Effects of pre-calving body condition and postpartum concentrate feed proportions of the ration on performance, mobilization of adipose tissue depots, ruminal pH parameters, microbial efficiency and animal health during the transition period in dairy cows.

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Für Mama und Papa.

Die Individualität ist die eigentliche Quelle allen Fortschritts.

Mohandas Karamchand Gandhi

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APP	Acute phase protein
BCS	Body condition score
BCS_H	High body condition score
BCS_L	Low body condition score
BHB	β-hydroxybutyrate
C	Concentrate feed proportion(s)
C_{35}	Concentrate feed proportion of 35%
C_{60}	Concentrate feed proportion of 60%
CLA	Conjugated linoleic acid
CNCPS	The Cornell Net Carbohydrate and Protein System
CO_2	Carbon dioxide
CRP	C-reactive protein
DIM	Days in milk
DMF	Dry matter flow
DMI	Dry matter intake
EB	Energy balance
fOM	Fermentable organic matter
GfE	Society of Nutrition Physiology (Gesellschaft für Ernährungsphysiologie)
GNB	Gram-negative bacteria
Hpt	Haptoglobin
IDO	Indolamine-2,3-dioxygenase
IL	Interleukine

ABBREVIATIONS

IndiKuh	Indikatoren im Bereich Stoffwechsel und Fütterung bei Milchkühen
Kyn	Kynurenine
LAVES	Lower Saxony State Office for Consumer Protection and Food Safety
LBP	Lipopolysaccharide-binding-protein
LPS	Lipoplysaccharides
LRCpH	Dascor Lethbridge Research Centre Ruminal pH Measurement System
mCP	Synthesized microbial crude protein
MD-2	Protein myeloid differentiation factor 2
ME	Metabolizable energy
MEE	Milk energy efficiency
N	Nitrogen
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
NE_L	Net energy for lactation
NH_3	Ammonia
OMF	Organic matter flow
peNDF	Physically effective neutral detergent fibre
RNB	Ruminal-nitrogen-balance
SARA	Subacute ruminal acidosis
SCFA	Short chain fatty acids
SD	Standard deviation
TDO	Tryptophan-2,3-dioxygenase
TLR4	Pattern recognition receptor
Trp	Tryptophan
UDP	Undegradable protein
USM	Ultrasonic measurements

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1. Introduction

During the last two decades the milk production in Germany increased by 8,000 tonnes, which represents an increment of about 15% (BLE 2018). However, a discrepancy occurs between high milk production on the one hand and animal health and welfare and thus the prevention of economic losses on the other hand. Farmers have always strived to ensure a good animal welfare which is defined as the absence of illness and injury. Over the past decade, science has been increasingly devoted to welfare of cattle, including the focus on healthy and well-nourished animals (von Keyserlingk et al. 2009). On average, every two to three cows develop health problems during the transition period (Jordan and Fourdraine 1993, Enting et al. 1997, Drackley 1999, Drackley et al. 2005). To reconcile both economic and welfare aspects, particular attention has to be paid to that period, which is defined as the period from three weeks before until three weeks after calving.

The transition from late gestation to lactation is accompanied with metabolic, endocrine and immunological changes. The energy requirements suddenly double overnight with the onset of lactation. However, feed intake and therefore energy consumption is known to decrease before calving which results in a negative energy balance (NEB). The resultant energy deficit can have major impacts on the animals' health (Grummer 1995, Veerkamp et al. 2003, Drackley et al. 2005).

To reduce the gap between energy requirements and energy intake, energy content of the rations is often increased and they are provided as concentrates. High concentrate feed proportions (C) in the rations were shown to reduce the energy deficit and enhance milk yield (Delaby et al. 2009, Xie et al. 2017, Dänicke et al. 2018). However, increasing the amount of concentrates presupposes several adaptions of the rumen. Due to an accompanied increase of short chain fatty acids (SCFA) production, the acid absorption capacity by the rumen epithelium must adapt equally to impede a decrease of the ruminal pH. A low ruminal pH and therefore a change in the rumen environment can have major implications on the microorganisms which colonize the rumen, degrade fibre and meet major parts of the cows' protein demands. Furthermore, high C together with an insufficient supply of physical effective fibre enhance the risk for developing a subacute ruminal acidosis (SARA) (Dirksen et al. 1984, Allen 1997, Goad et al. 1998, Bannink et al. 2012, Gao and Oba 2014).

The physiological solution to deal with the challenging transit situation is to mobilize energy which is stored in adipose tissue depots. However, an excessive mobilization of body fat is often accompanied with an increased concentration of ketone bodies in blood. When the hepatic capacity to handle the increased non-esterified fatty acids (NEFA) levels is exceeded, metabolic disorders, such as ketosis and further health problems can occur. In this context, a high pre-calving body condition score (BCS) is known to enhance post-calving lipolysis (Moe et al. 1972, Littledike et al. 1981, Tamminga et al. 1997, Roche et al. 2009). All the necessary adaptions during the transition period are energy consuming, while the energy balance is already negative. Additionally, the nutritive and energy requirements of the immune system increase as well. As lactations are energy prioritizing processes, immune suppression may occur (Bell 1995, Dänicke et al. 2018).

Apart from the composition of the ration and the respective body condition of the cow, individual differences to deal with the physiological changes were described. Cows seem to differ in their ability to deal with different feeding factors, as well as in the capability of their ruminal epithelia to absorb SCFA. Moreover, individual responses to inflammatory processes are different (Dado and Allen 1994, Jacobsen et al. 2004, Beauchemin and Penner 2009, Gao and Oba 2014).

Performance, lipolysis, rumen conditions, microbial efficiency and the immune system reveal a complex picture. Although the challenges for the cow during the transition period cannot be met just by animal nutrition, one of the main factors influencing these parameters is the C of the ration and additionally the BCS which are both easily to assess and therefore also play an active role in practice. Thus, the present thesis aims to examine the impacts of two rations differing in C in combination with different BCS.

2. Background

2.1 The transition period

The transition period, defined as the period from three weeks antepartum until three weeks postpartum, is the most challenging time for dairy cows (Drackley 1999). Energy intake increases more slowly than energy requirements for lactogenesis rise which results in a NEB (Grummer 1995, Veerkamp et al. 2003). The change from a late gestation stage to lactation comprises several physiological, metabolic, endocrine and immunological changes (Drackley et al. 2005).

2.1.1 Dry matter intake and negative energy balance

The transition period is characterized by a decrease in dry matter intake (DMI) before calving and a subsequent increase afterwards. The DMI is mainly regulated by physical parameters, such as rumen filling and passage rate as well as by chemical parameters, such as fatty acids. Another explanation for the decreased pre-calving DMI comprises the fetal growth as the unborn calf is supposed to restrict the abdominal space (Kaske and Groth 1997, Gruber et al. 2006). Drackley et al. (2005) declared a decrease of 10 to 30% compared to intake during the early dry period. The DMI increases relatively slow after calving, the most pronounced increase of DMI is observed approximately 2 weeks postpartum and it rises constantly afterwards. The maximum is reached around 8 and 22 weeks after calving. However, milk yield is highest during the fifth and seventh week after parturition (Ingvartsen and Andersen 2000). Thus, during this period the energy requirements cannot be met and cows experience a NEB. Consequently, concentrate proportions for lactation diets are often increased to meet the energy requirements of a lactating cow. Furthermore, concentrate is known to enhance the total feed consumption by around 0.4 – 0.6 kg per kg additional concentrate (Gruber et al. 2004).

The energy requirements are dependent on the cow's reproductive stage. In late gestation, only the energy requirement for maintenance and fetal growth have to be met (Dänicke et al. 2018). From the onset of lactogenesis, the energy requirements double overnight due to the onset of milk production (Drackley et al. 2005). Energy balance is estimated considering the factors of energy intake and energy expenditures, like those for maintenance, fetal growth and milk production. Therefore, the accuracy of the determined energy balance is dependent on the accuracy of the methods estimating the included factors. Energy intake and energy requirement

for maintenance represent sources of high variations (Dänicke et al. 2018). Gruber et al. (2007) claimed a general underestimation of energy requirements for maintenance, which is dependent on the chemical body composition as well as the metabolic stage.

2.1.2 Mobilization of adipose tissue depots and ketosis

The sole selection of cows for high milk yield is increasingly questioned as high milk yield is positively correlated to the incidences of several production diseases and appears to be closely related to the magnitude of NEB (von Keyserlink et al. 2009). Current breeding strategies are aimed at consideration of functional and health traits as relevant selection criteria while relevance of milk yield decreases in order to support the physiological adaptations in the transit period (Fleming et al. 2018).

During lactation mobilization of body reserves plays an important role as DMI and energy intake are not sufficient to meet the demands of a high yielding dairy cow (Dillon et al. 2003, Flachowsky et al. 2009). Body fat and protein are mobilized to compensate the energy requirements (Tamminga et al. 1997, von Soosten et al. 2012). This process is accompanied by an increase of circulating NEFA and ketone bodies, such as β-hydroxybutyrate (BHB) (Littledike et al. 1981, Schulz et al. 2014). The mobilization of body reserves is a physiologically necessary process in mammals. Nevertheless, excessive mobilization can lead to metabolic disorders and further health problems (Moe et al. 1972, Kaske et al. 2005).

Adipose tissue depots present the body's stored energy and consist of adipocytes which are cells filled by triacylglycerides. Triacylglycerides are broken down and resynthesized continually. The breakdown, known as lipolysis, leads to production of NEFA which can be released into the bloodstream and are taken up by the liver (Herdt 2000). NEFA are either resynthesized to triacylglycerides or further metabolized to acetyl-CoA via β-oxidation in the liver's mitochondria (Newsholme and Leech 1984). By means of oxaloacetate, acetyl-CoA is introduced into the citric-acid cycle and further oxidized to carbon dioxide (CO_2). With the onset of lactogenesis, the main parts of oxaloacetate are used to synthesize glucose. Consequently, oxaloacetate is not available for the introduction of acetyl-CoA into the citric-acid cycle. As a result, acetyl-CoA is instead rebuilt to ketone bodies. Thus, if an excessive release of NEFA exceeds the capacity of the citric-acid cycle, acetyl-CoA is primarily converted

to ketone bodies. Hence, concentration of BHB in blood is markedly increased (Newsholme and Leech 1984, Wilke 2011).

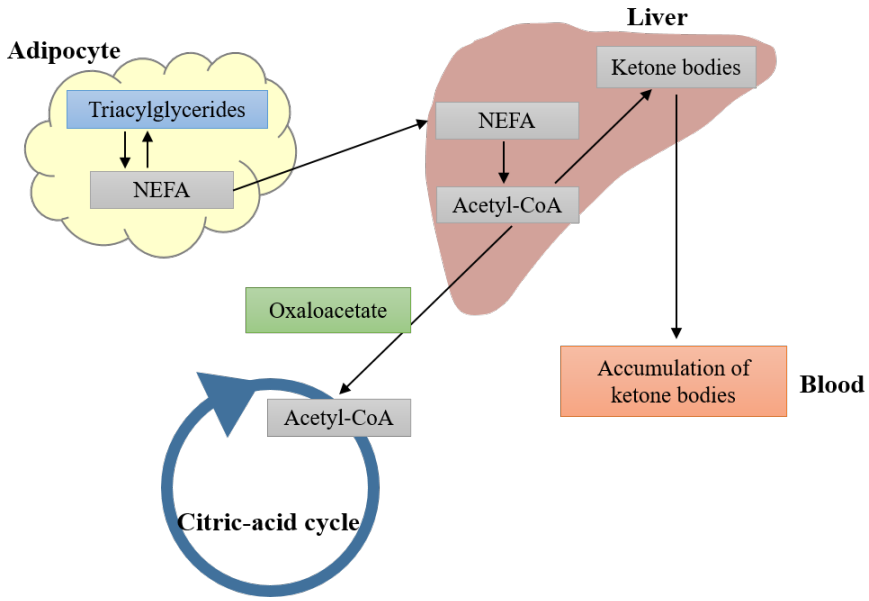

Figure 1. Simplified schematic presentation of degradation of triacylglycerides to ketone bodies during the process of body fat mobilization. Triacylglycerides are catabolized to non-esterified fatty acids (NEFA) in the adipocytes and further metabolized to acetyl-CoA in the liver. Acetyl-CoA is either introduced into the citric-acid cycle for energy generation by means of oxaloacetate or rebuilt to ketone bodies. Ketosis occurs when ketone body concentration exceeds the physiological thresholds.

This process, also known as ketogenesis, is a physiologically necessary pattern which provides additional energy. However, the risk of ketosis increases with increasing ketone body concentrations, when exceeding critical values (Wilke 2011). There are two types of ketosis: clinical ketosis includes symptoms, such as a general poor condition, a reduced DMI, as well as a reduced milk yield, excessive loss of body condition and diarrhoea (Dirksen 2006). Subclinical ketosis then again is characterized by the absence of clinical signs. However, production losses, reduced fertility and a predisposition for subsequent production diseases occur in association with subclinical ketosis (Geishauser et al. 2001). The incidence of subclinical ketosis is estimated to occur at >40% on average (Emery et al. 1964, McArt et al. 2012). According to Nielen et al. (1994) BHB concentrations in blood serum >1.2 mmol/L indicate a subclinical ketosis. Oetzel (2004) proposed that NEFA-values >0.4 mmol/L describe an imbalance in the energy supply and therefore an accelerated lipid mobilization. Therefore, subclinical as well as clinical ketosis are accompanied with a severe NEB (Herdt 2000). A high BCS before calving is known to be associated with a pronounced reduction in DMI and

consequently with a severe NEB, which leads to accelerated lipid mobilization (Samartín and Chandra 2001, Roche et al. 2009).

2.2 Dietary composition and its impacts on rumination behaviour and ruminal pH

2.2.1 Components and functions of the rumen

The rumen is part of the cows' forestomach system and comprises the major part, around 80%, of the total stomach volume. The rumen mucosa includes a dense vascular system and a multilayer squamous epithelium, which enables the exchange of substances. The papillae of the rumen mucosa lead to a sevenfold enlargement of the surface (Lebzien 2005). Microorganisms colonizing the rumen play an important role for degradation of feed stuff (Stern et al. 1994). As the rumen provides optimal conditions for bacterial growth, ruminants and microorganisms live in perfect symbiosis. The populations of bacteria, protozoa and fungi are able to digest fibre and carbohydrates and to supply proteins, vitamins and SCFA for the host (Nocek and Russell 1988, Russell and Rychlik 2001). SCFA represent important energy-providing degradation products for the animal and cover 80% of the cow's energy requirements (Wurm 2010). Acetic, propionic and butyric acid are the predominant forms of SCFA, whereby acetate covers the largest part with 60 – 70%, followed by propionate with 15 – 20% and butyrate with 10 – 15% (Bergman 1990, Breves and Lebzien 2009). However, different diets can influence the composition of the microbial population and therefore change the fermentation profile (Russell and Hespell 1981). High concentrate proportions in the diets increase the SCFA concentration and lead the production towards propionate, which is an important precursor for glucose synthesis. Propionate is taken up by the liver very efficiently and covers 60 – 65% of the glucose requirements for lactation (Bergman et al. 1966, Bauman et al. 1971, Bergman 1990, Breves and Lebzien 2009). In contrast, degradation of fibre increases the amounts of acetate and butyrate. Acetate is used as predominant carbon source for de novo synthesis of fatty acids, as well as for fatty acid elongation in the metabolism of the cow. Therefore, it represents a major substrate for lipogenesis. Butyrate is degraded to D-3-hydroxybutyrate by the rumen epithelium and the liver and it is used for lipogenesis and oxidation (Kristensen et al. 1998, López 2005, Roche et al. 2009).

The composition of the ration is crucial for rumen health. A high fibre content is known to increase salivation with its buffering effects. Therefore, long fibre particles are decisive, as they must be chewed intensively to reduce particle size and are therefore referred to as physically

effective neutral detergent fibre (peNDF) (Mertens 1997). In case of a disbalanced ration with a lack of roughage and thus a decrease in saliva production, increased amounts of concentrate lead to accumulation of SCFA, which affects ruminal pH negatively (Bannink et al. 2012). This in turn can impact the fibrolytic and increase lactate-utilizing bacteria (Goad et al. 1998). To impede the increase of SCFA and the resulting decrease of ruminal pH, SCFA are absorbed across the rumen wall (Dirksen et al. 1984). The predominant part of SCFA appear in the rumen in dissociated form within the physiological pH range. However, dissociated forms show hydrophilic properties, wherefore a simple diffusion by absorption through the rumen wall is not possible. The main mechanism to remove the dissociated SCFA is an exchange with bicarbonate which additionally buffers ruminal pH. Due to the lipid solubility of undissociated forms, these can be removed by means of simple diffusion (Allen 1997, Breves and Lebzien 2009). Consequently, the ruminal pH is determined by several factors, such as the production of SCFA and the acid removal by the rumen epithelium, the peNDF content of the ration, as well as the saliva production. The rumination behaviour and the accompanying saliva production with its buffering capacities is another important factor to balance the ruminal pH. Fibre rich diet compositions are known to increase chewing time. Salivary buffers such as bicarbonate neutralize 15 up to 40% of the SCFA produced in the rumen (Allen 1997, Gäbel et al. 2002). Moreover, an increased saliva production also increases particulate and fluid movement from the rumen to lower digestive tracts (Krause et al. 2002). Therefore, diet dependent morphological adaptions of the rumen mucosal papillae are necessary to increase absorption capacity. Especially in early lactation, epithelial cells are short and reveal a small surface, due to the previous reduction in DMI. The change from a dry period diet to a lactation diet, high in concentrate, requires physiological and structural adaptions of the rumen mucosa (Gäbel et al. 2002, Beauchemin and Penner 2009, Lohölter et al. 2013). It is assumed, that the rumen epithelium requires an adaption period of between 4 and 8 weeks for papillae growth to be completed after the change from a forage diet to a concentrate diet (Dirksen et al. 1984, Dirksen et al. 1985).

2.2.2 Subacute ruminal acidosis

Subacute ruminal acidosis is a widespread disease in dairy cows. Sudden changes to diets with high amounts of easily fermentable carbohydrates in combination with a lack of peNDF and therefore a decreased saliva production impose the highest risk for SARA, as well as a high

level of feed intake and sorting behaviour and a previous exposure. Hence, the number of cases is greatest during early lactation and may result in economic losses (Gröhn and Bruss 1990, Beauchemin and Penner 2009, Gao and Oba 2014). SARA implies a repeated decline of ruminal pH for a certain time period. An increased production of SCFA and the shift of the acetate:propionate ratio towards propionate due to high concentrate proportions in the ration cause a drop in the rumen pH. The accumulation of SCFA exceeds the capabilities of the rumen to balance production on the one hand and absorption and neutralization on the other hand (Enemark et al. 2002, Beauchemin and Penner 2009, Breves and Lebzien 2009). Consequences of SARA include decreased feed intake, decreased milk yield, milk fat depression, and subsequent health problems, such as diarrhoea, laminitis and increased incidences of inflammations (Britton and Stock 1987, Nocek 1997, Kleen et al. 2003, Khafipour et al. 2009, Gao and Oba 2014). However, the diagnosis of SARA has proved to be difficult due to the lack of clear clinical signs. The low ruminal pH affects fibre digestion and the absorptive capacity of the epithelium. It can further result in an increased translocation of bacteria, amines and endotoxins into the systemic circulation, due to rumen mucosa lesions (Harmon et al. 1985, Russell and Wilson 1996, Gozho et al. 2005). SARA might also have a major impact on the microbial population of the rumen and can lead to partial defaunation (Jørgensen et al. 1993). The above described relations are summarized in Figure 2.

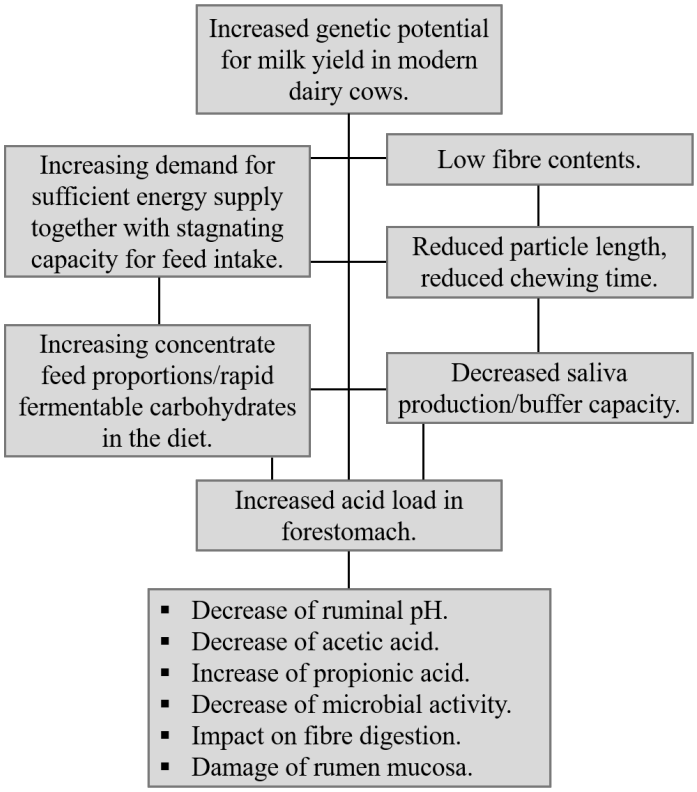

Figure 2. Impacts of genetic selection and increasing energy requirements as well diet compositions on rumen conditions. Modified according to Enemark et al. (2002) and Lebzien (2008).

To characterize SARA thresholds were developed as the exact revelation of an acidosis is the main difficulty when only the physiological range of the ruminal pH is known. Accordingly, a daily mean pH <6.16 in combination with the time per day with pH <5.8 for more than 5.24 hours are most frequently used to indicate SARA (Zebeli et al. 2008, Beauchemin and Penner 2009). The measurement of the ruminal pH is a challenge on its own. Due to high daily variations of ruminal pH, continuous pH measurements are most appropriate (Keunen et al. 2002). In rumen-cannulated cows, this is feasible by using intraruminal probes. The Lethbridge Research Centre Ruminal pH Measurement System (Dascor, Inc., Escondido, CA, USA, Figure 3) is advantageous due to the possibility of calibration in buffer solutions of pH 3 and 7 before and after each application which ensures highly accurate and reliable measurements.

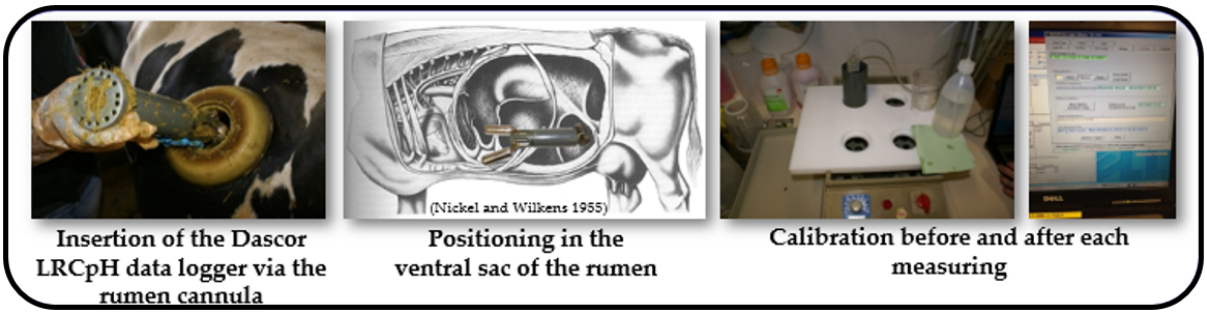

Figure 3. Insertion, positioning and calibration of the Dascor Lethbridge Research Centre Ruminal pH Measurement System (LRCpH) (own image files).

The predisposition to develop a SARA is supposed to differ between cows, even under the same conditions. The individual prevalence depends on several factors, including feeding behaviour, such as level of feed intake, meal frequency, sorting behaviour, as well as ruminating behaviour, saliva production, the ruminal microbial colonization and previous periods of SARA (Beauchemin and Penner 2009). Individual differences concerning tolerance for different feeding factors, as well as day to day variations in feeding behaviour and individual variations for rumination are known to exist. Furthermore, differences in the capability of the ruminal epithelia to absorb SCFA might explain the variation of ruminal pH among animals. Due to these large variations it is challenging to prevent the development of SARA (Dado and Allen 1994, Beauchemin and Penner 2009, Gao and Oba 2014).

2.2.3 Relation between subacute ruminal acidosis and ketosis

SARA and ketosis are closely linked, as both are related to energy intake and NEB. Thereby it is unclear whether ketosis or acidosis is the primary condition. The reduced feed intake due to SARA can result in ketosis, by reason of the unsatisfied energy requirements. On the other hand, ketosis caused by extensive lipid mobilization can impair feed intake and lead to secondary SARA, when the cow increases feed intake again (Enemark et al. 2002).

2.2.4 Ruminal pH, nutrient flows and microbial efficiency

The dominating factors to ensure maximum milk yield are on the one hand the energy supply and on the other hand the metabolizable protein. Metabolizable protein includes protein synthesized from the microbial population in the rumen, dietary protein that escapes ruminal degradation (undegradable protein, UDP) as well as endogenous protein (GfE 2001, Oba and Allen 2003). Feed protein is degraded to peptides, amino acids and ammonia. Parts of these degradation products are used by microorganisms to synthesize microbial protein. If the

concentration of ammonia (NH_3) exceeds the microbial demands, NH_3 is absorbed by the rumen wall and degraded to urea in the liver. Urea is either excreted with urine and milk or recirculated into the rumen via saliva or over the rumen wall if nitrogen (N) deprivation occurs (rumino-hepatic circulation) (Breves and Lebzien 2009).

Microbial protein, as well as UDP are digested and absorbed as amino acids over the small intestine. In this process, the microbial protein determines quantity and quality of the metabolizable protein. Dietary protein with low ruminal degradability can also display low digestibility in the small intestine compared to the protein synthesized by microorganisms. Therefore, microbial protein is less expensive for the cow and ensures an optimal amino acid composition which meets the animal´s demands for milk production (Clark et al. 1992, O'Connor et al. 1993, Oba and Allen 2003, Breves and Lebzien 2009).

An ammonia-N concentration of 2 – 5 mg/dL is proposed to be adequate for a maximum microbial protein synthesis. As the availability of energy is supposed to be the main limiting factor, increasing amounts of carbohydrates in the diet provide energy for this process. The ruminal-nitrogen-balance (RNB) indicates the supply of microorganisms in the rumen with N. An RNB higher than 50 g signals an N oversupply. N cannot be fully utilized by the microorganisms, which in turn can result in metabolic problems. A negative RNB reflects an undersupply and inhibits microbial growth. Therefore, an RNB-value of the ration between 0 and 50 g is considered ideal (Kamphues et al 2014, Spiekers et al 2009).

