased fecal nitrogen (N) and DM losses for SP-fed calves. However, apparent total tract crude fat disappearance tended to increase when replacing lactose with SP. The substantial SP fermentation indicates that only 10% of the SP intake was enzymatically hydrolyzed and absorbed as glucose. This was in agreement with the marginal increase in ^{13}C enrichment in peripheral plasma glucose after feeding naturally ^{13}C -enriched gelatinized starch and maltose, compared to a clear increase after feeding naturally ^{13}C -enriched lactose to control calves. It was concluded that fermentation, rather than enzymatic digestion, is the main reason for small intestinal starch disappearance in milk-fed calves. The expected decrease in growth performance with such extensive SP fermentation is partially compensated by the greater crude fat digestion and possibly by a reduced urinary glucose excretion when replacing lactose with SP.

Glucose, fructose and glycerol do not require enzymatic hydrolysis and can be absorbed directly from the small intestine. However, these lactose replacers might differentially affect glucose and insulin metabolism and with that energy partitioning. The effects of partly replacing lactose with glucose, fructose or glycerol on energy and N partitioning and glucose homeostasis and insulin sensitivity were, therefore, studied in [Chapter 4 and 5](#). Forty male Holstein-Friesian calves either received a lactose control MR or a MR in which one third of the lactose was replaced with glucose, fructose or glycerol ($n = 10$ per treatment). Energy and N retention were not affected by MR composition. Fructose absorption from the small intestine was incomplete resulting in fructose fermentation. This resulted in fecal losses of DM, energy and N and the lowest numerical energy and N retention for fructose-fed calves. Postprandial plasma concentrations of glucose exceeded the renal threshold for glucose in glucose-fed calves and control calves, which resulted in urinary glucose excretion. Glycerol was likely excreted with the urine of glycerol-fed calves. Oxidation of glucose, fructose and glycerol was quantified by feeding a single dose of $[\text{U-}^{13}\text{C}]\text{glucose}$, $[\text{U-}^{13}\text{C}]\text{fructose}$ or $[\text{U-}^{13}\text{C}]\text{glycerol}$ with the MR and subsequently measuring $^{13}\text{CO}_2$ production. Oxidation of lactose replacers did not differ between lactose replacers and averaged 72% of intake. However, the time at which the maximum rate of oxidation was reached was delayed for fructose-fed compared to glucose-fed and glycerol-fed calves, indicating that fructose was converted into other substrates before being oxidized. Conversion of fructose and glycerol into glucose was confirmed by an

increase in ^{13}C enrichment of peripheral plasma glucose after feeding [$\text{U-}^{13}\text{C}$]fructose and [$\text{U-}^{13}\text{C}$]glycerol, respectively. Insulin sensitivity did not differ between MR treatments, but was already low at the start of the experiment at 15 weeks of age and remained low throughout the experiment. It was concluded that glucose and glycerol can replace one third of the lactose from the calf MR, but that inclusion of fructose should be lower to prevent incomplete absorption from the small intestine.

In literature and the studies in this thesis, high inter-individual variation in growth performance was found in veal calves. The experiment described in [Chapter 6](#) was, therefore, designed to assess the predictability of later life growth performance by characterizing calves in early life. In addition, it was examined whether the ability of calves to cope with MR in which lactose is partially replaced by alternative energy sources can be predicted. From 2 to 11 weeks of age, male Holstein-Friesian calves were fed a lactose control MR and solid feed according to a practical feeding scheme and were characterized individually using targeted challenges related to feeding motivation, digestion, post-absorptive metabolism, immunology, behavior and stress. Based on the results in Chapter 4, a combination of glucose, fructose and glycerol in a 2:1:2 ratio was used to replace half of the lactose from the MR (GFG). From 11 to 27 weeks of age, calves received a lactose control MR or the GFG MR ($n = 65$ per treatment). Growth performance from 11 to 27 weeks of age tended to be lower for GFG-fed than for control calves (-25 g/d). Measurements in early life explained 12% of the variation in growth performance in later life. However, this was mainly related to variation in solid feed refusals. When growth performance was adjusted to equal solid feed intake, only 4% of the variation in standardized growth performance in later life, reflecting feed efficiency, could be explained by early life measurements. This indicates that > 95% of the variation in feed efficiency in later life could not be explained by early life characterization. It is hypothesized that variation in health status explains substantial variation in feed efficiency in veal calves. Significant relations between fasting plasma glucose concentrations, fecal dry matter and fecal pH in early life and feed efficiency in later life depended on MR composition. These measurements are, therefore, potential tools for screening calves in early life on their ability to cope with a MR in which half of the lactose is replaced by glucose, fructose and glycerol (in a 2:1:2 ratio).

