# Effects of intensive milk replacer feeding and butyrate on growth performance and intermediary metabolism in calves

Dörte Frieten

Inaugural-Dissertation zur Erlangung des Grades eines
**Dr. med. vet.**
beim Fachbereich Veterinärmedizin
der Justus-Liebig-Universität Gießen

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"The important thing is not to stop questioning."
*– Albert Einstein*

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# Abbreviations

| | |
|---|---|
| ADF | acid detergent fiber |
| ADG | average daily gain |
| Adl | ad libitum fed calves |
| ANOVA | analysis of variance |
| BHB | *β*-hydroxybutyrate |
| B+ | with butyrate supplementation |
| B- | without butyrate supplementation |
| BW | body weight |
| CON | concentrate |
| CP | crude protein |
| d | day(s) |
| DM | dry matter |
| DMI | dry matter intake |
| EDTA | ethylenediaminotetraacetate |
| eGP | endogenous glucose production |
| ELISA | enzyme-linked immunosorbent assay |
| FBPase | fructose-1,6-bisphosphatase |
| FPT | failure of passive transfer |
| G6Pase | glucose-6-phosphatase (protein) |
| G6PC | glucose-6-phosphatase (gene) |
| GCK | glucokinase |
| GfE | Gesellschaft für Ernährungsphysiologie |
| GH | growth hormone |
| GHBP | growth hormone-binding proteins |
| GHIH | growth hormone-inhibiting hormone |
| GHR | growth hormone receptor |
| GHRH | growth hormone-releasing hormone |
| GIT | gastrointestinal tract |
| GNG | gluconeogenesis |
| GP | glycogen phosphorylase (protein) |
| GS | glycogen synthase |
| h | hour(s) |

| | |
|---|---|
| Ig | immunoglobulins |
| IGF | insulin-like growth factor |
| IGFBP | insulin-like growth factor binding protein |
| IGFR | insulin-like growth factor receptor |
| INSR | insulin receptor |
| LSM | least squares means |
| ME | metabolizable energy |
| MR | milk replacer |
| mRNA | messenger ribonucleic acid |
| n | number of samples |
| NDF | neutral detergent fiber |
| NEFA | non-esterified fatty acids |
| NFE | nitrogen-free extract |
| NRC | National Research Council |
| OIE | World Organisation for Animal Health |
| PC | pyruvate carboxylase (gene or protein) |
| PCCA | propionyl-CoA carboxylase α (gene or protein) |
| PCK1 | phosphoenolpyruvate carboxykinase c (cytosolic; gene) |
| PCK2 | phosphoenolpyruvate carboxykinase m (mitochondrial; gene) |
| PCR | polymerase chain reaction |
| PEPCKc | phosphoenolpyruvate carboxykinase c (cytosolic; protein) |
| PEPCKm | phosphoenolpyruvate carboxykinase m (mitochondrial; protein) |
| PFK | phosphofructokinase |
| PK | pyruvate kinase |
| PYGL | glycogen phosphorylase (gene) |
| RIA | radioimmunoassay |
| Res | restricted fed calves |
| SCFA | short-chain fatty acids |
| SD | standard deviation |
| SE | standard error |
| wk | week(s) |

# 1 General Introduction

The responsibility of humans to protect the lives and well-being of animals is a fundamental concern and is proclaimed in the first article of the German Animal Welfare Act. Additionally, products from food-producing animals are one cornerstone of human nutrition and the basis of livelihood to many farmers worldwide. The dairy industry, next to swine and poultry industry, is the biggest animal agriculture sector in Germany.

From aspects of animal welfare, agricultural economics, and consumer acceptance rearing of vital and healthy calves is one of the main objectives of modern agriculture and may improve longevity and lifetime productivity in dairy cattle (Shamay et al., 2005; Drackley, 2008; Soberon et al., 2012).

The World Organisation for Animal Health (OIE) defines animal welfare in the *Terrestrial Animal Health Code* as a state where the animal is healthy, comfortable, well-nourished, safe, able to express natural behavior, and is not suffering from pain, fear, and distress, etc. (OIE, 2017). Three of these six principles might not be accomplished by conventional calf feeding methods. Feeding calves limited amounts of liquid feed (i.e., 4 – 6 L/d) during the first weeks of life results in a lack of expression in natural suckling behavior (Miller-Cushon and DeVries, 2015), followed by hunger (Hammon et al., 2002; De Paula Vieira et al., 2008; Borderas et al., 2009), and stress for the calves. To further improve the development of the calves, especially in artificial rearing systems, feed additives like organic acids (e.g., butyric acid) were studied (Guilloteau et al., 2010a).

The aim of this thesis was to investigate the influence of ad libitum milk and milk replacer (MR) feeding and the supplementation of butyrate on growth performance, metabolism, and maturation of the somatotropic axis in dairy calves. Thus, performance data, blood and liver samples were collected to analyze and examine the treatment effects.

In the first chapter, current scientific knowledge on calf feeding and its impact on growth and development during the preweaning period, as well as aspects of butyrate supplementation, are summarized.

The two following chapters contain the peer-reviewed publications for this doctoral thesis. The first publication intended to reveal the influence of ad libitum MR feeding and butyrate supplementation on growth performance and energy metabolism, and the second publication investigated effects on vital parts of the somatotropic axis.

Finally, the main results from the performed study will be discussed and compared to the literature research. A summary at the end provides a general overview of this thesis.

# 2 Literature Overview

## 2.1 Preweaning calf management

To establish a good and reliable calf management is the key factor for successful calf-rearing. Calf mortality during the rearing period is a fundamental welfare and economical problem for farmers, as the animal was bred and raised, but never reached a productive stage (i.e., fattening performance or lactation).

The incidence risk for perinatal mortality, defined as the duration from birth to 48 h after calving, ranges in dairy herds worldwide between 3 to 9 % (Compton et al., 2017). Furthermore, Compton et al. (2017) evaluated 5 to 11 % to be the postnatal (24 h to weaning) mortality incidence risk for dairy calves. In Germany, perinatal mortality rates (including stillbirth) of 14.4 % (Fröhner and Reiter, 2005) and postnatal rates of approx. 6 % (Sanftleben, 2009; Tautenhahn, 2017) were reported. These national and international rates are high compared to relatively low mortality risks of about 3 to 4 % in other countries (Svensson et al., 2006; Gulliksen et al., 2009) and feasibly lower incidence rates under good calf management (Torsein et al., 2014; Windeyer et al., 2014). Hence, Mee (2013) raised the question "Why do so many calves die on modern dairy farms and what can we do about calf welfare in the future?".

### 2.1.1 Colostrum intake

Perinatal mortality is associated with insufficient hygiene at birth, newborn calf care, and deficient calf nutrition (Fröhner and Reiter, 2005). Later calf care with good colostrum management cannot prevent the calf from diseases if the newborn is exposed to an unhygienic environment (Weaver et al., 2000). The first colostrum intake is the most important event for agammaglobulinemic born calves to absorb maternal immunoglobulins (Ig) and build up a passive immune system (Godden, 2008). Feeding calves colostrum in low amounts, poor quality or not within the first hours of life results in reduction or failure of passive transfer (FPT). In this case, calf immunity through passive immunization is low, while morbidity and mortality rates increase (Donovan et al., 1998; Quigley et al., 2013). Colostrum quality can be measured indirectly with on-farm tools using a colostrometer or Brix refractometer (Weaver et al., 2000; Quigley et al., 2013; Bartier et al., 2015).

Bartier et al. (2015) found high correlations between direct measurement of IgG content and the afore-mentioned on-farm tools.

Besides the passive immunization, colostrum supplies essential nutrients and energy, e.g., four-fold and approx. twofold greater protein and fat content than in whole milk, respectively, and provides further bioactive substances for growth and development to the newborn calf (Blum and Hammon, 2000; Godden, 2008). With respect to the importance of colostrum feeding, high morbidity and mortality rates are further associated with low nutrient intake (Khan et al., 2011). Improved nutrient and energy intake can only be achieved via enhanced supply of liquid feeds for the newborn calves.

### 2.1.2 Feeding practices

For decades in the last century, since animal production became more intensive and economical, rearing calves differed basically from rearing by their dams. Conventional feeding practice relies on early cow-calf separation and restrictive milk or MR feeding. Reduction in milk supply to calves became common practice to ensure early solid feed intake to fasten forestomach development. This feeding method is associated with early calf-weaning and facilitates management and costs of feedstuff. However, the intake and digestion of solid feed in the underdeveloped rumen is insufficient to meet the energy requirements during the first weeks of life (Khan et al., 2016). Moreover, the conventional feeding practice is considered non-natural, as calves, like all mammals, naturally consume milk ad libitum from their mothers. In semi-wild cattle breeds calves suckle about 8 times per day at their dam (Reinhardt and Reinhardt, 1981) and dairy calves have approx. 5 to 7 rewarded visits (with milk intake) in automatic feeders when fed ad libitum (De Paula Vieira et al., 2008; Miller-Cushon et al., 2013). Earlier (Hammon et al., 2002) and recent (Liang et al., 2016) studies declared that newborn dairy calves are able to absorb and digest high amounts of liquid feed in the first week of life. In a study from Maccari et al. (2015) ad libitum fed Holstein calves had mean intakes of 3.8 L of first colostrum. Considering the preweaning feeding period, ad libitum milk feeding studies confirmed that voluntary whole milk intake averages 10 L/d (Appleby, 2001; Jasper and Weary, 2002; Maccari et al., 2015) and voluntary MR intake was reported slightly higher with about 13.5 L/d (Borderas et al., 2009; Miller-Cushon et al., 2013). Hence, self-determined liquid feed intake is much greater than in conventional practices with about 6 L/d. Feeding these amounts is still common practice, whereas several studies indicated malnutrition and hunger are present in limited-fed calves (Khan et al., 2011; Miller-Cushon and DeVries, 2015; Rosenberger et

al., 2017). The failure of satiety could be seen in the high frequency of unrewarded visits to automatic feeders compared to ad libitum fed calves (Hammon et al., 2002; De Paula Vieira et al., 2008; Korst et al., 2017). Furthermore, ongoing studies indicate possible long-term effects. Bar-Peled et al. (1997) investigated an earlier calving age and a tendency for greater milk production, and Soberon et al. (2012) found greater milk yield during the first lactation, when calves had higher preweaning average daily gain (ADG). A meta-analysis published by Soberon and Van Amburgh (2013) acknowledged effects of elevated milk or MR intake and preweaning growth rate on first lactation milk yield. On the basis of previous studies the latter authors calculated a greater milk production of 1,550 kg in the first lactation for every kg of preweaning ADG and the effect was two times more likely to occur when calves fed intensive diets before weaning.

Another aspect of supplying the calves with milk or MR is the feeding method, either manual milk feeding with, e.g., teat buckets, or through automatic feeding systems. Bucket feeding usually takes place twice a day, whereas calves fed limited amounts of milk will rapidly consume their portions (Miller-Cushon and DeVries, 2015). With automatic feeders the meal size and duration of pauses can be set and calves can express a more natural behavior with more self-determined milk intake throughout the day. Jorgensen et al. (2017) also revealed the ability to predict upcoming health problems through data from milk feeders.

To prevent neonatal animals from health maladies, good hygiene in feeding practice is vital. A successful hygiene practice can be achieved when in bucket feeding, buckets and teats are cleaned after every meal, just one marked bucket per calf is used and a new cleaned and disinfected bucket is provided to each newborn calf. In automatic feeding systems, the programmed cleaning routine can be used and additionally regular cleaning of nipple-feeding stations and feeding devices is strongly recommended, but need further analysis (Jorgensen et al., 2017).

Focusing on supply sufficient amounts of digestible feed to young calves includes early offering of solid feed and transition from liquid to solid diets (weaning) later on. The process of weaning marks another important event in calf life, whereby the pseudo-monogastric animal matures to a functional ruminant. During weaning, the increasing solid feed intake increases the development of rumen volume and papilla size, which provide more volume for solid feed intake and absorptive capacity (Baldwin et al., 2004; Khan et al., 2016). As the process involves a complexity of cell differentiation and maturation and a completely new adjustment to another feedstuff, allowing more developmental time for

these critical changes should be provided to the calf (Huber, 2017). Weaning can take place when the ruminating system is mature to digest solid feeds and therefore able to maintain growth and well-being of the calves (OIE, 2017). Different weaning systems were studied (Khan et al., 2007; Eckert et al., 2015; de Passillé and Rushen, 2016) and a better transition than with conventional weaning was seen with step-down (feeding calves at high plane and step-down to medium plane before weaning), concentrate-dependent (weaning starts after calves consume a certain amount of solid feed), and 'late-weaned' systems (e.g., weaned at 8 vs. 6 wk of age). These weaning methods contribute to improved nutrient intake and growth rates, and stress-reduced transition to solid feed for the calves.

## 2.2 Butyrate as a feed additive

Feedstuff supplementation ranges from organic acids, minerals, pre- and probiotics, and enzymes to plant and herb extracts with the intended purposes of providing essential nutrients, accelerated animal health, increased feed intake and growth performance (Wenk, 2000; Jouany and Morgavi, 2007). This applies especially to food producing animals as consumers expect food from farm animals to be non-hazardous to health. Furthermore, the agriculture and environment sectors benefit from intended optimized productivity and nutrient utilization (Wenk, 2000).

Butyrate, widely known as a growth promoter, became an object of research interest when the European Union passed the regulation (Regulation (EC) No 1831/2003) and finally banned the use of antibiotics as promoters of growth in 2006 (Biagi et al., 2007; Jouany and Morgavi, 2007). However, the use of butyrate in MR for calves is rare (Hill et al., 2016) and generally MR have a deficiency in short-chain fatty acids (SCFA; Esselburn et al., 2013; Hill et al., 2016), which are naturally provided to newborn calves when fed colostrum (Contarini et al., 2014) and milk (Harper et al., 1961; Parodi, 1997). The supplementation of fatty acids (e.g., butyrate, medium-chain fatty acids, and linolenic acid) in MR improved the ADG and feed efficiency, and reduced medical treatment in preweaned calves, and effects partly persisted postweaning (Esselburn et al., 2013).

### 2.2.1 Characteristics of butyrate

Butyrate, besides acetate and propionate, is one of the predominant forms of SCFA. It is the end-product of microbial fermentation of dietary fibers, and is naturally present in the forestomach of ruminants and in the colon of monogastrics (Guilloteau et al., 2010a). Furthermore, butyric acid is contained in milk with 3 to 4 % of milk fat (Alais, 1984) and provided by utilization of milk lipids in the gastrointestinal tract (GIT; Guilloteau et al., 2010a).

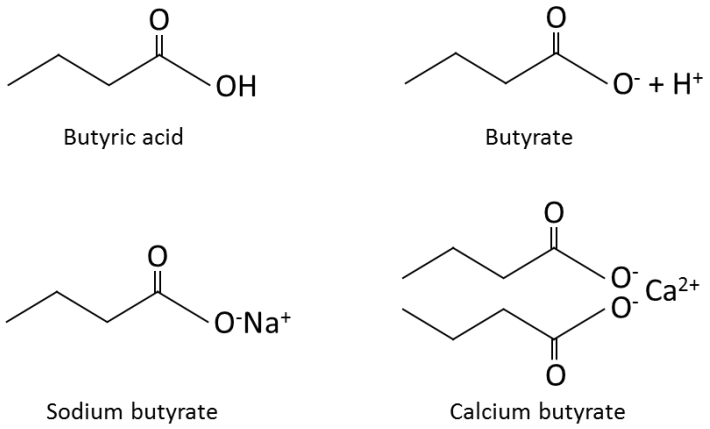


Figure 2.1. Biochemical formula of butyric acid, butyrate, sodium (Na-) butyrate, and calcium (Ca-) butyrate.

In Figure 2.1 the basic substances, and butyric acids sodium (Na) and calcium (Ca) salts can be seen. Butyric acid is associated with a penetrating smell, while salts are less odorous and the complex is more stable (Guilloteau et al., 2009). In our trial we used a mixture of Ca-/Na-butyrate that was supplemented with 0.33 % to the commercial MR product by the MR company. Deducting all minerals and additives the MR, as fed to the calves, contained 0.24 % butyrate on dry matter (DM) basis. The study design was in accordance with works of Guilloteau et al. (2009) and Górka et al. (2011) who also added butyrate salts in comparable concentrations to MR.

### 2.2.2 Biological effects in animals

In general, butyrate affects the postnatal development of the GIT as an energy supplier for the enterocytes and by increasing the epithelial cell proliferation, differentiation, and maturation rates (Guilloteau et al., 2010a). Butyric acid stimulates rumen papilla growth through an increase in mitotic index and compared to other SCFA, the apoptosis rate is much lower (Mentschel, 2001; Khan et al., 2011). In a study from Górka et al. (2014)

similar results of accelerated mitotic index and reduced apoptotic index were found, when sodium butyrate was supplemented to MR or starter feed.

Unlike the direct effects of butyrate as a stimulator of rumen papilla growth and in general, rumen epithelium development, there were no local effects on rumen maturation expected in this study because butyrate, when used as a feed additive in MR, bypasses the rumen and enters the abomasum directly through the esophageal groove. Evidence suggests that added butyrate is metabolized rapidly in the GIT as it was not found in the blood circulation (Guilloteau et al., 2010a).

Supplementation of 0.3 % Na-butyrate in MR enhances growth performance (Górka et al., 2011) and feed efficiency in calves (Guilloteau et al., 2004). The feed efficiency is presumably higher because of improved nutrient digestibility. Guilloteau et al. (2004) found an increase in daily pancreatic juice secretion and trypsin activity, whereas Guilloteau et al. (2010b) revealed in another study enhanced daily production for chymotrypsin and lipase (increased by 52 % and 40 % relative to body weight (BW), respectively). Similar to effects in rumen, butyrate fed in MR partly stimulated the proliferation, differentiation, and maturation in small intestine enterocytes (Górka et al., 2014). Guilloteau et al. (2010a) reviewed a delayed gastric emptying in animals that might contribute to an improved gastric digestion. Additionally, Bach et al. (2013) reported a decreased insulin sensitivity when milk feeding is unphysiologically applied in larger amounts only twice daily. The prolonged gastric phase reduces digesta flow and butyrate could indirectly affect plasma glucose and insulin levels. Oral administration of butyrate suppressed postprandial glucose and insulin concentrations resulting in an improved insulin sensitivity (Kato et al., 2011).

Through structural changes in intestinal tissue, butyrate improves the mucosal defense and immune system in animals (Guilloteau et al., 2009; Ma et al., 2012; Jiang et al., 2015) and humans (Pouillart, 1998; Kovarik et al., 2013). Recent studies revealed the ability of butyrate to promote health in growing animals by improving the GIT defense system (Guilloteau et al., 2009) and reducing the incidence of diarrhea in calves (Górka et al., 2009, 2011) and piglets (Huang et al., 2015). Ma et al. (2012) studied butyrate treatment in porcine epithelial cells *in vitro* and evaluated higher mRNA expression of intestinal tight junction proteins. In addition to the impact of butyrate in the mammalian intestine, Jiang et al. (2015) added butyrate to the diet and found partly improved effects of intestinal inflammatory response after challenging broiler chickens with Escherichia coli lipopolysaccharide.

Butyrate might also be involved in the regulation of the somatotropic axis which will be discussed in Chapter 2.3.3.

Because of the improvements in development and function of the GIT, nutrient digestibility, glucose metabolism, and immune status, this conditions led to an enhanced growth performance when butyrate was used as a feed additive (Górka et al., 2009, 2011). In fact, stimulation of postnatal growth was mainly seen in studies with early postnatal administration of butyrate (Niwińska et al., 2017).

## 2.3 Postnatal maturation of calves

Phenotypical development of neonatal calves consists of skeletal and muscle growth. Maturation can be determined noninvasively by regular weighing, and wither or hip heights measurements of the growing animal (Kertz and Chester-Jones, 2004). Data from blood and liver samples reveal further insights to intermediary metabolism and growth regulation.

### 2.3.1 Development of the gastrointestinal tract

Short- and long-term productivity of farm animals, like calves, depend on energy and nutrient intake, but also on maturation of GIT with increasing digestion and absorptive capacity (Baldwin et al., 2004; Huber, 2017).

Calves are born with an anatomically present, but physiological non-functional four-chambered stomach (reticulum, rumen, omasum, and abomasum; Figure 2.2).

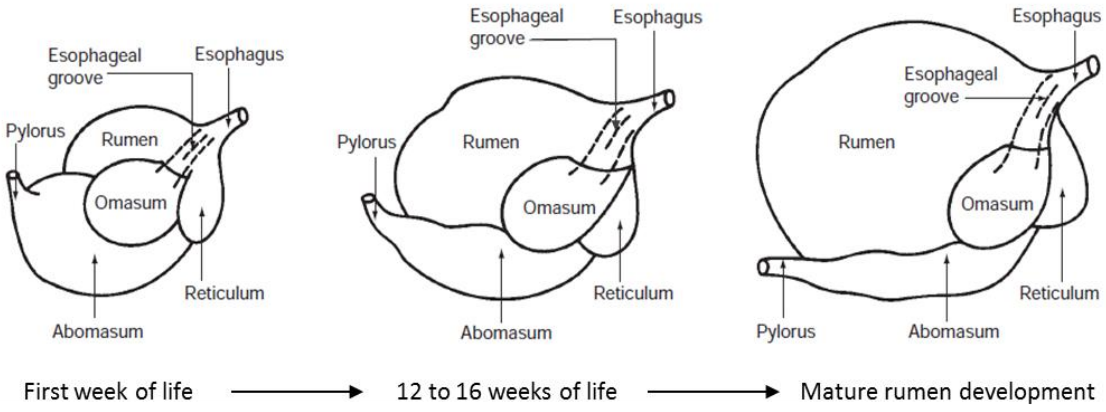


Figure 2.2. The ruminant forestomachs and abomasum in different stages of development. Modified from Heinrichs and Jones (2003).

The postnatal digestive system of ruminants is called pseudo-monogastric, because the only functioning stomach to digest milk is the abomasum, which is functionally comparable to the stomach of monogastric mammals. Milk enters the abomasum by bypassing the proximal forestomach through the esophageal groove.

Young calves digest feedstuff, almost exclusively liquid feed, in the abomasum and small intestine. With age and increasing intake of solid feed, digestion gradually and then completely shifts to digestion of solid feed in the forestomach, primarily in the rumen of the calf. Hence, the rumen must be mature enough to absorb and metabolize nutrients and maintain energy supply for growth (Suarez-Mena et al., 2017). Development of the rumen includes two major aspects: rumen papilla growth and muscular extension. Rumen volume and muscular growth is stimulated by increasing rumen filling (Tamate et al., 1962). Rumen epithelium development is mainly triggered by SCFA (Mentschel et al., 2001), but like other organs, rumen growth profits from accelerated nutrient and energy intake through liquid feed (Khan et al., 2016). As schematically demonstrated in Figure 2.2, the rumen proportion on the four-chambered stomach increases from 30 to 70 % during weaning (Baldwin et al., 2004).