However, improved energy supply increases not only the growth rate of microorganisms, but also fermentation and therefore lowers ruminal pH. Thus, the risk for the cow to develop a ruminal acidosis increases. Hence, a sufficient amount of peNDF ensures normal ruminal movements and digesta flows as well as saliva production with its buffering properties to maintain physiological pH values (Clark et al. 1992, Stern et al. 1994, Allen 1997, Yang et al. 2001). The above described relations are summarized in Figure 4.

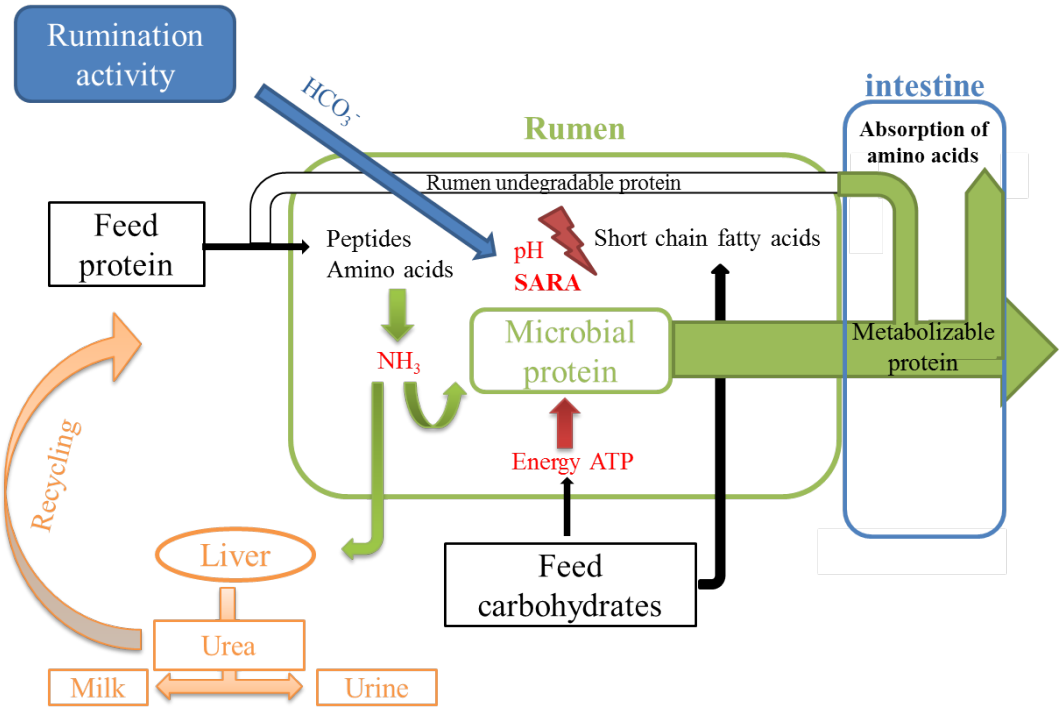

Figure 4. Schematic presentation of synthesis of microbial protein. Feed protein is degraded to ammonia (NH_3) by microorganisms, colonizing the rumen. By means of energy, provided by feed carbohydrates NH_3 is further used to synthesize microbial protein. Microbial protein as well as rumen undegradable protein are the two components of the metabolizable protein, which is digested and absorbed over the small intestine as amino acids. If the NH_3 concentration exceeds the microbial demands, it is degraded to urea in the liver and excreted with urine and milk. In periods of nitrogen deprivation, it can be resorbed again over the rumen wall. The increased amount of carbohydrates enhances the risk for developing a subacute ruminal acidosis (SARA), due to a high production of short chain fatty acids (SCFA) not compensated for by adequate SCFA absorption and/or buffering capacity which finally results in prolonged periods of low ruminal pH.

An increased DMI and thus an enhanced intake of carbohydrates leads to an increased passage rate. Although digestibility of organic matter in the rumen decreases due to a reduced retention time in the rumen, microbial growth rate increases. Rapidly degradable carbohydrates and proteins enable the provision of energy within a short period of time. Thus, energy from ruminal fermentation is used efficiently. Moreover, an increased passage rate reduces microbial lysis and enhances microbial efficiency (Rode et al. 1985, Clark et al. 1992, Oba and Allen 2003). However, a C >70% reduces the microbial protein synthesis. The high passage rate of non-structural carbohydrates leads to an uncoupled fermentation. Then, the release of energy is faster than the energy consumption of the microbes and an adequate amount of fibre is necessary, as it assures a consistent energy release throughout the day. However, too high fibre contents might result in a too low energy supply which would decrease microbial growth. Moreover, the slower passage rate would enhance lysis of microbial cells. This, in turn can lead

to recycling of energy and N which is used for microbial maintenance rather than growth (Hagemeister et al. 1981, Owens and Goetsch 1986, Clark et al. 1992).

The energy spilling or energy uncoupling is also used to keep the intracellular pH of the microbes stable, which at the same time reduces energy for microbial growth and therefore impacts microbial efficiency (Strobel and Russell 1986). Different models describe the relation between pH and microbial growth or microbial activity. The Cornell Net Carbohydrate and Protein System (CNCPS) proposes a pH-threshold of 6.2 below which the growth of fibrolytic bacteria is depressed. Another study revealed a decrease in microbial activity if the pH value is <5.8 for high quality pasture (Pitt et al. 1996, de Veth and Kolver 2001). However, not only the ruminal pH value itself, but also the time with an unfavourably low ruminal pH plays an important role. De Veth and Kolver (2001b) showed that the degradation of fibre decreases after 4 hours at a suboptimal pH of <5.8. Nevertheless, the concentration of fibrolytic bacteria in the rumen is higher than necessary so that the fibre degrading populations still remain, when the ruminal pH is in an optimal range for a sufficient period of time. Thus, fibre digestion is depressed due to a poor access to fibre rather than an actual decrease in microbial activity. Moreover, a depressed ruminal pH does not cause high levels of cell death, which permits a fast regeneration of fibrolytic bacteria and fibre degradation when regaining a sufficient pH value (Mould and Ørskov 1983, Hoover 1986, Shriver et al. 1986, Weimer 1998, de Veth and Kolver 2001b).

However, various factors affect microbial efficiency. Especially in combination with a slow passage rate, an extensive turnover of microorganisms in the rumen due to predation of protozoa might lower microbial efficiency, as the fibrolytic activity of protozoa is lower than that of bacteria. Nevertheless, if the passage rate increases, the availability of bacteria for the supply of protozoa decreases, which leads to an increased number of bacteria compared to the number of protozoa. Apart from that also the amount of nutrients plays a major role, as well as the synchronisation of food degradation to ensure a constant supply of nutrients for the microorganisms (Wallace and McPherson 1987, Clark et al. 1992, Oba and Allen 2003, Firkins et al. 2006, Firkins et al. 2007).

2.3 Impacts of body condition score and dietary composition on health parameters

2.3.1 The immune system

The immune system is subdivided into the innate and the adaptive immune system. To provide an optimal protection, the two systems work synergistically (Sordillo 2016).

2.3.1.1 The innate immune system

The innate immune system allows an early and rapid response within seconds. It reacts to a wide spectrum of pathogens and injuries. It represents the dominant defence mechanism, which is the nonspecific component of the immune system. The innate immune system is the so-called first line defence. It can be so efficient that no further noticeable changes of physiological functions occur (Sordillo 2016). The innate immune system includes a collection of proteins, such as cytokines, which interact with other components of the immune system, as well as cellular components, such as neutrophils, monocytes macrophages and also killer cells (Hajishengallis and Lambris 2010, Trevisi and Minuti 2018). The primary role of the cytokines is to mediate inflammatory processes, for example to enable the migration of leucocytes from blood into the affected tissue (Ryman et al. 2015).

2.3.1.2 The adaptive immune system

The adaptive immune system represents the specific immune response. The respective response can be delayed and take several days. It is characterized by specific responses to infectious pathogens and repeated exposures to the same microbe (Ryman et al. 2015). The activity of the adaptive immune system is triggered when the innate immune system is unable to eliminate the pathogen. Antigen-specific lymphocytes and memory cells build the memory immune response, which is known to be faster, stronger, longer lasting and more efficient than the innate immune response. The delayed onset is based on clonal expansion of B- and T-lymphocytes specific to the corresponding pathogen. Cytokines, produced by T-cells regulate duration and extent of the immune response (Sordillo 2016).

2.3.2 Immunological dysfunctions during the periparturient period

The time around calving is described as a proinflammatory period, which is accompanied with a decreased general immune-competence (Saad et al. 1989, Mallard et al. 1998, Hachenberg et al. 2007). The reasons for the immunosuppression around calving are unclear, but seem to be

multifactorial. Endocrine and metabolic changes during the periparturient period can partly explain this phenomenon. Other influencing factors of the functionality of the immune system are the adaption from a dry period diet to a lactation diet and a possible NEB (Bell 1995, Drackley et al. 2005). Thus, endogenous as well as exogenous factors impact the immune functions and can lead to further health problems, such as metabolic disorders and infections (Schulz et al. 2015).

The transition from late gestation to lactation results in high stress levels for the animals, visible in high cortisol concentrations (Burton et al. 2005). Cortisol as well as other steroid hormones are supposed to be associated with immunological changes. Cortisol receptors naturally occur in immunological cells and are increasingly expressed around calving and they are known to cause immunosuppression (Burton et al. 1995, Kulberg et al. 2002, González et al. 2003, Tienken et al. 2015). Moreover, tissue lesions and inflammatory reactions caused by the event of calving can lead to additional immunological stress (Hachenberg et al. 2007).

Since lactation is a resource-prioritized process, nutrient availability for immune reactions decreases. As a result, glucose deficiency is another factor influencing the provision of immune cells with energy. Glucose is the main energy source especially for monocytes, but it is mainly transported to the udder with the onset of lactation (Bell 1995, Pithon and Curi et al. 2004, Drackley et al. 2005, Dänicke et al. 2018). Eger et al. (2015) demonstrated the decrease of monocyte glucose uptake after parturition with a minimum reached at day 21 postpartum. Therefore, changes in the functionality of the immune system during the periparturient period – additionally to the increasing energy requirements of the immune cells – can be caused by fluctuating nutrient and energy availability (Tienken et al. 2015).

2.3.3 Diet composition and its impact on immunological processes

Nutrition and nutrient supply can have a major impact on the immune system and its functionality. The demands of the immune cells for both energy and nutrients need to be met additionally during a period where the energy balance is known to be already negative (Bell 1995, Ingvartsen and Moyes 2012). To what extent the demands are met is, in turn, dependent on the composition of the diet. It is difficult to estimate the energy costs for immune responses. However, acute-phase-responses are supposed to be more expensive than specific, lymphocyte-mediated reactions. If resources are limited, energy is directed away from the immune cells and

used for prioritized processes, such as milk production and maintenance (Bell 1995, Klasing 2004, Dänicke et al. 2018). Therefore, rations need to be adjusted to reduce the immunological challenges during the periparturient period by providing enough energy (Sordillo 2016).

2.3.4 Relation between diet composition, rumen health and immunological responses

The gastrointestinal tract represents an important component of the immune system, which can get out of balance by diet changes. To meet the animal's high energy requirements after parturition, the amount of concentrates is often increased. However, changes of diet compositions towards higher concentrate proportions are known to cause acid conditions in the rumen (Enemark et al. 2002). A low ruminal pH leads to lysis of gram-negative bacteria and therefore to an increased release of lipopolysaccharides (LPS) into the rumen. It is assumed that the increased production of SCFA and the resulting increased osmolality in the rumen lead to a high susceptibility of the rumen mucosa for injuries and tissue lesions, which enables the translocation of LPS into the blood stream (Nagaraja et al. 1978, Nocek et al. 1984, Khafipour et al. 2009, Hartwiger et al. 2018). Additionally, high concentrations of butyric acid are assumed to stimulate the proliferation of the rumen epithelium (Sakata und Tamate 1978). A severe proliferation can result in parakeratotic changes accompanied with a decrease in the epitheliums absorption capacity and an increased susceptibility for tissue damage (Hinders and Owen 1965, Tamate and Kikuchi 1978). The described processes facilitate the translocation of LPS into the portal blood. In a first step, LPS is degraded by Kupffer cells via non-stimulatory pathways in the liver. When LPS concentration exceeds the liver´s capacity, it is translocated into the systemic blood circulation (Erridge et al. 2002). The presence of LPS in blood can trigger systemic immune responses, lead to the production of proinflammatory cytokines and cause the development of an acute-phase response (Baumann and Gauldie 1994, Kushner and Rzewnicki 1994, Gozho et al. 2005, Khafipour et al. 2009). Once LPS is translocated into the blood, the endotoxins are recognized by the LPS-binding-complex via the surface protein of monocytes $CD14^+$, which binds free LPS to inactivate it. The complex consists of the LPS-binding-protein, membrane $CD14^+$, the pattern recognition receptor (TLR4) and the associated protein myeloid differentiation factor 2 (MD-2). The engaged complex transduces a signal, initiating a transcription of a proinflammatory cytokine cascade (da Silva Correia et al. 2001, Erridge et al. 2002). The above described relations are summarized in Figure 5.

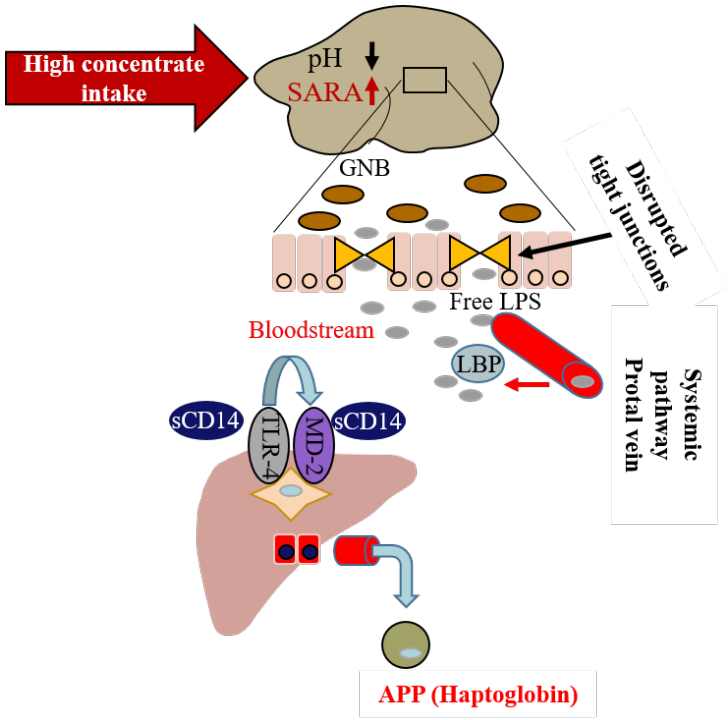

Figure 5. Association between increasing concentrate proportion in the ration, rumen conditions and increase of lipopolysaccharides (LPS). On the one hand, high amounts of concentrates can lead to lysis of gram-negative bacteria (GNB) and therefore to an increase of LPS. On the other hand, increased concentrate results in decreasing ruminal pH, which in turn impacts the susceptibility of the epithelium for injuries and enables the translocation of LPS into the blood. The LPS-binding-complex binds free LPS. The complex consists of the LPS binding protein (LBP), the surface protein of $CD14^{+}$, the pattern recognition receptor (TLR 4) and the associated protein myeloid differentiation factor 2 (MD-2). The complex initiates a transcription of a proinflammatory cytokine cascade, which triggers an acute-phase-response and results in production of acute phase proteins (APP), such as haptoglobin. Adapted from Zebeli and Metzler-Zebeli (2012).

However, results indicate, that mild episodes of a decreased ruminal pH do not affect the barrier functions of the epithelium and therefore do not increase LPS concentrations in blood (Rodríguez-Lecompte et al. 2014).

2.3.5 The acute-phase-response and inflammatory markers

The acute-phase-response belongs to the non-specific immune defence, which is triggered by stimulation of the innate immune system. Proinflammatory cytokines, such as tumor necrosis factor α, interleukine (IL)-1 and IL-6 stimulate the hepatic synthesis of positive acute phase proteins (APP), which increase rapidly, whereas negative APP decrease (Esposito et al. 2014, Trevisi et al. 2011). The most important APP, which are defined as inflammation markers, are serum amyloid A and haptoglobin (Hpt) (Horadagoda et al. 1999, Eckersall et al. 2001).

2.3.5.1 Haptoglobin

Hpt is the most important acute-phase-protein and one of the main inflammatory markers in ruminants (Hachenberg et al. 2007). The main function of Hpt is to bind free haemoglobin in the circulation (Eckersall and Connor 1988). In healthy cows, it is not detectable, whereas it increases after calving up to 5 mg/mL which is 4.5 times higher compared to the prepartal basal level (Hachenberg et al. 2007). A concentration of >0.2 mg/mL indicates early or mild inflammation in cattle (Skinner et al. 1991).

Hpt is mainly produced by the liver, but can also be classified as adipokine (Saremi et al 2012). The direct involvement of adipose tissue depots in immune functions is declared to be probable, as the subacute and mesenteric adipose tissue depots are capable to produce proinflammatory cytokines. Consequently, changes in adipose tissue depots can influence the Hpt level in the circulation (Murata et al. 2004, Mukesh et al. 2010, Saremi et al. 2012). However, Hpt concentrations are described as highly variable among individuals. The individual response is dependent on the animal's genotype on the one hand and the metabolic and health conditions on the other hand. Another explanatory approach are the differences between cytokines which are responsible for secretion and synthesis of the APP. Either way, the individuality of the cow as an explanatory variable can describe 22% of the variation (Baumann et al. 1989, Horadagoda et al. 1999, Jacobsen et al. 2004).

2.3.5.2 Tryptophan and Kynurenine

Another important marker for inflammatory processes is the kynurenine:tryptophan (Kyn:Trp)-ratio. It is an indicator for the activity of indolamine-2,3-dioxygenase (IDO) and immune activation (Hayaishi 1976).

IDO as well as tryptophan-2,3-dioxygenase (TDO) are important enzymes in the main metabolic pathway for the degradation of Trp via the Kyn-pathway. Trp is an essential amino acid, which needs to be obtained via food (Nichols et al. 2016). In blood serum of healthy organisms, it was shown to be positively correlated with Kyn. During periods of inflammation, Trp is degraded and therefore decreased, whereas the concentration of Kyn increases (Schröcksnadel et al. 2006, Le Floch'n et al. 2011). The catabolism-rate of Trp is regulated by IDO and TDO which are described as rate-limiting enzymes in Kyn-synthesis (Kanai et al. 2009, Wirthgen et al. 2018). Consequently, availability of Trp for the pathogen is reduced

(Schröcksandel et al. 2006). Kyn and its degradation products are known to fulfil several functions, such as the dilation of blood vessels and the regulation of immune reactions (Schröcksnadel et al. 2006, Wang et al. 2010). The decrease of Trp concentration and the resulting increase of the Kyn:Trp-ratio are also known to establish fetus-tolerance in bovine during the period of peri-implantation (Maes et al. 2001, Schröcksnadel et al. 2003, Groebner et al. 2011).

However, concentration of Trp is influenced by multiple factors. Nutrition, lactation phases as well as breed can lead to individual variations (Klein et al. 2013).

2.3.6 Association between body condition and impaired immune reactivity

On the one hand, the NEB during the periparturient period can also lead to an energy deficit in the immune cells, which might result in immune suppression (Ohtsuka et al. 2006). On the other hand, a severe energy deficit triggers mobilization of stored energy in body fat, which can lead to health problems, too. The consequences of a severe lipolysis are compounded in cows with a high pre-calving BCS, which is often accompanied by postpartum metabolic disorders. The decline of DMI before calving is greater, leading to a more pronounced NEB (Roche et al 2007, Roche et al. 2008). Consequently, the mobilization of body fat is more intense and the increase of ketone bodies exceeds the physiological range. Thus, a rapid loss of body condition after calving leads to metabolic disorders such as ketosis with marked impacts on the immune system (Bell 1995, Markusfeld et al. 1997, Roche et al. 2009, Ingvartsen and Moyes 2012, Dänicke et al. 2018).

However, a markedly low BCS (<3.0) before calving suggests malnutrition and can result in impairment of production and reproduction and immunological deficits, due to insufficient reserves of energy and protein (Roche et al. 2009, Eger et al. 2015). Therefore, Roche et al. (2009) proposed a pre-calving BCS between 3.0 and 3.5 to be optimal.

3. Scope of the thesis

During the transition period cows are exposed to metabolic, endocrine and immunological changes. Their energy requirement increases, whereas energy intake decreases before and increases only slowly after calving, which results in a NEB.

The composition of the ration is the one variable that can be changed most easily in practice. Therefore, the C has a major instrumental importance in dairy farming, as many benefits were awarded. These benefits imply an enhanced DMI and a less severe energy deficit, resulting in a reduced mobilization of body fat and higher milk production. However, several negative consequences, such as a reduction in ruminal pH and microbial efficiency are known.

Determining the BCS is a common method to assess the nutritional status of cows. However, a too high pre-calving BCS is supposed to increase complications at birth and further health problems.

Both pre-calving BCS and postpartum C are known to influence performance, lipolysis, microbial protein synthesis and immune parameters in dairy cows in an interactive manner. Furthermore, individual responsiveness to these factors and their combinations might further contribute to overall variations. Therefore, the present thesis paid special attention to the possibly continuing effects of the pre-calving BCS and effects of varying C of lactation diets on the mentioned relations.

Thus, the following hypotheses were examined in the current study:

1. The dynamics of adipose tissue around parturition along with parameters of energy metabolism and performance are triggered by varying concentrate proportions and different body conditions.
2. Ultrasound based methods characterize the dynamics of adipose tissue changes of transit cows on an individual basis more precisely than the simple determination of BCS and energy balance.
3. The impact of varying dietary C on diurnal ruminal pH-kinetics is higher than that on daily mean pH or time with pH <5.8.
4. Different dietary energy levels impact microbial efficiency.

5. The effect of individually different microbial efficiencies on duodenal nutrient flows and digestibility is greater than that of varying C.
6. Cows differing in BCS at calving show different immunological and haematological responses when supplied with varying C postpartum.

To clarify these issues, this study was done within the project "Evaluation of Animal Welfare in Dairy Farming – Indicators for the Metabolism and Feeding" (Indikatoren im Bereich Stoffwechsel und Fütterung bei Milchkühen, IndiKuh). The project was performed in compliance with the German legislation on animal protection (Animal Welfare Act) and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany) in consultation with an independent ethics committee.

In the present study, sixty pluriparous German Holstein cows were involved from 42 days before, until 120 days after calving. The sixty animals included 13 ruminally-fistulated and 10 additionally duodenally-fistulated cows. Before parturition, cows were either allocated into a high (BCS_H) or a low BCS (BCS_L) group. Both groups received the same total mixed ration consisting of 80% silage (70% maize silage, 30% grass silage on dry matter [DM] basis) and 20% concentrate on a DM basis. After calving, the two groups were further subdivided by diets varying in C. For this, the diet changed to two partial mixed rations consisting of 48% maize silage, 20% grass silage and 32% concentrate on a DM basis. For the groups with lower C (C_{35}) the ration contained 35% concentrate and an energy content of 6.9 MJ NE_L/kg on a DM basis. The groups with a higher C (C_{60}) received 60% concentrate, increasing from 35% to 60% during the first three weeks after calving and reaching an energy content of 7.3 MJ NE_L/kg on a DM basis. Additional concentrate was provided by automatic feeding stations until the required amounts were achieved. Thus, the following four experimental groups emerged: BCS_H/C_{60}, BCS_H/C_{35}, BCS_L/C_{60}, BCS_L/C_{35}.

The results of the described experiment were published in 3 original papers. **Paper I** deals with the dynamics of adipose tissue and energy metabolism as well as performance parameters in dairy cows during early lactation. **Paper II** focusses on the fistulated cows and on ruminal pH parameters, microbial efficiency, duodenal nutrient flows and digestibilities. Moreover, it examines the relation between ruminal pH and microbial efficiency from a new perspective, by additionally grouping the animals according to their individual microbial efficiency during the week of duodenal chyme sampling (week 13 postpartum ± 16 days). **Paper III** addresses

immunological and haematological parameters as well as inflammatory markers during the transition period.

4. Paper I

Bünemann, K., von Soosten, D., Frahm, J. Kersten, S., Meyer, U., Hummel, J., Zeyner, A., Dänicke, S. (**2019**)

Effects of Body Condition and Concentrate Proportion of the Ration on Mobilization of Fat Depots and Energetic Condition in Dairy Cows during Early Lactation Based on Ultrasonic Measurements.

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Effects of Body Condition and Concentrate Proportion of the Ration on Mobilization of Fat Depots and Energetic Condition in Dairy Cows during Early Lactation Based on Ultrasonic Measurements

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Simple Summary: During early lactation, cows face metabolic challenges. They experience a negative energy balance as energy intake increases more slowly than energy output with milk rises. To compensate for that energy deficit, higher amounts of concentrate are offered. Additionally, cows are able to extract energy from body fat by lipid mobilization. Excessive body fat mobilization, however, leads to metabolic disorders. Therefore, high-conditioned cows are suggested to have a more pronounced lipid mobilization. The intention of the present study was to examine the change of various fat depots during the transition period depending on body condition and energy supply with ultrasonic measurements. Body condition loss after calving usually interpreted as mobilization of subcutaneous adipose tissue was not different between cows with a higher or lower body condition score. However, ultrasonic measurements detected a more pronounced mobilization of subcutaneous adipose tissue in higher conditioned animals. In contrast, inner fat depots were mobilized similarly between cows. Higher concentrate feed proportions led to a less pronounced negative energy balance. A less pronounced negative energy balance would have been expected to decrease lipid mobilization. However, this relation could not be verified in the present study. This demonstrates that sonography-based methods provide a clearer picture of metabolic conditions.

Abstract: The aim of this study was to evaluate energy metabolism and lipid mobilization via ultrasonic measurements (USM), considering inner fat depots, in lactating dairy cows differing in body condition score (BCS) and fed rations with low (35% at dry matter basis; C_{35}) or high (60% at dry matter basis; C_{60}) concentrate feed proportions postpartum. Sixty pluriparous German Holstein cows were arranged in a 2 × 2 factorial design from d 42 antepartum (relative to calculated calving) until d 120 postpartum. Animals were divided into a group with a lower (initial BCS = 3.1 ± 0.38 SD; BCS_L) and a group with a higher (initial BCS = 3.83 ± 0.41 SD; BCS_H) BCS. Due to higher dry matter intake C_{60} groups reached the positive energy balance earlier, whereas C_{35} groups had a more pronounced negative energy balance. Although this would suggest a more pronounced mobilization of C_{35} groups the USM revealed no differences between feeding groups. Differences in BCS between both BCS groups remained almost the same over the trial. This was not reflected in ultrasonic data, as lipid mobilization was higher in higher conditioned cows. These findings demonstrate the extended possibilities of USM to depict metabolic processes.