The studies reported in this thesis demonstrate that glycerol, glucose and a combination of glucose, fructose and glycerol in a 2:1:2 ratio are promising lactose replacers. The effects of replacing lactose by other carbohydrate or energy sources described in this thesis are required to evaluate the potential of lactose replacers for inclusion in calf milk replacers and provide input for feed evaluation for calves and ruminants.

Acknowledgements

En dan zijn die 4 PhD-jaren ineens voorbij en schrijf je je dankwoord. Ik heb mijn PhD-tijd als uitdagend, leuk en leerzaam ervaren, en ik zou het zonder twijfel nog een keer doen! Het is lastig de juiste woorden te vinden voor iedereen die direct of indirect heeft bijgedragen aan mijn proefschrift, maar ik zal mijn best doen.

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Voor het verkrijgen van de data beschreven in dit boekje zijn drie grote kalverproeven uitgevoerd. Gelukkig heb ik hier veel hulp bij gehad. De eerste en derde proef zijn uitgevoerd bij de faciliteiten van de VanDrie Group in Scherpenzeel. Klaas, Bart, Ewart en Henrico, bedankt dat jullie het hebben volgehouden met mij. Wanneer je dacht dat het niet gekker kon, kwam ik met nóg een lijstje aan. Bedankt voor jullie hulp, inzet en het eindeloos invullen van mijn lijstjes! Tonnie, jij had het voeren van de kalveren in de eerste proef op je genomen. Het voeren van 40 kalveren op mijn manier ging net zo snel als het voeren van 400 kalveren in de afdeling ernaast. Bedankt voor je geduld en nauwkeurigheid! De tweede proef is bij Carus, de dierfaciliteiten van Wageningen Universiteit, uitgevoerd. In theorie waren slechts enkele dierverzorgers betrokken bij het voeren, maar iedere middag stond bijna iedereen van Carus "mijn" kalveren te voeren, zodat iedereen daarna naar huis kon. Ries, André, Rinie, Bert, Ben, Debbie, Ronald, Teus, Willem en Sabine, bedankt voor de goede zorgen! Tamme, Sven, Joswita en Marcel, bedankt voor jullie hulp bij de respiratiecellen en natuurlijk voor de gezelligheid tussen het verzamelen van monsters en het schoonmaken van de cellen door. Verder heb ik het genoeg gehad met studenten samen te werken; Henk-Jan, Rik, Sanne, Nuno, Lonneke en Lydia, bedankt voor jullie hulp en doorzettingsvermogen, ook wanneer ik bedacht had 5.00u 's ochtends te beginnen! Francine, in welk rijtje zal ik je noemen? Toch maar hier, aangezien je in de eerste proef regelmatig bent ingevallen voor het meten van droge stof

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Myrthe

Curriculum vitae

Myrthe Gilbert was born in Dordrecht, The Netherlands, on the 26th of March 1986. She graduated from secondary education (VWO) in 2004 at Insula College, location Thuredrecht, Dordrecht. Thereafter, she studied Animal Sciences at Wageningen University. After obtaining her BSc degree, she continued with her MSc education. Myrthe did a major thesis in both “Animal Health and Behaviour” and “Animal Nutrition”. For the specialization in Animal Health and Behaviour, she studied the behavioural differences between broilers hatched in a hatchery and broilers hatched in the Patio system, in collaboration with Vencomatic B.V. (Eersel, The Netherlands). For the specialization in Animal Nutrition, she studied the effect of diet on oxalate and calcium excretion in the urine of dogs and cats. Her MSc internship was conducted in collaboration with the University of Pretoria. Myrthe evaluated habitat, behaviour and sources of competition for Sable antelopes (*Hippotragus niger*) on Hannah Lodge game farm in South Africa.

In 2011, Myrthe started her PhD at the Animal Nutrition Group and the Laboratory of Food Chemistry of Wageningen University. In her PhD, she focused on the effects of replacing lactose from calf milk replacers by other energy sources on digestion and post-absorptive metabolism. Her PhD was conducted within the framework of the Carbohydrate Competence Center in collaboration with Wageningen University, University Medical Center Groningen, VanDrie Group and Tereos Syral. The results of her PhD project are presented in this thesis. In 2014, Myrthe received a WIAS scholarship, which allowed her to spend 3 months at the Center for Nutrition and Food Sciences at the University of Queensland, Australia and contribute to their ongoing research.