Meale et al. (2017) reviewed earlier adaptation and maturation processes in the calf´s rumen when enzyme activities of ruminal microbiota found in 4 d old calves and overall microbial populations were detected within 20 minutes postnatum.

The development of the rumen (Shen et al., 2004) and small intestine (Blättler et al., 2001) depends on nutrient and energy intake from liquid feed during the first weeks of life, and is stimulated by insulin-like growth factor (IGF)-I (Sparks et al., 2003). Primary stimulation of intestinal development results from colostrum intake (Hammon et al., 2013).

About 30 % of the total energy use and protein synthesis is needed for utilization in the GIT (Baldwin et al., 2004). Greater development of the GIT with enhanced absorptive capacity would improve the glucose uptake and therefore the glucose status of the young calf (Hammon and Blum, 1997a; Steinhoff-Wagner et al., 2011). Providing more energy and nutrients to preweaned calves contribute to their health status and survival rate, as over 50 % of calf mortality is caused by enteric diseases (Lorenz et al., 2011; Hulbert and Moisá, 2016). Hence, an early maturation of the GIT is important to manifest a barrier and prevent calves from pathogen-induced scours.

### 2.3.2 Systemic and hepatic metabolism

Birth represents the environmental transition from intrauterine to extrauterine life, including major metabolic and hormonal adaptations. While the fetus is nourished through continuous transplacental supply, the neonate must adapt to discontinuous nutritional and energy supply under different environmental conditions. Unlike other mammals (e.g., piglets, foals, lambs) and in contrast to suckling calves (Schiessler et al., 2002), artificial calf rearing management further limits the nutritional supply offered to the calf, when feeding restricted liquid diets during the first weeks after birth.

The concentrations of several blood plasma parameters change with birth and during the first weeks of life. $\beta$-Hydroxybutyrate (BHB) correlates with starter intake and is an accepted marker for rumen development (Kristensen et al., 2007). Hence, postnatal BHB levels are low and increase with increasing consumption of solid feed and development of the forestomach. Greater BHB concentrations before significant starter intake indicate hepatic ketogenesis due to an energy deficit (Sallmann and Fuhrmann, 2005). Plasma lactate, an important glucose precursor for preruminants, is high at birth and decreases during the first week of life (Blum and Hammon, 1999). Cortisol, similar to lactate, has elevated levels at birth and decreases thereafter, especially in unlimited-fed calves (Hammon et al., 2002). In metabolic profiles of fat metabolism, triglycerides increase due to high fat absorption from colostrum feeding, while non-esterified fatty acids (NEFA) decreases with sufficient energy supply and remain stable for the following week (Egli and Blum, 1998; Kühne et al., 2000; Hammon et al., 2002).

In general, a calf´s energy supply changes from a fetal, mainly carbohydrate-based diet to a neonatal diet with relatively high fat and protein content in colostrum (Hammon et al., 2000). Therefore, the intermediary metabolism, with the liver as the central metabolic organ (Sallmann and Fuhrmann, 2005), must adapt to the new environment. Adaptation to extrauterine life starts intrauterine and is among other things responsible for maintaining blood glucose levels under higher metabolic rates after birth (Morton and Brodsky, 2016).

Glucocorticoids, especially cortisol, play a vital role in fetal maturation in relation to the upcoming delivery (Hillman et al., 2012). Cortisol concentration increases during late gestation in cows (Breier et al., 2000), and other mammals (Morton and Brodsky, 2016) and account for maturation of hepatic gluconeogenic enzymes. Furthermore, high levels of catecholamines are present at birth in ruminants (Richet et al., 1985) and humans (Morton and Brodsky, 2016) and lead to increased postnatal glucose and free fatty acids levels.

At birth, calves have reduced plasma glucose levels and the intake of lactose via colostrum, and later milk or MR, enhance glucose concentrations but often fail to meet the permanent glucose requirements. Hence, the neonatal calf has to establish an autonomous glucose production, i.e., endogenous glucose production (eGP) by gluconeogenesis (Hammon et al., 2013), since the hepatic glycogen storage is limited to an instant energy supply and decreases after birth (Haga et al., 2008). Glucose is essential to all mammals as the main energy source for the brain and renal medulla cells, and erythrocytes (Nelson and Cox, 2001).

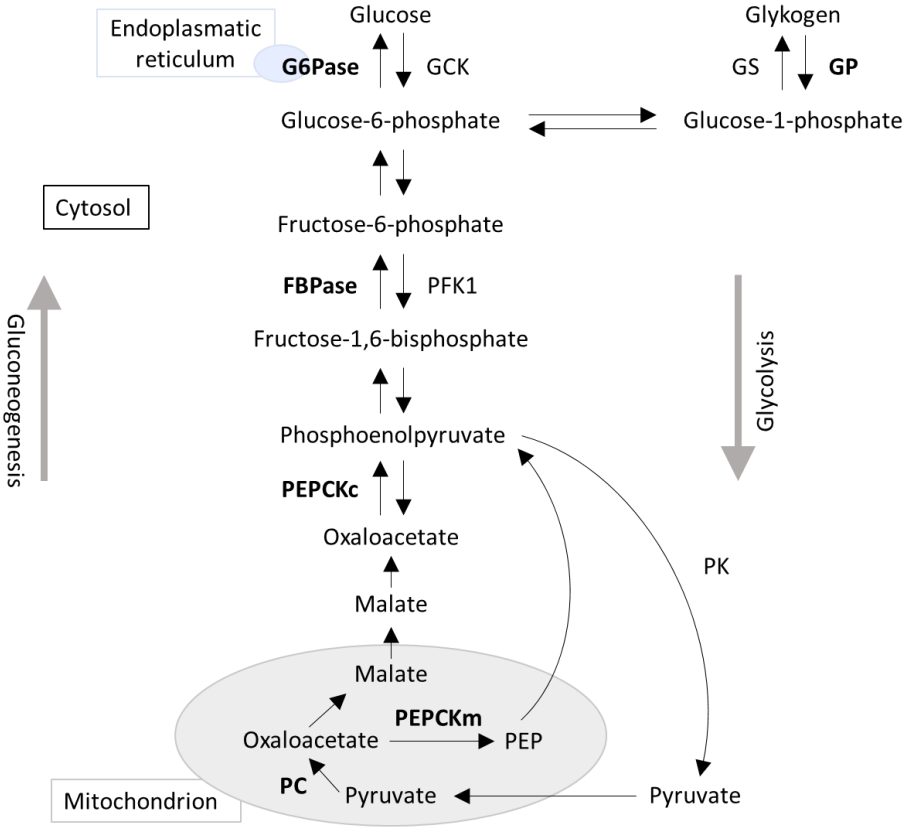


Figure 2.3. Overview of glucose metabolism in the liver. Abbreviations: G6Pase, glucose-6-phosphatase; GCK, glucokinase; GS, glycogen synthase; GP, glycogen phosphorylase; FBPase, fructose-1,6-bisphosphatase; PFK1, phosphofructokinase; PEPCK, phosphoenolpyruvate carboxykinase (c, cytosolic; m, mitochondrial); PC, pyruvate carboxylase; PK, pyruvate kinase. Modified from Jiang and Zhang (2003).

In Figure 2.3 the hepatic glucose metabolism is illustrated. The processes of gluconeogenesis (GNG) and glycogenolysis result in the intermediate glucose-6-phosphate that is hydrolyzed by glucose-6-phosphatase (G6Pase) to glucose (Van Schaftingen and Gerin, 2002). The gene glucose-6-phosphatase (G6PC) encodes the membranous enzyme G6Pase. The pathway of GNG in preruminating calves begins with pyruvate that originates from different precursors (i.e., lactate and glucogenic amino acids; Hammon et al., 2013)

and is converted to oxaloacetate by pyruvate carboxylase (PC) in mitochondria. PC is expressed by the eponymic gene pyruvate carboxylase. Inside the mitochondrion oxaloacetate can be converted by the mitochondrial phosphoenolpyruvate carboxykinase (PEPCKm) to phosphoenolpyruvate (PEP), or after intermediate steps, in the cytosol by PEPCKc (cytosolic). This process depends on the above mentioned precursors. Mitochondrial PEPCK catalyzes PEP mainly from pyruvate coming from lactate, while catalyzing with cytosolic PEPCK dominates when glucogenic amino acids (e.g., alanine) are the precursors of pyruvate (Aschenbach et al., 2010). The genes encoding for PEPCKc and PEPCKm are identically named and abbreviated with PCK1 and PCK2, respectively. After the following steps (summarized in Figure 2.3) G6Pase, which is increased after birth and during malnutrition, catalyzes glucose that can enter the circulation. Therefore, G6Pase contributes to the eGP and is partly responsible for glucose homeostasis (Van Schaftingen and Gerin, 2002; Hammon et al., 2013). The blood glucose level is further regulated by intestinal glucose absorption, whereby colostrum intake enhances absorption rates through increasing small intestinal surface and tended to enhance lactase activities (Steinhoff-Wagner et al., 2014). In the work from Steinhoff-Wagner et al. (2011) no treatment effects on eGP were seen when calves fed milk-formula instead of colostrum, but mRNA abundance and enzyme activity of gluconeogenic enzyme, i.e., PC, were higher in formula-fed calves.

With development of the functional rumen, the favored gluconeogenic substrate shifts from lactate to propionate in ruminating calves (Donkin and Armentano, 1995). Propionate originates from microbial fermentation in the rumen. Propionyl-CoA carboxylase α (PCCA) carboxylates the pathway from propionyl-CoA to methylmalonyl-CoA and conversion leads to succinyl-CoA. Succinyl-CoA enters the citric acid cycle or is converted to pyruvate and hence, a substrate for GNG (Nelson and Cox, 2001).

Intensive milk feeding results in greater plasma glucose and insulin concentrations compared to limited-fed calves (Schäff et al., 2016). A higher insulin:glucagon ratio is characteristic for anabolic metabolism (Hammon et al., 2012). Glucagon as the antagonist of insulin contributes to glucose homeostasis. During states of low blood glucose, glucagon promotes glycogenolysis and GNG in the liver (Jiang and Zhang, 2003).

Previously high glucose and insulin levels through intensive milk feeding did not lead to differences in pancreatic insulin response compared to limited-fed calves when tested via intravenous glucose tolerance test after weaning. Hence, an impaired glucose homeostasis was not seen in weaned dairy calves (Maccari et al., 2015).

### 2.3.3 Regulation of body growth

Postnatal growth is mainly regulated by the somatotropic axis. Parameters of the somatotropic axis can be measured postnatum (Cordano et al., 2000; Ontsouka et al., 2004), but subsequently significant changes occur (Hammon et al., 2012). Thus, Blum and Hammon (2000) stated the principal function, but questioned its maturation in neonates. The somatotropic axis is a neuroendocrine system which is affected by the supply of nutritional (Savage, 2013) and non-nutritional components (Cordano et al., 2000; Hammon et al., 2012). Colostrum compared to transition or mature milk contains the highest amount of bioactive components, such as growth factors, insulin, and glucagon (Blum, 2006). Interestingly and similar to colostral insulin, there is no evidence of significant absorption of bioactive factors (Hammon et al., 2013), but recent studies in neonatal calves indicated a significant absorption of colostral adiponectin, an adipocytokine with an insulin-sensitizing effect (Kesser et al., 2015).

The understanding of the scope and function of the somatotropic axis changed during the years from a more or less linear endocrine system (original somatomedin hypothesis, 1950s) to a complex system (somatomedin hypothesis, 2000) with endocrine, paracrine, and autocrine effects of different parameters synthesized in several tissues (Le Roith et al., 2001). The current understanding of the somatotropic axis is shown in a simplified scheme in Figure 2.4. Parallel research and increasing knowledge led to a variable terminology for parameters of the somatotropic axis. For simplicity the first mentioned terms will be used further on, while synonyms are shown in parentheses.

Signaling starts in the hypothalamus with releasing of growth hormone (GH)-releasing hormone (GHRH; *GH releasing factor*) or GH-inhibiting hormone (GHIH; *somatostatin*) to stimulate or suppress the episodic release of GH (*somatotropin*) from the anterior pituitary gland, respectively. In blood, GH binds to GH-binding proteins (GHBP) or circulates unattached (Veldhuis et al., 1993) to target tissues and promotes effects through binding to a hormone-receptor complex with two GH receptors (GHR; Clark, 1997; Breier et al., 2000). The pathway matures when the low pre- and perinatal abundance of GHR mRNA increases with age (Breier et al., 1988; Sauter et al., 2003). In response to GH binding at the liver-specific GHR1A, secretion of insulin-like growth factor–I (IGF-I; *somatomedin C*) increases. The IGF ligands are carried via IGF binding proteins (IGFBP) to the specific target tissue and bind to IGF receptors (IGFR) with distinct affinities (McGuire et al., 1992). Likewise, to the complex of GH-GHBP the half-life of IGF is prolonged when bound to IGFBP (Duan, 2002).

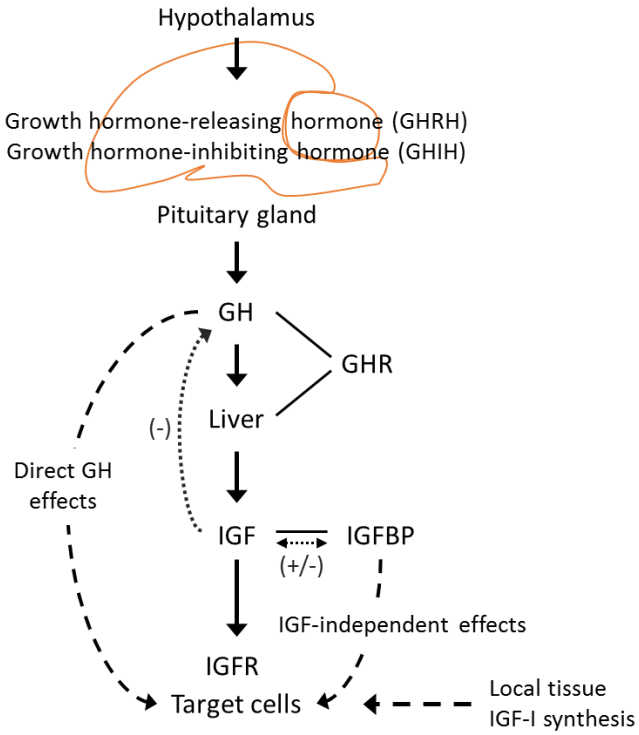


Figure 2.4. Simplified scheme of the somatotropic axis. Solid arrows illustrate secretion of GH and IGF. Direct effects of GH, IGF-I, and IGFBP on target cells are shown by dashed arrows, and dotted arrows indicate regulatory effects of IGF-I and IGFBP. Abbreviations: GH (growth hormone), GHR (growth hormone receptor), IGF (insulin-like growth factor), IGFBP (IGF binding proteins), IGFR (IGF receptor). Modified from Akers (2006).

These endocrine actions of the somatotropic axis are completed by paracrine and autocrine effects (Breier et al., 2000). IGF-I is regulated by GH through the stimulation of synthesis and release from the liver (Le Roith et al., 2001), but later on the local synthesis and GH-independent effects of IGF-I were evaluated. Furthermore, GH itself has direct effects on tissues but is mediated via locally synthesized IGF-I (Breier et al., 2000). Suppressing GH release is also initiated by SCFA which forms another feedback loop as GH enhances lipolysis (Le Roith et al., 2001).

A delay in postnatal response is reported by Hammon et al. (2003) where plasma IGF-I and IGFBP-3 concentrations increase after 2 wk of life. As IGF-I is mainly produced in the liver, a greater plasma IGF-I concentration results from an enhanced hepatic IGF-I expression (Jones and Clemmons, 1995; Cordano et al., 2000). IGF-II seems to regulate mainly prenatal growth where it is highly expressed and decreases after birth (Breier et al., 2000).

Colostrum intake soon after birth stimulates plasma IGF-I concentrations (Hammon and Blum, 1997b; Hammon et al., 2000), while plasma and hepatic levels of IGFBP-1 and IGFBP-2 were reduced after colostrum feeding (Sauter et al., 2003). Further maturation of

the somatotropic axis is supposed to be enhanced by glucocorticoids (Hammon et al., 2003), whereby decreased GH levels, but increased expression and function of the GHR after postnatal glucocorticoid treatment indicate elevated maturation of the somatotropic axis (Sauter et al., 2003). In fact, the frequently-used term 'maturation of the somatotropic axis' refers to the afore-mentioned changes, especially of GH and IGF with conversely high levels of GH perinatal and IGF-I postnatal, and the decline of high fetal IGF-II plasma concentrations after birth (Breier et al., 2000).

*In vitro* studies (Brameld et al., 1999) tested the effect of glucose deficiency through direct removal of glucose in pig hepatocytes resulting in depressed expression of GHR. The physiological state of the somatotropic axis in well-nourished animals indicate high levels of IGF-I, whereas GH concentration decreases through negative feedback of circulating IGF-I (Le Roith et al., 2001). States of negative energy balance in adult cattle (Butler et al., 2003; Gross et al., 2011) result in the uncoupling of the GH-IGF axis with low IGF-I concentrations, while GH levels are high.

In growing animals, plasma IGF-I and IGFBP-3, and hepatic GHR and IGF-I gene expressions are enhanced, while IGFBP-1 and -2 had lower plasma levels (Cordano et al., 2000; Hammon et al., 2003; Sauter et al., 2003). During times where the organism is exposed to mal- or undernutrition IGF-I and IGFBP-3 levels are decreased while concentrations of IGFBP-1 and IGFBP-2 increase (Clark, 1997). Therefore, Clark (1997) presumed nutritional stress due to the lack of nutrient supply to be a responsible factor, as stress stimulates glucocorticoid synthesis leading to GH resistance with decreased IGF-I and IGFBP-3 concentrations. In fasted rats the IGF-I mRNA expression was reduced in many tissues, except from the brain and heart. This indicates that the most vital organs are protected during catabolic metabolism (Breier et al., 2000). Higher nutrient supply resulted in elevated IGF-I levels in ad libitum fed calves (Maccari et al., 2015; Schäff et al., 2016).

Besides the growth promoting role, Clark (1997) revealed the positive effect of the somatotropic axis on the development and function of the GIT and presumed a protective role of somatogenic hormones in states of extreme stress and immunodepression.

Furthermore, the supplementation of butyrate was reported to affect parameters of the somatotropic axis. In groups without butyrate supplement GHIH peaked postprandial, but not in Na-butyrate fed calves (Guilloteau et al., 2009) and in the same study increased expression of IGF-I receptors in the jejunum were evaluated. Tsubaki et al. (2001) reported *in vitro* effects of Na-butyrate with stimulated expression of IGFBP in human mammary cells.

## 2.4 Objectives

For this doctoral thesis, a trial with 64 dairy calves fed liquid feed for the first 8 wk either ad libitum or restrictively, with or without butyrate supplementation, was conducted. Calves were examined immediately from birth until the relatively long-lasting intensive feeding period including a two wk step-down phase, and for one wk thereafter. Comprehensive care and data collection revealed a general view of postnatal development and intermediary metabolism of the experimental calves.

The aim of the study was to test the following hypothesis:

1. Ad libitum feeding of liquid diets to calves enhances their growth performance, anabolic metabolism, and maturation of the somatotropic axis
2. Butyrate supplementation in MR improves postnatal development and feed efficiency in calves
3. The combination of ad libitum feeding and butyrate supplementation accelerates calf development and energy metabolism in a synergistic manner

How ad libitum MR feeding and butyrate supplementation affected the calves will be revealed in the first and second publication (chapter 3 and 4, respectively). The overall context of the research and literature findings will be discussed in chapter 5.

# 3 Publication 1

## Ad libitum milk replacer feeding, but not butyrate supplementation, affects growth performance as well as metabolic and endocrine traits in Holstein calves

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## 3.1 Abstract

The enhanced growth performance of calves fed a higher plane of nutrition pre-weaning is well documented, and the effect of butyrate on the development of the gastrointestinal tract in calves has been evaluated. The aim of this study was to examine the synergistic effects of ad libitum milk replacer (MR) feeding and butyrate supplementation on growth performance and energy metabolism in calves. Sixty-four (32 male, 32 female) Holstein calves were examined from birth until wk 11 of life. Calves received MR either ad libitum (Adl) or restrictively (Res) with (AdlB+, ResB+) or without (AdlB-, ResB-) 0.24 % butyrate supplementation. Colostrum and transition milk were fed in predefined amounts (Res or Adl) for the first 3 d postpartum. Ad libitum and restrictive MR feeding with or without butyrate was performed from d 4 until wk 8 of age. From wk 9 to 10, all calves were gradually weaned and were fed 2 L/d until the end of the trial. Concentrate (CON), hay, and water were freely available. Intakes of MR and CON were measured daily. Calves were weighed at birth and weekly thereafter. Blood was drawn on d 1 before the first colostrum intake; on d 2, 4, and 7; and weekly thereafter until the end of the study to measure plasma concentrations of metabolites and hormones. Liver samples were taken at d 50 and at the end of the study to determine gene expression related to glucose metabolism. Milk, MR, and total nutrient intake were greater, but CON intake was lower in Adl than in Res calves, resulting in a greater body weight, but partially lower gain to feed ratio in Adl than in Res. Plasma concentrations of glucose and insulin were higher during the ad libitum milk-feeding period, whereas plasma β-hydroxybutyrate was lower in Adl than in Res. Plasma concentrations of non-esterified fatty acids, lactate, total bilirubin, and cortisol were lower, but triglyceride and cholesterol concentrations were higher in Adl than in Res at specific time points. Feed intake, growth performance, and metabolic and endocrine changes were insignificantly affected by butyrate, and hepatic gene expression of enzymes related to endogenous glucose production was barely influenced by ad libitum MR feeding and butyrate supplementation. Intensive MR feeding indicated greater stimulation of growth and anabolic metabolism, but butyrate supplementation did not further improve postnatal growth and anabolic processes either in intensive or restrictive MR-fed calves.

Key words: preruminant, ad libitum feeding, butyrate, glucose

## 3.2 Introduction

Several studies in pre-weaning calves have indicated improved development and body growth with the application of intensive milk feeding programs (Hammon et al., 2002; Schäff et al., 2016; Korst et al., 2017). This improved growth and development might have long-lasting consequences on individual dairy performance (Bar-Peled et al., 1997; Bach, 2012; Van Amburgh and Soberon, 2013); however, the discussion on the extent of the milk feeding level is still ongoing, and restricted milk feeding to less than 6 L/d is still common (Hill et al., 2016). Growing evidence suggests that insufficient liquid supply during the first weeks of postnatal life, fed either as milk or milk replacer (MR), compromises postnatal maturation and the health of calves and deviates significantly from the natural situation, as observed in beef calves (Schiessler et al., 2002; Khan et al., 2011, 2016). Intensive milk or MR feeding programs resulted in elevated DM and energy intake and body growth (Hammon et al., 2002; Jasper and Weary, 2002) and accelerated organ development (Daniels et al., 2009; Geiger et al., 2016) during the pre-weaning period. Metabolic and endocrine changes in blood plasma pointed toward enhanced anabolic metabolism (Bartlett et al., 2006; Maccari et al., 2015; Schäff et al., 2016). In addition, it has been suggested that calves with elevated milk or MR intake during the pre-weaning period are less susceptible to illness (Ollivett et al., 2012; de Passillé et al., 2016). However, intensive milk feeding programs often last for only a few weeks after birth, supply milk or MR only twice daily (Bartlett et al., 2006; Daniels et al., 2008; Davis Rincker et al., 2011), and do not correspond to the ad libitum milk intake observed in beef calves (Egli and Blum, 1998; Schiessler et al., 2002).