Keywords: dairy cows; animal health; body condition score; concentrate feed proportion; energy metabolism; lipid mobilization; postpartal period

1. Introduction

Cows are exposed to metabolic challenges during the transition period. The imbalance between an insufficient increase of energy intake on the one hand and the high energy requirements, on the other hand, induce a negative energy balance [1,2]. The organism attempts to compensate the energy deficit by mobilizing energy from adipose tissue depots [3]. This process leads to an increase of circulating non-esterified fatty acids (NEFA) such as β-hydroxybutyrate (BHB) after a certain delay to an enlargement of ketone body concentration when hepatic NEFA utilization is insufficient [4,5]. Spreading of ketone bodies involves the risk of ketosis when exceeding critical values. Nielen et al. [6] defined BHB-values in blood serum >1.2 mM as indicator for subclinical ketosis. According to Oetzel [7] NEFA-values >0.4 mM describe a stage were higher lipid mobilization takes place and, therefore, indicate an imbalance in the energy status.

Experimental determination of adipose tissue mobilization usually requires expensive comparative slaughter techniques, which limits the investigation of kinetics of mobilization of individual cows. Using the slaughter technique, Von Soosten et al. [8] determined a mean mobilization of 20 kg during the first 42 days (d) after parturition in heifers. Drackley et al. [9] measured values of 26.5–48.3 kg for mobilization of visceral adipose tissues and 31.8–57.2 kg for abdominal adipose tissues in dry cows. However, little is known about the quantity of adipose tissue mobilization in high lactating, pluriparous cows during the transition period. Using an ultrasound-based technique which was calibrated with simultaneously slaughter-based fat depots, facilitates tracking the development of fat depot masses and consequently of their mobilization and accretion on an individual basis [10]. Thus, this technique was used in the present study to characterize the dynamics of adipose tissue metabolism of transit cows.

Furthermore, the literature reveals a discrepancy about whether a higher body condition increases the body fat mobilization in ruminants [11,12] or not [13]. The same applies for the concentrate proportion and therefore the starch and energy content of the ration. Studies differ in their results when high energy levels of the diet decrease the mobilization of adipose tissues [12,14] or not [13,15]. Hence, a second aim of the experiment was to characterize the deflections in the dynamics of adipose tissue around parturition along with milking performance and parameters of energy metabolism as triggered by varying concentrate feed proportions and different body condition.

2. Materials and Methods

The experiment was performed in compliance with the German legislation on animal protection (Animal Welfare Act) and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany) in consultation with an independent ethics committee (AZ 33.19-42502-04-15/1858).

2.1. Experimental Design

Sixty pluriparous German Holstein cows were involved in the study from 42 d antepartum (a.p.) relative to calculated calving until 120 d postpartum (p.p.). The experimental design was a 2 × 2 factorial layout with body condition score (BCS) and concentrate proportion in the diet (C) as factors. Cows were either in a high or a low BCS group (BCS_H or BCS_L). Further criteria for allocating the animals were milk yield and milk composition of the previous lactation as well as body weight and number of lactation. Supply of energy and nutrients was ensured based on the recommendations of the Society of Nutrition Physiology [16]. Before parturition, both groups received the same total mixed ration (TMR) consisting of 80% silage (70% maize silage, 30% grass silage on dry matter DM

basis) and 20% concentrate on a DM basis. After parturition, the diet changed to two partial mixed rations (PMR) consisting of 48% maize silage, 20% grass silage and 32% concentrate. To subdivide the BCS groups, diets varied in C. Rations for the groups with lower concentrate proportion (C_{35}) contained 35% concentrate and an energy content of 6.9 MJ NE_L/kg DM. The lower C was chosen to stimulate lipolysis by means of an energetic undersupply [4]. For the groups with a higher amount of concentrate (C_{60}), C increased from 35–60% during the first three weeks p.p. The C_{60} ration contained an energy content of 7.3 MJ NE_L/kg DM. Additional concentrate was provided by automatic feeding stations (Insentec, B.V., Marknesse, The Netherlands) until the required amounts were achieved. All cows remained in treatment until day (d) 120 in milk (DIM). Cows in the BCS_H group started with an average BCS of 3.83 (±0.41 standard deviation SD). The BCS_L group had an average BCS of 3.1 (±0.38 SD). Cows of group BCS_H/C_{60} (n = 15) had an average parity of 3.4 (±1.1 SD). For the BCS_H/C_{35} (n = 15) group the average parity was 3.3 (±1.2 SD). Cows of the BCS_L/C_{60} (n = 15) group had an average parity of 2.6 (±0.9 SD) and for the BCS_L/C_{35} (n = 15) group the average parity was 2.5 (±0.6 SD). TMR and PMR were provided ad libitum by self-feeding stations (RIC, Insentec B.V., Marknese, The Netherlands). The components and the chemical compositions of the feedstuffs are presented in Tables 1 and 2.

Table 1. Composition of concentrates during the dry and the lactating periods.

Components, g/kg of Fresh Matter	Concentrates		
	Dry Period	C_{35}	C_{60}
Soybean meal	115		
Rapeseed meal	150	400	200
Wheat	330	150	213
Barley		144	213
Maize		200	290
Dried sugar beet pulp	296	50	50
Urea	30	8	
Calcium carbonate	24	13	12
Soybean oil	15	10	10
Vitamin-mineral premix [+]	40		
Vitamin-mineral premix [#]		25	12

[+] Mineral feed for dry cows, ingredients per kg according to the manufacturer's specification: 10 g Ca; 120 g Na; 60 g P; 60 g Mg; 6 g Zn; 4 g Mn; 1.25 g Cu; 100 mg I; 50 mg Se; 35 mg Co; 8,000,000 IU vitamin A; 1,000,000 vitamin D_3; 2500 mg vitamin E, [#] Mineral feed for lactating dairy cows, ingredients per kg according to the manufacturer's specifications: 140 g Ca; 120 g Na; 70 g P; 40 g Mg; 6 g Zn; 5.4 g Mn; 1 g Cu, 100 mg I; 40 mg Se; 25 mg Co; 1,000,000 IU vitamin A; 1,000,000 IU vitamin D_3; 1500 mg vitamin E.

Table 2. Chemical components of concentrates and roughage during the experimental period from day 42 antepartum until day 120 postpartum.

Chemical Composition	Concentrates			Roughage	
	Dry Period	C_{35} *	C_{60} [#]	Maize Silage	Grass Silage
Dry matter, g/kg	890	878	878	361	306
Nutrients, g/kg DM [§]					
Crude ash	90	76	55	40	94
Crude protein	277	239	170	82	127
Ether extract	42	48	48	32	33
Crude fiber	94	85	66	203	273
a[†]Neutral detergent $fiber_{om}$ [‖]	207	208	178	401	526
Acid detergent $fiber_{om}$ [‖]	125	125	96	230	297
Starch content	310	368	490	331	0
Energy [‡], MJ/kg of DM					
ME	11.9	12.3	12.8	10.8	10.4
NE_L	7.4	7.7	8.1	6.5	6.2

[‡] Calculation based on equations of GfE [16]. [§] Dry matter. [†] Assayed with a heat-stable amylase. [‖] Expressed exclusive of residual ash. * Total starch content of C_{35}-ration was 285 g/kg DM. Total energy content of C_{35}-ration was 11.3 MJ ME/kg DM and 6.9 MJ NE_L/kg DM. [#] C_{60}-ration contained a starch content of 353 g/kg DM. Total energy content of C_{60}-ration was 11.8 MJ ME/kg DM and 7.3 MJ NE_L/kg DM.

2.2. Sample and Data Collection

Samples of the mixed rations components were taken twice weekly and then pooled to a collective sample for periods of 4 weeks. Samples of concentrate were collected once a week and pooled to a collective sample every 4 weeks, as well. Through the whole experiment, dry matter intake (DMI) was recorded individually for both PMR and concentrate provided by computerized feeding stations. Live weight was recorded weekly a.p. and twice daily p.p. after each milking. Milk yield was quantified twice daily during milking at 0530 and 1530 h by automatic milk counters (Lemmer Fullwood GmbH, Lohmar, Germany). Milk samples were taken twice a week during morning and evening milking and stored at 4 °C until analysis. BCS was determined at noon in weekly intervals on a 5-point-scale according to Edmonson et al. [17].

Blood samples were taken at determined time points, whereby deviations of 2 d were tolerated (42 d a.p., 14 d a.p., 7 d a.p., 3 d a.p., 3 d p.p., 7 d p.p., 14 d p.p., 21 d p.p., 28 d p.p., 42 d p.p., 56 d p.p., 70 d p.p. 95 d p.p. and 120 d p.p.) from the vena jugularis externa. Blood samples were centrifuged (Heraeus Varifuge 3.0R Heraeus, Osterode, Germany; 2123× *g*, 15 °C, 15 min) and stored at −80 °C until further analysis.

Ultrasonic measurements (USM) of adipose tissues took place at defined points in time, too (d 3 p.p., d 28 p.p., d 70 p.p. and d 120 p.p.). Back fat thickness (BFT) was estimated according to Staufenbiel [18], while rib fat thickness (RFT), subcutaneous (SAT), retroperitoneal (RAT), mesenteric (MAT) and omental (OAT) adipose tissues were assessed according to Raschka et al. [10]. For this purpose, double measurement of each tissue were performed using a Mindray M5 Vet (Mindray, Shenzhen, China) diagnostic ultrasound system with a linear (g MHz, Mindray 6LE5Vs) and a convex probe (3 MHz, Mindray 3C5s). The thickness of the measured fat tissues was sized in millimeters. To calculate the adipose tissue depot masses in kg the measuring points established by Raschka et al. [10] were used (Table A1). For calculating the mobilization of adipose tissue depots, the experiment was divided in periods (period 1: weeks 1–4 postpartum, period 2: weeks 5–10 postpartum, period 3: weeks 11–17 postpartum), which were also used for analyzing the remaining parameters to receive a consistent statistical design.

One animal could not be evaluated due to erroneous, implausible values.

2.3. Analyses

PMR components and concentrate were analyzed for DM, crude ash, crude protein, ether extract, crude fiber, neutral detergent fiber (NDF_{om}) and acid detergent fiber (ADF_{om}) according to the standard methods of the Association of German Agricultural Analysis and Research Centres [19] (Table 2). Milk samples were analyzed for fat, protein and lactose by an infrared milk analyzer (Milkoscan FT 6000; Foss Electric, Hillerød, Denmark). Using an automatic photometric measurement system (Eurolyser, Type VET CCA, Salzburg, Austria) serum samples were analyzed for β-hydroxybutyrat (BHB), non-esterified fatty acids (NEFA), triglycerides and glucose. According to the classification by Nielen et al. [6] BHB-values in blood serum >1.2 mM were used as indicator for subclinical ketosis.

2.4. Calculations

Weekly means of DMI, net energy intake (NEI), net energy balance (NEB), milk yield, and milk components were used for further calculations. Computations of net energy requirements for maintenance (NE_M) and lactation (NE_L) as well as for milk energy concentration were based on equations published by the Society of Nutrition Physiology [16]:

$$NE_M \text{ (MJ of } NE_L/d) = 0.294 \times BW^{0.75} \quad (1)$$

$$NE_L \text{ (MJ of } NE_L/d) = [\text{milk energy concentration (MJ of } NE_L/d) + 0.086] \times \text{milk yield (kg/d)} \quad (2)$$

$$\text{Milk energy (MJ/kg)} = 0.38 \times \text{milk fat (\%)} + 0.21 \times \text{milk protein (\%)} + 0.95 \quad (3)$$

The equation published by Gaines [20] was used to calculate the fat corrected milk (FCM):

$$4\%\ \text{FCM (kg/d)} = \{[\text{milk fat (\%)} \times 0.15] + 0.4\} \times \text{milk yield (kg/d)} \quad (4)$$

Energy corrected milk (ECM) was calculated based on the equation by Sjaunja et al. [21]:

$$\begin{gathered}\text{ECM (kg/d)} = \text{milk yield (kg/d)} \times \\ \{[38.3 \times \text{milk fat (g/kg)} + 24.2 \times \text{milk protein (g/kg)} + 16.54 \times \text{milk lactose (g/kg)} + 20.7]/3140\}\end{gathered} \quad (5)$$

In order to calculate the energy intake per day, the energy content of the feedstuffs was multiplied by DMI.

To calculate the NEB, the following equation was used:

$$\text{NEB (MJ of NE}_\text{L}\text{/d)} = \text{NEI (MJ of NE}_\text{L}\text{/d)} - \text{NE}_\text{M}\text{ (MJ of NE}_\text{L}\text{/d)} - \text{NE}_\text{L}\text{ (MJ of NE}_\text{L}\text{/d)} \quad (6)$$

To regard the gestational requirements in the NEB 13 MJ of NE_L/d were subtracted from week 6-3 a.p. During the last 3 weeks until calving the requirements were assessed with 18 MJ of NE_L/d.

For determination of the different adipose tissue depot masses, equations published by Raschka et al. [10] were used based on the collected data of the ultrasonic measurements:

$$\text{Subcutaneous adipose tissue (SAT, kg)} = -6.66 + 0.72 \times \text{R12} + 0.31 \times \text{AW3c} \quad (7)$$

$$\text{Retroperitoneal adipose tissue (RAT, kg)} = -9.55 + 0.62 \times \text{R12} + 0.06 \times \text{KD3b} \quad (8)$$

$$\text{Omental adipose tissue (OAT, kg)} = -2.32 + 0.55 \times \text{BFT} + 0.37 \times \text{AW3b} \quad (9)$$

$$\begin{gathered}\text{Mesenteric adipose tissue (MAT, kg)} = -12.8 + 0.38 \times \text{AW1b} + 1.73 \times \text{AW3b} \\ - 1.45 \times \text{AW3c} + 0.07 \times \text{KD2c}\end{gathered} \quad (10)$$

$$\text{Sum of mobilized adipose tissues (SoM, kg)} = \text{SAT} + \text{RAT} + \text{OAT} + \text{MAT} \quad (11)$$

Time-dependent changes in the individual and total adipose tissue depots were calculated by the differences of the fat masses between different time points.

It is assumed that 1 g of body fat corresponds to 39.8 kJ gross energy [22] of which 16% is lost as heat when fat is mobilized [23]. The following equation was used to estimate the energy mobilized from the adipose tissue depots:

$$\text{Mobilized energy} = \text{Mobilized fat (kg)} \times 39.8\ \text{MJ/kg} \times 0.84 \quad (12)$$

We used the following equations according to Hurley et al. [24] to calculate the efficiency parameters feed efficiency (FE), energy conversion efficiency (ECE), metabolic efficiency (MEff) and residual energy intake (REI):

$$\text{FE} = \text{ECM/DMI (kg/kg)} \quad (13)$$

$$\text{ECE} = \text{Energy excretion with milk (MJ)/Energy intake (MJ NE}_\text{L}\text{)} \quad (14)$$

$$\text{MEff} = [\text{Energy intake (MJ NE}_\text{L}\text{)} - \text{Energy excretion with milk (MJ)}]/\text{Body weight}^{0.75}\ \text{(kg)} \quad (15)$$

$$\text{REI} = \text{Energy intake (MJ NE}_\text{L}\text{)} - \text{expected energy intake (MJ NE}_\text{L}\text{)} \quad (16)$$

Variables of each of the three periods were finally summarized according to the mobilization of the adipose tissue depots, the experimental weeks of the performance, the milk and blood parameters, as well as the results of the calculated efficiency.

2.5. Statistical Analyses

As animals were fed similar diets before parturition, we evaluated the data for the postpartum period only.

The statistical analyses were performed by utilizing the statistical software SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). Performance parameters, blood values, mobilization of adipose tissue depot masses and efficiency parameters were analyzed by using the MIXED procedure for repeated measures with a compound symmetry structure [25]. BCS classification (BCS_H, BCS_L), C (C_{35}, C_{60}) and period (1, 2, 3) were applied as fixed effects, as well as the interactions between them. Each cow within treatment was considered a random effect. The period of sampling was regarded to be a repeated measure. Milk parameters were analyzed with the first measured value in week 1 as covariate. For the remaining parameters, the first measured value before calving was used as covariate. p-values ≤ 0.05 were declared to be statistically significant and p-values ≤ 0.01 considered highly significant. For calculating correlations between parameters, we employed the statistical software TIBCO Statistica (Version 13.3, TIBCO Software Inc., Palo Alto, CA, USA) by using Pearson's correlation. The correlation coefficient (r) was considered statistically significant, when $p \leq 0.05$, and highly significant, when $p \leq 0.01$. In the following, results are presented as LSMean $\pm$ Standard error of means (SEM) unless otherwise stated.

3. Results

3.1. Performance Parameters

For DMI (Figure 1A) we observed a C $\times$ period interaction ($p = 0.042$). The C_{35} groups exhibited a lower DMI than the C_{60} groups over time. All groups had a lower DMI in the first period compared to the following.

The same is true for NEI (Table 3), where we also detected a C $\times$ period interaction ($p = 0.001$). C_{35} groups had a significantly lower NEI than C_{60} groups in periods 2 and 3. For NEB (Figure 1B) we observed a treatment x period interaction ($p = 0.020$) as it was more negative in BCS_H/C_{35} group than in BCS_H/C_{60} and BCS_L/C_{60} groups in period 1. Furthermore, NEB was in a positive range for the BCS_H/C_{60} and BCS_L/C_{60} groups, but in a negative range for the BCS_H/C_{35} and BCS_L/C_{35} groups in period 2. The development of the NEB outlines that the C_{60} groups reached a balanced NEB after four weeks p.p. and remained relatively stable then, whereas the C_{35} groups increased continuously and reached the settlement only at the end of the experiment.

For BCS (Figure 1C) we proved a treatment x period interaction ($p = 0.030$). The BCS_H/C_{60} differed from the BCS_L/C_{60} and BCS_L/C_{35} group in all three periods, due to higher mean values. Moreover, the BCS_H/C_{35} showed higher means in comparison to the BCS_L/C_{35} group in period 1.

Live weight (Table 3) decreased from period 1 to 2, whereas it increased from period 2 to 3 within the C_{35} and C_{60} groups. We found a C $\times$ period interaction ($p = 0.018$), but no differences within one period.

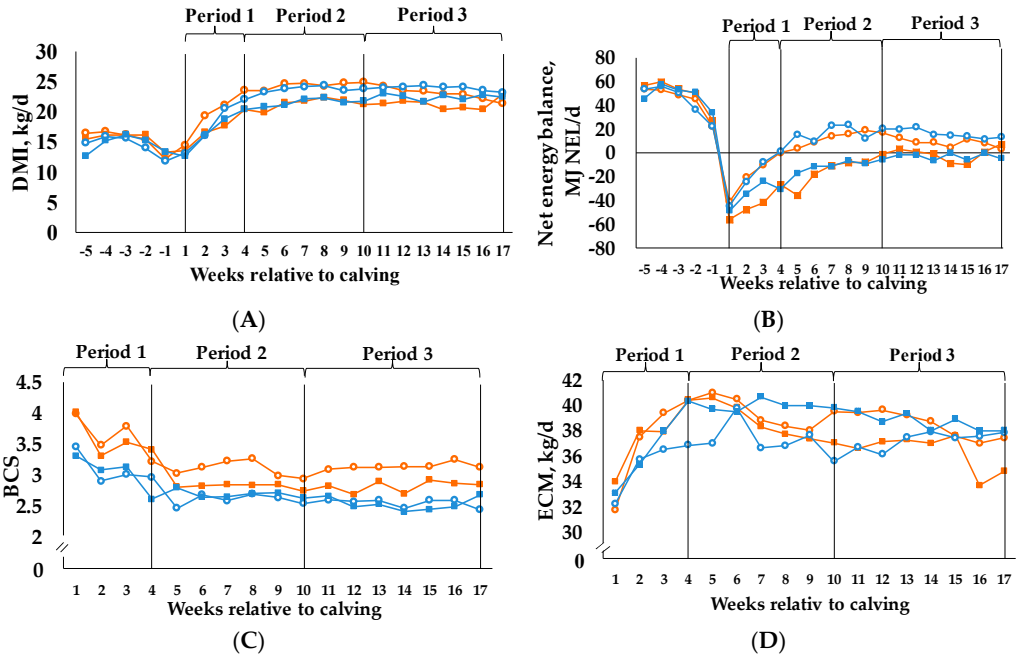

Figure 1. Development of (**A**) dry matter intake (DMI), (**B**) net energy balance, (**C**) body condition score (BCS) and (**D**) energy corrected milk yield (ECM) in the course of the experiment. Cows were categorized in high BCS (BCS_H) and low BCS (BCS_L). After parturition, these two groups were divided again, each into a group with a concentrate proportion of 60% (C_{60}) in the ration (increasing from 35–60% during the first three weeks after calving) and a group with a concentrate proportion of 35% (C_{35}) in the ration. Thus, four groups emerged: BCS_H/C_{60} (n = 15; ○), BCS_H/C_{35} (n = 15; ■), BCS_L/C_{60} (n = 15; ○), BCS_L/C_{35} (n = 15; ■), BCS, DMI and NEB were analyzed with first measured value before calving as covariate, ECM was analyzed with first measured value from week 1 as covariate, (**A**) *p*-values: BCS = 0.761, C = 0.069, period < 0.001, BCS × C = 0.836, BCS × period = 0.232, C × period = 0.042, BCS × C × period = 0.295; (**B**) *p*-values: BCS = 0.540, C < 0.001, period < 0.001, BCS × C = 0.985, BCS × period = 0.639, C × period < 0.001, BCS × C × period = 0.020; (**C**) *p*-values: BCS = 0.004, C = 0.252, period < 0.001, BCS × C = 0.998, BCS × period = 0.011. C × period = 0.182, BCS × C × period = 0.030; (**D**) *p*-values: BCS = 0.679, C = 0.579, period < 0.001, BCS × C = 0.217, BCS × period = 0.003, C × period = 0.043, BCS × C × period = 0.150.

Table 3. Effects of body condition, concentrate proportion in the diet (C) and period on dry matter intake (DMI), energy intake and live weight (LSM) during period 1 (weeks 1–4 postpartum), period 2 (weeks 5–10 postpartum) and period 3 (weeks 11–17 postpartum) in the treatment groups.

Item [+]	Treatment [§]				SEM [#]	*p*-Value *					
	BCS_H/C_{60} n = 15	BCS_H/C_{35} n = 15	BCS_L/C_{60} n = 15	BCS_L/C_{35} n = 15		BCS	C	BCS × C	BCS × Period	C × Period	BCS × C × Period
DMI											
Period 1	19.4	17.7	18.6	18.0	0.6	0.761	0.069	0.836	0.232	0.042	0.295
Period 2	24.3	22.6	24.4	22.9							
Period 3	23.4	23.3	24.3	23.6							
Energy intake, MJ of NE_L/day											
Period 1	143	124	131	128	4	0.949	<0.001	0.252	0.144	0.001	0.069
Period 2	182	157	179	163							
Period 3	175	163	177	166							
Live weight, kg											
Period 1	706	663	701	673	14	0.865	0.069	0.726	0.800	0.018	0.225
Period 2	694	662	688	670							
Period 3	699	677	703	682							

[§] Before calving cows were classified in high and low body condition score (BCS)-groups (BCS_H, BCS_L), after calving both groups were divided again, each into a group with 35% concentrate proportion (C_{35}) and a group with 60% concentrate proportion (C_{60}) in the ration (increasing from 35–60% during the first three weeks after parturition). Thus, four groups emerged: BCS_H/C_{60} (n = 15), BCS_H/C_{35} (n = 15), BCS_L/C_{60} (n = 15), BCS_L/C_{35} (n = 15). Values are presented as LSMeans, * Period ($p < 0.001$) for all variables, [+] Analyzed with first measured value before calving as covariate, [#] Pooled standard error of means.

3.2. Milk Parameters

For milk yield (Tables 4 and 5) we detected two interactions, BCS × period ($p = 0.023$) and C × period ($p < 0.001$). However, neither BCS nor C had an influence within the same period. For milk fat content (Tables 4 and 5) the same interactions were determined (BCS × period: $p < 0.001$, C × period: $p = 0.001$). The higher BCS led to a higher milk fat content in period 1, the same did a lower C in periods 2 and 3.

Table 4. Effects of body condition, concentrate proportion in the diet (C) and period on milk parameters (LSM) during period 1 (weeks 1–4 postpartum), period 2 (weeks 5–10 postpartum) and period 3 (weeks 11–17 postpartum) in the treatment groups.

Item [+]	Treatment [§]				SEM [#]
	BCS_H/C_{60} n = 15	BCS_H/C_{35} n = 15	BCS_L/C_{60} n = 15	BCS_L/C_{35} n = 15	
Milk yield, kg/day					
Period 1	32.8	32.2	32.1	33.8	1.1
Period 2	42.4	38.9	41.8	42.4	
Period 3	41.2	37.6	42.1	40.9	
Milk fat content, %					
Period 1	4.68	4.94	4.33	4.52	0.15
Period 2	3.23	3.79	3.19	3.83	
Period 3	3.26	3.58	3.06	3.73	
Milk fat yield, kg/day					
Period 1	1.62	1.63	1.49	1.61	0.06
Period 2	1.37	1.54	1.30	1.57	
Period 3	1.34	1.41	1.26	1.49	
Milk protein content, %					
Period 1	3.49	3.35	3.39	3.37	0.05
Period 2	3.23	3.18	3.24	3.15	
Period 3	3.30	3.27	3.24	3.27	
Milk protein yield, kg/day					
Period 1	1.21	1.14	1.18	1.20	0.03
Period 2	1.37	1.27	1.34	1.31	
Period 3	1.34	1.26	1.37	1.32	
Milk lactose content, %					
Period 1	4.77 ^{c,A}	4.58 ^{c,B}	4.72 ^{c,AB}	4.75 ^{c,AB}	0.03
Period 2	4.88 ^{b}	4.85 ^{b}	4.80 ^{b}	4.83 ^{b}	
Period 3	4.91 ^{a}	4.90 ^{a}	4.87 ^{a}	4.85 ^{a}	
Milk lactose yield, kg/day					
Period 1	1.67 ^{b}	1.60 ^{b}	1.64 ^{b}	1.71 ^{b}	0.05
Period 2	2.07 ^{a}	1.88 ^{a}	2.01 ^{a}	2.01 ^{a}	
Period 3	2.01 ^{a}	1.89 ^{a}	2.06 ^{a}	1.96 ^{a}	
Milk fat:protein ratio					
Period 1	1.35	1.43	1.25	1.31	0.04
Period 2	1.03	1.23	0.95	1.18	
Period 3	1.02	1.14	0.91	1.11	
Milk energy concentration, MJ/kg					
Period 1	3.65 ^{a}	3.74 ^{a}	3.48 ^{a}	3.51 ^{a}	0.06
Period 2	2.98 ^{b,AB}	3.23 ^{b,A}	2.88 ^{b,B}	3.14 ^{b,AB}	
Period 3	3.01 ^{b,AB}	3.16 ^{b,A}	2.84 ^{b,B}	3.13 ^{b,AB}	
Milk energy output, MJ/day					
Period 1	124.3	125.7	118.1	122.9	3.6
Period 2	131.7	129.4	125.7	133.9	
Period 3	128.0	122.0	125.4	129.3	
4% FCM, kg/day					
Period 1	37.5	38.4	35.3	37.0	1.2
Period 2	38.4	38.8	36.3	39.9	
Period 3	37.3	36.1	36.0	38.1	

^{a,b} Means with different superscripts differ within columns, ^{A,B} Means with different superscripts differ within row, [§] Before calving cows were classified in high and low body condition score (BCS)-groups (BCS_H, BCS_L), after calving both groups were divided again, each into a group with 35% concentrate proportion (C_{35}) and a group with 60% concentrate proportion (C_{60}) in the ration (increasing from 35–60% during the first three weeks after parturition). Thus, four groups emerged: BCS_H/C_{60} (n = 15), BCS_H/C_{35} (n = 15), BCS_L/C_{60} (n = 15), BCS_L/C_{35} (n = 15). Values are presented as LSMeans. [+] Analyzed with first measured value from week 1 as covariate, [#] Pooled standard error of means.