Peer reviewed scientific publications

- M.S. Gilbert, A.J. Pantophlet, H. Berends, A.M. Pluschke, J.J.G.C van den Borne, W.H. Hendriks, H.A. Schols, and W.J.J. Gerrits. 2015. Fermentation in the small intestine contributes substantially to intestinal starch disappearance in calves. *The Journal of Nutrition*; 145: 1147-1155.
- M.S. Gilbert, J.J.G.C van den Borne, H. Berends, A.J. Pantophlet, H.A. Schols, and W.J.J. Gerrits. 2015. A titration approach to identify the capacity for starch digestion in milk-fed calves. *Animal*; 9: 249-257.
- M.S. Gilbert, A.J. Pantophlet, J.J.G.C. van den Borne, W.H. Hendriks, H.A. Schols, and W.J.J. Gerrits. Effects of replacing lactose from milk replacer by glucose, fructose or glycerol on energy partitioning in veal calves. Accepted for publication by *Journal of Dairy Science*.
- M.S. Gilbert, J.J.G.C. van den Borne, C.G. van Reenen, and W.J.J. Gerrits. Predicting variation in growth efficiency in veal calves from a characterization of feeding motivation, digestion, metabolism, behavior and immunology in early life. To be submitted.
- A.M. Pluschke, M.S. Gilbert, B.A. Williams, J.J.G.C. van den Borne, H.A. Schols, and W.J.J. Gerrits. The effect of replacing lactose by starch on protein and fat digestion in milk-fed calves. Submitted to *Animal*.
- A.J. Pantophlet, M.S. Gilbert, J.J.G.C. van den Borne, W.J.J. Gerrits, H. Roelofsen, M.G. Priebe and, R.J. Vonk. Lactose can partly be replaced by glucose, fructose or glycerol without affecting insulin sensitivity in veal calves. Submitted to *Journal of Dairy Science*.
- E. Labussière, H. Berends, M.S. Gilbert, J.J.G.C. van den Borne, and W.J.J. Gerrits. 2014. Estimation of milk leakage into the rumen of milk-fed calves through an indirect and repeatable method. *Animal*; 8: 1643-1652.
- H. Berends, J.J.G.C. van den Borne, N. Stockhofe-Zurwieden, M.S. Gilbert, T. Zandstra, W.F. Pellikaan, C.G. van Reenen, E.A.M. Bokkers, and W.J.J. Gerrits. 2015. Effects of solid feed level and roughage-to-concentrate ratio on ruminal drinking and passage kinetics of milk replacer, concentrates, and roughage in veal calves. *Journal of Dairy Science*; 98:5621-5629

Conference and symposia proceedings

- M.S. Gilbert, A.J. Pantophlet, J.J.G.C. van den Borne, H.A. Schols, and W.J.J. Gerrits. 2013. Small intestinal fermentation contributes substantially to starch disappearance in milk-fed calves. Page 59-60 in Proceedings of the 4th International Symposium on Energy and Protein Metabolism and Nutrition, 9-12 September 2013, Sacramento, California, USA.
- M.S. Gilbert, J.J.G.C. van den Borne, A.J. Pantophlet, and W.J.J. Gerrits. 2013. A titration approach to identify the capacity for starch digestion in milk-fed calves. Page 233-234 in Proceedings of the 4th International Symposium on Energy and Protein Metabolism and Nutrition, 9-12 September 2013, Sacramento, California, USA.
- A.J. Pantophlet, M.S. Gilbert, J.J.G.C. van den Borne, R.J. Vonk, W.J.J. Gerrits, A.M. Pluschke, H.A. Schols, M.G. Priebe, and J. Roelofsen. 2013. Effects of starch-derived substrates on pancreatic and mucosal enzyme activities in milk-fed calves. Page 289-290 in Proceedings of the 4th International Symposium on Energy and Protein Metabolism and Nutrition, 9-12 September 2013, Sacramento, California, USA.
- M.S. Gilbert, A.J. Pantophlet, J.J.G.C. van den Borne, H.A. Schols, and W.J.J. Gerrits. 2014. Small intestinal fermentation contributes substantially to starch disappearance in milk-fed calves. Proceedings of the 39th Animal Nutrition Research Forum, 3 March 2014, 39th Animal Nutrition Research Forum (ANR), Utrecht, The Netherlands.
- M.S. Gilbert, A.J. Pantophlet, J.J.G.C. van den Borne, W.H. Hendriks, H.A. Schols, and W.J.J. Gerrits. 2014. The effect of replacing lactose by glucose, fructose or glycerol on the energy partitioning of milk-fed calves. Page 22 in Proceedings of the WIAS Science Day 30 April 2014.
- M.S. Gilbert, A.J. Pantophlet, J.J.G.C. van den Borne, W.H. Hendriks, H.A. Schols, and W.J.J. Gerrits. 2014. The effect of replacing lactose by glucose, fructose or glycerol in milk replacer on energy partitioning in Holstein-Friesian calves. Page 113 in Proceedings of the 30th Biennial Conference of the Australian Society of Animal Production. Australian Society of Animal Production.
- H. Berends, W.J.J. Gerrits, L.E. Webb, E.A.M. Bokkers, M.S. Gilbert, B. Engel, and C.G. van Reenen. 2014. Variation in dietary preferences, health, and performance in Holstein-Friesian Calves from 0 to 6 months of age provided free diet selection. Page 231 in Proceedings of the 30th Biennial Conference of the Australian Society of Animal Production. Australian Society of Animal Production.