Butyrate is known as a natural growth-stimulating substrate that enhances growth performance in young mammals (Guilloteau et al., 2010). In pre-weaning calves, butyrate supplementation in MR increases BW, structural growth, and health and affects insulin-dependent glucose metabolism (Górka et al., 2011; Kato et al., 2011). Butyrate deploys its stimulating effects on postnatal growth in young calves by accelerating postnatal maturation of the gastrointestinal tract and improving gut function (Guilloteau et al., 2009; Górka et al., 2014). The aim of the current study was to investigate feed intake, growth performance, and metabolic and endocrine changes in calves fed MR ad libitum supplemented with butyrate for 8 wk. We intended to combine the positive effects of intensive MR feeding and MR butyrate supplementation to test the hypothesis that both treatments stimulate postnatal growth and affect energy metabolism in calves related to

anabolic processes in an additive manner. The dose for butyrate supplementation in MR was adapted from the work of Guilloteau et al. (2009) and Górka et al. (2011).

## 3.3 Materials and Methods

The animal experiment was conducted from June 2014 to June 2015 at the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Germany. The experimental procedures in this study were performed in strict accordance with the German Animal Welfare Act and were approved by the relevant Department for Animal Welfare Affairs (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany; registration no. 23 177-07/G 13-20-069).

### 3.3.1 Animals and husbandry

Sixty-four German Holstein calves [n = 32 each for intact male (not castrated) and female] were used from birth until 80 ± 2 (mean ± SD) days of life. We used a birth monitoring system (iVET, Papenburg, Germany) to observe calving and to ensure that first blood sampling occurred prior to initial colostrum intake. The calves selected for this study met the following criteria: birth without assistance or with minor assistance (eutocia), pedigreed singletons, birth weight between 35 to 55 kg, and physiological health and vigor. After colostrum intake, all calves received 10 mL of an iron paste (115 mg Fe3+/mL and 108 mg dextran/mL, Sinta GmbH, Schwarzenborn, Germany) and were treated for navel disinfection with an iodine solution (Albrecht GmbH, Aulendorf, Germany). Within 2 to 3 h after birth, the calves were brought to individual straw-bedded calf hutches. This husbandry lasted for 10 ± 3 d (mean ± SD) before the calves were finally housed in an open straw-bedded stable. Calves that received no butyrate (ResB-, AdlB-) or butyrate supplementation (ResB+, AdlB+) were housed in opposite group pens and fed from two different automatic feeders. In each pen, two different feeding stations per feeder were available for calves younger than 3 wk of age and calves 4 wk of age and older. The health of the calves was evaluated by daily rectal temperature measurement and navel and feces examination until wk 3 of age. Health maladies such as diarrhea, pneumonia and navel ill were documented and treated by a veterinarian according to diagnosis. Clinical data from this portion of the study will be presented in a companion paper.

### 3.3.2 Feeding and diets

Within 2 h after birth, all calves received 2.5 ± 0.09 kg (mean ± SD) of colostrum from their dams via bottle. Because colostrum from their dams was not available, five calves received the same amount of colostrum from a high-quality colostrum reserve stored at -20°C, and quality was measured by specific gravity and optical Brix refractometers (Gross et al., 2016). Subsequently, the calves were allocated to one of the four feeding groups with respect to their sex, birth weight, and cow parity to create equal groups. For the following five meals (until d 3 of life), calves were fed acidified transition milk (2 mL acidifier/L milk, H. W. Schaumann GmbH, Pinneberg, Germany) from their dams with teat buckets either in amounts of 3 L per meal (Res; n = 32) or ad libitum (Adl; n = 32).

Table 3.1. Nutrient and chemical composition of the milk replacer (MR) and concentrate (CON)

| Item, % of DM unless noted otherwise | MR[1] | CON[2] |
|---|---|---|
| DM, g/kg | 968 | 866 |
| CP | 21.7 | 21.0 |
| Crude fat | 18.6 | 4.2 |
| Crude fiber | 0.2 | 5.9 |
| $ADF_{OM}$[3] | ND[6] | 8.1 |
| $aNDF_{OM}$[3] | ND | 16.5 |
| Ash | 7.3 | 7.0 |
| NFE[4] | 52.1 | 61.9 |
| Total sugar | 44.9 | ND |
| ME, [5] MJ/kg of DM | 18.34 | 13.15 |
| Ca | 1.17 | 1.22 |
| P | 0.65 | 0.66 |
| Na | 0.47 | 0.29 |
| K | 1.43 | 1.21 |
| Lysine | 1.8 | ND |
| Methionine | 0.48 | ND |

[1]Ingredients of MR: 50 % skim milk powder, 25 % whey powder, 16.5 % vegetable oil, 3 % wheat powder; MR fed to butyrate-supplemented restrictive (ResB+) and ad libitum (AdlB+) treatments contained 0.24 % butyrate of DM.
[2]Ingredients of CON: 35.0 % corn, 23.0 % soybean meal, 13.0 % beet pulp dried with molasses, 7.5 % wheat, 6.0 % barley, 3.2 % rapeseed meal, 3.0 % linseed meal, 3.0 % beet pulp, 2.0 % wheat bran, 0.09 % calcium carbonate, 0.05 % mono-calcium phosphate.
[3]Acid detergent fiber (ADF) and neutral detergent fiber (NDF) are expressed exclusive of residual ash. The NDF was assayed with a heat-stable amylase (Korst et al., 2017).
[4]Nitrogen-free extract (NFE), calculated as NFE = 100 - (CP + crude fat + crude fiber + ash).
[5]ME calculated using the equation: ME, MJ/kg of DM = (24.2 × CP + 36.6 × fat + 17.0 × total sugar)/100 × 0.97 × 0.96.
[6]ND = not determined.

From d 4 on, all calves were fed MR (12.5 % solids; Trouw Nutrition Deutschland GmbH, Burgheim, Germany) without supplementation or supplemented with calcium-sodium butyrate (0.24 % butyrate as fed; Benelux GmbH, Amel, Belgium) in amounts of either 6 L/d (ResB-; ResB+; n = 16, respectively) or ad libitum (maximum 25 L/d; AdlB-; AdlB+; n = 16, respectively). The ingredients and the chemical composition of the MR are given in Table 3.1.

The MR was stepped down linearly from d 57 to d 70 in all groups and was fed in amounts of 2 L/d until the end of the trial. During the first days of life, the feeding took place twice per day at 0700 and 1700 h in the hutches. Between feeding times, buckets for Adl calves were maintained with the calves and were refilled at noon if necessary. Teat buckets were covered with a lid to ensure that no rain or dirt contaminated the milk. In the stable, feeding was conducted using automatic feeding systems for MR and concentrate (Förster-Technik GmbH, Engen, Germany). Milk replacer was fed in small portions of a maximum of 2 L per meal for Res calves and a maximum of 5 L per meal for Adl calves, followed by an off time of 2 h for Res calves and 30 min for Adl calves after the end of the meal. Water and hay were freely available, and concentrate (pelleted starter; Raiffeisen Waren-Zentrale Rhein-Main eG, Köln, Germany; Table 3.1) was offered ad libitum in the stable.

### 3.3.3 Measurement of feed intake, feed analyses, and body weight

During the period in the calf hutches, the daily milk and MR intakes were documented by weighing residues with an electronic scale (Sartorius AG, Göttingen, Germany). When calves were fed with the automatic feeding system, data for MR and concentrate intake were sent automatically to the connected PC program (Förster-Technik GmbH).

The nutrient compositions of the MR and concentrate were analyzed by an accredited external laboratory (Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Speyer, Germany) according to the Weender standard procedure (Naumann and Bassler, 2004). Analyses of feedstuff were used to determine the ME content of concentrate according to GfE (2009). The composition of DM and energy from individual colostrum milkings were calculated using measurements from Kühne et al. (2000). The energy content of MR was calculated as gross energy on energy equivalents of 24.2, 36.6, and 17.0 MJ/kg DM for CP, crude fat, and total sugar, respectively. Gross energy for colostrum and MR was transformed into ME by ME = 0.97 × 0.96 × gross energy (NRC, 2001).

The birth weight was measured after the first colostrum intake. The exact amount of ingested colostrum was subtracted from the initial weight. Body weight was recorded weekly until the end of the trial using a mobile scale (Tru-Test Ltd., Auckland, New Zealand). The ADG was calculated from BW with the precise number of days between weighing.

### 3.3.4 Blood and liver sampling and analyses

Blood samples were collected from a jugular vein before first colostrum intake (d 1), 24 h after colostrum intake (d 2), before first MR intake (d 4), and subsequently on d 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, and 77. Ad libitum milk-fed calves were fasted for at least 1 h before blood sampling. Blood from Res calves was drawn before the morning feeding in the calf hutches or after 1 h fasting in group pens. After shaving and disinfecting the skin, the vein was punctured and blood was collected in evacuated tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) containing potassium-EDTA (1.8 mg/mL) and tubes containing sodium fluoride (2-4 mg/mL) and potassium oxalate (1-3 mg/mL). Blood samples were held on ice until centrifugation at 3,500 × *g* for 10 min. The supernatants from plasma were pipetted into aliquots and stored at –20°C until analyzed.

Analyses of metabolites [glucose, BHB, lactate, urea, non-esterified fatty acids (NEFA), triglyceride, cholesterol, and total bilirubin] were performed from sodium fluoride/potassium oxalate plasma. Insulin, glucagon, and cortisol concentrations were determined from EDTA plasma. All metabolites were analyzed spectrophotometrically (HORIBA ABX SAS Montpellier, France). The following kits were used: glucose (#A11A01667), lactate (#A11A01721), and triglyceride (#A11A01640; Axon Lab AG, Baden, Switzerland); BHB (#RB1008), urea (#LT-UR 0010), and total bilirubin (#LT-BR 0500; LABOR + TECHNIK Eberhard Lehmann GmbH, Berlin, Germany); NEFA (#434-91795, Wako Chemicals GmbH, Neuss, Germany); and cholesterol (#553-127, mti diagnostics GmbH, Idstein, Germany). Butyrate analysis in blood plasma was performed as recently described (Laeger et al., 2012).

Plasma concentrations of insulin (#RIA-1257) and glucagon (#RIA-1258) were determined by RIA using kits from DRG Instruments GmbH (Marburg, Germany), which were adapted to bovine subjects (Hammon et al., 2009). Intra- and interassay coefficients of variation were 3.7 and 5.5 % for insulin and 3.4 and 22.5 % for glucagon, respectively.

The plasma cortisol concentration was analyzed in duplicate after extraction with diethylether using a commercially available ELISA kit (#EIA1887, DRG Instruments GmbH; Gruse et al., 2016). The assay was validated for the use with bovine plasma. The test sensitivity was 3.4 µg/L, and the intra- and interassay coefficients of variation were 5.3 and 12.1 %, respectively.

On d 50 ± 2 and d 80 ± 2 (mean ± SD; d 80 females only), approximately 80 to 100 mg of liver tissue was collected from each calf by biopsy (modified from Swanson et al., 2000) with a Bard Magnum biopsy instrument and Bard Magnum core tissue biopsy needle (C.R. Bard Inc., Covington, GA). Liver samples of male calves on d 80 ± 2 (mean ± SD) were collected after calves were harvested at the end of the study. Liver samples were flushed in ice-cold 0.9 % NaCl and frozen in liquid nitrogen. For further analysis, the liver tissue was pulverized in liquid nitrogen. Glycogen content was determined with the use of a commercial kit based on amyloglucosidase-catalyzed glucose release (no. 10207748035; Roche Diagnostics GmbH, Mannheim, Germany). The relative mRNA abundance of genes related to glucose metabolism was quantified as previously described (Saremi et al., 2012; Schäff et al., 2016). Primer sequences and PCR conditions for reference genes [hippocalcin-like 1 (*HPCAL1*), low-density lipoprotein 10 (*LRP10*), and RNA polymerase II (*POLR2A*)] and target genes [glycogen phosphorylase (*PYGL*), glucose-6-phosphatase (*G6PC*); phosphoenolpyruvate carboxykinase (cytosolic: *PCK1*; mitochondrial: *PCK2*), pyruvate carboxylase (*PC*), and propionyl-CoA carboxylase α (*PCCA*)] were recently published (Saremi et al., 2012; Gruse et al., 2015; Schäff et al., 2016). The primer products were verified by sequencing applied with the BigDye Terminator v1.1 Cycle Sequencing kit and an ABI 3130 Genetic Analyzer (Thermo Fisher Scientific Inc., Waltham, MA). Real-time PCR was performed with the use of a LightCycler (F. Hoffman-La Roche AG, Basel, Switzerland). The fluorescent dye used was SYBR Green I. Melting curve analysis and agarose gel electrophoresis were used to confirm the specificity of the PCR products. Quantification cycle values and amplification efficiencies obtained with the use of LinRegPCR version 2013.0 (Ruijter et al., 2013) were imported into qBASE+ version 2.6.1 (Biogazelle NV, Zwijnaarde, Belgium) for all subsequent calculations and quality controls (Gruse et al., 2015; Schäff et al., 2016). The geometric mean of the reference gene abundances was applied for normalization. The data are presented as the ratio of the copy number of the respective gene of interest to the geometric mean of the reference gene abundance.

### 3.3.5 Statistical analyses

Data were evaluated by repeated-measures ANOVA using PROC MIXED in SAS for Windows (release 9.4, SAS Institute Inc., Cary, NC). The ANOVA model contained the fixed effects of feeding regimen (Res vs. Adl), butyrate supplementation, time, sex, and respective interactions. Repeated measures on each calf were considered using the repeated statement of the MIXED procedure using an unstructured type of block diagonal residual covariance matrix structure (SAS Institute Inc.). Least squares means (LSM) and their standard errors (SE) were computed for each fixed effect in the models, and all pair-wise differences of LSM were tested with the Tukey-Kramer procedure. The SLICE statement of the MIXED procedure was used to conduct partitioned analyses of the LSM for interactions. Differences in data with *P*-values < 0.05 were defined as significant, and *P*-values < 0.1 were considered as trends. Values are presented as LSM ± SE if not declared otherwise in the text.

## 3.4 Results

### 3.4.1 Feed intake and growth performance

Intake of first colostrum (first milking) was similar for all calves (2.5 ± 0.01 L, LSM ± SE). However, during the subsequent five meals, Adl calves consumed 25.4 % more transition milk than Res calves. Sum of first colostrum and transition milk intakes for all 3 d were 19.8 ± 0.5 L for Adl and 14.8 ± 0.5 L for Res. Furthermore, MR intake and hence ME, CP, and fat intake were higher ($P < 0.01$) from wk 1 to 10 in Adl than in limited milk-fed calves (Figure 3.1A; Table 3.2). The DMI of milk powder reached the highest amount of 1.7 kg/d in wk 5 for AdlB- and in wk 8 for AdlB+. These amounts were 2.3 times greater than the intake of the respective Res groups. Butyrate supplementation did not affect MR intake in a significant manner throughout the study, but butyrate-fed calves showed a trend ($P = 0.09$) of lower MR intake in wk 1. The concentrate intake, as shown in Figure 3.1B, was negligible for all calves in the first 3 wk of life. The concentrate intake increased, as did ME, CP, and fat intake of the concentrate, at the age of 4 wk in the Res groups, and the increase occurred earlier than for the Adl groups, resulting in a greater ($P < 0.01$) concentrate intake in Res than in Adl calves from wk 4 on throughout the study. In wk 5, concentrate intake was higher ($P = 0.03$) for butyrate-fed groups, but no other effects of butyrate supplement on concentrate intake were observed. The total DMI and

ME, CP, and fat intake were greater ($P < 0.01$) in Adl than in Res calves (Table 3.2). The ME intake by MR compared with concentrate was greater until wk 8 of age for Res groups and until wk 10 of age for Adl groups. At wk 11, 89, 85, 88, and 86 % of ME came from concentrate intake for ResB-, AdlB-, ResB+, and AdlB+, respectively.

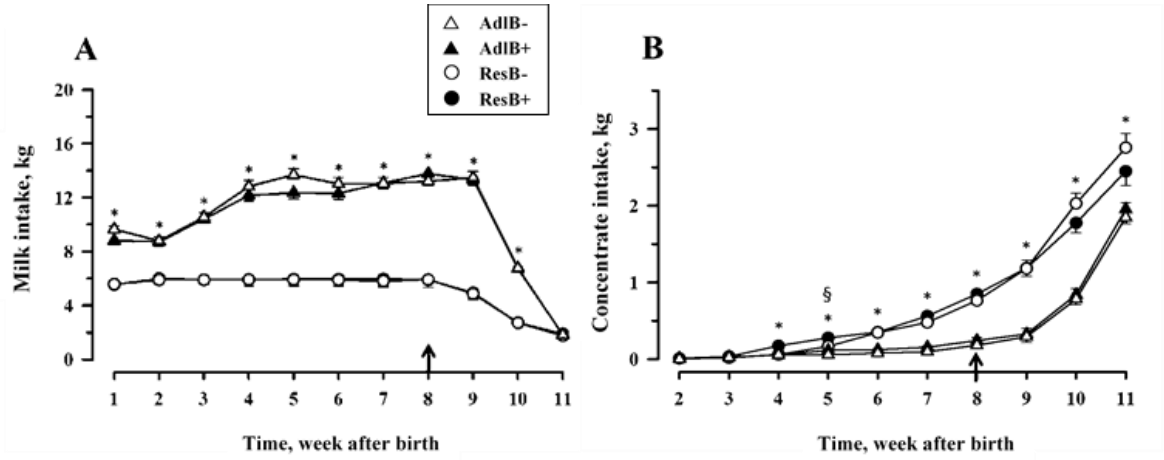


Figure 3.1. Milk and milk replacer (MR; A) and concentrate (B) intake in calves fed milk and MR either ad libitum or restrictively and supplemented MR without (△ AdlB-; ○ ResB-) or with 0.24 % butyrate (▲ AdlB+; ● ResB+). Arrow marks the start of weaning. Data are presented as weekly LSM ± SE; * indicates the effect of feeding regimen ($P < 0.05$); § indicates the effect of butyrate supplementation ($P < 0.05$).

Birth weights ranged from 36.5 to 53.5 kg. For our groupings, the mean birth weight (43.6 ± 0.6 kg) did not differ among the groups. The BW was greater ($P < 0.01$) in Adl than in Res groups for the entire trial (Figure 3.2A). Butyrate supplement resulted in a trend of lower BW in wk 5 ($P = 0.11$) and 6 ($P = 0.1$) and from wk 9 until the end of the study ($P < 0.1$). The ADG was approximately 1.8 times greater ($P < 0.01$) in Adl than in Res groups until wk 6 of age, greatest in wk 9 ($P < 0.01$) in AdlB-, but greater ($P < 0.01$) in wk 10 and 11 in Res than in Adl calves (Figure 3.2B). The ADG over the total experimental period was greater ($P < 0.01$) in Adl than in Res calves (980 ± 22 and 795 ± 22 g/d for Adl and Res calves, respectively). The gain to feed ratio changed with time and was higher in wk 1 ($P = 0.01$) but lower ($P < 0.01$) in wk 5, 7, 8, and 10 in Adl than in Res calves and was reduced ($P < 0.01$) by butyrate in wk 9 (Figure 3.2C).

Table 3.2. Dry matter, ME, CP, and fat intake of liquid, concentrate, and total feed (liquid and concentrate) intake of calves fed milk and milk replacer (MR) either ad libitum or restrictively and supplemented MR without (ResB-; AdlB-) or with 0.24 % butyrate (ResB+; AdlB+)

| | Dietary treatment | | | | | Fixed effect, *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|
| Item[1] | ResB- | AdlB- | ResB+ | AdlB+ | SEM | Milk | Butyrate | Time | Sex |
| Liquid intake | | | | | | | | | |
| DM, kg/d | 0.63 | 1.30 | 0.63 | 1.26 | 0.03 | < 0.001 | 0.6 | < 0.001 | 0.7 |
| ME, MJ/d | 11.0 | 22.4 | 11.1 | 22.0 | 0.54 | < 0.001 | 0.6 | < 0.001 | 0.7 |
| CP, g/d | 136 | 282 | 138 | 274 | 6.73 | < 0.001 | 0.6 | < 0.001 | 0.7 |
| Crude fat, g/d | 117 | 242 | 118 | 235 | 5.77 | < 0.001 | 0.6 | < 0.001 | 0.7 |
| Concentrate intake | | | | | | | | | |
| DM, kg/d | 0.67 | 0.30 | 0.67 | 0.35 | 0.05 | < 0.001 | 0.6 | < 0.001 | 0.4 |
| ME, MJ/d | 8.78 | 3.89 | 8.84 | 4.55 | 0.65 | < 0.001 | 0.6 | < 0.001 | 0.3 |
| CP, g/d | 140.2 | 62.1 | 141.0 | 72.6 | 10.39 | < 0.001 | 0.6 | < 0.001 | 0.3 |
| Crude fat, g/d | 28.0 | 12.4 | 28.2 | 14.5 | 2.08 | < 0.001 | 0.6 | < 0.001 | 0.3 |
| Total feed intake | | | | | | | | | |
| DM, kg/d | 1.24 | 1.57 | 1.25 | 1.58 | 0.06 | < 0.001 | 0.9 | < 0.001 | 0.5 |
| ME, MJ/d | 19.0 | 26.2 | 19.2 | 26.2 | 0.81 | < 0.001 | 1.0 | < 0.001 | 0.6 |
| CP, g/d | 264 | 338 | 266 | 340 | 11.72 | < 0.001 | 0.9 | < 0.001 | 0.5 |
| Crude fat, g/d | 142 | 253 | 144 | 248 | 6.14 | < 0.001 | 0.8 | < 0.001 | 0.7 |

[1]Values are presented as LSM for the whole experimental period.