Table 5. *p*-values of effects of body condition, concentrate proportion in the diet (C), period and interactions between them on milk parameters.

Item [+]	*p*-Value *					
	BCS	**C**	**BCS × C**	**BCS × Period**	**C × Period**	**BCS × C × Period**
Milk yield, kg/day	0.205	0.302	0.181	0.026	<0.001	0.229
Milk fat content, %	0.362	0.002	0.655	<0.001	<0.001	0.059
Milk fat yield, kg/day	0.580	0.013	0.296	0.165	0.001	0.738
Milk protein content, %	0.574	0.290	0.631	0.777	0.019	0.091
Milk protein yield, kg/day	0.480	0.069	0.267	0.129	0.083	0.320
Milk lactose content, %	0.642	0.244	0.083	0.010	0.107	0.013
Milk lactose yield, kg/day	0.372	0.187	0.268	0.533	0.006	0.005
Milk fat:protein ratio	0.05	0.001	0.749	0.211	<0.001	0.163
Milk energy concentration, MJ/kg	0.035	0.004	0.881	0.022	<0.001	0.018
Milk energy output, MJ/day	0.780	0.633	0.245	0.003	0.040	0.192
4% FCM, kg/day	0.566	0.278	0.288	0.009	0.046	0.169

Before calving cows were classified in high and low body condition score (BCS)-groups (BCS_H, BCS_L), after calving both groups were divided again, each into a group with 35% concentrate proportion (C_{35}) and a group with 60% concentrate proportion (C_{60}) in the ration (increasing from 35–60% during the first three weeks after parturition). Thus, four groups emerged: BCS_H/C_{60} (n = 15), BCS_H/C_{35} (n = 15), BCS_L/C_{60} (n = 15), BCS_L/C_{35} (n = 15). * Period ($p < 0.001$) for all variables, [+] Analyzed with first measured value from week 1 as covariate.

This is similar to the C × period interaction ($p = 0.001$) for milk fat yield (Tables 4 and 5), where lower C also led to higher means in period 2. For milk protein content (Tables 4 and 5), we found a C × period interaction ($p = 0.021$), too. There were no differences within the same period. Period ($p < 0.001$) had an effect on milk protein yield (Tables 4 and 5), as period 1 differed from periods 2 and 3 due to higher milk protein yields for the latter two periods in all four groups. We discovered a BCS × C × period interaction ($p = 0.013$) for milk lactose content (Tables 4 and 5). In period 1 the BCS_L/C_{60} group differed from the BCS_L/C_{35} group with regard to a higher mean. The same interaction was determined for milk lactose yield (BCS × C × period: $p = 0.005$, Tables 4 and 5), although the groups exhibited no differences within the same period. We observed a C × period interaction for milk fat:protein ratio (Tables 4 and 5), where lower C again led to higher values in all three periods. For milk energy concentration (Tables 4 and 5), we observed a BCS × C × period interaction ($p = 0.017$). In periods 2 and 3 the BCS_H/C_{35} group differed from the BCS_L/C_{60} group regarding a higher energy concentration. The BCS_L/C_{60} group and the BCS_L/C_{35} group exhibited different means in period 3 only, whereby the former showed a higher energy concentration. We determined a BCS × period interaction ($p = 0.003$) and a C × period interaction ($p = 0.040$) for milk energy output (Tables 4 and 5). However, no differences within the same periods were found. The same is true for the 4% FCM (Tables 4 and 5). We determined the same interactions (BCS × period: $p = 0.008$, C × period: $p = 0.044$), but did not observe any differences within same periods. We found those two interactions (BCS × period: $p = 0.003$, C × period: $p = 0.040$) again for ECM (Figure 1D), but once more there were no differences within the same periods quantifiable.

3.3. Mobilization of Adipose Tissue Depots and Energy

BCS influenced BFT (Table 6) over time as we found a BCS × period interaction ($p = 0.005$). However, we could not determine differences between groups within one period. For RFT (Table 6) a time effect ($p < 0.001$) was observed. In all groups RFT was more degraded in period 1 than in periods 2 and 3.

Table 6. Effect of body condition, C (Concentrate proportion in the diet) and period on change of back fat thickness and rib fat thickness (LSM) during period 1 (weeks 1–4 postpartum), period 2 (weeks 5–10 postpartum) and period 3 (weeks 11–17 postpartum) in the treatment groups. Negative values represent accretion of adipose tissue, positive values describe mobilization.

Item [+]	Treatment [§]				SEM [#]	*p*-Value [*]					
	BCS_H/C_{60} n = 15	BCS_H/C_{35} n = 14	BCS_L/C_{60} n = 15	BCS_L/C_{35} n = 15		BCS	C	BCS × C	BCS × Period	C × Period	BCS × C × Period
Back fat thickness, cm/day											
Period 1	0.14	0.15	0.07	0.10	0.02	0.750	0.384	0.563	0.003	0.549	0.819
Period 2	−0.02	0.00	0.01	0.03	0.02						
Period 3	−0.01	−0.02	0.01	0.01	0.02						
Rib fat thickness, cm/day											
Period 1	0.04	0.07	0.09	0.09	0.03	0.136	0.567	0.881	0.903	0.809	0.827
Period 2	0.01	0.01	0.02	0.04	0.03						
Period 3	−0.05	−0.05	−0.02	−0.02	0.03						

[§] Before calving cows were classified in high and low body condition score (BCS)-groups (BCS_H, BCS_L), after calving both groups were divided again, each into a group with 35% concentrate proportion (C_{35}) and a group with 60% concentrate proportion (C_{60}) in the ration (increasing from 35–60% during the first three weeks after parturition). Thus, four groups emerged: BCS_H/C_{60} (n = 15), BCS_H/C_{35} (n = 15), BCS_L/C_{60} (n = 15), BCS_L/C_{35} (n = 15). Values are presented as LSMeans, * Period ($p < 0.001$) for all variables, [+] Analyzed with first value measured before calving as covariate, [#] Pooled standard error of means.

The same effect is true for OAT (Figure 2A, $p_{period} < 0.001$) and RAT (Figure 2B, $p_{period} < 0.001$). In contrast, BCS had an influence over time concerning SAT (Figure 2C), as we detected a BCS × period interaction ($p = 0.002$). In period 1 we observed higher mobilization for BCS_H/C_{60} and BCS_H/C_{35} than for BCS_L/C_{60} and BCS_L/C_{35}. Apart from that we found a BCS × C interaction ($p = 0.028$) for MAT (Figure 2D).

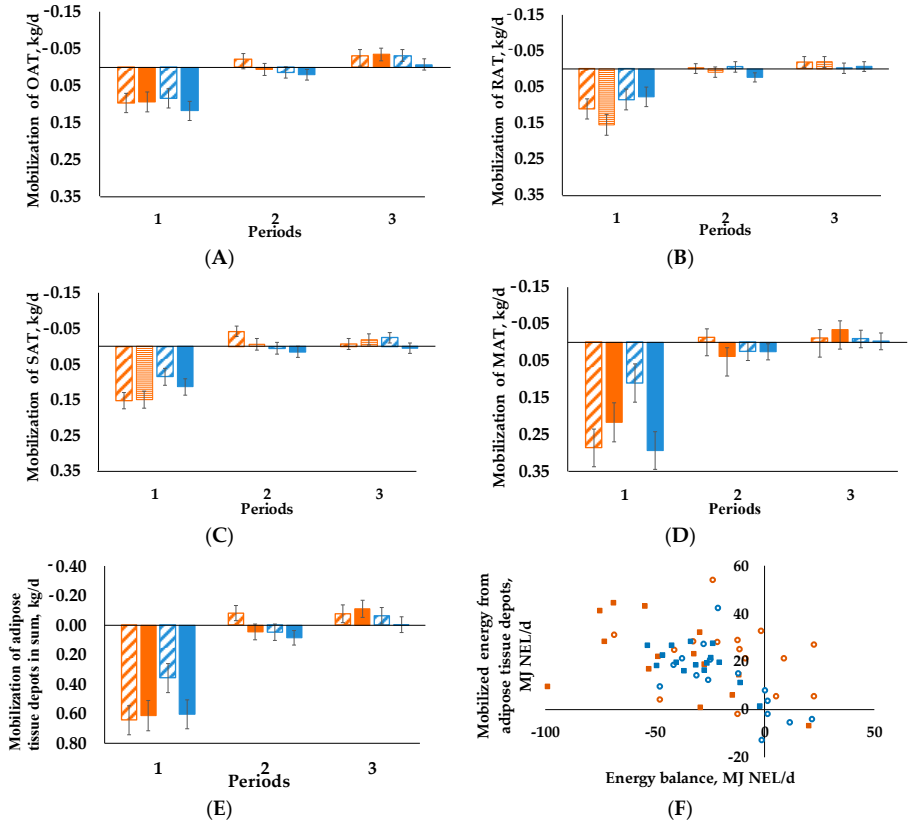

Figure 2. Mobilization of the single adipose tissues and the sum of the (**A**) omental (OAT), (**B**) retroperitoneal (RAT), (**C**) subcutaneous (SAT), (**D**) mesenteric (MAT), (**E**) adipose tissues, negative values represent accretion of adipose tissue, positive values describe mobilization, LSMeans) of the experimental groups during the three periods, period 1: weeks 1–4 postpartum, period 2: weeks 5–10 postpartum, period 3: weeks 11–17 postpartum); as well as the (**F**) correlation of mobilized energy from adipose tissues and energy balance for each individual cow in period 1 (correlation coefficient = −0.4634, $p < 0.05$). Cows were categorized in high BCS (BCS_H) and low BCS (BCS_L). After parturition, these two groups were divided again, each into a group with a concentrate proportion of 60% (C_{60}) in the ration (increasing from 35–60% during the first three weeks after calving) and a group with a concentrate proportion of 35% (C_{35}) in the ration. Thus, four groups emerged: BCS_H/C_{60} (n = 15; **A**–**E** orange striped bars, **F** ○) BCS_H/C_{35} (n = 14; **A**–**E** orange bars, **F** ■), BCS_L/C_{60} (n = 15; **A**–**E** blue striped bars, **F** ○), BCS_L/C_{35} (n = 15; **A**–**E** blue bars, **F** ■). Error bars indicate SEM. Mixed models are analyzed with first measured value before calving as covariate, (**A**) *p*-values: BCS = 0.216, C = 0.213, period < 0.001, BCS × C = 0.536, BCS × period = 0.734, C × period = 0.953, BCS × C × period = 0.382; (**B**) *p*-values: BCS = 0.428, C = 0.381, period < 0.001, BCS × C = 0.648, BCS × period = 0.089, C × period = 0.526, BCS × C × period = 0.382; (**C**) *p*-values: BCS = 0.740, C = 0.250, period < 0.001, BCS × C = 0.549, BCS × period = 0.002, C × period = 0.788, BCS × C × period = 0.244; (**D**) *p*-values: BCS = 0.703, C = 0.163, period < 0.001, BCS × C = 0.034, BCS × period = 0.480, C × period = 0.366, BCS × C × period = 0.065; (**E**) *p*-values: BCS = 0.984, C = 0.138, period < 0.001, BCS × C = 0.311, BCS × period = 0.087, C × period = 0.595, BCS × C × period = 0.232.

Neither C nor BCS had an influence on SoM (Figure 2E), only a time effect ($p_{period} < 0.001$) was visible. Period 1 differed from periods 2 and 3 concerning higher mobilization in all groups. For period 2 the BCS_H/C_{60} group exhibited accretion, which is also true for all groups in period 3.

3.4. Blood Parameters

Results for BHB (Figure 3A) and NFEA (Figure 3B) are presented in d, as the measuring points were determined in d relative to calving. Nevertheless, in accordance with the other data, the statistical analyses are calculated in periods and start at calving.

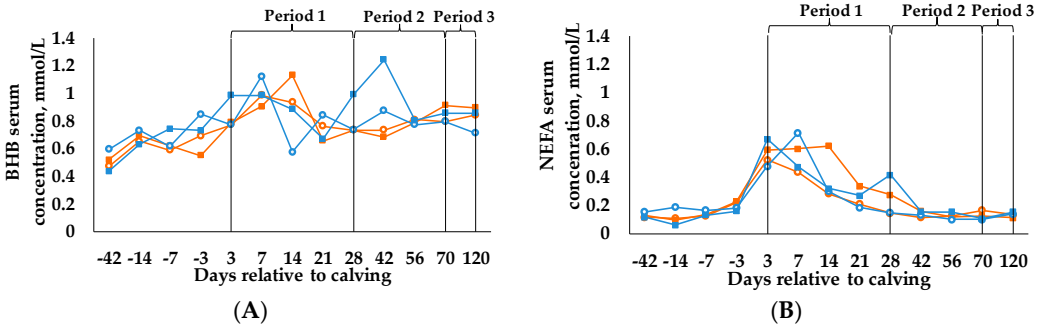

Figure 3. Concentration of (**A**) β-hydroxybutyrat (BHB) and (**B**) non-esterified fatty acids (NEFA) in blood serum (LSM) from d 42 antepartum until d 120 postpartum. Statistical analysis starts at calving (period 1: weeks 1–4 postpartum, period 2: weeks 5–10 postpartum, period 3: weeks 11–17 postpartum). Cows were categorized in high BCS (BCS_H) and low BCS (BCS_L). After parturition, these two groups were divided again, each into a group with a concentrate proportion of 60% (C_{60}) in the ration (increasing from 35–60% during the first three weeks after calving) and a group with a concentrate proportion of 35% (C_{35}) in the ration. Thus, four groups emerged: BCS_H/C_{60} (n = 15; ○), BCS_H/C_{35} (n = 15; ■), BCS_L/C_{60} (n = 15; ○), BCS_L/C_{35} (n = 15; ■), Parameters are analyzed with first measured value before calving as covariate, (**A**) *p*-values: BCS = 0.747, C = 0.346, period = 0.906, BCS × C = 0.621, BCS × period = 0.272, C × period = 0.762, BCS × C × period = 0.422; (**B**) *p*-values: BCS = 0.877, C = 0.208, period < 0.001, BCS × C = 0.909, BCS × period = 0.557, C × period = 0.131, BCS × C × period = 0.069.

Neither BCS and C, nor time affected the concentration of BHB in blood serum. Only the BCS_L/C_{35} group exceeded the threshold of 1.2 mmol/L declared as an indicator for subclinical ketosis according to Nielen et al. [6] in period 2.

For NEFA, we observed a time effect ($p < 0.001$) as concentration in blood serum was highest in period 1 and decreased in periods 2 and 3.

The same is true for glucose (Table A2) as a time effect ($p < 0.001$) was determined. Like in the case of NEFA, the concentration of glucose in blood serum was lower in period 1 than in periods 2 and 3, regardless of BCS and C.

Triglycerides (Table A2) were influenced by C, as C_{60} groups presented higher values than C_{35} groups. Furthermore, triglycerides exhibited a BCS × period interaction ($p = 0.034$). However, there were no differences within the same period. In accordance to the preceding parameter, the concentration of triglycerides was lowest in period 1 in all experimental groups.

3.5. Efficiency Parameters

To compare the different energy and feed efficiencies between groups, efficiency variables were calculated. The C affected FE (Tables A3 and A4), as we observed a C effect ($p = 0.003$) as well as a time effect ($p = 0.001$). Groups with a lower concentrate proportion were more efficient. In period 1 all groups showed the highest FE compared to periods 2 and 3. The same is true for ECE

(Tables A3 and A4) were we determined the same effects ($p_C < 0.001$, $p_{period} < 0.001$). Again, groups with lower concentrate proportion had higher ECE values because of a higher efficiency. In accordance with the FE, ECE values were higher in period 1 than in periods 2 and 3 in all groups.

MEff (Tables A3 and A4) exhibited a C × period interaction ($p < 0.001$). Groups with a lower concentrate proportion in the ration had lower means in all three periods.

We found the same results for REI (Tables A3 and A4). Similarly, to MEff, groups with lower concentrate availability had lower means during the whole trial.

3.6. Correlations

Relations of elevated parameters were computed using correlations. The following values were considered: total mobilization of adipose tissue depots both in sum and separately as well as NEB, BHB, NEFA and efficiency parameter FE.

For all animals NEB correlated negatively with BFT(r = −0.466, $p \leq 0.001$), RAT (r = −0.349, $p \leq 0.01$), SAT (r = −0.397, $p \leq 0.01$), MAT (r = −0.271, $p \leq 0.05$) and SoM (r = −0.375, $p \leq 0.01$), as well as with BHB (r = −0.502, $p \leq 0.001$), NEFA (r = −0.402, $p \leq 0.01$) and FE (r = −0.867, $p \leq 0.001$). By contrast BCS, RFT and OAT did not correlate. The mobilized energy from adipose tissue depots and NEB (Figure 2F) showed a significant negative correlation, as correlation coefficient was −0.4634 ($p < 0.05$) for period 1.

4. Discussion

The aim of this study was to investigate the body fat depot mobilization and energy metabolism of pluriparous cows during the first weeks after parturition depending on body condition before calving and on different amounts of concentrate in the ration after calving by combining a feeding trial with ultrasound-based estimation of various depot fat depots.

One outcome of our investigation was that the amount of concentrate in the ration influenced the DMI, as groups with higher supply of concentrate consumed more DM in period 2. This result is comparable to other studies. Schmitz et al. [26] showed that a high proportion of concentrates in the ration enhanced DMI and Gruber et al. [27] proposed an increase between 0.4–0.6 kg per kg additional concentrate, which was linked to a subsequent roughage displacement. In contrast, other findings pointed out that there were no differences in feed intake between cows fed rations with different proportions of concentrates during the lactation period [28]. A reason for these controversial results might be caused by other feeding factors, such as composition, energy density and quality of roughage, as well as NDF content.

The higher DMI of C_{60} groups of our trial also resulted in a higher energy intake compared to C_{35} groups. These relations might explain why low concentrate groups suffered from a more pronounced negative EB and reached the positive EB much later than high concentrate groups. As described in Dänicke et al. [29] the energetic dilution of the C_{35} ration leads to a qualitative decrease of NEB.

Differences in BCS between both BCS groups and between individuals remained more or less the same over the whole transition period, whilst higher concentrate feed supply after calving (C_{60}) failed to counterbalance the BCS loss observed in group with lower concentrate allowance (C_{35}). Other studies agree with our findings, that feeding has little effect on BCS loss after parturition [9,30]. As NEI was higher in group C_{60} and NEB less negative, the question was whether the mobilization of the internal fat depots was less pronounced. However, the individual groups of our trial did not differ when SoM was evaluated collectively. Having a closer look at individual fat depots, it becomes obvious that SAT was more extensively mobilized in BCS_H groups compared to their BCS_L counterparts. This finding is surprising as no corresponding differences in BCS losses were noticed and BCS changes are usually regarded to represent the changes in SAT [31]. This significant effect of BCS on SAT mobilization exclusively occurred in period 1 where mobilization of all measured fat depots was most pronounced. The metabolic circumstances and the pronounced negative EB during this time explain these expectable results, which are also proved by the negative correlation of mobilized energy from

body fat and NEB. Tamminga et al. [3] share our findings. With regard to the effect of body condition on mobilization of SAT Chilliard et al. [11] have shown that fat mobilization was physiologically related to body fatness. Nevertheless, we could not demonstrate the expected correlation between BCS and NEB, which would have indicated the higher potential of high-conditioned cows to mobilize body fat. This non-existent relationship could also explain why we determined no further association between BCS and mobilization. That is in line with Pedernera et al. [14] who had already pointed out, that BCS should not be used as an overall parameter to explain the energetic condition and metabolism of cows. It becomes apparent that USM show differences in lipid mobilization, which remain concealed by simply determining the BCS.

Due to the limited capability of BCS changes to indicate fat mobilization comprehensively, one could hypothesize that the mobilization of adipose tissues was related to energy balance, as proven by the highly significant overall correlations between NEB and mobilized energy from body fat, as well as those of NEB and the single adipose tissue depots and SoM in all animals of our investigation. Consequently, it should also depend on C whereby a decline of the energy balance would expectedly increase the lipolytic potential [11]. Reversely, high amounts of concentrate would then lead to a positive EB and consequently result in a decrease of adipose tissue mobilization. Our study could, however, not support those findings and other trials, which attempted to decrease body fat mobilization by increasing energy-rich diets, had neither been successful [15]. Considering the NEB alone does not duly reflect the metabolic processes. This suggests that other factors, such as genetic regulatory mechanisms may influence the mobilization relevantly.

Blood concentration of BHB is used as an indicator for lipolysis and ketosis whereby the literature states different thresholds. Nielen et al. [6] defined a value of BHB > 1.2 mmol/L in serum as the critical level for subclinical ketosis. Oetzel [7] declared a level of NEFA > 0.4 mmol/L as a stage where high lipomobilization takes place and therefore indicates an imbalance in energy state.

In the present study, none of the four trial groups exceeded the limit value for BHB in period 1, and we could not demonstrate any time or group effects, too. NEFA values were not affected by treatment either, but showed a significant time effect and also the characteristic curve during the transition period. NEFA values are typically higher in early lactation than in mid-lactation [32] which indicates higher mobilization of adipose tissue depots due to the particular metabolic challenges during this time. All four groups of our study exceeded the threshold for higher lipomobilization (>0.4 mmol/L) in period 1 [7]. This is in line with the negative energy balance during this timeframe as this threshold indicates a negative energy state. The negative EB was more pronounced in groups with lower concentrate allowance; due to a forced lipid mobilization, we would have also expected higher serum concentrations for both BHB and NEFA. However, the sonography-based determination of the adipose tissue mobilization did not verify the differences seen in NEB. A closer look at SAT, MAT and SoM indicated that regular conditioned animals with a lower concentrate proportion in the ration mobilized as much body fat as regular conditioned cows with a high concentrate availability. Only cows with an energetic oversupply mobilize less body fat. Van der Drift [33] stated that a high BCS and higher fat depot mobilization increased the risk for ketosis and hypothesized that high-energy diets compensated the cows' energy demand and made high mobilization of body fat unnecessary in order to avoid that. Other studies confirm this presumption [12,14,34]. Chilliard [35] pointed out that cows mobilize more body fat when their access to feed is limited. Other studies argue the converse. Cows with high-energy diets exhibited higher BHB and NEFA values compared with cows supplied with low-energy diets [4,34]. Yet other studies, in turn, go in line with our findings and could not determine any significant differences between treatments in the first weeks p.p. [26,36]. This suggests that NEB does not accurately reflect the grade of lipid mobilization. The examination of lipid mobilization by ultrasonic technology brings out physiological and metabolic relations that remain concealed by using BCS determination or NEB calculation only.

A possible explanation for our results might be the absence of lipolytic stimuli in the adipose tissue depots, for example, a low glucose level [37]. Propionate is necessary for synthesizing glucose.

Presumably, in the present study, there might have been sufficient DMI and also starch and energy content in both rations to generate adequate amounts of propionate in the rumen, so that glucose concentration did not decrease and therefore no lipolytic stimuli occurred. Another reason for detecting only concentrate tendencies and a single BCS effect on fat depot mobilization could be an adequate level of oxaloacetate for introducing NEFA into the citric acid cycle which prevents an increase of BHB beyond the physiological range [38]. Van der Drift [33] indicates that not only non-genetic, but also genetic variations of BHB concentration have to be considered. Those findings reflect our perceptions concerning SoM, where we could not prove any group differences either. Different studies had already pointed out that cows vary in their potential to deal with metabolic changes and to adapt to NEB and varying dietary energy [26,33,39].

In the present study, the mobilization of protein was not examined. Protein mobilization can reduce both, fat mobilization and NEFA and BHB concentration in blood [33]. It can be hypothesized that C_{35} and BCS_H groups mobilized sufficient protein to cover group effects in fat mobilization and serum NEFA and BHB concentration.

A positive relationship between C and milk yield would have been expected due to the additionally provided energy in the diet as had been proved in previous studies [12,26,40]. BCS had no influence on milk yield either. Other investigations underline our findings that body condition at calving has little effect on milk production of well-fed cows [35]. In the present study we failed to demonstrate a BCS effect both on DMI and on milk yield. Nevertheless, other milk parameters such as milk fat content differed between trial groups in the present study. The higher BCS might have increased the milk fat content caused by higher mobilization of body fat reserves in period 1 [12,34]. Furthermore, higher amounts of concentrates led to milk fat depression in periods 2 and 3, because of the associated decrease of the acetate-to-propionate ratio in the rumen [41,42]. Acetate is an important source for synthesis of milk fat in ruminants [43]. Lipid mobilization of BCS_H groups may have concealed the concentrate effect in period 1. These results are also reflected in ECM. Relating to NEB, lower ECM yield and higher DMI led to a positive EB in C_{60} groups.

In our trial, the C_{35} groups seem to be more efficient, as their FE was higher compared to C_{60} groups. This is, however, related to an equal milk production level of both groups, whereby animals of C_{35} groups consumed less DMI containing the lower energy content. Thus, C_{35} groups also exhibited a longer and more pronounced negative energy balance. In this case, higher milk production was accompanied by less feed intake and more body fat mobilization. Spurlock et al. [44] proposed a genetic correlation between high FE and a more pronounced negative energy balance. We could underline this due to the highly significant correlation between NEB and FE in the present study. It indicates that efficient cows might be more endangered to develop metabolic diseases. This hypothesis is confirmed by findings of Chilliard et al. [11], where NEFA concentrations were highly correlated with NEB, which could also be proven in the present study.

5. Conclusions

The results of this study confirm the benefits of higher amounts of concentrates concerning the DMI, and therefore the energy intake, whereas milk yield was unaffected. However, higher amounts of concentrate led to milk fat depression. Due to higher energy intake and equal milk yield, C_{60} groups reached the positive energy balance earlier than the C_{35} groups. Lower DMI and equal milk yield led to an improved efficiency in C_{35} groups. However, the term efficiency must be critically reviewed, as it is related to a high energy deficit.

The determination of body fat mobilization by ultrasonic recording revealed the differences between groups seen in the NEB, which suggested a need for higher lipid mobilization in C_{35} groups.