Training and supervision plan¹

Basic package (3 ECTS²)

WIAS Introduction Course	2012
WIAS Course on Philosophy of Science and/or Ethics	2012

International conferences (3 ECTS)

5 th International Veal Congress, Noordwijk, The Netherlands	2011
4 th International Symposium on Energy and Protein Metabolism and Nutrition, Sacramento, USA	2013
Joint ISNH/ISRP International Conference "Harnessing the Ecology and Physiology of Herbivores", Canberra, Australia	2014

Seminars and workshops (4 ECTS)

WIAS Science Day	2012-2015
Animal Nutrition Research Forum (ANR)	2012, 2014
Carbohydrate Competence Center (CCC) symposium	2012-2015
"Healthy Food and living environment" symposium	2013
WIAS Fiber seminar	2014
"Nutrition, Health and Welfare of Calves" symposium	2014
"Nutrient Requirements and Animal Health" symposium	2014

Presentations (10 ECTS)

"Approach for assessing the adaptability for starch digestion of veal calves", WIAS Science Day 2012, Wageningen, The Netherlands, poster presentation	2012
"Fermentation contributes substantially to starch disappearance in milk-fed calves", CCC symposium 2013, Groningen, The Netherlands, oral presentation	2013
"Small intestinal fermentation contributes substantially to starch disappearance in milk-fed calves", WIAS Science Day 2013, Wageningen, The Netherlands, poster presentation	2013
"Small intestinal fermentation contributes substantially to starch disappearance in milk-fed calves", 4 th International symposium on energy and protein metabolism and nutrition, Sacramento, USA, oral presentation	2013

¹ Completed in the fulfillment of the requirements for the education certificate of the Graduate School Wageningen Institute of Animal Sciences (WIAS).

² One ECTS equals a study load of 28 hours.

About the author

“A titration approach to identify the capacity for starch digestion in milk-fed calves”, 4 th International symposium on energy and protein metabolism and nutrition, Sacramento, USA, poster presentation	2013
“Glucose, fructose and glycerol are suitable replacers of lactose in a calf milk replacer”, WIAS Science Day 2014, Wageningen, The Netherlands, oral presentation	2014
“Small intestinal fermentation contributes substantially to starch disappearance in milk-fed calves”, ANR 2014, Utrecht, The Netherlands, oral presentation	2014
“Glucose, fructose and glycerol are suitable replacers of lactose in a calf milk replacer”, CCC symposium 2014, Groningen, The Netherlands, oral presentation	2014
“The effect of replacing lactose by glucose, fructose or glycerol in milk replacer on energy partitioning in Holstein-Friesian calves”, Joint ISNH/ISRP International conference “Harnessing the ecology and Physiology of Herbivores”, Canberra, Australia, poster presentation	2014
“Replacing lactose by processed plant polysaccharides for calf nutrition”, CCC symposium 2015, Groningen, The Netherlands, oral presentation	2015
In-depth studies (7 ECTS)	
Food and Biorefinery Enzymology Course, Graduate School VLAG, Wageningen, The Netherlands	2011
Summer Course Glycosciences, Graduate Schools VLAG and GBB, Groningen, The Netherlands	2012
Advances in Feed Evaluation Science Course, Wageningen Academy, Wageningen, The Netherlands	2013
Indirect Calorimetry and Selected Applications Course, University of California, Davis, USA	2013
In Vitro Cumulative Gas Production Course, University of Queensland, Brisbane, Australia	2014
Animal Immunology and Health discussion group	2015
Professional skills support courses (3 ECTS)	
PhD competence assessment, Wageningen Graduate Schools	2011
Mobilising your - scientific - network, Wageningen Graduate Schools	2011
Workshops Creative Thinking and Social Media, NWO Talent Day	2011
Techniques for Writing and Presenting a Scientific Paper, Wageningen Graduate Schools	2012
PhD career assessment, Wageningen Graduate Schools	2015

Research skills training (8 ECTS)

Preparing own PhD research proposal	2011
External training period, Center for Nutrition and Food Sciences, University of Queensland, Australia	2014

Didactic skills training (11 ECTS)

Supervising farmers project, Inleiding Dierwetenschappen	2011
Guest lecture, Principles of Animal Nutrition	2012
Supervising practicals, Principles of Animal Nutrition	2012
Supervising BSc thesis	2012
Supervising intern	2013
Guiding practical Respiration Chambers, Indirect Calorimetry and Selected Applications Course, University of California, Davis, USA	2013
Supervising MSc thesis (4x)	2013-2014
Supervising practicals, Animal Nutrition and Physiology	2015

Total: 49 ECTS

Colophon

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