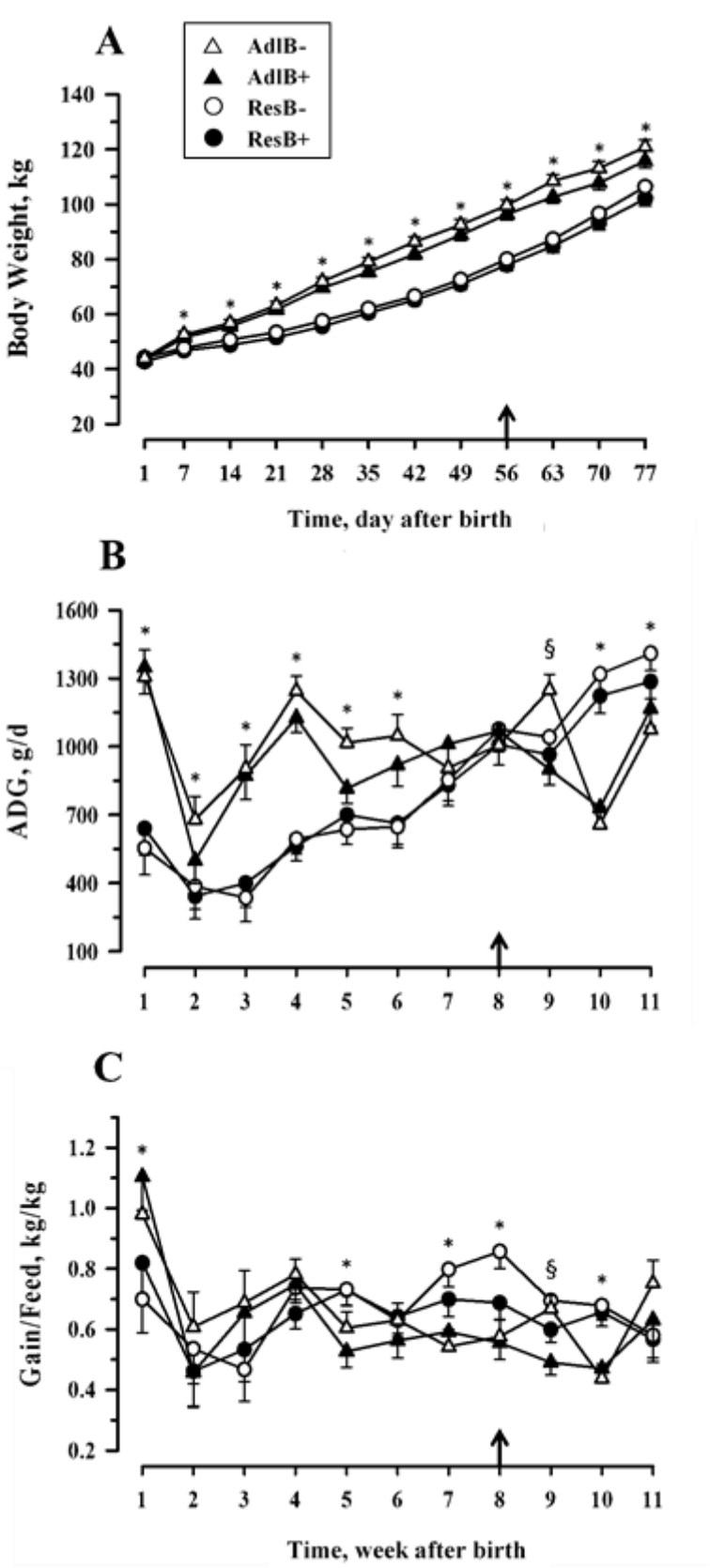


Figure 3.2. Body weight (A), ADG (B), and gain to feed ratio (C) in calves fed milk and milk replacer (MR) either ad libitum or restrictively and supplemented MR without (△ AdlB-; ○ ResB-) or with 0.24 % butyrate (▲ AdlB+; ● ResB+). Arrow marks the start of weaning. Data are presented as LSM ± SE; * indicates the effect of feeding regimen ($P < 0.05$); § indicates the effect of butyrate supplementation ($P < 0.05$).

### 3.4.2 Metabolic profiles

The results of selected metabolites that reflect carbohydrate metabolism and urea are presented in Figure 3.3. The concentration of butyrate in blood plasma was below the detection limit in all calves (data not shown). The glucose concentration in blood plasma increased ($P < 0.01$) in all groups after first feed intake and was higher ($P < 0.01$) until d 56 but lower ($P < 0.01$) on d 70 and 77 in Adl than in Res calves (Figure 3.3A). Butyrate supplementation decreased plasma glucose on d 14, 21, and 42 ($P < 0.05$). Plasma glucose tended to be greater ($P < 0.06$) in female than in male calves (data not shown).

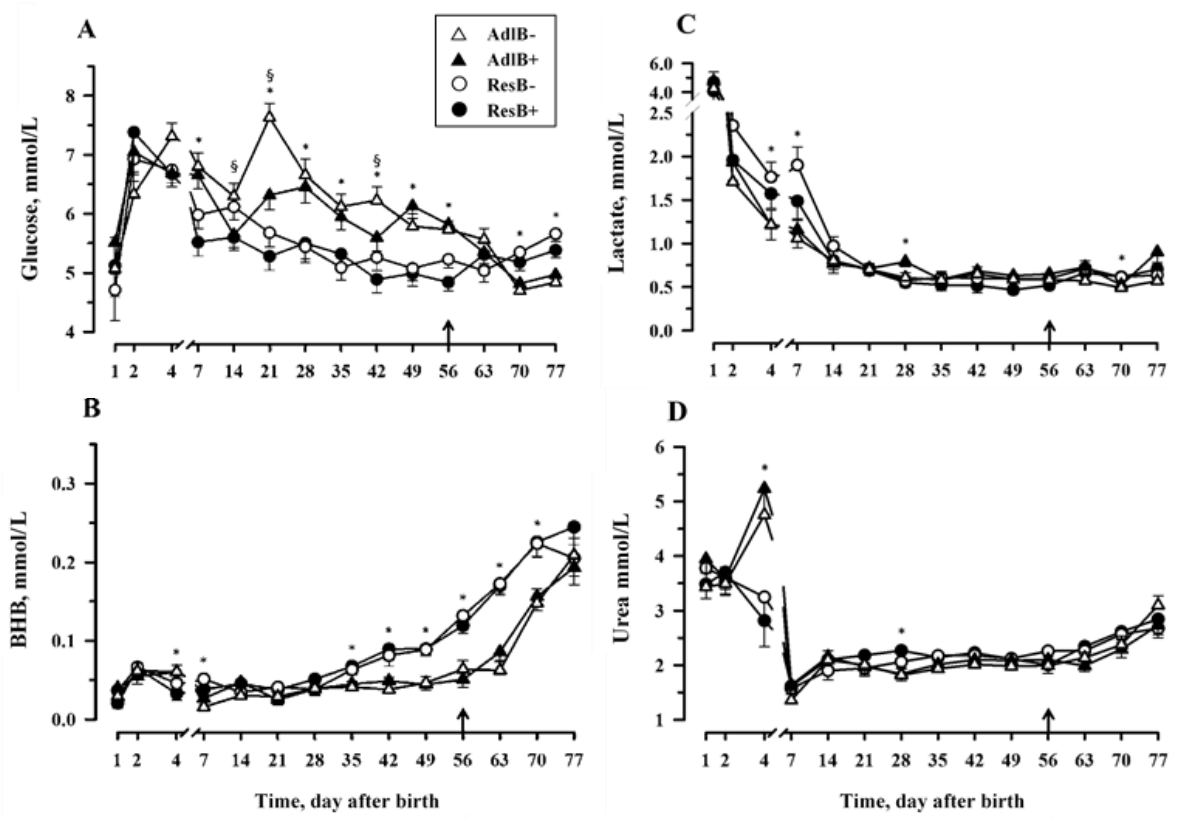


Figure 3.3. Blood plasma concentrations of glucose (A), BHB (B), lactate (C), and urea (D) in calves fed milk and milk replacer (MR) either ad libitum or restrictively and supplemented MR without (△ AdlB-; ○ ResB-) or with 0.24 % butyrate (▲ AdlB+; ● ResB+). Arrow marks the start of weaning. Data are presented as LSM ± SE; * indicates the effect of feeding regimen ($P < 0.05$); § indicates the effect of butyrate supplementation ($P < 0.05$).

Plasma BHB concentration was lower ($P < 0.05$) on d 4 but was higher ($P < 0.01$) on d 7 in Res than in Adl calves (Figure 3.3B). The plasma concentration of BHB increased from d 35 on in Res calves and was higher ($P < 0.01$) from d 35 to 70 in Res than in Adl calves. In Adl calves, the increase in plasma BHB concentration was delayed starting on d 70. Plasma lactate concentration decreased ($P < 0.01$) from birth to the end of the first month

of age in all groups and was higher ($P < 0.01$) on d 4, 7, and 70 but lower ($P < 0.05$) on d 28 in Res than in Adl calves (Figure 3.3C). Plasma urea concentration increased ($P < 0.01$) from d 2 to 4 in Adl calves but showed the lowest concentration in all groups on d 7 (Figure 3.3D). Urea concentration was higher ($P < 0.01$) on d 4 but lower ($P = 0.01$) on d 28 in Adl than in Res calves.

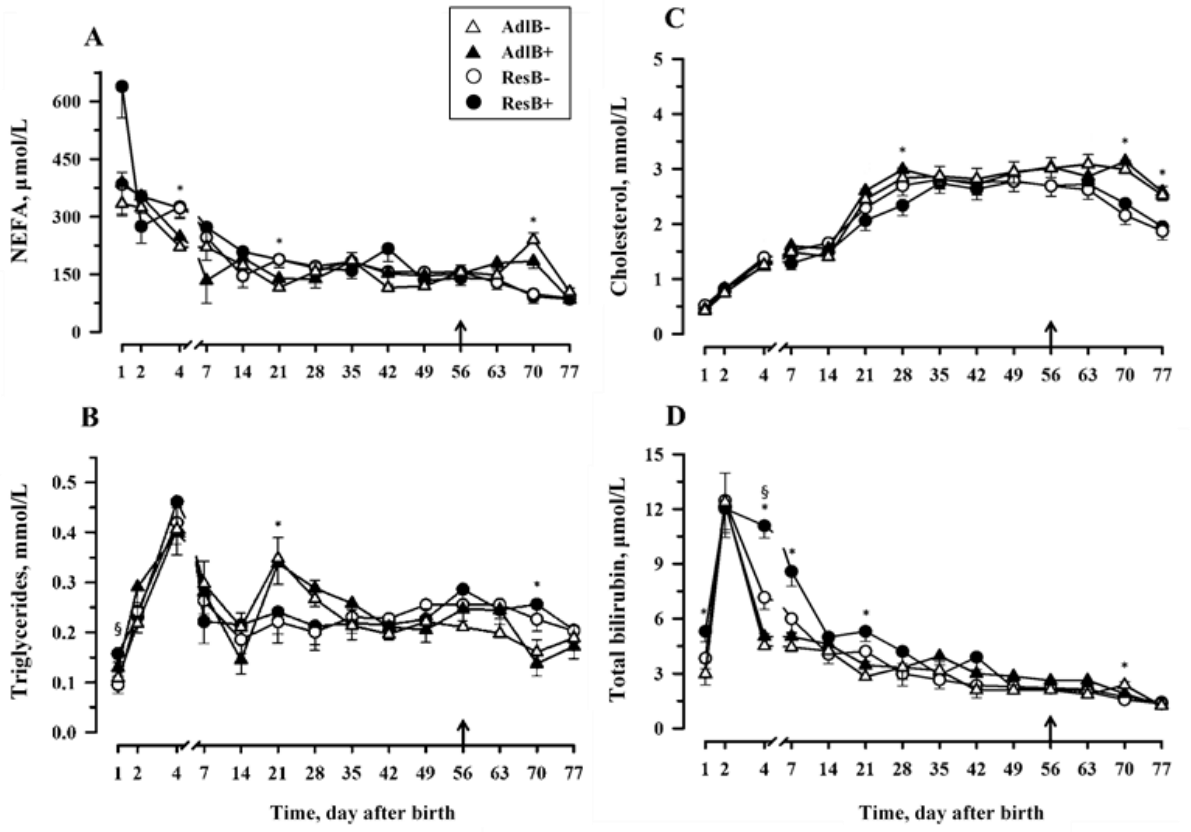


Figure 3.4. Blood plasma concentrations of non-esterified fatty acids (NEFA; A), triglycerides (B), cholesterol (C), and total bilirubin (D) in calves fed milk and milk replacer (MR) either ad libitum or restrictively and supplemented MR without (△ AdlB-; ○ ResB-) or with 0.24 % butyrate (▲ AdlB+; ● ResB+). Arrow marks the start of weaning. Data are presented as LSM ± SE; * indicates the effect of feeding regimen ($P < 0.05$); § indicates the effect of butyrate supplementation ($P < 0.05$).

Figure 3.4 presents data from fat metabolism. Plasma NEFA concentration decreased ($P < 0.01$) with time during the first 2 wk and was lower ($P < 0.01$) on d 4 and 21 but higher ($P < 0.01$) on d 70 in Adl than in Res calves (Figure 3.4A). Plasma triglyceride concentration increased ($P < 0.01$) in all groups from d 1 to 4 and increased ($P < 0.01$) again in Adl calves on d 21 (Figure 3.4B). Triglyceride concentration was higher on d 21 but lower on d 70 in Adl than in Res calves. The triglyceride concentration before first colostrum intake was higher in calves that were later supplemented with butyrate. Plasma cholesterol concentration increased ($P < 0.01$) up to wk 4 of life in all groups

(Figure 3.4C), remained constant for 5 wk in Res and for 6 wk in Adl calves, and subsequently decreased to the end of the study in all groups. The Adl calves had a higher cholesterol concentration ($P < 0.05$) than Res calves on d 28, 70 and 77. Plasma total bilirubin concentration increased in all calves after colostrum intake on d 2 and decreased until the end of the trial ($P < 0.01$; Figure 3.4D). The Res calves showed a slower decrease than Adl calves from d 2 to 14 ($P < 0.01$), and ResB+ calves had the highest concentrations on d 1, 4, 7, and 21, resulting in milk feeding effects on these days ($P < 0.01$) and a butyrate effect ($P < 0.05$) on d 4. Total bilirubin concentration was higher ($P < 0.01$) on d 70 in Adl than in Res calves.

### 3.4.3 Hormone profiles

The plasma insulin concentration was higher ($P < 0.05$ or less) from d 4 to 63 but lower ($P < 0.01$) on d 70 in Adl than in Res calves and was higher ($P < 0.05$) on d 28 in calves that were fed butyrate (Figure 3.5A).

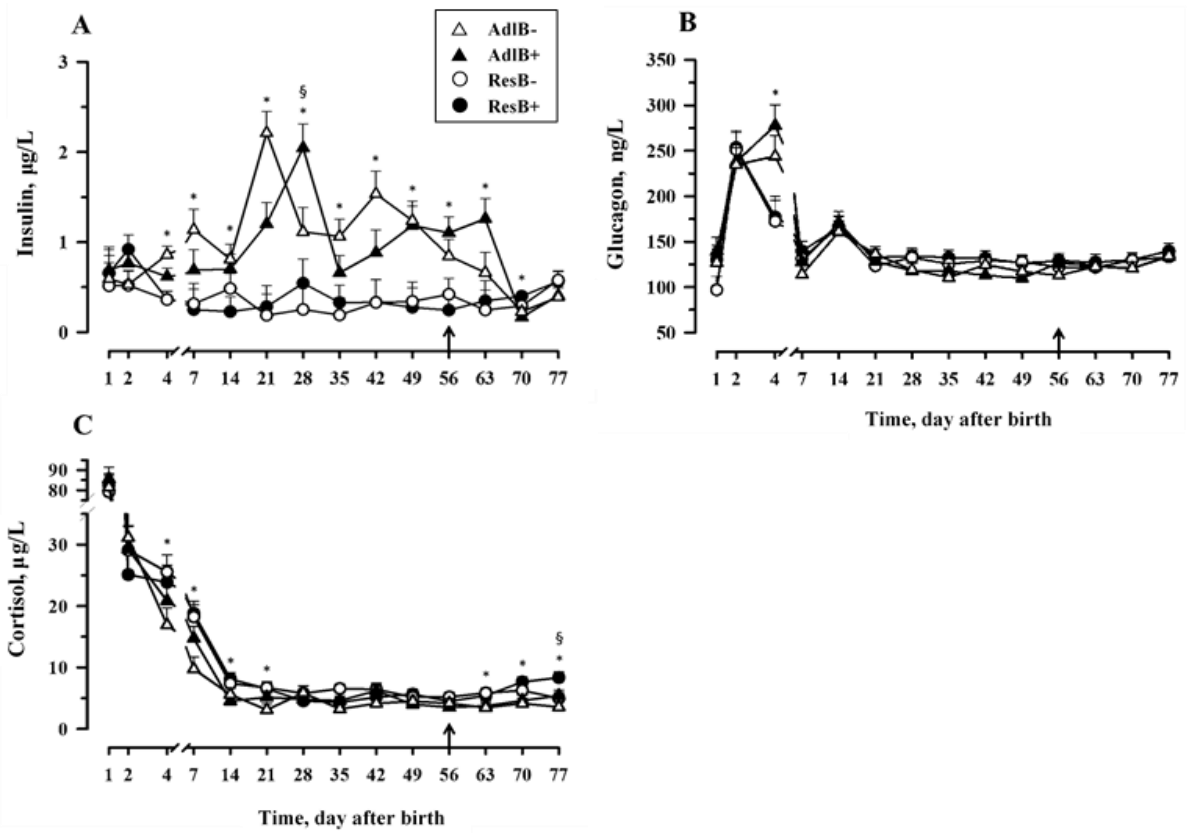


Figure 3.5. Blood plasma concentrations of insulin (A), glucagon (B), and cortisol (C) in calves fed milk and milk replacer (MR) either ad libitum or restrictively and supplemented MR without (△ AdlB-; ○ ResB-) or with 0.24 % butyrate (▲ AdlB+; ● ResB+). Arrow marks the start of weaning. Data are presented as LSM ± SE; * indicates the effect of feeding regimen ($P < 0.05$); § indicates the effect of butyrate supplementation ($P < 0.05$).

Plasma glucagon concentration increased ($P < 0.01$) after first colostrum intake in all groups, remained high in Adl calves, and decreased ($P < 0.01$) in Res calves on d 4 and decreased ($P < 0.01$) in all groups to d 7 (Figure 3.5B). Glucagon concentration was higher ($P < 0.01$) on d 4 in Adl than in Res calves. A trend of higher glucagon concentration in Res than in Adl calves ($P < 0.1$) was observed on d 28, 35, and 49. Plasma concentrations of insulin and glucagon were greater ($P < 0.01$) in female than in male calves (data not shown). The plasma cortisol concentration decreased from birth until d 14 of life in all groups and remained stable thereafter (Figure 3.5C). The plasma cortisol concentration was lower ($P < 0.01$) in Adl than in Res calves from d 4 to 21 and on d 63 and 70 and was highest on d 77 in ResB+.

### 3.4.4 Hepatic glycogen concentration and gene expression related to glucose metabolism

The hepatic glycogen concentration decreased ($P < 0.01$) in all groups from d 50 to 80 and was greater ($P < 0.01$) in Adl than in Res calves, especially when butyrate was supplemented (Table 3.3). The glycogen concentration was greater ($P < 0.05$) in female than in male calves (data not shown). The mRNA abundance of *PYGL* and *G6PC* increased ($P < 0.01$) with time and was greater ($P < 0.05$) in male than in female calves (data not shown). The mRNA abundance of *PCK1* and *PCK2* increased ($P < 0.01$) with time, and for *PCK1*, mRNA abundance on d 50 tended to be greater but on d 80 tended to be lower in B- than B+ calves (time × butyrate, $P = 0.06$). The mRNA abundance of *PC* on d 80 tended to be greater ($P = 0.1$) in B+ than in B- calves. The mRNA abundance of *PCCA* increased with time ($P < 0.001$), tended to be slightly lower ($P < 0.1$) in B+ than in B- calves on d 50, and tended to be greater ($P < 0.1$) in Adl than in Res calves on d 80.

Table 3.3. Hepatic glycogen concentration and relative mRNA expression ($log_2$) of gluconeogenic enzymes on d 50 and 80 in calves fed milk and milk replacer (MR) either ad libitum or restrictively and supplemented MR without (ResB-; AdlB-) or with 0.24 % butyrate (ResB+; AdlB+)

| | Dietary treatment | | | | | Fixed effect, *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|
| Item[1,2] | ResB- | AdlB- | ResB+ | AdlB+ | SEM | Milk | Butyrate | Time | Sex |
| Glycogen, mg/100 mg wet weight | | | | | | | | | |
| d 50 | 2.25[ab] | 2.86[ab] | 2.03[b] | 3.03[a] | 0.28 | < 0.01 | 0.6 | < 0.001 | 0.05 |
| d 80 | 1.53[ab] | 1.53[ab] | 1.07[b] | 1.65[a] | 0.18 | | | | |
| Relative mRNA expression related to reference genes | | | | | | | | | |
| *PYGL* | | | | | | | | | |
| d 50 | 4.57 | 4.68 | 3.91 | 4.47 | 0.40 | 0.2 | 0.5 | < 0.001 | 0.05 |
| d 80 | 5.62 | 5.91 | 5.46 | 6.04 | 0.40 | | | | |
| *G6PC* | | | | | | | | | |
| d 50 | 6.69 | 7.29 | 6.47 | 6.06 | 1.21 | 0.6 | 0.7 | < 0.001 | 0.05 |
| d 80 | 15.2 | 11.6 | 14.4 | 15.4 | 1.96 | | | | |
| *PCK1* | | | | | | | | | |
| d 50 | 8.90 | 10.3 | 6.72 | 6.41 | 1.58 | 0.7 | 1.0 | < 0.001 | 0.4 |
| d 80 | 12.4 | 12.3 | 16.6 | 14.1 | 1.59 | | | | |
| *PCK2* | | | | | | | | | |
| d 50 | 3.84 | 3.41 | 3.84 | 3.32 | 0.33 | 0.5 | 1.0 | < 0.001 | 0.3 |
| d 80 | 4.91 | 5.31 | 5.28 | 5.04 | 0.39 | | | | |
| *PC* | | | | | | | | | |
| d 50 | 5.27 | 5.80 | 5.74 | 5.29 | 0.66 | 0.9 | 0.11 | 0.3 | 0.5 |
| d 80 | 5.24 | 5.82 | 7.60 | 6.55 | 0.93 | | | | |
| *PCCA* | | | | | | | | | |
| d 50 | 2.28 | 2.48 | 2.23 | 2.06 | 0.20 | 0.15 | 0.07 | < 0.001 | 0.3 |
| d 80 | 3.04 | 3.66 | 2.78 | 3.12 | 0.27 | | | | |

[a,b]Different letters within the same row indicate significant differences ($P \leq 0.05$).
[1]Values are presented as LSM.
[2]*PYGL* = glycogen phosphorylase; *G6PC* = glucose-6-phosphatase; *PCK1* = phosphoenolpyruvate carboxykinase (cytosolic); *PCK2* = phosphoenolpyruvate carboxykinase (mitochondrial); *PC* = pyruvate carboxylase; *PCCA* = propionyl-CoA carboxylase α.