Furthermore, we could not find differences between groups concerning BCS loss. However, BCS affected the mobilization of SAT over time, as high-conditioned cows had a more pronounced SAT mobilization. Although BCS is based on changes in SAT, the determination failed to detect them in the present study. Therefore, BCS determination and NEB calculation should be used carefully as indicators

for general mobilization or overall metabolic processes and conditions. The sonography-based method revealed boundaries of BCS determination and NEB calculation and visualized physiological and metabolic relations that remain concealed in other methods. Physiological processes, such as protein mobilization and hormone levels, might also influence fat loss. Further research is necessary to clarify causal interrelations.

Author Contributions: The experiment's conceptualization was initiated by J.H., S.D., U.M., D.v.S., K.B.; Methodology and validation of data was done by K.B., D.v.S.; Formal analysis was performed by K.B.; Investigation was done by S.K., J.F., D.v.S., K.B.; Resources were looked up by K.B.; Curation and preparation, visualization of data was done by D.v.S. and K.B.; Writing original draft preparation was performed by K.B.; Supervision and writing of the review was done by D.v.S., J.F., S.K., U.M., S.D., J.H., A.Z.; Editing was done by K.B.; Project was administrated by J.H., U.M., S.D.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Ultrasonic measuring points and its descriptions.

Measuring Points	Description
R12 [+]	Subcutaneous fat over 12th rib
AW1b [§]	Distance from skin to distal muscle margin above the peritoneum at the point of interception of a vertical line through the last lumbar vertebra and a horizontal line through the patella
AW3b [§]	Distance from the skin to the distal muscle margin above the peritoneum at the center of the paralumbar fossa
AW3c [§]	Thickness of the abdominal wall at the center of the paralumbar fossa
KD3b *	Distance from the skin to the peritoneum in the intertransverse space directly cranial to intertransverse space where the caudal pole of the kidney is visible
KD2c *	Distance from the skin to the distal kidney margin in the intertransverse space directly cranial to KD2c

[+] R = rib, [§] AW = abdominal wall, * KD = kidney.

Table A2. Effects of body condition, concentrate proportion in the diet (C) and period on glucose and triglyceride concentrations in blood serum (LSM) during period 1 (weeks 1–4 postpartum), period 2 (weeks 5–10 postpartum) and period 3 (weeks 11–17 postpartum) in the treatment groups.

Item [+]	Treatment [§]				SEM [#]	*p*-Value *					
	BCS_H/C_{60} n = 15	BCS_H/C_{35} n = 15	BCS_L/C_{60} n = 15	BCS_L/C_{35} n = 15		BCS	C	BCS × C	BCS × Period	C × Period	BCS × C × Period
Glucose, mmol/L											
Period 1	2.68	2.72	2.63	2.71	0.12	0.439	0.609	0.276	0.446	0.425	0.625
Period 2	3.06	2.99	2.85	2.88							
Period 3	3.23	2.88	3.04	3.07							
Triglycerides, mmol/L											
Period 1	0.13	0.13	0.12	0.13	0.01	0.066	0.044	0.149	0.034	0.151	0.077
Period 2	0.15	0.16	0.16	0.16							
Period 3	0.17	0.24	0.16	0.16							

[§] Before calving cows were classified in high and low body condition score (BCS)-groups (BCS_H, BCS_L), after calving both groups were divided again, each into a group with 35% concentrate proportion (C_{35}) and a group with 60% concentrate proportion (C_{60}) in the ration (increasing from 35–60% during the first three weeks after parturition). Thus, four groups emerged: BCS_H/C_{60} (n = 15), BCS_H/C_{35} (n = 15), BCS_L/C_{60} (n = 15), BCS_L/C_{35} (n = 15). Values are presented as LSMeans, * Period ($p < 0.001$) for all variables, [+] Analyzed with first measured value before calving as covariate, [#] Pooled standard error of means.

Table A3. Effects of body condition, concentrate proportion in the diet (C) and period on efficiency parameters (LSM) during period 1 (weeks 1–4 postpartum), period 2 (weeks 5–10 postpartum) and period 3 (weeks 11–17 postpartum) in the treatment groups.

Item [+]	Treatment [§]				SEM [#]
	BCS_H/C_{60} n = 15	BCS_H/C_{35} n = 15	BCS_L/C_{60} n = 15	BCS_L/C_{35} n = 15	
Feed efficiency [†], kg/kg					
Period 1	1.93	2.27	1.92	2.11	0.14
Period 2	1.58	1.84	1.54	1.81	0.14
Period 3	1.66	2.07	1.56	1.71	
Energy conversion efficiency [#], MJ/MJ NE_L					
Period 1	0.88	1.04	0.89	0.97	0.03
Period 2	0.71	0.85	0.70	0.84	
Period 3	0.73	0.80	0.70	0.79	
Metabolic efficiency [‡], MJ NE_l/kg body weight$^{0.75}$					
Period 1	0.17 [c]	0.00 [c]	0.16 [c]	0.07 [c]	0.03
Period 2	0.38 [b,AB]	0.20 [b,CD]	0.43 [b,A]	0.22 [b,C]	
Period 3	0.35 [a,A]	0.30 [a,B]	0.42 [a,A]	0.28 [a,AB]	
Residual energy intake [¶], MJ NE_L					
Period 1	27.9 [a,A]	7.5 [a,B]	21.7 [a,A]	10.7 [a,B]	3.8
Period 2	18.3 [b,A]	−5.5 [b,B]	17.1 [b,A]	−3.3 [b,B]	
Period 3	5.5 [c,A]	−5.2 [c,B]	6.7 [c,A]	−6.5 [c,B]	

[a,b] Means with different superscripts differ within columns, [A,B] Means with different superscripts differ within row, [§] Before calving cows were classified in high and low body condition score (BCS)-groups (BCS_H, BCS_L), after calving both groups were divided again, each into a group with 35% concentrate proportion (C_{35}) and a group with 60% concentrate proportion (C_{60}) in the ration (increasing from 35–60% during the first three weeks after parturition). Thus, four groups emerged: BCS_H/C_{60} (n = 15), BCS_H/C_{35} (n = 15), BCS_L/C_{60} (n = 15), BCS_L/C_{35} (n = 15). Values are presented as LSMeans, [+] Analyzed with first measured value from week 1 as covariate, [#] Pooled standard error of means, [†] Feed efficiency (FE) = energy-corrected milk, kg/DMI, kg, [#] energy conversion efficiency (ECE) = energy excretion with milk, MJ/energy intake, MJ NE_L, [‡] Metabolic efficiency (MEff) = (energy intake, MJ NE_L − energy in milk, MJ)/body weight$^{0.75}$, kg, [¶] Residual energy intake (REI) = energy intake, MJ NE_L − expected energy intake, MJ NE_L.

Table A4. *p*-values of effects of body condition, concentrate proportion in the diet (C), period and interaction between them on efficiency parameters.

Item [+]	*p*-Value [*]					
	BCS	C	BCS × C	BCS × Period	C × Period	BCS × C × Period
Feed efficiency [†], kg/kg	0.240	0.009	0.509	0.491	0.997	0.718
Energy conversion efficiency [#], MJ/MJ NE_L	0.291	<0.001	0.617	0.718	0.190	0.310
Metabolic efficiency [‡], MJ NE_l/kg body weight$^{0.75}$	0.182	<0.001	0.672	0.787	<0.001	0.002
Residual energy intake [¶], MJ NE_L	0.920	<0.001	0.629	0.771	<0.001	0.04

Before calving cows were classified in high and low body condition score (BCS)-groups (BCS_H, BCS_L), after calving both groups were divided again, each into a group with 35% concentrate proportion (C_{35}) and a group with 60% concentrate proportion (C_{60}) in the ration (increasing from 35–60% during the first three weeks after parturition). Thus, four groups emerged: BCS_H/C_{60} (n = 15), BCS_H/C_{35} (n = 15), BCS_L/C_{60} (n = 15), BCS_L/C_{35} (n = 15), * Period ($p < 0.001$) for all variables, [+] Analyzed with first measured value from week 1 as covariate, [†] Feed efficiency (FE) = energy-corrected milk, kg/DMI, kg, [#] energy conversion efficiency (ECE) = energy excretion with milk, MJ/energy intake, MJ NE_L, [‡] Metabolic efficiency (MEff) = (energy intake, MJ NE_L − energy in milk, MJ)/body weight$^{0.75}$, kg, [¶] Residual energy intake (REI) = energy intake, MJ NE_L − expected energy intake, MJ NE_L.

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5. Paper II

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Effects of Different Concentrate Feed Proportions on Ruminal Ph Parameters, Duodenal Nutrient Flows and Efficiency of Microbial Crude Protein Synthesis in Dairy Cows During Early Lactation

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Simple Summary: Around calving, cows exhibit a depression in feed intake. An imbalance between energy intake and energy demands occurs, which results in a negative energy balance. Concentrate feed proportions of the ration are increased to compensate that energy deficit. The accompanying increase in concentrate intake leads to higher production of short chain fatty acids, which in turn might lower the ruminal pH. A ruminal pH < 5.8 for a certain period of time can lead to subacute ruminal acidosis. Keeping the ruminal pH within the physiological range is important for microorganisms colonizing the rumen. Those microorganisms metabolize feed protein via ammonia or amino acids to microbial protein, which is then available for the host. Microbial efficiency is limited by availability and balance of nitrogen and energy, the latter is mostly provided as starch. The aim of the present study was to examine influences of different concentrate feed proportions and of microbial efficiencies on ruminal pH parameters, nutrient flows and digestibilities. Therefore, cows were additionally grouped according to their individual microbial efficiency. The concentrate treatment effect did not cause differences in the mentioned parameters. However, more microbial efficiency cows exhibited higher nutrient flows but lower digestibilities.

Abstract: The aim of the study was to examine different pH parameters, such as variations throughout the day, depending on differing concentrate feed proportions. Moreover, special attention was payed to individual variation in microbial efficiencies (microbial crude protein/fermented organic matter) and their relation to ruminal pH, nutrient flows and digestibilities. For this, cows were grouped according to microbial efficiency (more, n = 5, vs. less efficient cows, n = 4). After calving, thirteen ruminally cannulated pluriparous cows, including nine duodenally cannulated animals, were divided into groups offered rations with a lower (35% on dry matter basis, n = 7) or a higher (60% on dry matter basis, n = 6) concentrate feed proportion. Ruminal pH parameters were assessed continuously by using intraruminal probes. Nutrient flows, nutrient digestibility and microbial efficiency were determined for duodenally cannulated cows. For most ruminal pH parameters it seemed that individual variability was higher than the

treatment effect. However, a positive relationship between actual concentrate intake and diurnal pH fluctuations was found. Besides, the effect of individually different microbial efficiencies was assessed. Again, there were no group differences for pH parameters. However, nutrient flows were significantly higher in more efficient cows, whereas digestibilities were lower in in more efficient cows.

Keywords: dairy cows; concentrate feed proportion; ruminal pH; nutrient flows; microbial efficiency; postpartal period

1. Introduction

Cows display a depression in feed intake during the transition period, which leads to an imbalance between energy intake and energy demands [1,2]. To balance this discrepancy, concentrate feed proportions of the rations (C) are often elevated post partum (p.p.). The increase of concentrate intake is accompanied by an increased production of short chain fatty acids (SCFA) in the rumen, which can affect the ruminal pH negatively [3,4]. To keep the ruminal pH within the physiological range, the absorption of SCFA through the ruminal epithelium has to increase simultaneously [5,6]. Another factor to compensate the elevated SCFA concentration is an increased chewing activity, which in turn increases saliva production and buffer secretion. Disappearance of SCFA is also determined by passage rate to the lower digestive tract [7]. If the imbalance between production and elimination of SCFA impacts the ruminal pH in a way that it drops below an appropriate threshold for a certain period of time, this can result in subacute ruminal acidosis (SARA) [7–9]. The risk of developing SARA is particularly high during the transition period, with a shift from a dry period to an early lactation diet [10]. Detection of SARA is difficult. Nevertheless, for the attempt of definition, thresholds according to Zebeli et al. [9] are used most frequently. These are described as a daily mean pH < 6.16 and the time per day with pH < 5.8 for more than 5.24 hours. However, several studies assume high individual variability in ruminal pH and in susceptibility to develop SARA [1,11,12].

A low ruminal pH can also reduce fiber digestion and therefore lower microbial efficiency, which is defined as synthesized microbial protein per fermented organic matter (mCP/fOM) [13–15]. A decrease of fiber digestion can impede an even energy provision throughout the day, which is necessary for an optimal microbial growth. The release of energy from non-structural carbohydrates is faster than the energy consumption of the microorganisms and can result in an uncoupled fermentation. This again can lead to energy spilling [16,17]. Energy is used for the so-called non-growth processes, such as maintaining the intracellular pH on an optimal level instead of using it for cell growth under low pH conditions [16,18]. However, most studies assumed stable ruminal pH conditions while variations throughout the day received little attention [19]. Microbial efficiency depends on availability and balance of nitrogen (N) and fermentable energy [15,18,20]. Fermentability of the diet might in turn influence not only the ruminal pH, but also the passage rate [18]. The fermentability of the diet is primarily influenced by fermentability of the contained starch. Starch fermentation is accompanied with an increase of SCFA concentration, which lowers ruminal pH. In addition, a high amount of starch can increase passage rate, due to a smaller particle size of feedstuffs with higher starch contents compared to forage [5,18,21]. Yet, few studies have been conducted on the relationship between ruminal pH, nutrient flows and microbial efficiency. Firkins et al. [22] demonstrated an improvement of 39% in microbial efficiency, due to a 15% decrease of organic matter digestibility. Therefore, they considered the increasing passage rate to be a general explanation. They proposed that substrate supply increases with enhanced passage rate, which would improve cell growth, due to an increase of growth related enzymes in the microorganisms. Additionally, an increased passage rate can decrease microbial turnover in the rumen [23]. However, investigations differ in their results concerning this relation, as higher passage rates can also lead to microbial washout [24,25].

The objective of the present study was, on the one hand, to examine different ruminal pH parameters depending on differing concentrate feed proportions, thereby, paying special attention to diurnal fluctuations. Besides, the concentrate effect on microbial efficiency, nutrient flows and digestibility was assessed. On the other hand, the study intended to investigate the relation between ruminal pH and microbial efficiency from a new perspective for gaining further information. Therefore, the microbial efficiency was chosen as starting point, by grouping the cows according to their individual microbial efficiency (more vs. less efficient). For this, the average microbial efficiency of 156 g mCP/kg fOM according to GfE [15] was used as threshold. Moreover, the effect of microbial efficiency on nutrient flows and digestibilities was examined. For this reason, different statistical evaluations were applied in order to appraise both the effects of varying dietary energy concentration as well as those of microbial efficiency on the parameters mentioned.

2. Materials and Methods

The experiment was performed in compliance with the German legislation on animal protection (Animal Welfare Act) and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany) in consultation with an independent ethics committee (AZ 33.19-42502-04-15/1858).

2.1. Experimental Design

Two diet types were created in order to induce distinct differences in energy supply and presumably ruminal pH parameters. While the high-caloric diet was designed to contain 60% concentrate feed, the low-caloric ration was contrasted with 35% concentrate feed.

The current study was based on 13 ruminally cannulated, pluriparous German Holstein cows, including nine additionally duodenally cannulated animals. Rumen cannula enables the access to the reticulo-rumen via the dorsal sac of the rumen. The duodenal cannula was inserted in the proximal duodenum. The experiment covered the period from 3 weeks before calving until 70 days in milk (DIM) and one additional week of duodenal chyme sampling (cows were on average in the 13th week of lactation ± 16 days). Before parturition, all cows received the same standardized total mixed ration (TMR) consisting of 80% silage (70% maize silage, 30% grass silage on dry matter (DM) basis) and 20% concentrate on DM basis. After calving, animals were divided into two groups and assigned to two different concentrate feed proportions. Cows received a partial mixed ration (PMR) consisting of 48% maize silage, 20% grass silage and 32% concentrate feed. Rations for the groups with lower concentrate feed proportion (C_{35}) were adjusted to 35% concentrate feed by using automatic feeding stations (Intensec, B.V., Marknesse, The Netherlands). For the groups with a higher amount of concentrate (C_{60}), C was also provided by the automatic feeders and increased from 35% to 60% during the first three weeks p.p. The components and the chemical compositions of the feedstuffs are presented in Tables 1 and 2.

Table 1. Composition of concentrates during the dry and the lactating period.

Components, g/kg of Fresh Matter	Concentrates		
	Dry Period	Lactating Period	
		C_{35}	C_{60}
Soybean meal	115		
Rapeseed meal	150	400	200
Wheat	330	150	213
Barley		144	213
Maize		200	290
Dried sugar beet pulp	296	50	50
Urea	30	8	
Calcium carbonate	24	13	12
Soybean oil	15	10	10
Vitamin-mineral premix +	40		
Vitamin-mineral premix #		25	12

+ Mineral feed for dry cows, ingredients per kg according to the manufacturer's specif cation: 10 g Ca; 120 g Na; 60 g P; 60 g Mg; 6 g Zn; 4 g Mn; 1.25 g Cu; 100 mg I; 50 mg Se; 35 mg Co; 800,000 IU vitamin A; 100,000 vitamin D_3; 1500 mg vitamin E. # Mineral feed for lactating dairy cows, ingredients per kg according to the manufacturer's specif cations: 140 g Ca; 120 g Na; 70 g P; 40 g Mg; 6 g Zn; 5.4 g Mn; 1 g Cu, 100 mg I; 40 mg Se; 25 mg Co; 1,000,000 IU vitamin A; 100,000 IU vitamin D_3; 1500 mg vitamin E.

Table 2. Chemical components of the dry period diet, as well as of the C_{35}- and C_{60}-ration during the experimental period from day 21 antepartum until day 70 postpartum.

Chemical Composition	Dry Period Diet	C_{35}-ration	C_{60}-ration
Dry matter, g/kg	378	425	526
Nutrients, g/kg DM §			
Crude ash	63	96	59
Crude protein	131	144	153
Ether extract	34	38	42
a † Neutral detergent $fiber_{om}$ $	327	353	294
Acid detergent $fiber_{om}$ $	225	204	169
Starch content	247	285	353
Energy ‡, MJ/kg of DM			
ME	10.9	11.3	11.8
NE_L	6.6	6.9	7.3

‡ Calculation based on equations of GfE [16]. §Dry matter. † Assayed with a heat-stable amylase for maize silage and concentrates. $ Expressed exclusive of residual ash. C_{35} group comprised seven ruminally cannulated cows, including four duodenally cannulated animals with an average parity of 3.8 (±1.5). The C_{60} group contained six ruminally and five duodenally cannulated animals with an average parity of 3.4 (±1.4).

2.2. Measurements and Sample Collections

2.2.1. Dry Matter Intake and Milk Yield

Dry matter intake (DMI) was recorded for both PMR and concentrate individually by computerized feeding stations (Insentec, B.V., Marknesse, The Netherlands). Milking took place at 05:30 a.m. and 03:30 p.m. and milk yield was determined by automatic milk counters throughout the 70 DIM (Lemmer Fullwood GmbH, Lohmar, Germany).

2.2.2. Feed and Milk Samples

Samples of the mixed ration components were taken twice a week and pooled to a collective sample every four weeks. Samples of concentrate were collected once a week and also pooled to a collective sample monthly. Milk samples were taken twice a week during morning and evening milking and stored at 4 C until analysis.

collective sample monthly. Milk samples were taken twice a week during morning and evening milking and stored at 4 °C until analysis.

2.2.3. Rumen Fluid Samples

Rumen fluid samples were taken after morning milking at eight time points, after calving (days p.p.: 3, 7, 14, 21, 28, 42, 56, 70) as well as in the week of duodenal chyme sampling. Rumen fluid samples were collected through the rumen cannula using a manual pump. With every sample, about 200 mL of rumen fluid were taken. Samples were stored at 4 °C until further analysis.

2.2.4. Rumen pH and Rumination Behavior Measurements

A ruminal pH measuring device (Lethbridge Research Centre Ruminal pH Measurement System, Dascor, Escondido, CA, USA) was used to record the pH values in the ventral sac of the rumen continuously. The pH values were recorded every minute and measured during several consecutive 24-h periods each week (2 ± 1.16; mean ± SD) from week -3 to week 10 relative to calving, as well as in the week of duodenal chyme sampling. At some periods data of individual cows were missing, due to either insufficient capacity of instruments or technical problems. However, it was ensured that at least three cows of every group were recorded in every period. Before and after each measuring period the system was calibrated in buffer solutions of pH 4 and 7 at 39 °C.

2.2.5. Duodenal Chyme and Faeces Samples

Each duodenally cannulated cow received a chromium oxide marker (Cr_2O_3) for 16 consecutive days. Marker administration started when cows were 73 DIM (± 16) on average. During the first 10 days, the marker was inserted into the rumen through the rumen cannula in two portions of 50 g each at 05:00 a.m. and 5:00 p.m. During the last 6 days, the marker was inserted in four portions of 25 g every 6 h. The chromium oxide marker dosing schedule was performed according to Schäfers et al. [26]. During the last 5 days of the marker administration period, samples of duodenal chyme were collected in intervals of 10 h. Samples were collected from the duodenal cannula and pooled to a collective sample for the five sampling days and stored at −20 °C until further analysis.

Faeces samples were taken rectally with every duodenal chyme sampling and then pooled to a collective sample.

2.3. Analyses

2.3.1. Feed and Milk Analyses

Feed samples were analyzed according to the standard methods of the Association of German Agricultural Analysis and Research Centers (VDLUFA, method numbers are given hereafter) [15]. PMR components and concentrate were analyzed for DM (3.1), crude ash (8.1), crude protein (Dumas method, 4.1.2), starch (7.2.1, polarimetric method), ether extract (5.1.1), neutral detergent fiber ($aNDF_{om}$, 6.5.1) and acid detergent fiber (ADF_{om}, 6.5.2) (Table 2).

Milk samples of 20 morning and 20 evening samples were analyzed for fat, protein, and lactose by an infrared milk analyzer (Milkoscan FT 6000; Foss Electric, Hillerød, Denmark).

2.3.2. Rumen Fluid Analyses

Short chain fatty acids (SCFA) were determined according to Geissler et al. [27]. For this, rumen fluid samples were centrifuged (5× 2400 *g*) (Beckman J2-H2, Beckman Coulter Inc., Brea, CA, USA), 10 mL of the fluid phase were added to 1 mL of 25% sulphuric acid and centrifuged again (20× 2700 *g*) (Eppendorf 5417 R, Eppendorf AG, Hamburg, Germany). The supernatant was filled into GC-vials. Afterwards it was separated by gas chromatography (Clarus 680 CG, Perkin Elmer, Waltham, MA, USA) with a polyethylene glycol capillary column (Zebron ZB-FFAP, 30 m × 0.32 mm i.d., 0.5 µm film thickness, Phenomenex LTD, Aschaffenburg, Germany) and a flame ionization detector.

Ammonia-N was analyzed using a steam distillation according to DIN38406-E5–2 within two hours after fluid sampling [28].

2.3.3. Duodenal Chyme and Faeces Analyses

After thawing, 2 x 60 mL of each duodenal chyme sample were filled in 100 mL Kautex bottles. To ensure a representative proportion of solid and fluid components, the sub-sampling was done under constant stirring. The remainders of the duodenal chyme samples, as well as the pooled faeces samples were freeze dried (CHRIST, Osterode am Harz, Germany). Analysis of DM of duodenal chyme and faeces samples was performed according to VDLUFA method 3.1 [29]. Faeces samples were also analyzed for $aNDF_{om}$ and ADF_{om}. Furthermore, the 60 mL samples of duodenal chyme were used to determine the total nitrogen content according to Kjeldahl method (VDLUFA method 4.1.1) and the ammonia content according to DIN 38406-E5-2 [28,29]. The freeze dried samples of duodenal chyme and faeces were, besides, used to analyze the chromium concentration. For this, samples were prepared according to Williams et al. [30]. Chromium content of the freeze dried duodenal chyme and faeces samples was determined using an optical emission spectrometer with inductively coupled plasma (ICP-OES Quantima; GBC Scientific Equipment Pty. Ltd., Melbourne, VIC, Australia). The proportion of microbial nitrogen in duodenal chyme was determined at a wavelength of 800-2400 nm by using a NIR spectroscopy according to Lebzien and Paul [31].

2.4. Calculations

Computation of daily duodenal dry matter flow (DMF) was based on the equation according to Pappritz et al. [32]:

$$\mathrm{DMF}\left(\frac{\mathrm{kg}}{\mathrm{day}}\right) = \left(\frac{\frac{\text{application of } Cr_2O_3 \text{ in mg}}{\mathrm{cow}/\mathrm{day}}}{Cr_2O_3 \text{ in duodenal chyme } \left(\frac{\mathrm{mg}}{\mathrm{g}}\mathrm{DM}\right)}\right)/1000 \quad (1)$$

The following formulas were used to calculate non-ammonia-nitrogen (NAN) proportion at the duodenum:

$$\text{Total N in duodenal chyme (\% of DM)} = \frac{\text{Total N (\% of fresh matter (FM))}}{\text{DM of duodenal chyme (\%)}} \times 100 \quad (2)$$

$$NH_3N \text{ in duodenal chmye (\% of DM)} = \frac{NH_3N \text{ in duodenal chyme (\% of FM)}}{\text{DM of duodenal chyme (\%)}} \times 100 \quad (3)$$

$$\text{NAN proportion in duodenal chyme (\% of DM)} = \text{Total N in duodenal chyme (\% of DM)} - NH_3N \text{ in duodenal chyme (\% of DM)} \quad (4)$$

Duodenal NAN flow (kg/day) was calculated by multiplying the NAN proportion in duodenal chyme (% of DM) by DMF (kg/day).

The microbial crude protein (mCP) was calculated using the following formula:

$$\text{mCP (g/day)} = [\text{duodenal NAN flow (kg/day)} \times (\text{microbial N proportion of NAN (\%))}/100] \times 6.25 \quad (5)$$

According to GfE [15] and Pappritz et al. [32]microbial organic matter (mOM), and ruminal fOM were calculated as follows:

$$\text{mOM (kg/day)} = 11.8 \times \text{microbial N (kg/day)} \quad (6)$$

$$\text{fOM (kg/day)} = \text{OM intake (kg/day)} - [\text{duodenal OM flow (kg/day)} - \text{microbial OM (kg/day)}] \quad (7)$$

Microbial efficiency was calculated as mCP per fOM according to GfE [15].

Digestibility quotient of $aNDF_{om}$ and ADF_{om} was calculated according to Simon [33] for the total digestive tract and at the duodenum. An example for the calculation is given for digestibility of $aNDF_{om}$ at the duodenum.

$$\text{digestibility quotient of aNDFom (\%)} = [(\text{aNDFom intake (kg/day)} - \text{aNDFom at the duodenum(kg/day))/aNDFom intake (kg/day)}] \times 100 \quad (8)$$

The equation according to McGinn et al. [34] was used to calculate total digestive tract digestibility of DM (tdDM):

$$\text{tdDM (\%)} = 1 - [(Cr_2O_3 \text{ in marker (mg/day))/DM intake (kg/day)}]/ Cr_2O_3 \text{ in faeces} \times (\text{mg/kg} \times \text{DM}) \quad (9)$$

2.5. Statistical Analysis

For ruminal pH data were summarized to means of every measuring period for each cow and hence weekly means were calculated.