## 3.5 Discussion

### 3.5.1 Effects of milk feeding intensity and butyrate supplementation on feed intake and growth performance

Allowing calves to drink unlimited amounts of milk and MR during the pre-weaning period more than doubles liquid feed intake in Adl calves compared with that in Res calves, which supports previous findings (Hammon et al., 2002; Maccari et al., 2015; Schäff et al., 2016). The MR intake in ad libitum fed calves was greater than in common intensive milk-feeding programs for pre-weaning calves (Bartlett et al., 2006; Davis Rincker et al., 2011). However, the greater CP and fat content of the MR used in previous studies than that used in the present study might partially account for the higher protein and ME intake in intensive MR-feeding programs than that in the ad libitum MR feeding of the current study (Brown et al., 2005; Daniels et al., 2008; Geiger et al., 2016).

The overall concentrate intake was much lower in Adl than in Res calves. Even at the end of the study, Adl calves did not reach the concentrate intake measured in Res calves, although the increase in concentrate intake at the end of the study (i.e., when MR intake was reduced in all calves to 2 L) was greater in Adl calves. This finding was a consequence of the long-lasting ad libitum MR feeding period in the current study. The Adl calves received unlimited amounts of MR up to wk 9 of age because the feeding program began reduction of MR intake from 25 L/d in wk 8. The MR reduction in wk 8 had no consequences for MR intake in most of the Adl calves because maximal MR intake was mostly 14-15 L/d. However, certain calves reached a milk intake of 25 L/d for several days. Time for reduction of MR feeding in Adl calves was probably too short in our study. It is well know that too abrupt decrease of milk feeding in intensive milk-fed calves leads to depression of feed intake and growth during weaning (Khan et al., 2011). Previous studies with a shorter ad libitum milk-feeding period indicated no negative effect or even a greater concentrate intake after the ad libitum milk-feeding period in pre-weaning calves (Khan et al., 2011; Schäff et al., 2016; Korst et al., 2017). In calves fed milk at 20 % of BW for the first 25 d of life, concentrate intake was greater after the intensive milk-feeding period than in calves fed milk at 10 % of BW (Khan et al., 2007). Nevertheless, reduced solid feed intake during ad libitum or intensive milk feeding is a common phenomenon, illustrating the preference for milk or MR instead of concentrate in pre-weaning calves (Jasper and Weary, 2002; Davis Rincker et al., 2011; Miller-Cushon et al., 2013).

However, at the end of the study, when MR intake was reduced to 2 L for all calves, the portion of ME intake delivered by the concentrate was comparable among all calves. This observation shows that Adl calves immediately switch to concentrate intake when MR is reduced. In addition, plasma concentration of BHB, which is a result of concentrate digestion in the rumen, did not differ between Adl and Res calves at the end of the study.

Overall, total nutrient intake was much greater in Adl than in Res calves, and the nutrient intake was determined by the elevated MR intake in Adl calves, which supports previous studies (Khan et al., 2011, 2016). Total ME intake was 27 % less and total CP and fat intake were 22 and 43 % less in Res than in Adl calves, showing that the greater concentrate intake in Res calves could not compensate for the lower MR intake in these calves. The greater nutrient intake in Adl calves caused improved body growth and resulted in Adl calves that were 14 kg heavier at the end of the study. The ADG per week was greater until wk 6 of age but was lower at the end of the study (especially in wk 10) in Adl calves. Therefore, the elevated concentrate intake in Res calves led to comparable weekly ADG with age but could not compensate for the impaired body growth during the first weeks of age, which is consistent with previous studies (Khan et al., 2011, 2016; Schäff et al., 2016). The reduced ADG in wk 10 mirrored the lower feed intake in Adl calves during weaning because the reduction of MR intake during wk 10 was not compensated by the concentrate intake. A more gentle MR reduction in Adl calves could probably avoid such a drop in ADG in Adl calves. However, our findings indicate the greatest potential for stimulation of body growth via ad libitum milk feeding during the first 6 wk of age, which supports previous findings (Korst et al., 2017). Subsequently, an impaired gain to feed ratio in ad libitum milk-fed calves became obvious. This observation contrasts with previous studies in which intensive milk/MR feeding improved the feed efficiency in pre-weaning calves (Barlett et al., 2006; Khan et al., 2007; Davis Rincker et al., 2011), but a reduced feed efficiency was recently also found in calves fed MR ad libitum for the first 5 wk of age (Schäff et al., 2016). Obviously, the prolonged ad libitum milk feeding period in the current study compromises feed efficiency, but the calculated feed efficiency does not validate organ maturation and organ development, such as mammary gland development, in pre-weaning heifer calves (Geiger et al., 2016). In addition, the enhanced gain to feed ratio in Res calves from wk 7 of age on might partly be a result of greater solid feed intake in Res calves leading to enhanced gut filling.

Butyrate supplementation of MR insignificantly affected feed intake and growth performance in the current study. At wk 5 of age, concentrate intake was slightly greater in calves fed butyrate, and a trend of slightly reduced growth was noted in both butyrate-fed groups. In addition, the gain to feed ratio was reduced by butyrate supplementation in wk 9 of age. These findings of the effect of butyrate supplementation in MR on feed intake and growth performance were not expected because, in previous studies, butyrate supplementation in MR with a comparable dose led to improved growth performance (Górka et al., 2011) and feed efficiency (Guilloteau et al., 2009, 2010). Reasons for these different findings are presently not obvious because butyrate dose in MR and MR feeding intensity were comparable among studies (Guilloteau et al., 2009, 2010; Górka et al., 2014), but butyrate content was lower than in cow's milk (Chilliard et al., 2009). There might be differences in MR origin and calf management between the studies that lead to the divergent results. Butyrate fed to pre-weaning pigs stimulated growth and maturation of the small intestine enterocytes, which was barely observed in calves fed MR with butyrate (Guilloteau et al., 2010; Górka et al., 2014). Intestinal mucosa growth was stimulated by butyrate supplementation in the male calves of the current study [C. Gerbert, D. Frieten, C. Koch, G. Dusel, R. Zitnan (Research Centre Nitra, Kosice, Slovakia), H. M. Hammon, unpublished observation]. Surprisingly, the elevated mucosa growth owing to butyrate treatment did not cause enhanced body growth or did not improve the gain to feed ratio.

Feed intake and growth performance was not affected by sex, although birth weight was numerically greater in male than female calves (data not shown). These findings are unexpected because birth weight and growth rates are typically greater in male than in female calves (Kerr et al., 1991; Kertz et al., 1997; Egli and Blum, 1998), but not all studies indicate sex differences in birth weight and growth performance in calves during the milk-fed period (Govoni et al., 2004; Schäff et al., 2016).

### 3.5.2 Effects of milk feeding intensity and butyrate supplementation on systemic and hepatic metabolic changes

In the current study, ad libitum MR feeding primarily affected glucose metabolism. Plasma glucose usually decreases with age and increasing ruminant function (Quigley et al., 1991a, b; Hugi and Blum, 1997; Daniels et al., 2008). The decrease in plasma glucose with age was delayed in Adl calves compared with that of Res calves, and Adl calves showed a

greater plasma glucose concentration during the intensive milk feeding period, which supports previous findings (Maccari et al., 2015; Schäff et al., 2016; Kesser et al., 2017). Elevated plasma glucose in Adl calves most likely resulted from increased glucose intake because no differences were observed in gene expression of gluconeogenic enzymes with respect to intensive MR feeding on d 50 of age. However, gene expression of gluconeogenic enzymes increased (with the exception of *PC*) with age in all calves, which fits the elevated ruminant function after increasing concentrate intake and elevated substrate supply for gluconeogenesis (Quigley et al., 1991b; Hammon et al., 2005; Kesser et al., 2017). However, the time pattern of gluconeogenic gene expression in growing calves is more variable and depends on the age of weaning and nutrient intake (Hammon et al., 2005; Haga et al., 2008). The increased gene expression of gluconeogenic enzymes observed on d 80 was necessary to increase endogenous glucose production to maintain glucose homeostasis when oral glucose supply diminished. Indeed, hepatic glycogen concentration, which was elevated because of intensive MR feeding on d 50 of age, decreased in all calves on d 80. Hepatic gluconeogenic capacity was probably reduced in Adl calves at the end of the study because plasma glucose concentration was lower in Adl than in Res calves. The lower concentrate intake in Adl calves at the end of the study likely created less substrate for gluconeogenesis (Zhang et al., 2016).

The elevated plasma glucose concentration in ad libitum milk-fed calves caused an increase in plasma insulin during intensive milk feeding, as also indicated in previous studies (Daniels et al., 2008; Maccari et al., 2015; Schäff et al., 2016). Elevated insulin concentration in Adl calves was not a result of impaired insulin function (MacPherson et al., 2016; Kesser et al., 2017). The rise in plasma insulin might stimulate anabolic processes in muscle and fat tissue, leading to enhanced tissue growth in intensive milk-fed calves (Schäff et al., 2016). In contrast, plasma glucagon and cortisol decreased after the first week of life in all calves. The slower decrease of plasma cortisol during the first month of age in Res calves might indicate certain catabolic processes in these calves that were also observed in calves with impaired colostrum supply (Hammon and Blum, 1998; Kühne et al., 2000), and this observation fits with partly greater plasma NEFA and lactate concentrations in Res calves. The reasons for the higher plasma cortisol concentration at the end of the study in Res calves are currently not known and are inconsistent with the lower NEFA concentration in these calves. However, the cortisol concentration is low in general, and cortisol might be less metabolically effective during this time period. The rise

in plasma glucagon during the first 24 h of age in all calves and the elevated glucagon concentration in Adl calves on d 4 mirror the high protein intake with colostrum feeding on d 1 and the elevated protein intake owing to transition milk until d 4 in Adl calves (Hammon and Blum, 1998; Kühne et al., 2000). The elevated protein intake during the first days of age in Adl calves might also explain the increase in plasma urea concentration on d 4 of age (Rauprich et al., 2000). In addition, glucagon sensitivity decreases with age and rumen development (Donkin and Armentano, 1995; Baldwin et al., 2004), and the low plasma glucagon concentration after 3 wk of age might be a component of the reduced glucagon action.

Owing to greater concentrate intake and rumen function, the increase in BHB plasma concentration occurred much earlier in Res than in Adl calves (Quigley et al., 1991b). Therefore, the BHB time pattern in blood plasma followed the concentrate intake, and in Adl calves, the increase in plasma BHB occurred only after the reduction of MR feeding and the increase in concentrate intake at the end of the study, which led to a comparable plasma BHB concentration between Adl and Res calves in wk 11 of age. This finding indicates that rumen functioning is quickly enhanced when MR intake decreases and concentrate intake increases. Supporting this assessment, papilla growth of the ruminal mucosa was measured in the male calves of this study and was not different between Res and Adl calves at d 80 of age [C. Gerbert, D. Frieten, C. Koch, G. Dusel, R. Zitnan (Research Centre Nitra, Kosice, Slovakia), and H. M. Hammon, unpublished observation].

Plasma triglycerides were enhanced only at d 21 during intensive milk feeding in Adl calves. This observation was in contrast to previous findings in which plasma triglycerides were elevated during the entire intensive milk-feeding period (Schäff et al., 2016). In wk 10, the reduced concentrate intake led to a lower plasma triglyceride concentration in Adl than in Res calves. The plasma cholesterol concentration increased with age, consistent with previous studies (Hammon et al., 2002; Maccari et al., 2015; Schäff et al., 2016), indicating an elevated concentration at certain time points because of intensive milk feeding, but was greater in Adl than in Res at the end of the study, when MR was low in all calves and concentrate intake was higher in Res calves. It is likely that the greater concentrate intake reduced cholesterol synthesis in the rumen epithelium and led to lower plasma cholesterol in Res calves (Steele et al., 2011). Plasma concentration of total bilirubin increases after birth in neonates as a consequence of destruction of fetal

hemoglobin in the liver and spleen (Egli and Blum, 1998; Chowdhury and Chowdhury, 2009). The elevated plasma total bilirubin during the first week of age in Res calves might be a consequence of increased plasma NEFA in these calves. Plasma NEFA likely competes with plasma albumin binding and might affect hepatic bilirubin metabolism (Hadorn et al., 1997; Chowdhury and Chowdhury, 2009).

Overall, butyrate insignificantly affected metabolic and endocrine changes in calves in the current study. As mentioned previously, butyrate was not detected in blood plasma of calves fed MR with butyrate, which was also the case in previous studies (Guilloteau et al., 2010). An elevated plasma glucose concentration owing to butyrate feeding by MR (as reported by Górka et al., 2011) or enhanced insulin sensitivity (as stated by Kato et al., 2011) could not be supported herein. The opposite was the case because plasma glucose was lower at certain time points in calves fed MR with butyrate. Minor effects of oral butyrate supplementation were noted in the liver on gene expression of *PC* and *PCCA*. However, these data are difficult to interpret because hepatic *PCCA* mRNA was reduced and *PC* mRNA was slightly increased by butyrate treatment, but no differences in feed intake were observed because of butyrate supplementation, assuming the same gluconeogenic substrate supply in calves fed butyrate or not. Therefore, depressed plasma glucose because of butyrate feeding might result from impaired glucose uptake, but less is known as to whether butyrate affects lactose digestion and glucose absorption in pre-weaning calves.

Although growth performance was not affected by sex, female calves indicated greater plasma glucose, insulin, and glucagon concentrations and had a greater hepatic glycogen concentration than male calves. Sex effects on glucose metabolism in neonatal calves were also reported in previous studies (Sasaki et al., 2002; Schäff et al., 2016; Kesser et al., 2017). Interestingly, gene expression of *PYGL* and *G6PC* were greater in male than female calves. Obviously, greater endocrine glucose production occurs in male than in female calves, which compensates for the lower glucose status in male calves. However, further studies are needed to comprehend the sex differences in glucose metabolism in pre-weaning calves.

## 3.6 Conclusions

Ad libitum MR feeding for 8 wk resulted in elevated nutrient intake and accelerated growth but did not improve the feed conversion ratio. The metabolic and endocrine status mirrored the stimulation of anabolic processes to support body growth. Surprisingly and contrary to our hypothesis, the combination of an intensive milk-feeding program and allocation of butyrate with the MR did not result in an improvement in body growth beyond the growth stimulation owing to ad libitum MR feeding. In our study, butyrate was not able to provide additional stimulation on body growth, either in the ad libitum or in the restrictive MR feeding groups.

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# 4 Publication 2

## Influence of ad libitum milk replacer feeding and butyrate supplementation on the systemic and hepatic insulin-like growth factor I and its binding proteins in Holstein calves

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## 4.1 Abstract

Ad libitum milk-feeding and butyrate (B) supplementation have the potential to stimulate postnatal growth and development in calves. The somatotropic axis is the main endocrine regulator of postnatal growth and may be affected by both ad libitum milk replacer (MR) feeding and B supplementation in calves. We hypothesized that ad libitum MR feeding and B supplementation stimulate systemic and hepatic insulin-like growth factor (IGF)-I and IGF binding proteins (IGFBP) in preweaning calves. Sixty-four (32 male, 32 female) Holstein calves were examined from birth until wk 11 of life. Calves received MR either ad libitum (Adl) or restrictively (6 L/d; Res). In each feeding group half of the calves received a MR with 0.24 % butyrate and the other half received same MR without butyrate. Ad libitum MR feeding was performed from d 4 until wk 8 of age. From wk 9 to 10, Adl and Res calves were gradually weaned and were fed 2 L/d until the end of the trial. Concentrate, hay, and water were freely available. Feed intake was measured daily and body weight weekly. Blood samples for analyzing plasma concentrations of glucose, insulin, IGF-I, and IGFBP-2, -3, and -4 were taken on d 1, 2, 4, and 7, then weekly or every other week (IGFBP) until wk 11 of life. Liver samples were taken on d 50 and at the end of the study (d 80) to measure gene expression of the growth hormone receptor 1A (*GHR1A*), *IGF1*, *IGFBP1 to 4,* and of the IGF Type 1 and insulin receptor in the liver. Intake of MR and body weight were greater, but concentrate intake was lower in Adl than in Res. Plasma concentrations of IGF-I and IGFBP-3 were greater and plasma concentration of IGFBP-2 was lower in Adl than in Res during the ad libitum milk feeding period. After reduction of MR in both groups to 2 L/d plasma concentrations of IGF-I and IGFBP-4 were lower and plasma concentration of IGFBP-2 was higher in Adl than in Res. Supplementation of B depressed plasma IGF-I from wk 1 to 4 and in wk 9. On d 50, mRNA abundance of the *GHR1A* and *IGF1* was greater and of *IGFBP2* mRNA was lower in Adl than in Res. At d 80, *IGFBP2* mRNA was greater in Adl than in Res and *IGFBP2* mRNA increased with B supplementation. Ad libitum MR feeding stimulated the systemic and hepatic IGF system and mirrored the greater growth rate during the ad libitum MR feeding, whereas butyrate supplementation partly reduced the systemic and hepatic IGF system.

Key words: calves, ad libitum feeding, butyrate, somatotropic axis

## 4.2 Introduction

Intensive milk or milk replacer (MR) feeding programs could improve energy and nutrient intake, body growth (Hammon et al., 2002; Jasper and Weary, 2002; Bartlett et al., 2006), and organ development in calves (Geiger et al., 2016; Schäff et al., 2016; Soberon and Van Amburgh, 2017). A greater growth and development early in life could potentially affect the lifetime performance of dairy cows (Bar-Peled et al., 1997; Bach, 2012; Van Amburgh and Soberon, 2013); in addition, these feeding programs might promote robustness during the critical time period before weaning (Ollivett et al., 2012; de Passillé et al., 2016; Khan et al., 2016). Intensive or ad libitum milk feeding, starting in the colostral period, may enhances the synthesis and release of IGF-I; IGF-I belongs to the somatotropic axis and is an important factor stimulating postnatal growth (Breier et al., 2000; Khan et al., 2011; Hammon et al., 2012). The postnatal interaction of growth hormone (GH) and IGF-I, together with its binding proteins (IGFBP), affect body growth and organ development in mammals (Etherton and Bauman, 1998; Breier et al., 2000), including the development of the mammary gland (Akers, 2006; Weaver and Hernandez, 2016) and immune function (Clark, 1997). Because the plasma concentrations of IGF-I and IGFBP depend on nutrient intake (Thissen et al., 1994; Savage, 2013), the increased protein and energy intake from elevated milk or MR feeding might affect the blood and tissue level of IGF-I and its binding proteins (Bartlett et al., 2006; Maccari et al., 2015; Schäff et al., 2016). Therefore, changes of the components of the somatotropic axis due to an intensive MR feeding program may at least partly explain the accelerated mammary gland development in preweaning calves (Geiger et al., 2016; Soberon and Van Amburgh, 2017).

Butyrate is known as a natural growth-stimulating substrate that enhances growth performance in young mammals and that has the potential to interfere with parameters of the somatotropic axis (e.g., by stimulation of GH and IGFBP release; Tsubaki et al., 2001; Guilloteau et al., 2010; Miletta et al., 2014). In preweaning calves, butyrate supplementation in MR increases BW, structural growth, and health and affects insulin-dependent glucose metabolism (Guilloteau et al., 2010; Górka et al., 2011; Kato et al., 2011). Recent findings in preweaning calves indicate that ad libitum MR feeding stimulates body growth and anabolic metabolism, but butyrate supplementation did not further improve postnatal growth either in ad libitum or restrictive MR-fed calves (Frieten et al., 2017). The objective of the present study was to verify the previously published effects on body growth due to ad libitum MR feeding and butyrate supplementation by

investigating the systemic and hepatic IGF-I system including IGFBP as main regulators of postnatal body growth in calves.

## 4.3 Materials and Methods

### 4.3.1 Animals, feeding, and diets

The animal experiment at the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Germany, was recently described in a companion paper (Frieten et al., 2017). The experimental procedures were performed in accordance with the German Animal Welfare Act (Federal Republic of Germany, 2014) and were approved by the relevant Department for Animal Welfare Affairs [Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany (23 177-07/G 13-20-069)].

Briefly, 64 Holstein calves (n = 32 each for male and female) were used from birth until 80 ± 2 (mean ± SD) days of life. After birth, the calves were brought to individual straw-bedded calf hutches. This husbandry lasted for 10 ± 3 d (mean ± SD) before the calves were finally housed in an open, straw-bedded stable. In each pen, young calves were separated from the older group for 2 to 3 wk, with an own self-feeding station and separate access to concentrate, hay, and water. The health of the calves was evaluated by daily rectal temperature measurement and navel and feces examination until 3 wk of age. Health maladies, such as diarrhea, pneumonia, and navel ill were documented and treated by a veterinarian. Clinical data from this study will be presented in a companion paper.

During the first 2 h after birth, all calves received 2.5 ± 0.09 kg (mean ± SD) of colostrum via bottle. Subsequently, the calves were allocated to one of the four feeding groups regarding their sex, birth weight, and cow parity to create equal groups. For the following five meals (until d 3 of life), calves were fed acidified transition milk (2 mL of acidifier/L milk, H. W. Schaumann GmbH, Pinneberg, Germany) with teat buckets either in amounts of 3 L per meal (Res; n = 32) or ad libitum (Adl; n = 32). From d 4 on, all calves were fed MR (12.5 % solids; 21.9 % CP, 18.6 % crude fat; Trouw Nutrition Deutschland GmbH, Burgheim, Germany) either without (B-) or with (B+) supplementation of calcium-sodium butyrate (0.24 % as fixed to the MR powder; Benelux GmbH, Amel, Belgium) in amounts of either 6 L/d (ResB-; ResB+; n = 16, respectively) or

ad libitum (maximum 25 L/d; AdlB-; AdlB+; n = 16, respectively). The ingredients and the chemical composition of the MR were presented in the companion paper (Frieten et al., 2017) and are given in the Table 3.1. The dose for butyrate supplementation in MR was adapted from the work of Górka et al. (2011) and the butyrate intake in ResB+ and AdlB+ is presented in the Results section. The MR allowance to all calves was reduced linearly between d 57 and 70, and thereafter calves continue to receive 2 L of MR/d until the end of the trial. In the calf hutches, feeding took place twice a day at 0700 and 1700 h. To guarantee ad libitum feed intake, buckets for Adl calves were maintained and calves had the chance to drink MR all the time. Buckets were refilled at noon, if necessary. Teat buckets were covered with a lid to ensure that no rain or dirt contaminated the milk. In the stable, feeding was conducted using automatic feeding systems for MR and concentrate (Förster-Technik GmbH, Engen, Germany). Milk replacer was fed in small portions, with a maximum of 2 L per meal for Res calves and 5 L per meal for Adl calves, followed by an off time of 2 h for Res calves and 30 min for Adl calves after the end of the meal. Water and hay were freely available, and concentrate as pelleted starter (Raiffeisen Waren-Zentrale Rhein-Main eG, Köln, Germany; Table 3.1) was offered ad libitum in the stable.

### 4.3.2 Measurement of performance data

During the period in the calf hutches, the daily milk and MR intake was documented by weighting residues with an electronic scale (Sartorius AG, Göttingen, Germany). In the stable, data of MR and concentrate intake were sent automatically from the automatic feeding system to the connected computer program (Förster-Technik GmbH). The nutrient compositions of MR and concentrate were analyzed by an accredited external laboratory (Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Speyer, Germany) according to the Weender standard procedure (Naumann and Bassler, 2004).

The birth weight was measured on a platform scale after the first colostrum intake. The exact amount of ingested colostrum was subtracted from the initial weight. The BW in wk 7 and 11 was recorded using a mobile scale (Tru-Test Ltd., Auckland, New Zealand). The ADG was calculated from BW with the precise number of days between weekly weight measurements.

### 4.3.3 Blood and liver sampling and analyses

Blood samples were taken from the jugular vein before first colostrum intake (d 1), 24 h after colostrum intake (d 2), before first MR intake (d 4), and then weekly from d 7 to 77, or once every other week from d 21 for IGFBP. All calves were fasted for at least 1 h before blood sampling. The blood was collected in evacuated tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) containing potassium-EDTA (1.8 mg/mL) to determine plasma concentrations of insulin (on d 49 and 77), IGF-I, and IGFBP-2, -3, and -4; and tubes containing sodium fluoride (2-4 mg/mL) and potassium oxalate (1-3 mg/mL) to determine plasma glucose concentration on d 49 and 77. Blood samples were cooled in ice water until centrifugation at 3,500 × *g* for 10 min at room temperature. The supernatants from plasma were pipetted into aliquots and stored at –20°C until analyzed.

The glucose concentration in blood plasma was analyzed spectrophotometrically (HORIBA ABX SAS, Montpellier, France) using the kit #A11A01667 (Axon Lab AG, Baden, Switzerland). Plasma concentration of insulin (#RIA-1257) was determined by RIA using kits from DRG Instruments GmbH (Marburg, Germany), which were adapted to bovine (Hammon et al., 2009). Intra- and interassay coefficients of variation for insulin were 3.7 and 5.5 %, respectively. Plasma concentration of IGF-I was measured by ELISA adapted from Daxenberger et al. (1998) using an anti-human IGF-I polyclonal antiserum (GroPep, Adelaide, Australia) that showed 100 % cross-reactivity to bovine IGF-I. For standard preparation recombinant human IGF-I (receptor grade, GroPep) was used and IGFBP were blocked with excess of human IGF-II (GroPep). Biotinyl human IGF-I was obtained from Ibt GmbH (Binzwangen, Germany). Intra- and interassay coefficients of variation were 6.4 and 9.1 %, respectively. Recoveries of three IGF-I concentrations added to different samples were 93.4 % ± 1.7, 90.8 % ± 1.8 and 90.8 % ± 2.7. The IGFBP-2, -3, and -4 were analyzed in plasma using quantitative Western ligand blot analysis, as previously described (Laeger et al., 2014; Schäff et al., 2016).

On d 50 ± 2 and 80 ± 2 (mean ± SD; d 80 females only), about 80 to 100 mg of liver tissue was collected from each calf by biopsy (modified from Swanson et al., 2000) with a Bard Magnum biopsy instrument and Bard Magnum core tissue biopsy needle (C.R. Bard Inc.; Covington, GA). The sample notch of a 12-gauge biopsy needle contained about 20 to 30 mg of liver tissue. To collect several biopsies while avoiding additional stress and abdominal pain for the calves, we used a Bard TruGuide coaxial biopsy needle that remained in the abdomen for the duration of biopsy. Liver samples of male calves on

d 80 ± 2 (mean ± SD) were collected after calves were harvested at the end of the study. Liver samples were flushed in ice-cold 0.9 % NaCl and frozen in liquid nitrogen. For further analysis, the liver tissue was pulverized in liquid nitrogen. The relative mRNA abundance of genes related to the IGF system was quantified as previously described (Saremi et al., 2012; Schäff et al., 2016). Primer sequences and PCR conditions for reference genes [hippocalcin-like 1 (*HPCAL1*), low-density lipoprotein 10 (*LRP10*), and RNA polymerase II (*POLR2A*)] and target genes [growth hormone receptor 1A *(GHR1A)*, IGF-I *(IGF1)*, IGFBP-1 *(IGFBP1)*, -2 *(IGFBP2)*, -3 *(IGFBP3)*, and -4 *(IGFBP4)*, IGF Type 1 receptor *(IGF1R)*, and insulin receptor *(INSR)*] were recently published (Kendall et al., 2003; Saremi et al., 2012; Schäff et al., 2016). The primer products were verified by sequencing applied with the BigDye Terminator v1.1 Cycle Sequencing kit and an ABI 3130 Genetic Analyzer (Thermo Fisher Scientific Inc., Waltham, MA). Real-time PCR was performed with the use of a LightCycler (F. Hoffman-La Roche AG, Basel, Switzerland); SYBR Green I (F. Hoffman-La Roche AG) was used as the fluorescent dye. Melting curve analysis and agarose gel electrophoresis were used to confirm the specificity of the PCR products. Quantification cycle values and amplification efficiencies obtained with the use of LinRegPCR version 2013.0 (Ruijter et al., 2013) were imported into qBASE+ version 2.6.1 (Biogazelle, Zwijnaarde, Belgium) for all subsequent calculations and quality controls (Schäff et al., 2016). The geometric mean of the reference gene abundances was applied for normalization. The data are presented as the ratio of the copy number of the respective gene of interest to the geometric mean of the reference gene abundance.

### 4.3.4 Statistical analyses

Data were evaluated by repeated-measures ANOVA using PROC MIXED in SAS for Windows (release 9.4; SAS Institute Inc., Cary, NC). The ANOVA model contained the fixed effects of feeding regimen (milk; Res vs. Adl), butyrate supplementation, time (wk 1-7 and 8-11 for performance data; d 49 and 77 for BW and for plasma glucose and insulin; d 50 and 80 for tissue data; time of frequent blood samples for plasma IGF-I and IGFBP), sex, and respective interactions. Repeated measures on each calf were considered using the repeated statement of the MIXED procedure with an unstructured type of the block diagonal residual covariance matrix structure (plasma IGF-I and IGFBP) or with an autoregressive residual covariance structure (performance data, plasma glucose and insulin, gene expression data; SAS Institute Inc.). Least squares means (LSM) and their

standard errors (SE) were computed for each fixed effect, and all pair-wise differences of LSM were tested with the Tukey-Kramer procedure. The SLICE statement of the MIXED procedure was used to conduct partitioned analyses of the LSM for interactions. Differences in data with *P* values < 0.05 were defined as significant, and *P* values < 0.1 were considered as trends. Values are presented as LSM ± SE if not declared otherwise in the text.

## 4.4 Results

### 4.4.1 Feed intake and growth performance

Data for feed intake and growth performance are presented in Table 4.1 and focused on the time periods of wk 1 to 7 (before MR reduction) and 8 to 11 (MR reduction), respectively. In both time periods, milk intake was greater in Adl than in Res calves (*P* < 0.001). On the contrary, concentrate intake in wk 8 to 11 was greater in Res than in Adl calves (*P* < 0.001). Total DMI in wk 1 to 7 was greater (*P* < 0.001) in Adl than in Res calves. The DMI was not affected by butyrate supplementation and by sex. In B-supplemented groups, B intake was greater (*P* < 0.001) in AdlB+ than in ResB+. Butyrate intake was 3.3 ± 0.1 g/d for wk 1 to 7 and 2.6 ± 0.09 g/d for wk 8 to 11 in AdlB+, and was 1.7 ± 0.1 g/d for wk 1 to 7 and 1.1 ± 0.09 g/d for wk 8 to 11 in ResB+.

The BW was greater (*P* < 0.001) at the end of wk 7 (d 49) and at the end of wk 11 (d 77) in Adl than in Res (Table 4.1). The ADG was higher from wk 1 to 7 (*P* < 0.001) but lower from wk 8 to 11 in Adl than Res groups (*P* < 0.001). Body weight (*P* < 0.06) and ADG (P = 0.1) at wk 11 tended to be higher in B- than B+ calves (Table 4.1).

Table 4.1. Dry matter intake of liquid and concentrate feed, BW, and ADG of calves fed milk and milk replacer (MR) either ad libitum or restrictively and supplemented MR without (ResB-; AdlB-) or with 0.24 % butyrate (ResB+; AdlB+)[1]

| | Dietary treatment | | | | | Fixed effect, *P*-value[3] | | | |
|---|---|---|---|---|---|---|---|---|---|
| Item[2] | ResB- | AdlB- | ResB+ | AdlB+ | SEM | Milk<br>Milk × time | Butyrate<br>Milk × butyrate | Time<br>Butyrate × time | Sex |
| Liquid intake, kg | | | | | | | | | |
| wk 1 – 7 | 34.5[b] | 68.8[a] | 34.9[b] | 65.8[a] | 1.36 | < 0.001 | 0.7 | < 0.001 | 0.8 |
| wk 8 – 11 | 12.9[b] | 29.9[a] | 13.1[b] | 30.2[a] | | < 0.001 | 0.5 | 0.2 | |
| Concentrate intake, kg | | | | | | | | | |
| wk 1 – 7 | 6.85 | 1.89 | 8.93 | 3.11 | 2.07 | < 0.001 | 0.6 | < 0.001 | 0.4 |
| wk 8 – 11 | 39.7[a] | 18.6[b] | 38.1[a] | 20.9[b] | | < 0.001 | 0.7 | 0.6 | |
| Total DMI, kg | | | | | | | | | |
| wk 1 – 7 | 41.4[b] | 70.6[a] | 43.8[b] | 68.9[a] | 2.44 | < 0.001 | 0.8 | < 0.001 | 0.4 |
| wk 8 – 11 | 52.6 | 48.5 | 51.2 | 51.0 | | < 0.001 | 1.0 | 0.9 | |
| BW, kg | | | | | | | | | |
| d 49 | 72.7[b] | 92.5[a] | 70.9[b] | 88.9[a] | 2.30 | < 0.001 | 0.11 | < 0.001 | 0.12 |
| d 77 | 106.2[b] | 120.6[a] | 102.0[b] | 115.7[a] | | < 0.001 | 0.8 | 0.12 | |
| ADG, g | | | | | | | | | |
| wk 1 – 7 | 572.6[b] | 1015.1[a] | 591.9[b] | 941.8[a] | 37.0 | < 0.01 | 0.2 | < 0.001 | 0.2 |
| wk 8 – 11 | 1212.1[a] | 997.6[b] | 1121.0[ab] | 966.4[b] | | < 0.001 | 0.8 | 0.4 | |

[a,b]Different letters within the same row indicate significant differences ($P \leq 0.05$).
[1]Modified from Frieten et al., 2017.
[2]Values are presented as LSM.
[3]Main fixed effects are presented in two rows: first row indicates *P*-values for milk (ad libitum versus restrictive), butyrate supplementation, time, and sex; second row indicates *P*-values for interaction milk × time, milk × butyrate, and butyrate × time.

### 4.4.2 Plasma concentrations of glucose, insulin, IGF-I, and IGF binding proteins

The results of glucose and insulin concentrations in blood plasma are presented in detail in the companion paper (Frieten et al., 2017). Plasma glucose and insulin concentrations decreased in Adl calves but increased in Res calves from d 49 to 77 ($P < 0.001$; Table 4.2). Plasma glucose concentration was higher on d 49 ($P < 0.001$) in Adl than in Res calves, but was lower on d 77 ($P < 0.05$) in Adl calves than in ResB-. Plasma insulin concentration was much higher in Adl than in Res calves at d 49 ($P < 0.001$), but did not differ between Adl and Res calves on d 77 of age.

The plasma concentration of IGF-I slightly increased ($P < 0.05$) from d 1 to 2 and decreased ($P < 0.001$) from d 4 to 14 of age in all groups. Plasma IGF-I increased ($P < 0.001$) in Adl groups from wk 3 on, but in Res calves increased from wk 7 on ($P < 0.001$; Figure 4.1A). We noted a distinct decrease of plasma IGF-I in Adl calves from wk 9 to 10 ($P < 0.001$).

Table 4.2. Blood plasma concentrations of glucose and insulin in calves fed milk and milk replacer (MR) either ad libitum or restrictively and supplemented MR without (ResB-; AdlB-) or with 0.24 % butyrate (ResB+; AdlB+)[1]

| Item[2] | Dietary treatment | | | | SEM | Fixed effect, *P*-value[3] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ResB- | AdlB- | ResB+ | AdlB+ | | Milk<br>Milk × time | Butyrate<br>Milk × butyrate | Time<br>Butyrate × time | Sex |
| Glucose, mmol/L | | | | | | | | | |
| d 49 | 5.07[b] | 5.79[a] | 4.99[b] | 6.13[a] | 0.18 | 0.3 | 0.8 | 0.01 | 0.7 |
| d 77 | 5.66[a] | 4.84[b] | 5.39[ab] | 4.97[b] | 0.18 | < 0.001 | 0.14 | 0.3 | |
| Insulin, µg/L | | | | | | | | | |
| d 49 | 0.34[b] | 1.24[a] | 0.28[b] | 1.19[a] | 0.17 | < 0.01 | 0.8 | 0.03 | 0.3 |
| d 77 | 0.58 | 0.39 | 0.54 | 0.41 | 0.17 | < 0.001 | 0.9 | 0.8 | |

[ab]Different letters within the same row indicate significant differences ($P \leq 0.05$).
[1]Modified from Frieten et al., 2017.
[2]Values are presented as LSM.
[3]Main fixed effects are presented in two rows: first row indicates *P*-values for milk (ad libitum versus restrictive), butyrate supplementation, time, and sex; second row indicates *P*-values for interaction milk × time, milk × butyrate, and butyrate × time.

Plasma IGF-I was higher from wk 1 to 9 ($P < 0.01$), but was lower ($P = 0.02$) at wk 11 in Adl than in Res calves. Butyrate feeding depressed plasma IGF-I from wk 1 to 4 and at wk 9 ($P < 0.05$). Plasma IGFBP-2 concentration decreased ($P < 0.001$) during first week of age and then increased ($P < 0.01$) until wk 2 in all groups (Figure 4.1B). Thereafter, plasma IGFBP-2 remained high in Res calves, but decreased ($P < 0.1$) and remained low until wk 9 in Adl calves. Plasma IGFBP-2 concentration significantly differed from wk 3 until the end of the trial, with higher concentrations in Res calves up to wk 9. At wk 11, plasma IGFBP-2 decreased ($P < 0.1$) in Res calves and increased ($P < 0.001$) in Adl calves, indicating higher concentrations in Adl than Res calves at wk 11 ($P < 0.01$).

Plasma IGFBP-3 concentration decreased ($P < 0.001$) during first week of age in all groups, and was greater ($P < 0.01$) in Adl than in Res calves from wk 3 to 9 (Figure 4.1C). We observed a trend for a greater IGFBP-3 plasma concentration at wk 11 in Adl than Res calves ($P = 0.07$). Calves fed butyrate had a lower IGFBP-3 plasma concentration on d 1 and 7 of age ($P \leq 0.05$). Plasma IGFBP-4 concentration increased ($P < 0.001$) after birth, but decreased ($P < 0.001$) from d 4 to 21 in all groups (Figure 4.1D) and was greater ($P < 0.05$) in Res than in Adl calves on d 1, 63, and 77 of age.

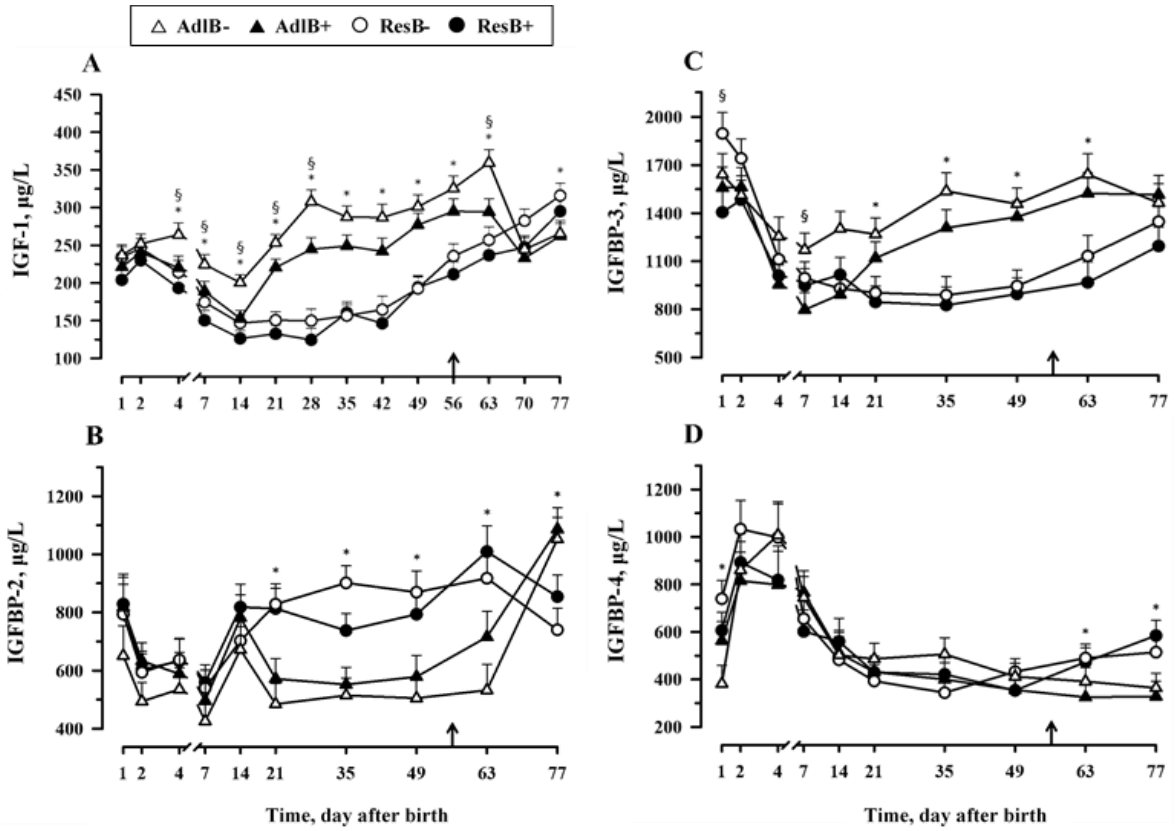


Figure 4.1. Blood plasma concentrations of IGF-I (A), IGF binding protein (IGFBP)-2 (B), IGFBP-3 (C), and IGFBP-4 (D) in calves fed milk and milk replacer (MR) either ad libitum or restrictively and supplemented MR without (△ AdlB-; ○ ResB-) or with 0.24 % butyrate (▲ AdlB+; ● ResB+). Arrows mark the start of weaning. Data are presented as LSM ± SEM; * indicates the effect of feeding regimen ($P < 0.05$); § indicates the effect of butyrate supplementation ($P < 0.05$).

### 4.4.3 Hepatic gene expression related to the IGF system

The hepatic gene expression of *GHR1A*, *IGF1*, *IGFBP1*, *-2*, *-3*, and *-4*, *IGF1R*, and *INSR* are presented in Table 4.3. The mRNA abundance of *GHR1A* and *IGF-I* on d 50 was greater ($P < 0.02$) in Adl than in Res calves, with highest abundance for *IGF1* in AdlB-, but did not differ on d 80. The abundance of *IGF1* mRNA was greater ($P = 0.03$) in male than in female calves. Male calves had a greater ($P < 0.001$) *IGFBP1* mRNA abundance and tended to have a greater (P = 0.07) *IGFBP3* mRNA abundance than female calves. The mRNA encoding for *IGFBP2* changed with time ($P = 0.02$), and was greater ($P < 0.001$) on d 50 but was lower ($P = 0.01$) on d 80 in Res than in Adl calves. On d 50, *IGFBP2* mRNA was greatest ($P < 0.05$) in ResB+ and on d 80 butyrate supplementation affected *IGFBP2* mRNA, with greatest expression in AdlB+. The abundance of *IGFBP4* mRNA increased with time ($P < 0.01$) in all feeding groups. The mRNA abundance of *IGF1R* tended (P = 0.07) to be greater in Res than in Adl calves on d 50, and the mRNA abundance of *INSR* showed a tendency (P = 0.06) for the butyrate × time interaction with an increasing abundance from d 50 to 80 in butyrate-fed calves.

Table 4.3. Relative mRNA expression ($log_2$) of liver samples in calves fed milk and milk replacer (MR) either ad libitum or restrictively and supplemented MR without (ResB-; AdlB-) or with 0.24 % butyrate (ResB+; AdlB+)

| Relative mRNA expression related to reference genes [1,2] | Dietary treatment | | | | SEM | Fixed effect, *P*-value[3] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | ResB- | AdlB- | ResB+ | AdlB+ | | Milk<br>Milk × time | Butyrate<br>Milk × butyrate | Time<br>Butyrate × time | Sex |
| *GHR1A* | | | | | | | | | |
| d 50 | 0.55 | 0.69 | 0.57 | 0.73 | 0.06 | 0.03 | 0.4 | 0.9 | 0.3 |
| d 80 | 0.59 | 0.63 | 0.63 | 0.68 | 0.06 | 0.2 | 0.9 | 0.8 | |
| *IGF1* | | | | | | | | | |
| d 50 | 15.3$^{b}$ | 24.9$^{a}$ | 16.3$^{b}$ | 21.0$^{ab}$ | 2.22 | 0.15 | 0.6 | 0.2 | 0.03 |
| d 80 | 24.6 | 18.3 | 20.5 | 21.6 | 2.20 | < 0.01 | 0.7 | 0.7 | |
| *IGFBP1* | | | | | | | | | |
| d 50 | 228 | 239 | 342 | 372 | 157 | 0.3 | 0.2 | 0.2 | < 0.001 |
| d 80 | 361 | 389 | 331 | 655 | 125 | 0.5 | 0.4 | 1.0 | |
| *IGFBP2* | | | | | | | | | |
| d 50 | 7.07$^{ab}$ | 4.25$^{b}$ | 7.86$^{a}$ | 4.21$^{b}$ | 0.90 | 0.7 | 0.09 | 0.02 | 0.7 |
| d 80 | 4.98$^{b}$ | 7.02$^{ab}$ | 6.78$^{ab}$ | 9.92$^{a}$ | 1.00 | < 0.001 | 0.9 | 0.07 | |
| *IGFBP3* | | | | | | | | | |
| d 50 | 3.65 | 3.92 | 4.22 | 4.06 | 0.46 | 0.5 | 0.4 | 0.2 | 0.07 |
| d 80 | 3.19 | 3.91 | 3.73 | 3.95 | 0.34 | 0.3 | 0.5 | 0.9 | |
| *IGFBP4* | | | | | | | | | |
| d 50 | 2.71 | 2.81 | 2.69 | 2.71 | 0.22 | 0.5 | 0.9 | < 0.01 | 0.6 |
| d 80 | 2.90 | 3.15 | 3.03 | 3.20 | 0.26 | 0.6 | 0.9 | 0.6 | |
| *IGF1R* | | | | | | | | | |
| d 50 | 2.74 | 2.69 | 4.09 | 2.54 | 0.43 | 0.2 | 0.2 | 0.5 | 0.6 |
| d 80 | 3.08 | 2.98 | 3.43 | 3.17 | 0.42 | 0.2 | 0.2 | 0.5 | |
| *INSR* | | | | | | | | | |
| d 50 | 6.43 | 6.53 | 5.56 | 5.50 | 0.61 | 0.7 | 0.5 | 0.3 | 0.9 |
| d 80 | 6.50 | 5.91 | 6.62 | 6.41 | 0.58 | 0.5 | 0.9 | 0.06 | |

[a,b]Different letters within the same row indicate significant differences ($P \leq 0.05$).