Weekly means were also calculated for performance parameters, milk parameters and proportion of SCFA and ammonia concentration in rumen fluid. For statistical analyses, two weeks were merged to one period for every parameter, which resulted in five periods.

For statistical analyses of duodenal chyme samples, mean values for the five sampling days were calculated for each cow and each parameter.

The statistical evaluation with the SAS software package (version 9.4.; SAS Institute Inc., Cary, NC, USA) included the data collected after parturition when cows received different diets. Performance parameters, proportion of SCFA and ammonia concentration values in rumen fluid, as well as rumen pH parameters were analyzed by using the MIXED procedure for repeated measures with a compound symmetry structure [35]. C and period were applied as fixed effects, as well as the interaction between them. Each cow within treatment was considered to be a random effect. The period of sampling was treated as a repeated measure.

For the analysis of the duodenal chyme samples with regard to the estimated parameters, we wanted to assess both the effect of rations differing in C, as well as individually different microbial efficiencies (defined as mCP/fOM, according to GfE [15]) concerning nutrients flows, digestibilities, and ruminal pH. As duodenal chyme was sampled in only one period, the statistical evaluation included just a simple t-test with two different grouping strategies. First, concentrate feed proportion (C_{60} vs. C_{35}) was used for grouping. Secondly, cows were grouped according to microbial efficiency (more and less efficient, see Figure 1). For this, the mean microbial efficiency of 156 g mCP/kg fOM according to GfE [15] was used as threshold. Cows with an individual microbial efficiency < 156 g/kg were considered to be less efficient, whereas cows with an individual microbial efficiency > 156 g/kg were considered to be more efficient. The individual microbial efficiencies for the week of duodenal chyme sampling are presented in Table A2. The statistical evaluations with either C or microbial efficiency as fixed factors are presented in Figure 1.

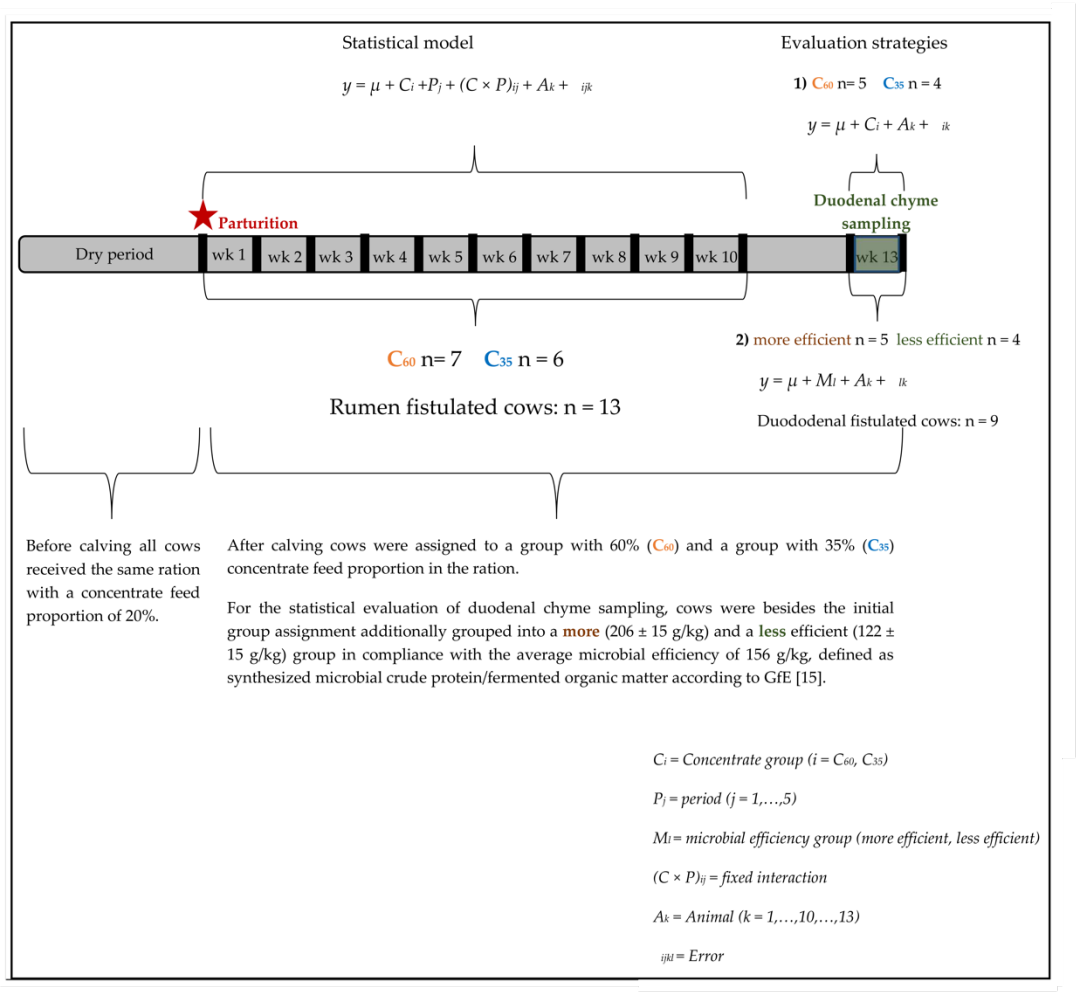


Figure 1. Experimental setup and statistical evaluations.

By assessing Pearson's correlation coefficient, we examined relations between parameters applying the statistical software TIBCO Statistica (Version 13.3, TIBCO Software Inc., Palo Alto, CA, USA) for both approaches. Furthermore, we performed linear regression analysis, if suitable.

The MIXED procedure, the t-Test and the correlation coefficient (r) were considered statistically significant when $p \leq 0.05$ and highly significant when $p \leq 0.01$ while a trend was assumed for $0.05 < p < 0.1$.

In the following, results are presented as LSMean ± standard error of means (SEM) for the MIXED procedure, and as means ± standard deviation of means (SD) for the t-Test, unless otherwise stated.

To analyze the diurnal ruminal pH variation, the values β0 and β1 were estimated by using PROC NLMIXED in SAS 9.4 (version 9.4; SAS Institute Inc., Cary, NC, USA). A logistic curve was fitted for every 24 hour interval, whereby β0 illustrates the slope of the regression line at the inflection point and therefore displays the variation of rumen pH over 24 h (the greater the values the more constant the ruminal pH) while β1 represents the inflection point of the curve and is an indicator for the average pH of the 24 h period [36]. The logistic curve depends on three parameters, namely the slope (β0) of the upper limit (β2) and the inflection point (β1) of the curve. The following formula describes this association:

$$y = \frac{\beta 2}{1 + \exp \times [-\beta 0 \times (x - \beta 1)]} \quad (10)$$

The accumulated time (min/day) spent below each corresponding pH point on the x-axis, is represented on the y-axis. The ruminal pH values are represented on the x-axis. The upper limit of the curve was kept constant at 1,440 min/day. Therefore, the logistic curve is only described by β0 and β1 Colman et al. [37] .

The time per day with pH <5.8 (min/day) was evaluated as described in Colman et al. [37]. Thresholds of 5.24 hours/day at pH <5.8 and a daily average pH <6.16 were set for a higher SARA risk according to Zebeli et al. (2008). For assessing the SARA risk, a $SARA_{risk}$-Score was calculated for which the following equation according to Schären et al. [38] was used.

$$\mathrm{SARA_{risk}} - \mathrm{Score} = \frac{\sum\left(\frac{\text{Number of positive observations per cow in period } i}{\text{Total number of observations per cow in period } i}\right)}{\text{Total number of cows assessed in period } i} \quad (11)$$

For the intergroup comparison, weekly means of each parameter were calculated.

3. Results

3.1. Performance Parameters

DMI increased over time irrespective of concentrate feed proportion of the ration ($p_{period} < 0.001$, Table A1). Net energy intake, as well as starch intake increased over time more pronounced in group C_{60} compared to $C_{35.}$ ($p_{C \times period}$ net energy intake = 0.016, Table A1, $p_{C \times period}$ starch intake < 0.001, Table A1).

Milk yield increased over time in both groups, with a steeper rise in group C_{35}, but an approximation of group C_{60} to group C_{35} during the last part of the trial ($p_{C \times period} < 0.001$). Energy corrected milk tended to be higher for the C_{35} group ($p_C = 0.093$) and enhanced within each group over the trial ($p_{period} < 0.001$). Milk fat content enhanced in both groups, with a more pronounced increase in group C_{35} ($p_{C \times period} < 0.001$). The same is true for milk fat yield ($p_{C \times period} = 0.042$). Milk protein content increased in both groups during the trial, without any group differences ($p_{period} < 0.001$). The same is true for milk protein yield ($p_{period} < 0.001$) and lactose concentration ($p_{period} = 0.002$). Milk lactose yield increased more pronouncedly in group C_{60} compared to C_{35} ($p_{C \times period} <0.001$). In contrast, milk fat:protein ratio decreased in both groups, but remained more stable in group C_{35} over time ($p_{C \times period} = 0.002$). (The appropriate results are shown in Table A1).

3.2. pH Parameters

We determined different pH parameters in order to describe the conditions in the rumen. For the SARA defining thresholds according to Zebeli et al. [9], including the daily mean pH (Figure 2A, Table 3) and the time per day with pH < 5.8 (Figure 2B, Table 3) we could not prove any significant effects between the two trial groups receiving different concentrate proportions. Regarding the $SARA_{risk}$-Score (Figure 2C, Table 3), values for the C_{60} group ranged between 0.33 and 0.86 (±0.17 SD). The C_{35} group reached values between 0.36 and 0.57 (±0.08 SD).

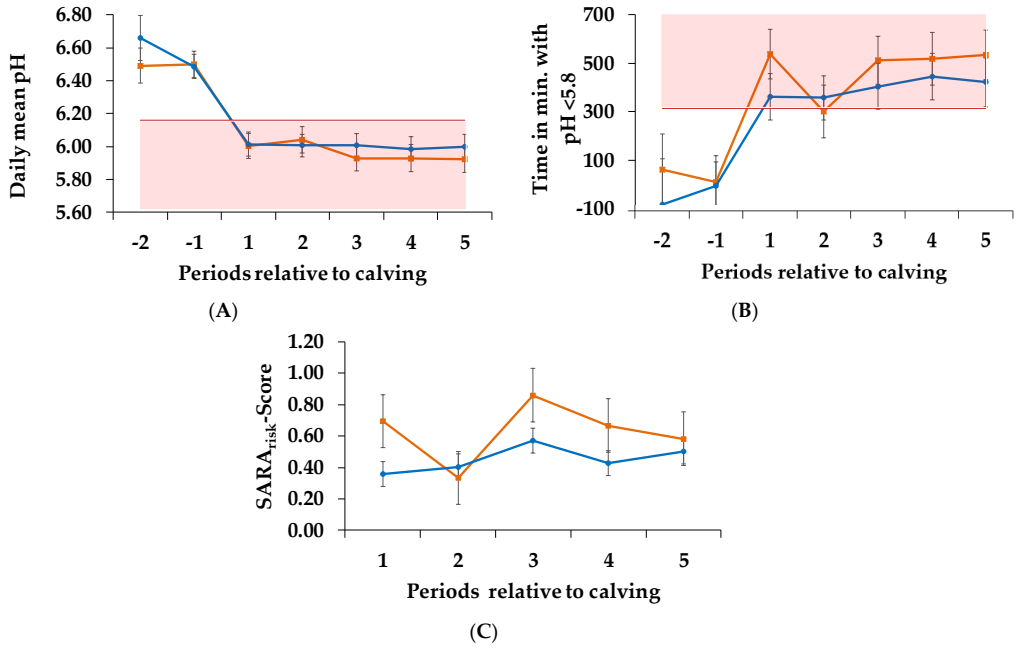


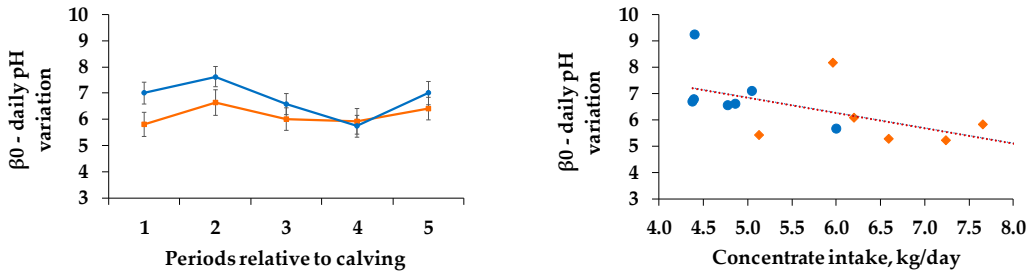
Figure 2. Daily mean pH of the rumen (**A**), time per day with pH < 5.8 (**B**) from week 3 antepartum until week 10 postpartum; red areas indicate SARA risk. Statistical analysis started at calving (period 1: weeks 1–2 postpartum, period 2: weeks 3–4 postpartum, period 3: weeks 5–6 postpartum, period 4: weeks 7–8 postpartum and period 5: weeks 9–10 postpartum) and SARA$_{risk}$-Score (C) was calculated as quotient of the sum of the number of positive observations per cow in period *i* and total number of observations per cow in period *i* divided by total number of cows assessed in period *i*, during period 1–5 in the treatment groups. After calving, cows were assigned to a group with 60% concentrate feed proportion (increasing from 35 to 60% during the first three weeks after parturition, **C_{60}, orange**, n = 6) and a group with 35% concentrate feed proportion (**C_{35}, blue**, n = 7) in the ration.

Table 3. *p*-values of effects of concentrate proportion of the diet (C), period and the interaction between them on daily mean pH and time in minutes with pH < 5.8.

	***p*-Values**		
	C	**Period** §	**C × Period**
(A) Daily mean pH	0.700	0.506	0.724
(A) Time in min. with pH < 5.8	0.557	0.205	0.545

After calving, cows were assigned to a group with 60% concentrate feed proportion (increasing from 35 to 60% during the first three weeks after parturition, C_{60}, n = 6) and a group with 35% concentrate feed proportion (C_{35}, n = 7) in the ration. § Period 1: weeks 1–2 postpartum, period 2: weeks 3–4 postpartum, period 3: weeks 5–6 postpartum, period 4: weeks 7–8 postpartum and period 5: weeks 9–10 postpartum.

C (p = 0.099) tended to affect β0 (Figure 3A, Table 4), as the group with the lower C exhibited higher values and thus had lower pH variations over the day.



(A) (B)

Figure 3. β0 (**A**: illustrates the slope of the regression line at the inflection point and therefore displays the variation of rumen pH over 24 h; the greater the values the more constant the ruminal pH) during period 1 (weeks 1–2 postpartum), period 2 (weeks 3–4 postpartum), period 3 (weeks 5–6 postpartum), period 4 (weeks 7–8 postpartum) and period 5 (weeks 9–10 postpartum) as well as regression of β0 and actual concentrate intake in period 1 (**B**: $y = -0.558x + 9.589$, $r^2 = 0.311$, $p < 0.05$) for each individual cow in period 1. After calving, cows were assigned to a group with 60% concentrate feed proportion (increasing from 35 to 60% during the first three weeks after parturition, C_{60}, **orange**, n = 6) and a group with 35% concentrate feed proportion (C_{35}, **blue**, n = 7) in the ration.

Table 4. *p*-values of effects of concentrate proportion of the diet (C), period and the interaction between them on daily pH variation (β0).

	p-Values		
	C	**Period** §	**C × period**
(A) β0 $	0.099	0.032	0.517

$ β0 illustrates the slope of the regression line at the inflection point and therefore displays the variation of rumen pH over 24 h; the greater the values the more constant the ruminal pH. After calving, cows were assigned to a group with 60% concentrate feed proportion (increasing from 35 to 60% during the first three weeks after parturition, C_{60}, n = 6) and a group with 35% concentrate feed proportion (C_{35}, n = 7) in the ration. § Period 1: weeks 1–2 postpartum, period 2: weeks 3–4 postpartum, period 3: weeks 5–6 postpartum, period 4: weeks 7–8 postpartum and period 5: weeks 9–10 postpartum.

β0 increased during the first part of the trial to decrease during period 3 and 4, with a second peak in period 5 in both groups ($p = 0.032$). However, none of the factors affected β1 (Table 5).

Table 5. Effects of concentrate feed proportion in the diet (C) and of period β1 (describes the inflection point of the curve and represents the average pH of the 24 h period) during period 1 (weeks 1–2 postpartum), period 2 (weeks 3–4 postpartum), period 3 (weeks 5–6 postpartum), period 4 (weeks 7–8 postpartum) and period 5 (weeks 9–10 postpartum) comparing the treatment groups.

Item	**Grouping** §		**SEM** #	***p*-Value**		
	C_{60} n = 6	**C_{35} n = 7**		**C**	**Period**	**C × Period**
β1						
Period 1	5.93	6.02	0.08	0.654	0.306	0.668
Period 2	6.07	6.02				
Period 3	5.96	6.01				
Period 4	5.92	5.98				
Period 5	5.94	5.99				

§ After calving cows were assigned to a group with 60% concentrate feed proportion (increasing from 35 to 60% during the first three weeks after parturition, C_{60}) and a group with 35% concentrate feed proportion (C_{35}) in the ration. Values are presented as least square means. # Pooled standard error of means.

When assessing the results of β1 on an individual basis (Figure 4) it also becomes apparent that animals within the same group differ. Both groups contain cows with lower or higher levels of average pH.

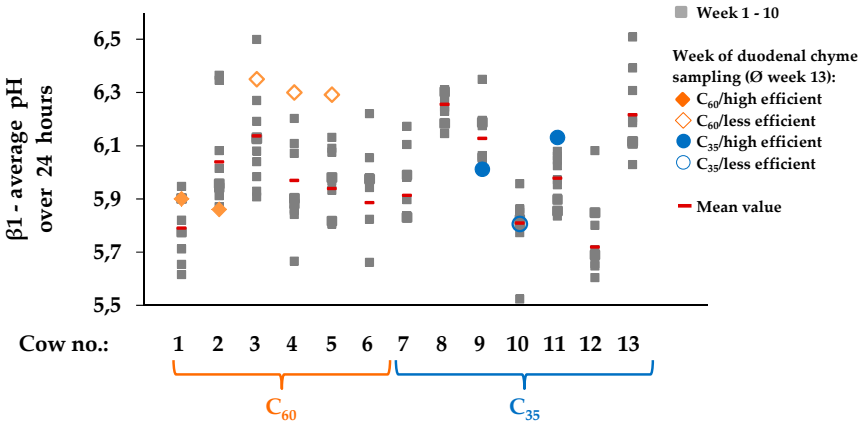


Figure 4. β1 (describing the inflection point of the curve and representing the average pH of the 24 h period) for individual cows and each of the 10 experimental weeks as well as of the week of duodenal chyme sampling (on average week 13 p.p. ± 16 days)) reflecting the individual variability independent of grouping. After calving cows were assigned to a group with 60% concentrate feed proportion (increasing from 35 to 60% during the first three weeks after parturition, **C_{60}, orange**, n = 6) and a group with 35% concentrate feed proportion (**C_{35}, blue**, n = 7) in the ration. For the week of duodenal chyme sampling, cows were additionally grouped into a **more** (●/◆ filled, 206 ± 17 g/kg, n=5) and a **less efficient** (○/◊, blank, 122 ± 17 g/kg, n=4) group in compliance with the average microbial efficiency of 156 g/kg defined as synthesized microbial crude protein/fermented organic matter according to GfE [15].

3.3. Short Chain Fatty Acids

Increase of total volatile fatty acids (Table 6) was observed in both groups over the trial (p_{period} = 0.020). Acetic acid (Table 6) tended to increase more pronounced in group C_{35} compared to C_{60} over time ($p_C \times period$ = 0.053). In contrast, propionic acid (Table 6) increased more pronounced in group C_{60} and decreased in group C_{35} during the last part of the trial ($p_C \times period$ = 0.064). Butyric acid (Table 6) decreased over time without any differences between groups (p_{period} = 0.001). For acetate:propionate ratio (Table 6), both groups achieved the highest values in period 1. Values then decreased during the first half of the study and increased again during the last part (p_{period} < 0.001).

Table 6. Effects of concentrate feed proportion in the diet (C) and period on short chain fatty acids, as well as on acetate:propionate ratio and ammonia-nitrogen (ammonia-N) concentration during period 1 (weeks 1–2 postpartum), period 2 (weeks 3–4 postpartum), period 3 (weeks 5–6 postpartum), period 4 (weeks 7–8 postpartum) and period 5 (weeks 9–10 postpartum) in the treatment groups.

Item	Grouping §		SEM#	*p*-Value		
	C_{60} n = 6	C_{35} n = 7		C	Period	C × period
Total short chain fatty acids, mmol/L						
Period 1	76.1	71.1	5.7	0.883	0.020	0.107
Period 2	79.7	73.5				
Period 3	72.9	90.3				
Period 4	94.8	83.5				
Period 5	79.5	80.7				
Acetic acid, Mol%						
Period 1	56.9	57.9	1.8	0.117	0.001	0.053
Period 2	51.5	56.6				
Period 3	53.4	54.1				
Period 4	50.6	57.3				
Period 5	53.4	56.3				
Propionic acid, Mol%						
Period 1	23.7	22.9	1.7	0.053	<0.001	0.064
Period 2	29.5	25.3				
Period 3	29.2	27.3				
Period 4	32.6	25.5				
Period 5	31.3	25.9				
Butyric acid, Mol%						
Period 1	14.7	14.9	0.9	0.224	0.001	0.422
Period 2	13.5	13.3				
Period 3	12.1	13.8				
Period 4	11.9	13.0				
Period 5	10.9	13.1				
Acetate:propionate ratio						
Period 1	2.49	2.63	0.22	0.103	<0.001	0.131
Period 2	1.82	2.33				
Period 3	1.90	2.04				
Period 4	1.57	2.34				
Period 5	1.73	2.30				

§ After calving cows were assigned to a group with 60% concentrate feed proportion (increasing from 35 to 60% during the first three weeks after parturition, **C_{60}**) and a group with 35% concentrate feed proportion (**C_{35}**) in the ration. Values are presented as least square means. # Pooled standard error of means.

3.4. Evaluations of The Week of Duodenal Chyme Sampling by Different Statistical Evaluations

3.4.1. Microbial Efficiency, pH Parameters, Nutrient Flows and Digestibiliy at the Duodenum

For the analysis of the estimated parameters in the duodenal chyme samples, we wanted to assess both the effect of rations differing in C, as well as individually different microbial efficiencies. For this, different statistical evaluations were applied and cows were assigned to different C according to the initial experimental design and additionally grouped according to microbial efficiency (mCP/fOM, more and less efficient). This attempt was made in order to gain more information about the relation between pH parameters and microbial efficiency, as well as on parameter such as nutrient flows and digestibility.

3.4.2. pH Parameters

We compared daily mean pH, time per day with pH < 5.8, as well as β0 and β1 during the week of duodenal sampling between the C_{60} and C_{35} groups, as well as between more and less efficient groups. However, we could not detect any significant differences (Table 7).

In this trial, concentrate had no significant effect on mCP, fOM or mCP/fOM (Table 8).

The same is true for DMF, organic matter flow (OMF) and rumen ammonia-N (Table 8).

mCP (Table 8) tended to be higher for the more efficient group compared to the less efficient group ($p_{mCP/fOM}$ = 0.081). FOM (Table 8) was significantly higher for less efficient groups ($p_{mCP/fOM}$ = 0.024). mCP/fOM was significantly higher for more efficient animals ($p_{mCP/fOM}$ <0.001). For DMF (Table 8), as well as for duodenal organic matter flow (OMF, Table 8), we detected a significant difference between groups ($p_{mCP/fOM}$ DMF = 0.032, $p_{mCP/fOM}$ OMF = 0.037) as more efficient groups had higher values in both parameters.

Rumen ammonia-N (Table 8) did not differ between groups.

3.4.3. Digestibility

We assessed both the total digestive tract digestibility of dry matter (tdDM) and the digestibility at the duodenum. None of the nutrient digestibilities at the duodenum (Table 9) was influenced by C.

Table 7. Effects of concentrate feed proportion in the ration (C) and microbial efficiency (synthesized microbial crude protein/fermented organic matter) on ruminal pH parameters.

Item [+]	Grouping model 1 [$]				Grouping Model 2 [§]			
	C_{60} n = 5	C_{35} n = 4	SD [#]	*p*-Value	More Efficient n = 5	Less Efficient n = 4	SD [#]	*p*-Value
Daily mean pH	6.01	6.08	0.20	0.674	6.00	6.06	0.21	0.700
Time in min. with pH <5.8	445	266	250	0.400	395	361	288	0.873
β0 [*]	6.09	5.54	0.87	0.391	5.84	5.93	0.85	0.893
β1 [†]	6.14	5.98	0.20	0.359	5.98	6.19	0.19	0.186

[+] Intergroup comparison for the week of duodenal chyme sampling (on average week 13 postpartum ± 16 days) - for both assignments (C_{60} vs. C_{35}, **more** vs. **less efficient**, respectively). [$] After calving cows were assigned to a group with 60% (increasing from 35 to 60% during the first three weeks after parturition, C_{60}) in the ration and a group with 35% concentrate feed proportion (C_{35}). [§] For the week of duodenal chyme sampling cows were additionally grouped into a **more** (206 ± 17 g/kg, n = 5) and a **less efficient** (122 ± 17 g/kg, n = 4) group according to the mean microbial efficiency of 156 g/kg, defined as synthesized microbial crude protein/fermented organic matter in compliance with GfE [15]. [*] illustrates the slope of the regression line at the inflection point and therefore displays the variation of rumen pH over 24 h; the greater the values the more constant the ruminal pH. [†] describes the inflection point of the curve and represents the average pH of the 24 h period. Values are presented as means, [#] Pooled standard deviation.

Table 8. Effects of concentrate feed proportion in the ration (C) and microbial efficiency (synthesized microbial crude protein/fermented organic matter) on synthesized microbial crude protein (mCP), fermented organic matter (fOM) and microbial efficiency (mCP/fOM), and on duodenal nutrient flows and nitrogen sources, and on ruminal nitrogen balance (RNB).