[1]Values are presented as LSM.

[2]*GHR1A* = growth hormone receptor 1A; *IGFBP1 – 4* = IGF binding protein-1 to -4; *IGFR1* = IGF-I receptor; *INSR* = insulin receptor.

[3]Main fixed effects are presented in two rows: first row indicates *P*-values for milk (ad libitum versus restrictive), butyrate supplementation, time, and sex; second row indicates *P*-values for interaction milk × time, milk × butyrate, and butyrate × time.

## 4.5 Discussion

Calves with unlimited milk and MR intake for 8 wk postnatal more or less doubled their MR intake compared with Res calves fed 6 L of MR/d. Such a great liquid feed intake was previously reported in other studies with dairy calves that were allowed to drink milk or MR ad libitum for a distinct time period after calving (Hepola et al., 2008; Maccari et al., 2015; Korst et al., 2017). On the other hand, concentrate intake was lower in Adl than in Res calves and the increase in concentrate intake was delayed in Adl calves (Frieten et al., 2017). An impaired concentrate intake in calves due to unrestricted milk feeding (Jasper and Weary, 2002) or enhanced MR feeding (Kristensen et al., 2007; Davis Rincker et al., 2011) was previously observed in some, but not in all studies (Maccari et al., 2015; Schäff et al., 2016; Korst et al., 2017). In addition, calves receiving elevated amounts of milk, but no ad libitum milk feeding, during the first weeks of life had a greater concentrate intake after the intensive milk feeding period and after weaning (Khan et al., 2007). Therefore, the management of the intensive milk feeding period might have an important effect on the concentrate intake in preruminant calves. In any case, ad libitum milk feeding (Maccari et al., 2015; Schäff et al., 2016; Korst et al., 2017) or enhanced milk feeding programs using MR with a greater CP content (Diaz et al., 2001; Bartlett et al., 2006; Khan et al., 2011) resulted in an elevated body growth during the preweaning period in calves. On the other hand, the supplementation of butyrate with the MR did not affect MR or concentrate intake in a consistent manner (Frieten et al., 2017).

The enhanced growth in Adl calves during the ad libitum milk feeding period coincided with elevated plasma concentrations of insulin, IGF-I, and IGFBP-3 and reduced plasma concentrations of IGFBP-2 during this time period. The stimulation of the postnatal somatotropic axis depends on the nutrient supply, starting in the neonatal period (Breier et al., 2000; Hammon et al., 2012; Savage, 2013), and reflects the glucose and insulin status of the animal (Breier et al., 1988; Thissen et al., 1994; Brameld et al., 1999). The nutrient intake was much greater in Adl than in Res calves during the ad libitum milk feeding period. Elevated nutrient intake affects the plasma IGF-I concentration in mammalian (Thissen et al., 1994; Savage, 2013), including the preweaning calf (Smith et al., 2002; Daniels et al., 2008) and ad libitum milk feeding (Maccari et al., 2015; Schäff et al., 2016). The stimulating effect of elevated nutrient supply by milk or MR intake on the IGF-I plasma concentration starts postnatum (Hammon and Blum, 1997; Hammon et al., 2000;

Sauter et al., 2003) and is related to the greater nutrient availability due to milk feeding (Breier et al., 1988).

The stimulation of the somatotropic axis due to ad libitum milk feeding in the present study was supported by the elevated hepatic gene expression of the liver-specific *GHR1A* and *IGF1*. Both were higher expressed in liver of Adl than Res calves at d 50 of age. The IGF-I originating from the liver contributes in a significant manner to the IGF-I in the blood plasma (Le Roith et al., 2001; Savage, 2013), hepatic *IGF1* gene expression correlated with IGF-I in blood plasma of calves (Cordano et al., 2000), and its gene expression depends on the GH action in the liver (Le Roith et al., 2001; Renaville et al., 2002; Savage, 2013). The elevated glucose and insulin status at the time of ad libitum milk feeding may have stimulated *GHR1A* gene expression in the liver of Adl calves, as insulin treatment promotes hepatic *GHR1A* mRNA abundance in dairy cows (Butler et al., 2003; Weber et al., 2017).

The reduced insulin and IGF-I concentrations in blood plasma after the MR reduction period reflected the lower concentrate intake in Adl calves (Frieten et al., 2017). The gene expression of *IGF1* as well as *GHR1A* in liver was not affected by the nutrient intake on d 80 of age in a significant manner. The increase in hepatic *IGF1* mRNA from d 50 to 80 in Res, but not in Adl calves, indicated that Res calves gained on nutrient intake with time due to elevated concentrate intake; Adl calves reached an elevated *IGF1* gene expression level on d 50 of age and did not further increase *IGF1* mRNA abundance in liver on d 80 of age. The differences in concentrate intake at the end of the study between groups (Frieten et al., 2017) were, however, not large enough to cause changes in hepatic *GHR1A* and *IGF1* mRNA abundances between groups, but the greater concentrate intake in Res calves at the end of the study resulted in a greater plasma IGF-I concentration. Therefore, tissues other than liver that synthesize IGF-I (e.g., muscle tissue) may contribute to the elevated plasma IGF-I concentrations in Res calves on d 80 of age (Le Roith et al., 2001).

The IGFBP-3, which is part of the 150-kDa complex and binds most of the IGF-I in blood plasma (Jones and Clemmons, 1995; Murphy, 1998), did not show an association between hepatic gene expression and plasma concentration at d 50 and 80 of age. In general, IGFBP are important regulators of the IGF-I action, and inhibitory and stimulating effects of IGFBP have been reported (Jones and Clemmons, 1995; Le Roith et al., 2001;

Savage, 2013). After a decrease during the first week of postnatal life, the plasma IGFBP-3 concentration increased with age during the MR feeding period (Skaar et al., 1994; Hammon et al., 2003; Schäff et al., 2016). The increase of plasma IGFBP-3 was more dominant in Adl than in Res calves, and the dynamics of plasma IGFBP-3 concentration were similar to those of plasma IGF-I during the ad libitum milk feeding period. Although plasma GH was not measured in our study, it is well known that the IGFBP-3 status depends on GH action (Jones and Clemmons, 1995; Murphy, 1998; Savage, 2013). Obviously, GH does not directly stimulate *IGFBP3* gene expression, because *IGFBP3* gene expression is located in non-parenchymal hepatic cells, but not in hepatocytes, and these cells do not express GH receptors (Murphy, 1998; Le Roith et al., 2001). However, both GH and IGFBP-3 are stimulated by enhanced nutrient intake in calves and growing ruminants (Renaville et al., 2002; Daniels et al., 2008). The fact that *IGFBP3* gene expression in liver did not correspond to IGFBP-3 plasma concentration is probably due to IGFBP-3 synthesis in other tissues than the liver (e.g., fibroblast, endothelial cells, bone; Jones and Clemmons, 1995), and possibly due to the low number of cells in the liver (non-parenchymal cell) with *IGFBP3* gene expression (Murphy, 1998; Le Roith et al., 2001). In addition, the IGFBP-3 plasma concentration depends on the proteolytic cleavage activity in the blood plasma and is probably less regulated at the transcriptional level (Jones and Clemmons, 1995). Overall, the greater plasma IGFBP-3 concentration in Adl calves corresponded to the elevated IGF-I concentration in these calves and mirrored their greater nutrient intake, but was not related to the hepatic *IGFBP3* gene expression.

The IGFBP-2 status in the calves indicated corresponding changes of plasma concentration and hepatic gene expression with respect to nutrient intake. The concentration of IGFBP-2 in blood plasma and liver was lower in the Adl than in the Res calves during the ad libitum milk feeding period, but increased to a higher level in Adl calves at the end of the study when concentrate intake was greater in Res than Adl calves. The plasma IGFBP-2 concentration indicated an inverse relationship with respect to the insulin and IGF-I status in calves (Hammon and Blum, 1997; Hammon et al., 2000; Daniels et al., 2008) and dairy cows (McGuire et al., 1995), and IGFBP-2 is elevated during the catabolic state (Thissen et al., 1994; Breier et al., 2000; Renaville et al., 2002). Therefore, elevated plasma IGFBP-2 concentration may reflect the inadequate nutrient supply in Res calves during the ad libitum MR feeding period and the impaired nutrient status in Adl calves after the MR step-down period, which corresponds to findings in humans (Savage, 2013). Surprisingly, hepatic *IGFBP1* gene expression did not behave in the same way as hepatic *IGFBP2* gene

expression, but elevated IGFBP-1 status during impaired nutrient supply was shown in human patients (Thissen et al., 1994; Savage, 2013) and in neonatal calves (Sauter et al., 2003).

Plasma concentration of IGFBP-4 increased from d 1 to 2 of life, as recently reported in calves (Schäff et al., 2016), but decreased thereafter and remained more or less constant in all calves until d 63 of age, indicating no effect of ad libitum MR feeding on plasma IGFBP-4; this was in contrast to previous findings (Schäff et al., 2016), but in agreement with findings of Daniels et al. (2008) in calves. In addition, ad libitum MR feeding did not affect hepatic *IGFBP4* gene expression, but *IGFBP4* gene expression increased from d 50 to 80 of age in all calves. A corresponding increase of plasma IGFBP-4 during the last weeks of the study was only seen in Res calves. The IGFBP-4 is one of the smaller IGFBP, with a significant plasma concentration that is decreased by IGF-I and inhibits IGF-I action (Clemmons, 1997; Duan, 2002; Firth and Baxter, 2002). Therefore, the simultaneous increase of IGFBP-4 and IGF-I in blood plasma at the end of the study in Res calves may indicate a modulation of the IGF-I activity by IGFBP-4. In addition, the MR feeding levels did not affect hepatic gene expression of *IGF1R* and *INSR* in the current trial.

Ad libitum MR-fed calves supplemented with butyrate partly indicated a lower plasma IGF-I concentration. The body growth was not stimulated by butyrate, in contrast to the finding of Górka et al. (2011), who reported greater BW in calves fed MR supplemented with butyrate. A recent review of studies with butyrate treatment in preweaning calves indicated inconsistent effects on body growth when butyrate was supplied by MR (Niwińska et al., 2017). Reasons for these different findings are not obvious, but the age of the calf when starting the butyrate supplementation could be of importance (Niwińska et al., 2017). Hepatic gene expression of *GHR1A* and *IGF1* did not respond to butyrate supplementation in a consistent manner, but *IGFBP2* gene expression was elevated and *INSR* gene expression increased considerably in butyrate-treated groups at the end of the study. These changes on the parameters of the somatotropic axis due to butyrate supplementation may suggest that nutrient supply in butyrate-treated calves was impaired (Thissen et al., 1994; Breier et al., 2000; Renaville et al., 2002). Reasons for these findings are rather speculative; because butyrate treatment did not result in elevated plasma butyrate concentration in these calves (Frieten et al., 2017), a direct effect of butyrate on the GH-IGF-axis is not likely, as described in previous studies (Tsubaki et al., 2001; Miletta et al., 2014). Studies in calves and heifers could not find a stimulating effect of

butyrate on plasma GH and IGF-I concentrations (Nosbush et al., 1996; Kato et al., 2011). In agreement with the findings on the somatotropic axis, the results on growth performance in the current study do not support data from literature that propose an improved growth rate in calves after butyrate feeding (Guilloteau et al., 2010), but do support previous findings on the lack of a growth promoting effect due to butyrate feeding in calves (Kato et al., 2011). However, intestinal mucosa growth was stimulated by butyrate supplementation in the male calves of the current study (Gerbert et al., 2017). Interestingly, this local growth-promoting effect in the small intestine did not cause an enhanced body growth in the calves of the present study.

We found no sex effects on DMI in these calves, and sex effects on growth performance were of minor importance. The lack of sex effects on plasma IGF-I concentration fits into this context. The lack of gender effects on growth performance and plasma IGF-I was recently described in another study of our laboratory (Schäff et al., 2016), but disagrees with earlier findings of sex effects on growth and plasma IGF-I in calves (Kerr et al., 1991; Kertz et al., 1997; Egli and Blum, 1998). However, main sex effects on growth and plasma IGF-I as well as plasma IGFBP-3 may occur when calves are older than 12 wk (Govoni et al., 2003). In this context, it was surprising to find a greater hepatic gene expression of *IGF1*, *IGFBP1*, and *IGFBP3* in male than female calves, which corresponds to the overall picture of a greater somatotropic activity in males than in females (Gatford et al., 1998). Female gonadal steroids, such as estrogen, inhibit gene expression of *IGF1* and *IGFBP* in liver (Leung et al., 2004) and may explain the lower hepatic gene expression of *IGF1* and *IGFBP1* and *IGFBP3* in female calves; however, the interaction of gonadal sex steroids and the somatotropic axis might become more clear in older calves.

## 4.6 Conclusions

Feeding milk and MR ad libitum for the first 8 wk of life affected IGF-I and IGFBP plasma concentration and hepatic gene expression. These changes mirrored the greater growth rate in Adl calves during the ad libitum MR feeding. At the end of the study, the greater IGF-I and lower IGFBP-2 in blood plasma in Res calves corresponded with the greater ADG in these calves. Butyrate supplementation did not stimulate growth performance but partly depressed the IGF-I status in the calves. As a consequence, the combination of an ad libitum milk-feeding program and allocation of butyrate with the MR did not result in an additional stimulation of IGF-I and IGFBP in calves.

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# 5 General Discussion

The advantages of enhanced growth performance and on metabolism, further the contributions to well-being, development, and long-lasting productivity, became clear in intensive milk or MR fed calves (Kertz et al., 2017). The feed additive butyrate demonstrated an improved development in growing animals when it was used in artificial systems with MR feeding. In several studies butyrate additionally enhanced the development, integrity, and efficiency of the GIT, and achieved an improved growth rate (Guilloteau et al., 2009; Kato et al., 2011; Górka et al., 2014).

The effects of the main objectives in the presented thesis (i.e., ad libitum feeding and butyrate supplementation) were evaluated, while the used calves were generally healthy and vital throughout the study (Gerbert, Frieten, and Hammon, unpublished data).

Calves have the greatest chances of survival if they are born in eutocia without assistance in a clean environment and are provided high-quality colostrum as soon as possible. We used a birth monitoring system to observe every calving process of which 25 % occurred at night (i.e., between 2000 to 0400 h). Through applying this method in practice a sufficient colostrum feeding, which relies on three major aspects: time, quantity and quality (McGuirk and Collins, 2004), could be ensured for the tested calves. All calves received sufficient amounts of Ig and no cases of FPT were observed (Gerbert, Stefaniak, and Hammon, unpublished observations). In addition and as stated above, colostrum also provides high amounts of nutrients and non-nutrient, biologically active factors (Blum and Hammon, 2000; Blum, 2006; Ontsouka et al., 2016). Subsequent colostrum milkings, after the first milking also referred to as transition milk, were offered for the first 3 d after birth. In a trial from Hammon and Blum (1998) calves fed six times colostrum instead of one colostrum meal or no colostrum had significant improved anabolic parameters.

Improved energy and nutritional supply in terms of intensive or ad libitum milk or MR feeding enhances growth performance, anabolic metabolism and development in calves. In our study Adl calves were provided with milk or MR up to 25 L/d. This was the highest adjustable amount for the automatic feeder. Although two calves were limited onetime by this volume when they reached 25 L intake per day, for all the other days and calves this method can be referenced as an authentic ad libitum feeding. With MR intakes of 13 L/d during wk 4 to 9 of life, our calves consumed almost 30 % of their birth BW, which is three times more than in conventional feeding recommendations (FAWC, 2015).

In practice, some general concerns in addition to incremental effort and expense, question the benefit of ad libitum feeding of calves. For example, intensive liquid feeding leads to diarrhea. In some trials a softer fecal consistency without impact on medical treatment were seen (Raeth-Knight et al., 2009; Davis Rincker et al., 2011; Liang et al., 2016). Hence, this is more likely a result of the enhanced fluid intake and probably lower fiber consumption than of pathogenic infections. Another concern is that greater liquid meals could exceed the volume of the abomasum and reflow into the rumen (Schwarz, 2011). Typical symptoms (e.g., depression, anorexia, abdominal pain, etc.) may occur similar to the ruminal drinking syndrome, due to rumen acidosis and dyskeratosis (Dirksen, 2002). None of this was seen in our trial or in the study of Ellingsen et al. (2016), whereas milk meals up to 6.8 L did not enter the rumen or indicate medical conditions which is the result of a greater distension of the abomasum. Moreover, MacPherson et al. (2016) found that an elevated MR intake resulted in slower abomasal emptying, and therefore an adapted nutrient supply to the small intestine.

As the voluntary liquid feed intake doubles compared to restrictive feedings, our results are in accordance with earlier ad libitum studies (Borderas et al., 2009; Miller-Cushon et al., 2013; Maccari et al., 2015). The use of automatic feeders where fresh meals are supplied in small amounts throughout the day could be a reason for the reported higher MR intake (Borderas et al., 2009; Miller-Cushon et al., 2013) compared to ad libitum milk intake by teat feeding (Appleby, 2001; Jasper and Weary, 2002). Furthermore, a longer ad libitum feeding period in the latter studies (i.e., 7 vs. 4 wk) may contribute to higher intakes, as Adl calves in our study reached their highest MR intake in wk 8 and 9 of life. A significantly greater milk intake began in the first week of age. Calves fed intensive diets beginning after 1 wk postnatum (Rosenberger et al., 2017) had lower milk intakes than milk allowances (9.4 vs. 12.0 L/d, respectively). Conclusively, calves fed intensive or ad libitum diets from birth appear better adapted to high quantities and may consume more milk or MR and have lesser feed refusals overall.

Planes of MR diets are often presented as kg/d, which is required to determine the nutrient and energy intake. In consideration of calf welfare by expressing their natural behavior it is equally important to prospectively determine the total amount of liquid feed so calves can satisfy their suckling need. To enable natural feeding and suckling behavior, best practice would be to feed the calves ad libitum milk or MR during the first weeks of life (Miller-Cushon and DeVries, 2015). And as we saw a steadily increasing MR intake up to wk 9

of life, duration of the intensive feeding period should be prolonged. In accordance with our results, Meale et al. (2017) proclaimed that the calf´s rumen is not mature enough for sole nutrient digestion before 8 wk of age. Nevertheless, the extended milk feeding period would continue to be an artificial feeding practice, as natural weaning occur around 10 month of age (Reinhardt and Reinhardt, 1981; Hulbert and Moisá, 2016).

New recommendations for feeding practices start to include intensive feeding at least, e.g., 20 % of BW for the first 28 days of life (Dairy Farmers of Canada, 2009). This new dietary recommendations become incorporated into practice. A recent Canadian survey (Medrano-Galarza et al., 2017) evaluated the offered plane of liquid feed averaged 10 L/d in automated milk fed calves. Interestingly, this amount was 2 L/d higher than in manual milk feeding systems. From personal experience, the amount of work involved in manual ad libitum feeding compared to restrictive feeding is equally achievable in the first days after birth. The prolonged ad libitum feeding was realized with automatic feeding systems that eased the effort of manpower. Hence, the use of technical improvements in calf feeding can be recommended for intensive MR feeding.

Hulbert and Moisá (2016) reviewed circumstances of very low body weight gain in the first two wk of age. When calves are fed limited amounts of milk or MR they often cannot maintain the energy requirements for thermogenesis (Hammon et al., 2012), maintenance and growth (Drackley, 2008), hence they lose or gain just little weight (Geiger et al., 2016). Negative ADG in the first week of life was shown when dairy calves fed restricted quantities of MR (Liang et al., 2016). In our study (1327.8 vs. 597.3 g/d ADG for Adl vs. Res in wk 1, respectively) and in studies from Maccari et al. (2015) and Schäff et al. (2016) distinct and significant weight gains were possible in the first weeks of life. Therefore, calves can metabolize high quantities of liquid feed and gain weight soon after birth when fed ad libitum or an enhanced plane of liquid feed compared to strongly limited-fed calves.

Adl compared to Res calves had a much higher body growth rate. The accelerated growth performance appeared with completely voluntary, and not forced, MR intake about twice as much in Adl than in Res, respectively. Our data match results from Davis Rincker et al. (2011) where calves fed with intensified MR rates (2.1 % of BW on DM basis) revealed significantly higher BW during wk 1 to 8 of life. Hence, the energy for growth and development after birth is mainly supported from liquid feed intake.

With the loss of maternal glucose supply, calves had low postnatal glucose levels. After colostrum intake, glucose concentrations increased for all calves with insignificant differences between groups. Significant differences, with higher glucose levels at d 7, and from wk 3 to 8, were seen in Adl groups as a result of elevated nutrient intake. Plasma insulin concentration followed the same metabolic pattern, with low levels in neonatal calves and greater levels ($P < 0.05$) from d 4 until wk 9 of age. Higher glucose and insulin blood concentrations might be considered physiological in growing animals and do not account for pathological states, as hyperglycemia and hyperinsulinemia. Although, Bach et al. (2013) investigated a decrease of insulin sensitivity in calves fed higher planes of MR (4 L/meal) twice daily. We observed insulin concentrations adapted to the glucose intake, but in accordance with MacPherson et al. (2016) we assume no decrease in insulin sensitivity.

Glucagon levels increased after colostrum intake as reported previously (Hammon et al., 2002, 2012). Interestingly, without evidence of insufficient glucose supply plasma glucagon concentrations peaked at d 4 in Adl, but decreased in Res. Hammon et al. (2013) assumed higher protein intakes accountable for increased glucagon levels. Plasma BHB concentrations were greater for Res compared to Adl ($P < 0.001$) indicating an accelerated rumen maturation. However, before significant CON intake Res calves had higher BHB levels on d 7 ($P < 0.01$) and d 21 ($P = 0.08$). This indicates hepatic ketogenesis due to malnutrition in these calves. During the first week postnatum and at specific time points throughout the trial, lactate and cortisol concentrations were greater in Res groups, respectively. Lactate serves as a substrate for GNG and hepatic uptake is stimulated by insulin (Donkin and Armentano, 1995), whereby cortisol levels stimulate GNG and might reflect lower feed intake (Hammon et al., 2002). Intensive feeding with higher fat intake is reflected by enhanced plasma triglycerides (Kühne et al., 2000). Greater triglyceride concentrations were seen on d 21 ($P = 0.01$) and d 28 ($P = 0.06$) in Adl groups. In addition, Res groups had greater NEFA levels on d 4 and d 21, indicating catabolic states with enhanced fat mobilization. Furthermore, it is worth mentioning that the specific peak in plasma NEFA immediately after birth, also affected the total bilirubin concentration in ResB- during the first week postnatum. This correlation was described by Hadorn et al. (1997) as a competition in substrate-binding to plasma albumin and conjugation of bilirubin in the liver which leads to elevated plasma bilirubin concentrations.

Surprisingly, no significant dietary treatment effects of the hepatic genes coding for the gluconeogenic enzymes were observed is this trial. Expression of G6Pase is described to be directly affected by increasing glucose concentrations (Van Schaftingen and Gerin, 2002). However, we only investigated time effects with greater expressions for every gene at the end of the trial, except from PC. The elevated abundance of genes related to the glucose metabolism from d 50 to 80 of life reveals the increasing impact of GNG on eGP. Adult cattle compared to neonates receive 90 % vs. 75 % of their glucose demands from GNG, respectively (Nafikov and Beitz, 2007). With decreasing nutrient supply of glucose and increasing solid feed intake and fiber carbohydrate digestion in the rumen, the enzymatic pathway, regulated by gene expression, accelerates in ruminating calves (Aschenbach et al., 2010).

The importance of maturation of the incipiently functioning somatotropic axis at birth has been reported (Hammon et al., 2002; Daniels et al., 2008; Brickell et al., 2009). The influence of sufficient nutrient and energy supply during the first weeks of age could be seen in our trial. Certain parameters like IGF-I and IGFBP-3 were enhanced in blood and liver in Adl calves, whereas IGFBP-2 was enhanced in Res groups. In accordance with the literature, this reflects the more mature somatotropic axis in Adl compared to Res calves and confirms the stimulating effect of intensive feeding (Breier et al., 2000; Renaville et al., 2002). Additionally, maturation is regulated due to increasing abundance of GHR (Smith et al., 2002; Sauter et al., 2003). We measured the liver-specific GHR1A that was enhanced in Adl groups.

Alongside higher quantities of liquid feed preweaning, the transition from liquid to solid feed is another important process as proven in many studies (Khan et al., 2011, 2016). The goal is to ease the transition from preruminants to functionally ruminants without metabolic deficiencies and body weight loss. This transition management might be more complex in intensively reared calves but data are inconsistent. Maccari et al. (2015) fed milk either ad libitum or restrictively for the first 3 wk of age, used a step-down weaning from wk 5 to 10, and reported even greater starter intake preweaning for the Adl group. In addition, ad libitum fed calves in the study from Schäff et al. (2016) tended to have greater concentrate intakes after MR step-down in wk 8 of age. To smooth the transition, as recommended in the review of Khan et al. (2011), we chose a gradual weaning over a two-week period with daily reduction of MR allowances at 7.2 % for Adl and 5.0 % for Res.

In a review from Costa et al. (2016), group-housed calves had increased solid feed intakes compared to single-housed calves. This is thought to be a reaction of mimicking feeding behavior of other calves. In our study, we group-housed the calves from wk 2 or 3 of age, but neither gradual weaning nor group housing could prevent Adl from distinct reduction in growth performance with specific declines in ADG, and anabolic metabolism and IGF-I axis (i.e., plasma concentrations of glucose, insulin, triglycerides, and IGF-I). While the latter parameters were decreased, plasma concentration and hepatic gene expression of IGFBP-2 increased in Adl after weaning. Therefore, the weaning period might still have been too short for a smooth transition and the group housing possibly was not so effective, because calves consumed concentrates from an automatic feeder with only one feeding place that was not visible for the other calves. For all mentioned parameters that were affected by weaning in Adl, at least a numerical improvement could be seen in wk 11, when all calves were fed 2 L MR/d. Overall, the deficiency in Adl occurred for about 2 wk when the calves were over 2-month old. This contrasts with Res who showed no impairment during weaning, but had deficiencies in anabolic parameters, as well as the systemic and hepatic IGF-I and IGFBP status, and body weight gain from wk 1 to 9, and wk 1 to 6 of age, respectively.

A smoother transition approach might not be linked to a specific time but rather to individual animal recordings of solid feed intake. Weaning can be realized when calves consume > 1 kg of solid feed per day (FAWC, 2015). This recommendation occurred for 6-wk old calves, whereas Res and Adl groups failed to reach this amount before wk 9 and wk 10 to 11 of age, respectively. The successful weaning reported from Maccari et al. (2015) is possibly connected to the longer individual housing (3 wk) and an extended weaning (6 wk) period in intensively reared calves. Moreover, the relative short ad libitum milk feeding period of 3 wk postnatum might be another reason for the elevated starter intake. Nevertheless and as stated before, rumen maturation is inadequate before 8 wk of age (Meale et al., 2017) and Khan et al. (2016) reviewed negative impacts on rumen health and rumination when a high-starch starter was fed to young calves. Additionally, our results showed that the ME intake through MR was greater than by concentrate intake for Res and Adl calves until wk 8 and 10 of life, respectively.

If intensive feeding rates along with longer preweaning liquid feeding can be established, the enhanced calf growth should be recognized and accompanied with adjustment in calf husbandry with respect to dimensions of individual housing systems or group sizes.

The application of MR with Ca-/Na-butyrate was unproblematic in practice. The odor between the commercial and trial MR was scarcely notable and no differences between solubility in manual or automatic feeding were recognized. Guilloteau et al. (2010a) reviewed further improvements of butyrate supplementation if it was fed soon after birth, and recommended an effective dose between 1 to 4 % of DMI. Interestingly, calves reared naturally by their dams would have a fivefold higher butyrate intake, as butyric acid is contained in cow´s milk with 3 to 4 % of milk fat (Alais, 1984; Boeckaert et al., 2008; Chilliard et al., 2009). The presumed amount of butyric acid in milk vs. the tested volume of butyrate in the MR of the study were 12.8 vs. 2.4 g/kg DM, respectively.

We fed MR with 0.24 % butyrate from d 4 of age, but the effects of butyrate were rare in this trial, except from higher concentrate intakes in wk 5 of age. In contrast to previous studies in calves (Górka et al., 2009; Guilloteau et al., 2010b) and piglets (Kotunia et al., 2004), we found no increase in growth performance, instead butyrate tendentially decreased body growth ($P = 0.1$). The lack of significant effects on growth performance was also reported in weaned piglets (Biagi et al., 2007), whereas the piglets from that work at least had numerically greater BW that we did not observe in our calves. The importance of early postnatal supply of butyrate was seen in trails where butyrate was orally supplemented after 1 wk of age and no growth-stimulation could be revealed (Niwińska et al., 2017). Calves from our trial received butyrate added in MR after 3 d of life, but nevertheless were fed milk with the above assumed high amounts of butyric acid prior to MR feeding. Hence, a delay in butyrate intake was not assumed.

Furthermore, parameters of the somatotropic axis, namely IGF-I and IGFBP-3, were impaired by butyrate feeding. Both parameters are recognized for stimulating cell proliferation and body growth. Although butyrate intake was higher in Adl compared to Res and compared to studies with oral butyrate supplementation in restricted diets (Górka et al., 2009; Guilloteau et al., 2009), the overall intake was much lower than in milk-fed calves as mentioned above. Guilloteau et al. (2010a) assumed negative effects on cell proliferation when high amounts of butyrate are absorbed, whereas Niwińska et al. (2017) questioned the sufficiency of the butyrate amount (~ 0.3 % of DM) used in calf trails. From the literature review no studies with higher percentage of butyrate supplementation in MR have been conducted, although Adl calves with higher butyrate intakes in total showed no stimulating improvement compared to Res calves in our study.

The results from the presented study clearly indicate major benefits of ad libitum MR feeding for calves during the first weeks of life. Butyrate supplementation in the MR did not lead to improved effects in restricted or ad libitum MR planes and no synergistic effects in ad libitum feeding could be seen. However, butyrate slightly suppressed growth performance and maturation of the somatotropic axis. According to this, ad libitum feeding, but not butyrate supplementation, can be recommended from the outcome of this study.

The feeding management would contribute to animal welfare that is based on the "five freedoms", whereas the freedom from hunger and malnutrition, freedom from distress, and freedom to express normal patterns of behavior might be fulfilled in ad libitum or at least intensive liquid feeding to calves in an adequate period before weaning (FAWC, 2015).

Furthermore, the overall issue of high mortality rates that continue to pose problems in calf rearing and result in poor welfare and economic losses, might be improved by intensive feeding and more precise focus on rearing management. Mee (2013) stated that international high mortality rates are often not recognized as a primary issue in the dairy industry. For example, there are no German national studies on overall calf mortality rates which may be contribute to deficient awareness of the problem. On the other hand, studies from Germany (Tautenhahn, 2017), Canada (Windeyer et al., 2014), and New Zealand (Cuttance et al., 2017) showed that postnatal, but not perinatal mortality rates were 0 % at some farms. Hence, Cuttance et al. (2017) assumed a strong potential to achieve greater reduction of postnatal mortality rates.

The presented trial examined 68 calves in total. Four calves had incomplete data sets or were excluded from the trial. In particular, one calf could not adjust to the automatic feeder, one calf had recurrent abomasal displacements, one calf died postoperative after liver biopsy, and one calf died because of various health deficiencies. This particular calf was the only one to contribute to postnatal mortality until 11 wk of age. Therefore, the postnatal mortality rate for this trial was 1.5 %. No differences of feeding levels or butyrate supplementation were seen on the relatively low mortality rate.

Nevertheless, a critical view on calf management, including calf nutrition, and the willingness to improve existing rearing systems, might help to diminish mortality on farms and increase welfare for the animal.

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# 7 Summary

Calf-rearing management is a particularly important field in animal production as the calf will be the future dairy or beef cattle. Therefore, a healthy, robust, and productive animal will ensure successful farming. High morbidity and mortality rates elucidate the need and potential for improvements in calf rearing. One of the key factors is the nutrition of the calf. Beginning with the essential colostrum feeding to provide the newborn with nutrients, immunoglobulins, and additional bioactive factors, the further provision of sufficient energy and nutrients will improve the calf´s development in short-term and possible long-term effects.

Like all mammals, neonatal calves depend on the supply of liquid feed, i.e., milk or milk replacer (MR), before the rumen development is adequate to absorb and digest solid feed for maintenance and growth. In conventional feeding regimes calves are fed restricted amounts of liquid feed (e.g., 4 – 6 L/d) for a few weeks, whereas ad libitum intake of milk or MR is at least twice as high and naturally lasts for several month. Hence, restricted-fed calves are not able to express their natural feeding behavior and cannot exploit their full growth potential. Furthermore, feed additives might increase the growth performance and feed efficiency. The short-chain fatty acid, butyrate, is known as a natural growth promoter and affected body growth, development of the gastrointestinal tract, and health in different species (i.e., calves, piglets, and chickens). Therefore, in the critical rearing stage where young stock is prone to health maladies, feed additives are used to improve health and robustness. An improved maturation of the intestinal tissue would contribute to the postnatal development of the calves through a greater and more viable absorptive surface.

In the present study, the effects of ad libitum MR feeding and butyrate supplementation on the postnatal development, energy metabolism, and the regulation of growth through the somatotropic axis were evaluated. The trial was conducted with 64 Holstein calves (32 female, 32 male) that were studied from birth until wk 11 of life. All calves received the same amount of colostrum (2.5 kg) from their dam within 2 hours postnatum. Subsequent meals of transition milk were either offered in restricted amounts of 6 L/d (Res; n = 32) or ad libitum (Adl; max. 25 L/d; n = 32) until d 3 of age. From d 4 until d 56, dietary planes continued with MR (12.5 % dry matter) feeding, whereby feeding groups were subdivided and received MR either without (ResB-; AdlB-) or with 0.24 % butyrate supplementation (ResB+; AdlB+). All calves were gradually weaned to 2 L/d from wk 9 to 10, and 2 L

MR/d were offered until the end of the trial. Concentrate, hay, and water were freely available.

Performance data were determined by daily feed intake and weekly weighing. On four time points in wk 1, then weekly or once every other wk (insulin-like growth factor binding protein; IGFBP) until wk 11 of age, blood samples were taken to evaluate plasma concentrations of specific metabolites, hormones, and the systemic IGF system. Liver samples were collected on d 50 and 80 of age to determine gene expression related to glucose metabolism and to the hepatic IGF system.

Calves fed liquid feed ad libitum had lower concentrate intake but higher milk and MR intakes which resulted in elevated metabolic and endocrine plasma levels, e.g., of glucose and insulin. The enhanced preweaning nutrient and energy intake positively affected the IGF system, especially with higher IGF-I and IGFBP-3 but lower IGFBP-2 concentrations. The effect of accelerated anabolic metabolism and stimulation of the somatotropic axis reflected the overall greater growth performance in Adl compared to Res calves. The supplementation of butyrate in MR had no additional effects on feed intake and body growth in the treatment groups. However, butyrate supplementation suppressed glucose status and the IGF system at specific time points throughout the study.

In conclusion, butyrate supplementation had no additional effects, but preweaned calves benefit from ad libitum milk or MR feeding that stimulated the intermediary metabolism and growth performance. Furthermore, the intensive feeding practice allowed the expression of a more natural feeding behavior.

# 8 Zusammenfassung

Das Management der Kälberaufzucht hat eine große Bedeutung, da das Kalb die zukünftige Milchkuh oder das Mastrind darstellt und somit gesunde, robuste und produktive Tiere den Erfolg eines landwirtschaftlichen Betriebes sichern. Die hohen Krankheits- und Sterblichkeitsraten verdeutlichen die Notwendigkeit und das Potenzial, die Kälberaufzucht zu verbessern. Ein Schlüsselfaktor hierfür ist die Kälberfütterung. Angefangen mit der essenziellen Kolostrumgabe, um das Neugeborene mit Nährstoffen, Immunglobulinen und weiteren bioaktiven Substanzen zu versorgen, verbessert eine weitere ausreichende Energie- und Nährstoffversorgung die unmittelbare und möglicherweise langfristige Entwicklung der Kälber.

Neugeborene Kälber sind wie alle Säugetiere auf die Aufnahme von Flüssignahrung in Form von Milch oder Milchaustauscher (MAT), angewiesen. Erst wenn der Pansen ausreichend entwickelt ist, um Festfutter zu verdauen, kann der Grundumsatz und das Wachstum hierüber generiert werden. In konventionellen Fütterungsstrategien werden Kälber restriktiv und für wenige Wochen mit Tränke versorgt (z.B. 4 – 6 l/d), wohingegen ad libitum getränkte Kälber mind. doppelt so viel Milch oder MAT aufnehmen und in der Natur erst nach mehreren Monaten abgetränkt werden. Somit sind restriktiv gefütterte Kälber weder in der Lage ihr physiologisches Trinkverhalten auszuleben noch können sie ihr Wachstumspotential ausschöpfen.

Des Weiteren ist es möglich, die Wachstumsleistung und Fütterungseffizienz durch den Einsatz von Futterzusatzstoffen zu erhöhen. Die kurzkettige Fettsäure Butyrat ist bekannt als natürlicher Wachstumsstimulator und beeinflusst das Körperwachstum, die Entwicklung des Magen-Darm-Traktes und die Gesundheit in verschiedenen Tierarten, z.B. Kälber, Ferkel, oder Küken. Zusatzstoffe im Futter sollen den Gesundheitsstatus und die Robustheit der Jungtiere verbessern, v.a. in der kritischen Aufzuchtphase, wenn sie besonders anfällig für Krankheiten sind. Im Allgemeinen führt eine ausgereifte und gesunde Darmmukosa zu einer verbesserten postnatalen Entwicklung der Kälber, da es eine größere und funktionsfähigere Absorptionsoberfläche bietet.

In der präsentierten Studie wurden die Effekte einer ad libitum Fütterung von MAT und eines Buttersäurezusatzes auf die postnatale Entwicklung, den Energiestoffwechsel und die Wachstumsregulation durch die somatotrope Achse untersucht. Der Versuch wurde mit 64 Kälbern (32 weiblich, 32 männlich) der Rasse Deutsche Holstein von ihrer Geburt bis zur 11. Lebenswoche durchgeführt. Alle Kälber erhielten die gleiche Menge an Erstkolostrum

(2,5 kg) von der Mutter innerhalb von zwei Stunden nach der Geburt. Anschließend wurde Transitmilch bis zum 4. Lebenstag entweder restriktiv mit 6 l/d (Res; n = 32), oder ad libitum (Adl; max. 25 l/d; n = 32) vertränkt. Ab dem 4. bis zum 56. Lebenstag wurde die Tränkeintensität mit einem MAT (12,5 % Trockenmasse) fortgesetzt, wobei die Fütterungsgruppen entweder einen MAT ohne Butyrat (ResB-, AdlB-) oder mit 0,24 % Butyratzusatz (ResB+, AldB+) erhielten. Alle Kälber wurden in den Lebenswochen 9 bis 10 linear auf 2 l/d abgetränkt, wobei sie bis zum Versuchsende 2 l/d erhielten. Kraftfutter, Heu und Wasser standen zur freien Verfügung.

Für die Leistungsdaten wurden die täglichen Futteraufnahmen und wöchentlichen Gewichte erfasst. Blutproben wurden an vier Zeitpunkten in der ersten Lebenswoche, dann wöchentlich oder zweiwöchentlich (*insulin-like growth factor binding protein*; IGFBP) bis zum Versuchsende entnommen. Im Blutplasma wurden spezifische Metaboliten und Hormone, sowie Parameter des IGF-Systems analysiert. Am 50. und 80. Lebenstag wurden Leberbiopsien durchgeführt, um die Genexpression in Bezug auf den Glukosestoffwechsel und das leberspezifische IGF-System zu untersuchen.

Ad libitum gefütterte Kälber hatten geringere Kraftfutteraufnahmen, aber höhere Milch- und MAT-Aufnahmen, was zu erhöhten metabolischen und endokrinen Plasmawerten, z.B. von Glukose und Insulin, führte. Die erhöhte Nährstoff- und Energiezufuhr vor dem Abtränken zeigte eine positive Beeinflussung des IGF-Systems mit höheren Konzentrationen von IGF-I und IGFBP-3, bei gleichzeitig niedrigeren IGFBP-2 Werten. Der Effekt des verbesserten anabolen Stoffwechsels und die Stimulation der somatotropen Achse spiegelten sich in dem insgesamt höheren Gewichtszuwachs der Adl gegenüber den Res Kälbern wider. Der Zusatz von Butyrat im MAT hatte keine zusätzlichen Effekte auf die Futteraufnahme und das Wachstum der Kälber. Jedoch minderte der Butyratzusatz den Glukosespiegel und das IGF-System zu bestimmten Zeitpunkten während des Versuchs.

Zusammenfassend zeigt diese Studie, dass der Zusatz von Butyrat keine weiteren, das Wachstum oder die Entwicklung stimulierenden Effekte hatte, aber Tränkekälber von der ad libitum Milch- oder MAT-Fütterung profitieren, die den Intermediärstoffwechsel und das Wachstum stimuliert. Darüber hinaus ermöglicht das intensive Tränkemanagement die Ausübung eines natürlicheren Trinkverhaltens der Kälber.

# 9 Appendix

## List of Publications

### Peer-reviewed publications

D. Frieten, C. Gerbert, C. Koch, G. Dusel, K. Eder, E. Kanitz, J. M. Weitzel, and H. M. Hammon. 2017. Ad libitum milk replacer feeding, but not butyrate supplementation, affects growth performance as well as metabolic and endocrine traits in Holstein calves. *Journal of Dairy Science* (100:6648-6661). https://doi.org/10.3168/jds.2017-12722.

D. Frieten, C. Gerbert, C. Koch, G. Dusel, K. Eder, A. Hoeflich, B. Mielenz, and H. M. Hammon. 2018. Influence of ad libitum milk replacer feeding and butyrate supplementation on the systemic and hepatic insulin-like growth factor I and its binding proteins in Holstein calves. *Journal of Dairy Science* (101:1661-1672). https://doi.org/10.3168/jds.2017-13603.

### Conference proceedings

D. Frieten, C. Gerbert, C. Koch, G. Dusel, K. Eder, B. Mielenz, A. Hoeflich, and H. M. Hammon
Ad libitum milk feeding and butyrate supplementation differently affect the somatotropic axis in dairy calves
2017 ADSA Annual Meeting, June 2017, Pittsburgh, Pennsylvania, USA.

C. Gerbert, D. Frieten, C. Koch, G. Dusel, K. Eder, R. Zitnan, and H. M. Hammon
Impact of ad libitum milk feeding and butyrate supplementation on organ and epithelial growth in the gastrointestinal tract of dairy calves
2017 ADSA Annual Meeting, June 2017, Pittsburgh, Pennsylvania, USA.

D. Frieten, C. Gerbert, C. Koch, G. Dusel, K. Eder und H. M. Hammon
Einfluss einer ad libitum Fütterung und von Butyrat auf das Wachstum und den Intermediärstoffwechsel von Holstein Kälbern
16. BOKU-Symposium Tierernährung, April 2017, Vienna, Austria.

Frieten, D., Gerbert, C., Koch, C., Dusel, G., Eder, K., Mielenz, B., Höflich, A., Hammon H. M.

Effects of intensive milk feeding and butyrate supplementation on the somatotropic axis in German Holstein calves

71st Conference of the Society of Nutrition Physiology, March 2017, Göttingen, Germany.

D. Frieten, C. Gerbert, C. Koch, G. Dusel, K. Eder und H. Hammon

Einfluss einer intensiven Milchfütterung und von Buttersäure auf das Wachstum und den Intermediärstoffwechsel beim Kalb

113. Tagung des Arbeitskreises der Futterberater für die Länder Hessen, Rheinland-Pfalz und Saarland, April 2016, Alsfeld, Germany.

Frieten, D., Gerbert, C., Koch, C., Dusel, G., Eder, K., Hammon, H. M.

Effects of intensive milk feeding and butyrate on metabolic and endocrine traits in blood and growth performance in German Holstein calves

70th Conference of the Society of Nutrition Physiology, March 2016, Hanover, Germany.

# Erklärung

Ich erkläre: „Ich habe die vorgelegte Dissertation selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Mainz, 05.12.2017
Dörte Frieten

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Wer an erster Stelle steht, kommt nur formal zum Schluss. Wie wäre es wohl gelaufen, wenn ich nicht regelmäßig von meinem Schreibtisch weggeholt worden wäre, nicht einen so starken und genau im richtigen Maße verständnisvollen Partner gehabt hätte, der noch dazu ganz versiert die Probleme mit allen Office-Anwendungen lösen kann? Ja, das wäre nicht so gut gewesen, darum gilt mein größter Dank für die Aufrechterhaltung der lebens- und liebenswerten Zeit während der Doktorarbeit dir, mein geliebter Matthias.

*Für die Kälber*
Mainz, 2018