Item [+]	Grouping model 1 [§]				Grouping model 2 [§]			
	C_{60} n = 5	C_{35} n = 4	SD [#]	*p*-Value	More efficient n = 5	Less efficient n = 4	SD [#]	*p*-Value
Microbial crude protein, g/day	2129	2280	409	0.806	2479	1922	356	0.081
Fermented organic matter, kg/day	14.4	12.9	3.0	0.457	12.1 [B]	15.8[A]	2.1	0.034
Microbial crude protein/fermented organic matter, g/kg	155	186	47	0.355	206 [A]	122[B]	17	<0.001
Duodenal dry matter flow, kg/day	15.8	15.9	2.9	0.954	17.7 [A]	13.6[B]	2.2	0.032
Duodenal organic matter flow, kg/day	13.6	13.5	2.6	0.956	15.1 [A]	11.7[B]	2.0	0.037
Rumen ammonia-N [*], mg/100 g	3.0	7.5	4.0	0.176	5.12	5.44	3.95	0.932

[+] Intergroup comparison for the week of duodenal chyme sampling (on average week 13 postpartum ± 16 days) - for both assignments (C_{60} vs. C_{35}, **more** vs. **less efficient**, respectively). [AB]least square means with different superscripts differ within row. [§] After calving cows were assigned to a group with 60% (increasing from 35–60% during the first three weeks after parturition, C_{60}) in the ration and a group with 35% concentrate feed proportion (C_{35}). [§] For the week of duodenal chyme sampling cows were additionally grouped into a **more** (206 ± 17 g/kg, n=5) and a **less efficient** (122 ± 17 g/kg, n=4) group according to the mean microbial efficiency of 156 g/kg, defined as synthesized microbial crude protein/fermented organic matter in compliance with GfE [15]. [*] Nitrogen. Values are presented as means, [#] Pooled standard deviation.

Table 9. Effect of concentrate feed proportion of the ration and microbial efficiency (synthesized microbial crude protein/fermented organic matter) on apparent nutrient digestibility at the duodenum and the total digestive tract digestibility at faecal level.

Item [+]	Grouping Model 1 [$]		SD [#]	*p*-Value	Grouping Model 2 [§]		SD [#]	*p*-Value
	C_{60} n = 5	C_{35} n = 4			More Efficient n = 5	Less Efficient n = 4		
Duodenal nutrient digestibility, %								
Neutral detergent fiber	37	40	12	0.750	32	46	11	0.086
Acid detergent fiber	31	35	13	0.711	27	40	12	0.144
Organic matter	43	38	10	0.490	33 [B]	51 [A]	5	0.001
Total digestive tract nutrient digestibility, %								
Neutral detergent fiber	46	42	6	0.298	41 [B]	49 [A]	4	0.036
Acid detergent fiber	40	35	6	0.338	34 [B]	43 [A]	4	0.017
Organic matter	67 [A]	60 [B]	4	0.003	61 [B]	68 [A]	3	0.027

[+] Intergroup comparison for the week of duodenal chyme sampling (on average week 13 postpartum ± 16 days), for both assignments (C_{60} vs. C_{35}, **more** vs. **less efficient**, respectively) [AB] least square means with different superscripts differ within row. [$] After calving cows were assigned to a group with 60% (increasing from 35–60% during the first three weeks after parturition, C_{60}) in the ration and a group with 35% concentrate feed proportion (C_{35}). [§] For the week of duodenal chyme sampling cows were additionally grouped into a **more** (206 ± 17 g/kg, n=5) and a **less efficient** (122 ± 17 g/kg, n = 4) group according to the mean microbial efficiency of 156 g/kg, defined as synthesized microbial crude protein/fermented organic matter in compliance with GfE [15]. Values are presented as means. [#] Pooled standard deviation.

In the same manner digestibility of ADF_{om} (Table 9) did not differ between more and less efficient animals. However, digestibility of $aNDF_{om}$ (Table 9) tended to be higher in the less efficient group compared to the more efficient group ($p_{mCP/fOM}$ = 0.086). Furthermore, digestibility of OM at the duodenum (Table 9) was significantly lower in more efficient animals ($p_{mCP/fOM}$ = 0.001).

The tdDM differed significantly between C_{60} and C_{35} groups (p_c = 0.030, Figure 5) as well as between more and less efficient groups ($p_{mCP/fOM}$ = 0.026, Figure 5). In the first case, higher C led to higher digestibility. In the second case, less efficient cows exhibited higher values.

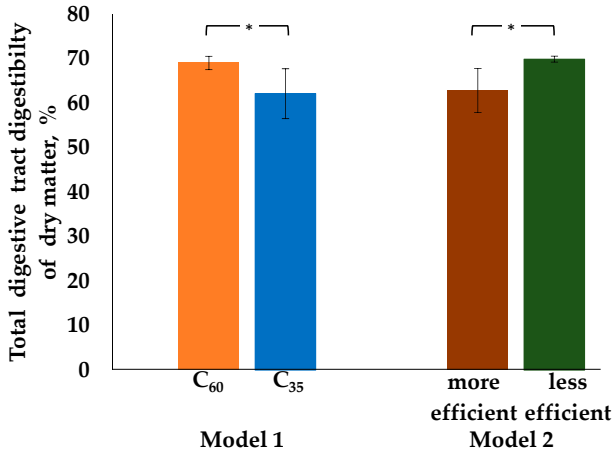


Figure 5. Total digestive tract digestibility of dry matter for the week of duodenal chyme sampling (on average week 13 postpartum ± 16 days). After calving cows were assigned to a group with 60% concentrate feed proportion (increasing from 35 to 60% during the first three weeks after parturition, C_{60}, **orange**, n = 5) and a group with 35% concentrate feed proportion (C_{35}, **blue**, n = 4) in the ration (model 1). For the week of duodenal chyme sampling, cows were additionally grouped into a **more** (206 ± 17 g/kg, **red**, n=5) and a **less** efficient (122 ± 17 g/kg, **green**, n = 4) group in compliance with the average microbial efficiency of 156 g /kg, defined as synthesized microbial crude protein/fermented organic matter according to GfE [15] (model 2). *Indicating significant group differences. C_{60} vs. C_{35}: *p*-value = 0.030, more efficient vs less efficient: *p*-value = 0.026.

The same is true for the total digestive tract digestibility quotient of OM (p_c = 0.003, $p_{mCP/fOM}$ = 0.027, Table 9). C_{60} and C_{35} animals did not differ in their total digestive tract digestibility quotient of $aNDF_{om}$ and ADF_{om} (Table 9). Furthermore, the less efficient group had a significantly higher total digestive tract digestibility quotients of $aNDF_{om}$ ($p_{mCP/fOM}$ = 0.036, Table 9) and ADF_{om} ($p_{mCP/fOM}$ = 0.017, Table 9) compared to the more efficient group.

3.5. Correlations and Regression Analysis

However, as we found a tendency of β0 being influenced by different concentrate feed proportions of the ration, we attempted to examine this relation more closely. Therefore, we correlated the β0 values with the actual concentrate intake of period 1 and then detected a significant relation (r^2 = 0.311, $p < 0.05$, Figure 4B). Animals which consumed more concentrate feed exhibited smaller β0 values and therefore had a more fluctuating ruminal pH over the day.

3.5.1. Ruminal pH, Duodenal Nutrient Flows, and Microbial Crude Protein

We calculated further correlations and performed regression analysis with measured and calculated variables of the duodenal chyme collection to further assess the assumed pH influence on microbial efficiency, which was not verifiable in the intergroup comparison.

Hereby, we could detect a positive relation between β1 and DMF (r^2 = 0.598, $p < 0.01$, Figure 6A), as well as between β1 and OMF (r^2 = 0.594, $p < 0.01$, Figure 6B) for the week of duodenal chyme

collection. Furthermore, increasing DMF was associated with a higher flow of microbial crude protein ($r^2 = 0.646$, $p < 0.05$, Figure 6C). The same is true for OMF ($r^2 = 0.536$, $p = 0.061$, Figure 6D).

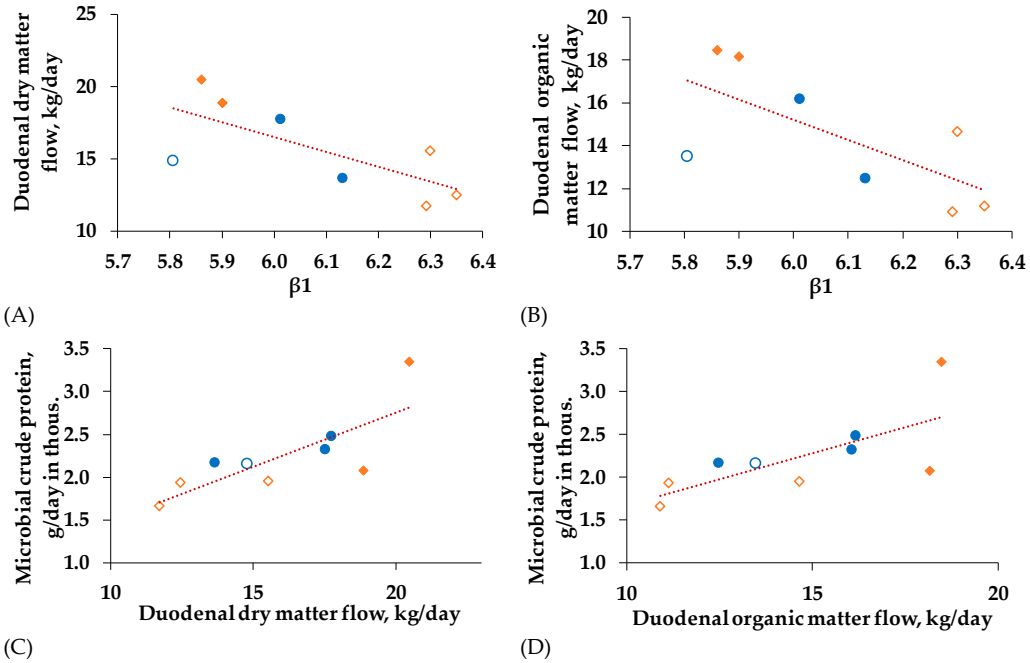


Figure 6. Regression of β1 (describing the inflection point of the curve and representing the average pH of the 24 h period) on duodenal dry matter flow (DMF, **A**, y = -10.25x + 77.97, $r^2 = 0.598$, $p < 0.05$) and of β1 on duodenal organic matter flow (OMF, **B**, y = -9.42x + 71.74, $r^2 = 0.594$, $p < 0.05$), as well as of DMF microbial crude protein (**C**, y = 0.13x + 0.23, $r^2 = 0.646$, $p < 0.05$) and of OMF on microbial crude protein (**D**, y = 0.12x + 0.47, $r^2 = 0.536$, $p = 0.061$) for the duodenal fistulated cows (n = 9) for the week of duodenal chyme sampling (in average week 13 postpartum ± 16 days). After calving cows were assigned to a group with 60% concentrate feed proportion (increasing from 35-60% during the first three weeks after parturition, C_{60}, **orange**, n = 5) and a group with 35% concentrate feed proportion (C_{35}, **blue**, n = 4) in the ration. For the week of duodenal chyme sampling cows were additionally grouped into a **more** (206 ± 17 g/kg, ●/◆, n = 5) and a **less efficient** (122 ± 17 g/kg, ○/◊, n = 4) group in compliance with the average microbial efficiency of 156 g/kg, defined as synthesized microbial crude protein/fermented organic matter according to GfE [15]. Correlation of (**A**) and (**B**) was conducted with eight animals, due to a missing β1 value for one more efficient cow of C_{35}.

4. Discussion

The aim of the present study was to examine the ruminal pH caused by differing concentrate feed proportions, thereby paying special attention to diurnal pH-kinetics. The second objective of the study was to gain information on the interplay between ruminal pH parameters and microbial efficiency. For this, cows were additionally (retrospective) grouped according to their individual microbial efficiency. Therefore, the mean microbial efficiency of 156 g mCP/kg fOM according to GfE [15] was used as threshold for allocating the cows to more (>156 g/kg) or less (<156 g/kg) efficient. Moreover, efficiency related parameters, such as nutrient flows and digestibilities were examined.

In the transition period, cows decrease dry matter intake (DMI) and net energy intake (NEI) during the last days prior to calving and increase it again gradually after parturition [39]. This finding is substantiated by the significantly stronger increase in NEI and starch intake of group C_{60} resulting both from the slightly enhanced DMI and the higher NE_L- and starch content of the ration.

We had expected that the higher starch intake of this group would lower the ruminal pH. A higher starch intake had been shown to increase the production of SCFA and thus decrease ruminal pH [5]. However, in the present study, we could not prove this inverse relation to pH parameters

such as daily mean pH, time per day with pH < 5.8 and β1. Considering the SARA defining thresholds according to Zebeli et al. [9], described as a daily mean pH < 6.16 and the time per day with pH < 5.8 for more than 5.24 h, we would have expected that cows fed higher C would decrease daily mean pH and increase time with pH < 5.8 more drastically than the C_{35} group. Surprisingly both groups achieved the critical ranges defined by Zebeli et al. [9]. However, high standard errors suggest a high individual variability, which probably prevented the detection of significant differences between groups. Other studies showed the opposite by observing a decrease in ruminal pH resulting from higher concentrate intake [40,41]. Possibly, difference of starch content between the rations in the present study must have been higher to provoke extremes. On the contrary, studies of Ueda et al. [5] and Beauchemin and Penner [1] confirm our results. Ruminal pH is not only influenced by ration composition, but also by the functionality of the epithelium and thus by absorption capacity to eliminate SCFA. Neutralization by buffers and passage to lower digestive tract are considered to be further factors influencing ruminal pH for compensating an increased SCFA production [7]. Beauchemin and Penner [1] assumed that the feed depression before calving already reduces the functionality of rumen epithelium and increases the susceptibility to SARA. Consequently, the rumen mucosa would be incapable of even dealing with lower C proportions, which might already result in critical pH values. Our study supports these findings.

Gao and Oba [42] suggest that cows can be either tolerant towards, or susceptible to SARA. Other studies confirm this hypothesis by assuming a high individual variability in ruminal pH, due to individual variability in absorption capacity and adaptability of the epithelium as well as in feeding behavior [1,3,12]. Nevertheless, in the present study, cows with higher concentrate intake exhibited lower β0 values, reflecting a more fluctuating ruminal pH independent from group classification, which supports the assumption that individuality might have covered treatment effects De Veth and Kolver [19] already highlighted that a comparison of daily mean pH presupposes stable pH conditions and that the daily pH variation is at least equally relevant. Other studies endorse the view that pH variations over the day are more relevant than the daily mean pH [19,43]. Consequently, the relation between concentrate amount and pH parameters must not be neglected.

The susceptibility to SARA in early lactation is supposed to influence microbial efficiency by destabilizing the population of microorganisms [44]. Rapidly fermentable carbohydrates decrease the growth of cellulolytic bacteria and stimulate growth of lactobacilli [45]. These alterations might influence microbial efficiency to synthesize microbial protein related to fermented organic matter. Based on these assumptions, microbial efficiency might also be associated with pH variations. For a better understanding of these associations, we retrospectively grouped the cows according to their microbial efficiency irrespective of the initial diet-based grouping.

Different studies had already indicated a missing effect of C on microbial efficiency [5,18]. Our results support these findings, as we could not detect any differences between the C_{60} and the C_{35} groups in mCP, fOM and mCP/fOM, supporting the idea to re-group the cows according to their microbial efficiency, to gain more information.

We have hypothesized a direct relation between pH parameters and microbial efficiency. A low pH is considered to inhibit fiber digestion and thus decrease microbial efficiency in ruminants [13,14]. However, our results could not identify differences in pH parameters, neither between the more and less efficient groups nor between the C_{60} and C_{35} groups. The time with an unfavorable pH might have been too short to affect the cellulolytic bacteria [46]. Other studies have neither been successful to reduce celullose digestion by decreasing pH in ruminants or to find a direct relation between pH and microbial efficiency [18,47,48]. Oetzel [8] supports our findings and suggests that microbial response on a low pH or even SARA is slow and that multiple acidotic impacts are necessary to inhibit microbial activity. Additionally, concentration of cellulolytic bacteria is considered to be higher than necessary and thus this population remains as long as pH is not unfavorable for too long [49]. Nevertheless, in the present trial, negative relationships between β1 and DMF, as well as OMF were observed. Both parameters decreased with increasing β1 values. In

turn, DMF and OMF were positively associated with microbial efficiency, possibly due to an accompanied decrease of predation by protozoa [50].

Oba and Allen [18] already indicated that a higher passage rate could increase microbial efficiency. A higher passage rate limits the microbial lysis as well as the use of energy for non-growth processes [18,51]. Rode et al. [52] demonstrated a higher passage rate with increasing concentrate proportions. Our study could not support these findings, neither for DMF nor for OMF. However, independent from C, more efficient cows showed higher DMF and OMF. These higher flows also explain the lower tdDM digestibility for more efficient cows in the present study [22]. Another possible explanation for lower digestibilities in the more efficient group might be that energy and substrate could not be used efficiently as digestibility increased, due to a fermentation rate that exceeded the microbial growth rate [18]. Sutton et al. [4] and Faichney et al. [53] already explained the missing relations in digestibility quotients at the duodenum and indicated that digestion in the colon partially compensates ruminal digestion in sheep. Conversely, missing differences in nutrient flows between C_{60} and C_{35} groups were accompanied by missing effects in digestibility. Only digestibility of OM analyzed at the duodenum was positively influenced by C, which is in line with the study of Yang et al. [12] and supports the idea of compensation in the lower digestive tract. The missing C effects in nutrient flows and digestibility in the present study might be due to an undersized difference of starch between the experimental rations.

Unexpectedly, the more efficient group exposed lower fOM values, in agreement with the study of Oba and Allen [18]. Energy from fOM is considered to be limiting factor for microbial efficiency [18]. That indicates that factors other than energy limited microbial efficiency additionally [17]. According to Clark et al. [17] these factors include synchronization of degradation of feed to permanently provide nutrients for microorganisms, content of nutrients and rumen conditions. Thus, a high individual variation for microbial efficiency can be assumed [17]. The second main limiting parameter for microbial crude protein synthesis is ammonia-N. In the present study, ammonia-N did neither differ between the C_{60} and C_{35} group nor between the more and less efficient group. Oba and Allen [18] could neither observe a direct relation to microbial efficiency. One appropriate explanation might be that a certain level of ammonia saturation was achieved in the present study. It was demonstrated that microbial crude protein synthesis exceeds the maximum with an amount of 5 mg/dL and does not increase further with increasing ammonia concentration [54]. For the present study, it may seem obvious that DMF and OMF mainly influenced microbial efficiency by decreasing predation by protozoa and energy spilling.

5. Conclusions

Higher amounts of concentrate did not affect daily mean pH or time with pH < 5.8. It may be assumed that individual differences among cows in ruminal pH impeded detection of significant group differences, due to high standard errors. However, we could prove a positive relationship between concentrate intake and $\beta 0$ values, reflecting larger pH fluctuations. It seems that the ability to smoothly adapt to the rapid drop in pH decreases with increasing concentrate feed intake, whereby individual differences to cope with this challenge become more obvious.

Comparing the cows grouped by microbial efficiency did not reveal differences in pH parameters. However, a relation between daily pH fluctuation and DMF as well as OMF was found. DMF and OMF in turn, were positively associated with microbial protein synthesis. Consequently, microbial efficiency was at least indirectly influenced by daily pH variation.

Further research is necessary to complete and improve the understanding of the ruminal processes. Important influencing factors, such as feeding behavior, comprising meal sizes and number of meals per day, rumen functionality, including absorption capacity of the rumen epithelium, as well as saliva production and its buffering capacity and furthermore the microbiome itself were not assessed in the present study, but should also be considered.

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Appendix A

Table A1. Effects of concentrate feed proportion in the diet (C) and period on dry matter intake (DMI), net energy intake (NEI), milk yield, energy corrected milk (ECM) and milk components during period 1 (weeks 1–2 postpartum), period 2 (weeks 3–4 postpartum), period 3 (weeks 5–6 postpartum), period 4 (weeks 7–8 postpartum) and period 5 (weeks 9–10 postpartum) in the treatment groups.

Item	Grouping §		SEM#	*p*-Value		
	C_{60} n = 6	C_{35} n = 7		C	Period	C × Period
DMI, kg/day						
Period 1	14.9	15.9	1.2	0.298	<0.001	0.103
Period 2	20.3	19.4				
Period 3	23.3	21.0				
Period 4	24.2	21.6				
Period 5	24.5	22.7				
NEI, MJ NE_L/day						
Period 1	106 ^{c}	113 ^{b}	8	0.133	<0.001	0.016
Period 2	146 ^{b}	135 ^{a}				
Period 3	169 ^{a}	146 ^{a}				
Period 4	176 ^{a}	150 ^{a}				
Period 5	178 ^{a}	158 ^{a}				
Starch intake, kg/day						
Period 1	4.6 ^{a}	4.5^{a}	0.4	0.004	<0.001	<0.001
Period 2	6.8 ^{b}	5.7^{b}				
Period 3	8.3 ^{Ac}	6.1 ^{Bc}				
Period 4	8.9 ^{Ad}	6.2 ^{Bd}				
Period 5	8.8 ^{Ae}	6.6 ^{Be}				
Milk yield, kg/day						
Period 1	23.3 ^{e}	29.0 ^{b}	1.9	0.365	<0.001	<0.001
Period 2	32.8 ^{d}	36.9 ^{a}				
Period 3	36.8 ^{cd}	40.0 ^{a}				
Period 4	40.8 ^{b}	40.0 ^{a}				
Period 5	41.0 ^{a}	40.2 ^{a}				
Milk fat content, %						
Period 1	4.88 ^{a}	4.82 ^{a}	0.37	0.233	<0.001	<0.001
Period 2	3.93 ^{b}	4.35 ^{ab}				

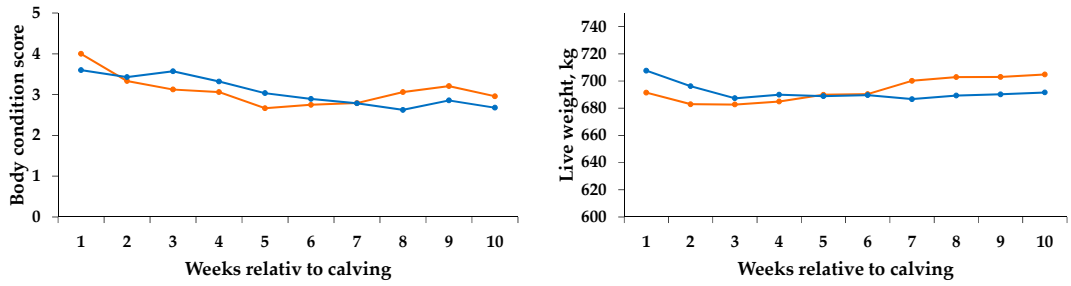
Period 3	3.32 [bc]	4.07 [b]				
Period 4	2.78 [c]	3.82 [b]				
Period 5	2.81 [c]	3.79 [b]				
Milk fat yield, kg/day						
Period 1	1.19 [c]	1.28 [c]	0.12	0.148	0.372	0.049
Period 2	1.30 [bc]	1.35 [bc]				
Period 3	1.22 [ab]	1.51 [ab]				
Period 4	1.14 [a]	1.53 [a]				
Period 5	1.17 [a]	1.50 [a]				
Milk protein content, %						
Period 1	3.90	3.52	0.17	0.297	<0.001	0.363
Period 2	3.34	2.96				
Period 3	3.26	3.04				
Period 4	3.23	3.16				
Period 5	3.29	3.18				
Milk protein yield, kg/day						
Period 1	0.94	1.04	0.08	0.944	<0.001	0.162
Period 2	1.11	1.09				
Period 3	1.20	1.22				
Period 4	1.33	1.27				
Period 5	1.36	1.27				
Milk lactose content, %						
Period 1	4.49	4.26	0.21	0.560	0.002	0.312
Period 2	4.74	4.31				
Period 3	4.76	4.61				
Period 4	4.79	4.80				
Period 5	4.80	4.85				
Milk lactose yield, kg/day						
Period 1	1.10 [c]	1.23 [b]	0.17	0.762	<0.001	<0.001
Period 2	1.57 [b]	1.59 [a]				
Period 3	1.75 [b]	1.71 [a]				
Period 4	1.97 [a]	1.72 [a]				
Period 5	1.97 [a]	1.73 [a]				
Milk fat:protein ratio						
Period 1	1.26 [a]	1.31 [a]	0.12	0.175	<0.001	0.002
Period 2	1.18 [ab]	1.34 [a]				
Period 3	1.03 [bc]	1.29 [a]				
Period 4	0.86 [c]	1.21 [a]				
Period 5	0.86 [c]	1.19 [a]				
ECM, kg/day						
Period 1	27.69	34.61	2.17	0.093	<0.001	0.210
Period 2	32.85	38.77				
Period 3	33.62	39.80				
Period 4	34.87	38.93				
Period 5	35.36	38.64				

[abcde] least square means (LSMeans) with different superscripts differ within columns. [AB]LSMeans with different superscripts differ within row. [§] After calving cows were assigned to a group with 60% concentrate feed proportion (increasing from 35-60% during the first three weeks after parturition, **C$_{60}$**) and a group with 35% concentrate feed proportion (**C$_{35}$**) in the ration. Values are presented as LSMeans. [#] Pooled standard error of means.

Table A2. Group allocation based on the individual microbial efficiency.

Cow Number	Group Allocation	Individual Microbial Efficiency
1	More efficient	191.86

2	More efficient	215.75
3	Less efficient	114.65
4	More efficient	198.03
5	More efficient	193.02
6	Less efficient	122.20
7	More efficient	231.77
8	Less efficient	145.98
9	Less efficient	106.92



(A) (B)

Figure A1. Progress of body condition score **(A)** and live weight **(B)** during the first 10 weeks postpartum. After calving cows were assigned to a group with 60% concentrate feed proportion (increasing from 35-60% during the first three weeks after parturition, C_{60}, **orange**, n = 5) and a group with 35% concentrate feed proportion (C_{35}, **blue**, n = 4) in the ration.

Table A3. Effects of microbial efficiency (synthesized microbial crude protein/fermented organic matter) on dry matter intake (DMI), milk yield, energy corrected milk (ECM) and milk components during the week of duodenal chyme sampling.

Item [+]	Grouping [§]		SD [#]	*p*-Value
	More Efficient n = 5	Less Efficient n = 4		
DMI, kg/day	23.9	25.3	2.7	0.515
Milk yield, kg/day	41.2	40.3	4.7	0.783
Milk fat content, %	3.32	3.16	0.51	0.731
Milk fat yield, kg/day	1.35	1.30	0.21	0.781
Milk protein content, %	3.31	3.26	0.20	0.711
Milk protein yield, kg/day	1.36	1.35	0.18	0.923
Milk lactose content, %	4.77	4.88	0.08	0.100
Milk fat:protein ratio	0.99	0.97	0.15	0.864
ECM, kg/day	37.4	37.1	4.4	0.919

[+] Intergroup comparison for the week of duodenal chyme sampling (on average week 13 postpartum ± 16 days), for the **more** and the **less efficient** groups. [§] For the week of duodenal chyme sampling cows were grouped into a **more** (206 ± 17 g/kg, n=5) and a **less efficient** (122 ± 17 g/kg, n=4) group according to the mean microbial efficiency of 156 g/kg, defined as synthesized microbial crude protein/fermented organic matter in compliance with GfE [15]. Values are presented as means. [#] Pooled standard deviation.

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6. Paper III

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Effects of Pre-Calving Body Condition and Different *post partum* Concentrate Feed Proportions on Immune-Associated and Hematological Parameters in Pluriparous Dairy Cows.

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Effects of Pre-Calving Body Condition and Different *post partum* Concentrate Feed Proportions on Immune-Associated and Hematological Parameters in Pluriparous Dairy Cows

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Simple Summary: Dairy cows have to cope with physiological, metabolic and endocrine challenges during the transition from late gestation to lactation. This period is characterized by a negative energy balance. Concentrate feed proportion of the rations is often increased to compensate the energy deficit. This can lead to acidotic conditions in the rumen, which might trigger the release of lipopolysaccharides from bacteria and result in inflammatory responses. An unfavorable pre-calving body condition is known to cause health problems after calving. Cows with a higher body condition (expressed as score, BCS, at a five-point scale) prior to calving might respond differently to either low or high concentrate feed proportions after calving with regard to hematological traits as indicators for possible interactions between nutritional status and immune system. For testing this hypothesis, cows were split into two classes (higher and adequate BCS) six weeks prior to calving according to their actual BCS and fed either a low- or high-energy-dense diet after calving. Neither BCS class nor dietary energy concentration influenced markedly hematological traits, including immune cell phenotypes, and inflammatory markers, such as haptoglobin. The overall covered individual BCS range and the *post partum* concentrate feed challenge did not overstress physiological adaptability.

Abstract: The present study aimed at evaluating the influences of different concentrate feed proportions in the ration offered to dairy cows *post partum* with different body condition scores (BCS) before calving. Therefore, 60 pluriparous cows were divided 42 days before expected calving into two groups with a higher or an adequate BCS. After calving, both groups were further subdivided into a group fed a ration with either a low concentrate feed proportion (C, 35% at dry matter basis) or a high (60% at dry matter basis) one. It was hypothesized that different BCS would lead to different reactions concerning varying concentrate feed proportions. Isolated BCS effects were detected in the white blood profile only before calving. Neither low nor high concentrate feed proportions affected hematological, blood immune cell phenotypes and inflammatory markers consistently irrespective of BCS group. It was concluded, that the assessed BCS span covered a range in which the capability of cows to cope with different dietary *post partum* energy supply remained unchanged.

Keywords: dairy cow; pre-calving body condition score; lactation diet; immune system; hematology; peripheral blood monocular cells; inflammatory markers

1. Introduction

Due to the transition from late gestation to lactation, the cow has to cope with physiological, metabolic and endocrine changes, which can be accompanied by high stress levels [1,2]. The individual body condition is known to influence the endocrine status, which in turn affects the immunological condition. Stress hormones, such as cortisol were proposed to change granulocyte (GR) count levels in blood [3]. Cortisol again, was shown to be negatively related to body condition [4]. However, several endogenous adaptions are physiological necessary, such as the degradation of tryptophan (Trp), which prevents a maternal immune reaction against the fetus [5]. All necessary adaptions are energy consuming processes at a time when the energy balance is already negative [6]. In prepartal cows over conditioning can increase the risk for complications at calving and thereafter [7]. Obesity was demonstrated to cause a high incidence of infections and alterations of immunity in both, humans and animals [8]. Cows with a higher pre-calving BCS exhibit a higher risk for accelerated lipolysis and consequently a more pronounced negative energy balance (NEB) *post partum* [7]. The suboptimal health status predispose for further health problems [9]. Haptoglobin (Hpt) is a major acute-phase-protein in cattle and used as indicator for an inflammatory response [10]. Hpt can also be regarded as a bovine adipokine and is therefore strongly related to body condition [11].

In experimental models, a low concentrate proportion of the ration is often used to increase lipolysis and enhance the risk of ketosis [12]. The energetic dilution of the ration would also increase the NEB [13]. This was confirmed in our previous study [14]. However, those experimental groups that were fed rations with lower concentrate feed proportions were not clearly prone to develop an excessive lipid mobilization. The non-esterified fatty acid (NEFA) concentrations of those groups exceeded the threshold for indicating an accelerated lipolysis. However, β-hydroxybutyrate (BHB) concentrations did not achieve the appropriate threshold [14] suggesting that NEFA utilization was not significantly compromised. In the present study, we aimed to investigate these relations more closely in the context of further immunological parameters.

Increasing concentrate proportions as an effort to counteract the NEB may also have some negative consequences for the cow. The change from a dry period diet to a lactation diet requires the adaptation of the rumen. Thus, an increasing concentrate proportion of the ration (C) can be considered as an additional challenge for the transition cow. Higher C may cause acidotic conditions in the rumen, which may result in subacute ruminal acidosis (SARA) [15,16]. These conditions lead to an increased lysis of Gram-negative bacteria, which release lipopolysaccharides (LPS) [17]. Additionally, the epithelium may be stressed and therefore more susceptible to injuries [18]. This would enable the translocation of LPS from the rumen into the blood stream [19] and the subsequently increased LPS transfer to the liver would trigger an acute-phase-response [20].

Several studies investigated the influence of *ante partum* BCS in combination with dry cow energy supply. These models often included different C during the dry period, in particular, to achieve an energy oversupply before calving and to stimulate *post partum* lipolysis [12,21,22]. However, to our knowledge, little is known about the possibly dynamic relation between pre-calving BCS variation covering a normal range and varying post-calving concentrate feeding regimen. Thereby, the pre-calving BCS of the animals in the current study ranged within a narrow range but separated cows into groups still significantly differing in BCS. Therefore, we aimed at evaluating the effects and interactions of different concentrate feed proportions in early lactating cows with different pre-calving body condition for that, we assessed hematological parameters, such as GR. It was hypothesized that if cows with higher BCS are characterized by a lower cortisol level an increased GR count level in blood would be expectable. Hüther et al. [23] described lower Kynurenine (Kyn): Trp-ratios in

cows with higher BCS *post partum* compared to cows with lower BCS. Therefore, the question was, whether different concentrate proportions would dynamically change the BCS effect on that parameter. Another inflammation indicator assessed in the present study is Hpt, which was expected to be higher in higher conditioned cows due to its role as adipokine. Moreover, the CD14+ epitope is part of the LPS receptor complex and mainly present on the surface of monocytes. It belongs to the innate immune system, which can be triggered by endotoxins [24]. The crucial information we wanted to obtain by determining specific, immune-relevant surface markers such as CD4, CD8, CD14 and CD21 in peripheral blood leukocytes was to get an indication of immune-regulatory processes related to our experimental questioning.

2. Materials and Methods

The experiment was performed in compliance with the German legislation on animal protection (Animal Welfare Act) and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany) in consultation with an independent ethics committee (AZ 33.19-42502-04-15/1858). Milking performance and evaluation of nutritional status through ultrasonic-based estimation of the transition period-related dynamics of various adipose tissues were previously reported [14].

2.1. Experimental Design

The experiment included 60 pluriparous German Holstein cows and lasted over a time range from 42 days *ante partum* until 70 days in milk (DIM). Before parturition, all animals were either allocated to a group with a higher or an adequate BCS (BCS_H, BCS_A) based on the available BCS range of the herd. BCS was determined on a 5-point-scale according to Edmonson et al. [25]. Further criteria of classification were milk yield and milk composition of the previous lactation, as well as body weight and number of lactation. Until the day of calving, all animals received the same total mixed ration (TMR) with 80% silage (70% maize silage, 30% grass silage) and 20% concentrate on a dry matter (DM) basis. Supply of energy and nutrients was ensured based on the recommendations of the Society of Nutrition Physiology [26]. From the first day of lactation, all animals were supplied with a partial mixed ration (PMR) consisting of 48% maize silage, 20% grass silage and 32% concentrate on a DM basis.

After calving, cows were additionally assigned to a group with a concentrate feed proportion of 60% (increasing from 35–60% during the first three weeks after calving, C_{60}) and a group with a concentrate feed proportion of 35% (C_{35}). Hence, the following four groups were formed: BCS_H/C_{60}, n = 15, BCS_H/C_{35}, n = 15, BCS_A/C_{60}, n = 15, BCS_A/C_{35}, n = 15).

The components and the chemical compositions of the feedstuffs, as well as the experimental groups are described in detail elsewhere [14]. In brief, cows in the BCS_H group had an initial mean BCS of 3.83 (±0.41 standard deviation) while the BCS_A group started the experiment with a mean BCS of 3.10 (±0.38). Taking into account the fulfillment of the other criteria of classification, the initial BCS difference was 0.73 assessed as significant (p = 0.030). The average BCS during the experiment from week 1 to week 10 for the experimental groups were the following: BCS_H/C_{60} = 3.44 (±0.72), BCS_H/C_{35} = 3.23 (±0.85), BCS_A/C_{60} = 2.70 (±0.67), BCS_A/C_{35} = 2.66 (±0.53). A more detailed presentation of the BCS values over the course of the experiment was reported elsewhere [14].

2.2. Sample and Data Collection

Blood samples were taken at defined time points relative to calving (*ante partum*: −42 day, −14 day, −7 day, −3 day; *post partum*: 3 day, 7 day, 14 day, 21 day, 28 day, 42 day, 56 day, 70 day, with tolerated deviation of 2 days) after morning milking from a *Vena jugularis externa*.

EDTA blood samples were collected for flow cytometry and hematology. Serum samples were collected for Hpt and serum metabolites, such as Trp and Kyn. Blood for serum was centrifuged (Heraeus Varifuge 3.0R Heraeus, Osterode, Germany; 2123× *g*, 15 °C, 15 min) and stored at −80 °C until further analysis within 12 months.

2.2.1. Hematology

For analyzing hematological parameters, EDTA-blood was used and a blood cell count was performed (Celltac MEK-6450, Nihon Kohden Corporation, Tokyo, Japan). Hematology included the white blood cell count with the count of total leukocytes (WBC), as well as cell counts of lymphocytes (LY), monocytes (MO), GR (comprising basophil and neutrophil GR) and eosinophils (EO), as well as their proportions of total WBC. Furthermore, the red blood cell profile was assessed, including the count of red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), the mean corpuscular volume (MCV), the mean corpuscular hemoglobin (MCH), the mean corpuscular hemoglobin concentration (MCHC) and the red cell distribution width (RDW).

2.2.2. Flow Cytometry—Leukocytes Phenotyping

In order to assess the types of leukocytes, we performed flow cytometry using monoclonal antibodies. Thus, percentage of CD4+ cells represented T-helper lymphocytes, that of CD8+ represented cytotoxic T-cells, proportion of CD14+ reflected monocytes and finally, percentage of CD21+ represented B-cells. Blood-cell phenotyping was performed using whole blood. EDTA was used as anticoagulant for blood samples. Samples were incubated for 30 min at room temperature with monoclonal antibodies for CD4 and CD8 (mouse anti-bovine CD4: FITC; mouse anti-bovine CD8: PE; Bio-Rad, Hercules, CA, USA), as well as for CD14 and CD21 (mouse anti-bovine CD14: FITC; mouse anti-bovine CD21: PE; Bio-Rad) or their corresponding isotype controls (mouse IgG2a negative control: RPE and mouse IgG2b: FITC negative control, BioRad, Hercules, CA, USA).). To lyse the red blood cells, samples were subsequently incubated with a lysis buffer (BD Bioscience, San Jose, CA, USA) for 10 min at room temperature. Afterwards, samples were centrifuged, supernatant was removed and samples were resuspended in HEPES-buffered saline and then measured by FACS Canto II (BD Biosciences, San Jose, CA, USA).). By means of side- and forward-scattering properties, peripheral mononuclear blood cell (PBMC) subpopulations were identified. At least 10,000 PBMCs were counted and stored in list mode data files. The spillover of both fluorochromes (FITC, PE) was compensated using the BD FACS Diva^{TM} Software (BD Biosciences, San Jose, CA, USA).

2.2.3. Haptoglobin and Serum Metabolites

Serum concentration of Hpt was measured from −42 d until 28 d, using an ELISA according to Hiss et al. [27]. Microplates were coated with bovine serum, blocked with casein and stored at 4 °C. The polyclonal antiserum against Hpt was added and incubated with samples, standard or controls for 2 hours at room temperature, after decanting the plates. The second enzyme-labeled antibody was added, after three time washing. Tetramethylbenzidin was used as chromogene. The optical density was determined at 450 nm. The limit of detection was set at 0.07 mg/mL. The intra- and inter-assay covariation was 9.99 and 11.67% respectively.

Trp and its degradation product Kyn were analyzed as described by Hüther et al. [23]. Fat extraction was performed by using hexane, and for protein precipitation, samples were mixed with ice cold ethanol. After centrifugation (20,800× *g*), the supernatant was quantitatively transferred into a flask and evaporated in a nitrogen stream at 40 °C. The residue was dissolved in aqueous mobile phase A and after filtration (amcro filter, PVDF, 0.45 µm) 20 µL were injected into a HPLC system (Shimadzu, Kyoto, Japan). Metabolites were separated by means of a reversed phase C18-column (Inertsil ODS-2, 150 × 3 mm i.d., 5 µm, Agilent, Böblingen, Germany), with a flow rate of 0.5 mL/min and a gradient elution. The mobile phase A consisted of 10 mM sodium 1-hexanesulfonate monohydrate, 0.5% (*v/v*) o-phosphoric acid and 0.5% (*v/v*) acetonitrile in ultrapure water; the mobile phase B consisted of 100% acetonitrile. For Trp and Kyn, the detection wavelengths were 278 nm and 360 nm, respectively. The intra- and inter-assay covariations were 3.1 and 6.3% (kynurenine) and 1.9 and 5.2% (tryptophan), respectively.

2.3. Statistical Analyses

For statistical analyses the software SAS was used (version 9.4; SAS Institute Inc., Cary, NC, USA). Variables of hematology and flow cytometry, as well as Hpt and the serum metabolites were evaluated using the MIXED procedure for repeated measurements with a compound symmetry structure [28]. The statistical analysis of the trail was divided into the time before and the time after parturition. BCS classification (BCS_H, BCS_A) and sampling day (−42, −14, −7, −3) were applied as fixed effects *ante partum*, as well as the interactions between them. The C effect (C_{35}, C_{60}) appeared *post partum* additionally to BCS classification and sampling day (3, 7, 14, 21, 28, 42, 56, 70). Each cow within treatment was considered a random effect. The sampling day was regarded to be a repeated measure. The statistical models are presented in Figure 1.

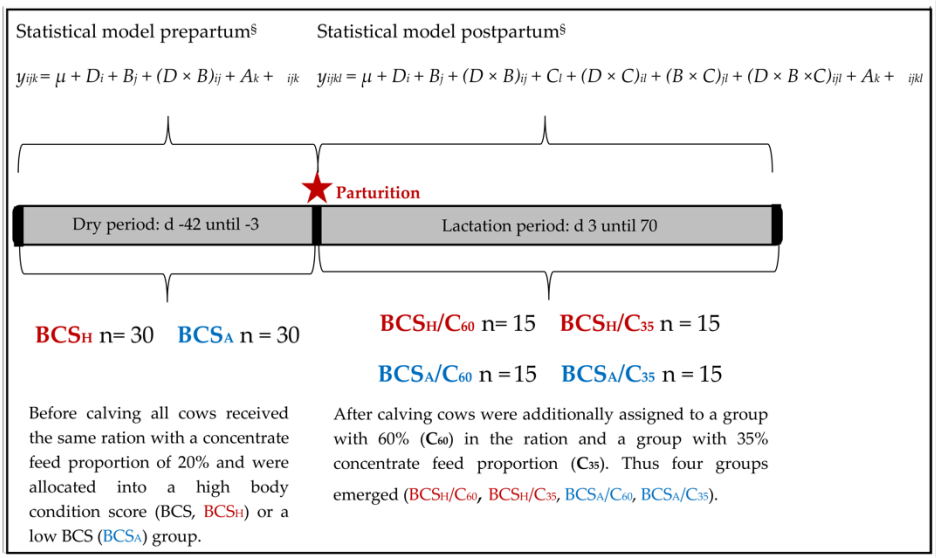

Figure 1. Statistical models. BCS: body condition scores; D_i = fixed effect of day (i = −42, . . . , −3/3, . . . , 70); B_j = fixed effect of BCS (j = BCS_H, BCS_A); A_k = effect of animal (k = 1, . . . ,30,...,60); C_l = fixed effect of concentrate feed proportion (l = C_{60}, C_{35}); $(D \times B)_{ij}$, $(D \times C)_{il}$, $(B \times C)_{jl}$, $(D \times B \times C)_{ijl}$ = fixed effects of respective interactions, ε_{ijkl} = error.

By assessing Pearson's correlation coefficient, we examined relations between the individual BCS of each cow and selected parameters applying the statistical software TIBCO Statistica (Version 13.3, TIBCO Software Inc., Palo Alto, CA, USA). Furthermore, we performed linear regression analysis. The *p*-values ≤ 0.05 were declared to be statistically significant. Results are presented as LSMean ± Standard error of means (SEM) unless otherwise stated.

3. Results

3.1. Hematology

The WBC (Figure 2A, Table 1) showed a gradual and continuous increase before and a decrease after calving. The BCS_A group developed *ante partum* higher WBC counts over time compared to the BCS_H group (effect Day × BCS *ante partum*, $p = 0.047$). *Post partum*, BCS_H/C_{60} showed the lowest values, whereas BCS_A/C_{60} developed the highest (effect BCS × C *post partum*, $p = 0.046$).

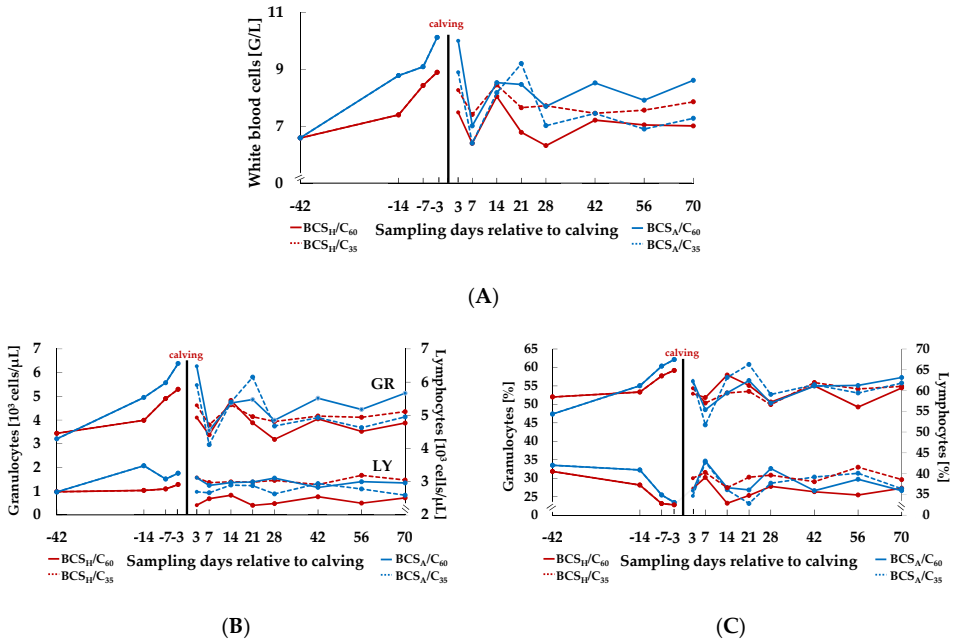

Figure 2. Characteristics of white blood cell counts (WBC, (**A**)), as well as counts (**B**) and percentages (**C**) of granulocytes (GR) and lymphocytes (LY) in the course of the experiment. BCS: body condition scores. *Ante partum*, cows were categorized in high body condition score (BCS, BCS_H,) and adequate BCS (BCS_A,). *Post partum*, the two groups were subdivided again, each into a group with a concentrate proportion of 60% (C_{60}, solid line) in the ration (increasing from 35–60% during the first three weeks after calving) and a group with a concentrate proportion of 35% (C_{35}, dashed line) in the ration. Thus, four groups emerged: BCS_H/C_{60} (n = 15), BCS_H/C_{35} (n = 15), BCS_A/C_{60} (n = 15), BCS_A/C_{35} (n = 15).

Table 1. Model corresponding to Figure 2.

	p-Values (before Calving) *						
	Day	**BCS**	**Day × BCS**				
A WBC	<0.001	0.063	0.047				
B GR count	<0.001	0.029	0.006				
B LY count	0.004	0.084	0.054				
C GR%	<0.001	0.679	0.028				
C LY%	<0.001	0.225	0.637				
	***p*-Values (after Calving) ***						
	Day	**BCS**	**Day × BCS**	**C**	**Day × C**	**BCS × C**	**Day × BCS × C**
A WBC	<0.001	0.124	0.091	0.811	0.761	0.046	0.905
B GR count	<0.001	0.084	0.099	0.988	0.927	0.172	0.749
B LY count	0.968	0.421	0.815	0.243	0.899	0.018	0.050
C GR%	<0.001	0.415	0.388	0.959	0.965	0.909	0.552
C LY%	<0.001	0.888	0.436	0.376	0.855	0.266	0.848

* before calving: −42 day, −14 day, −7 day, −3 day; after calving: 3 day, 7 day, 14 day, 21 day, 28 day, 42 day, 56 day, 70 day (with tolerated deviation of 2 days). WBC: blood cell counts; BCS: body condition scores; GR: granulocytes; and LY: lymphocytes.

The GR counts (Figure 2B, Table 1) increased before calving, whereby BCS_A animals showed higher GR counts compared to BCS_H animals over time (effect Day × BCS *ante partum*, $p = 0.006$), the same applies for GR proportion (effect Day × BCS *ante partum*, $p = 0.028$). After calving, both GR counts (effect Day *post partum*, $p < 0.001$) and GR proportion (Figure 2C, Table 1, effect Day *post partum*, $p < 0.001$) changed over time with an initial decrease shortly after calving.

The increase of percentage of GR *ante partum* was paralleled by a decrease of percentage of LY (Figure 2C, Table 1, effect Day *ante partum*, $p < 0.001$), whereas LY proportion increased *post partum*

(effect Day *post partum*, $p < 0.001$). LY count (Figure 2B, Table 1) increased before and decreased after calving in all groups, except for BCS_H/C_{35} animals, where values still increased after calving (effect Day *ante partum*, $p = 0.004$, effect Day × BCS × C *post partum*, $p = 0.050$). The MO count (Figure 3A, Table 2) and percentage (Figure 3C, Table 2) were not affected by treatment before calving. However, percentage of MO increased after calving (effect Day *post partum*, $p = 0.001$).

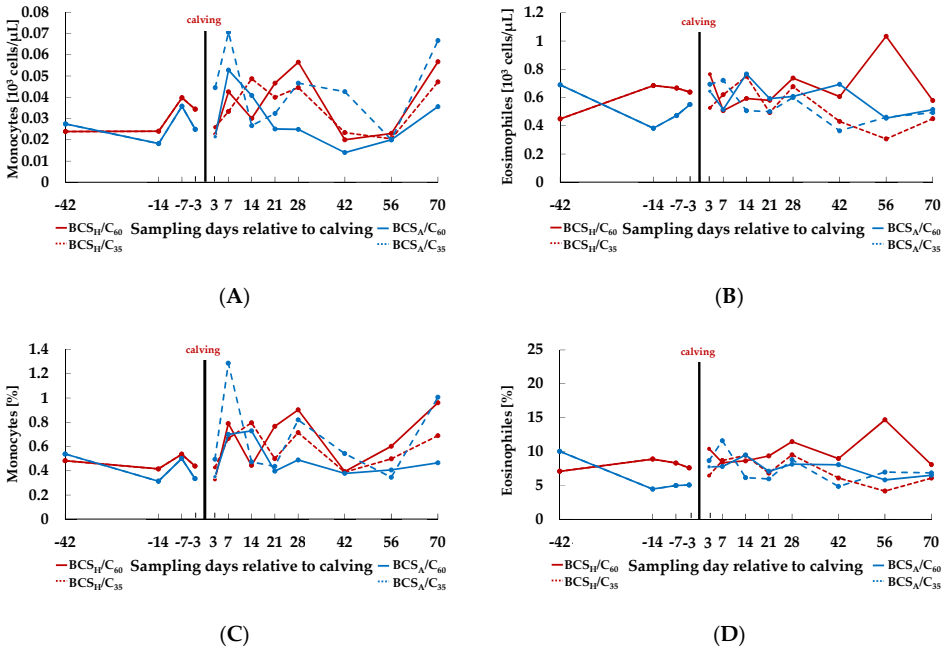

Figure 3. Characteristics of counts of monocytes (MO, (**A**)) and eosinophils (EO, (**B**)), as well as of percentages of MO (**C**) and EO (**D**) in the course of the experiment. *Ante partum*, cows were categorized in high body condition score (BCS, BCS_H) and adequate BCS (BCS_A). Post partum, the two groups were subdivided again, each into a group with a concentrate proportion of 60% (C_{60}, solid line) in the ration (increasing from 35–60% during the first three weeks after calving) and a group with a concentrate proportion of 35% (C_{35}, dashed line) in the ration. Thus, four groups emerged: BCS_H/C_{60} ($n = 15$), BCS_H/C_{35} ($n = 15$), BCS_A/C_{60} ($n = 15$), BCS_A/C_{35} ($n = 15$).

Table 2. Model corresponding to Figure 3.

	p-Values (before Calving) *						
	Day	**BCS**	**Day × BCS**				
A MO count	0.465	0.677	0.921				
B EO count	0.975	0.392	0.051				
C MO%	0.280	0.606	0.786				
D EO%	0.252	0.145	0.017				
	***p*-Values (after Calving) ***						
	Day	**BCS**	**Day × BCS**	**C**	**Day × C**	**BCS × C**	**Day × BCS × C**
A MO count	0.011	0.988	0.686	0.520	0.964	0.210	0.824
B EO count	0.467	0.164	0.663	0.070	0.152	0.408	0.135
C MO%	0.001	0.552	0.692	0.473	0.939	0.089	0.229
D EO%	0.435	0.047	0.741	0.079	0.311	0.062	0.201

* before calving: −42 day, −14 day, −7 day, −3 day; after calving: 3 day, 7 day, 14 day, 21 day, 28 day, 42 day, 56 day, 70 day (with tolerated deviation of 2 days). MO: monocytes; EO: eosinophils.

There was no treatment effect for EO count (Figure 3B, Table 2), neither before nor after calving. BCS_H animals developed *ante partum* higher percentages of EO (Figure 3D, Table 2) over time (effect Day

× BCS *ante partum*, $p = 0.017$). After parturition, percentage of EO was also affected by BCS (effect BCS *post partum*, $p = 0.047$). The red blood profile is presented in the Appendix A.

3.2. Flow Cytometry—Leukocytes Phenotyping

In all experimental groups the percentage of $CD4^+$ (Figure 4A, Table 3) decreased *ante partum* (effect Day *ante partum*, $p = 0.013$) and increased *post partum* (effect Day *post partum*, $p < 0.001$).

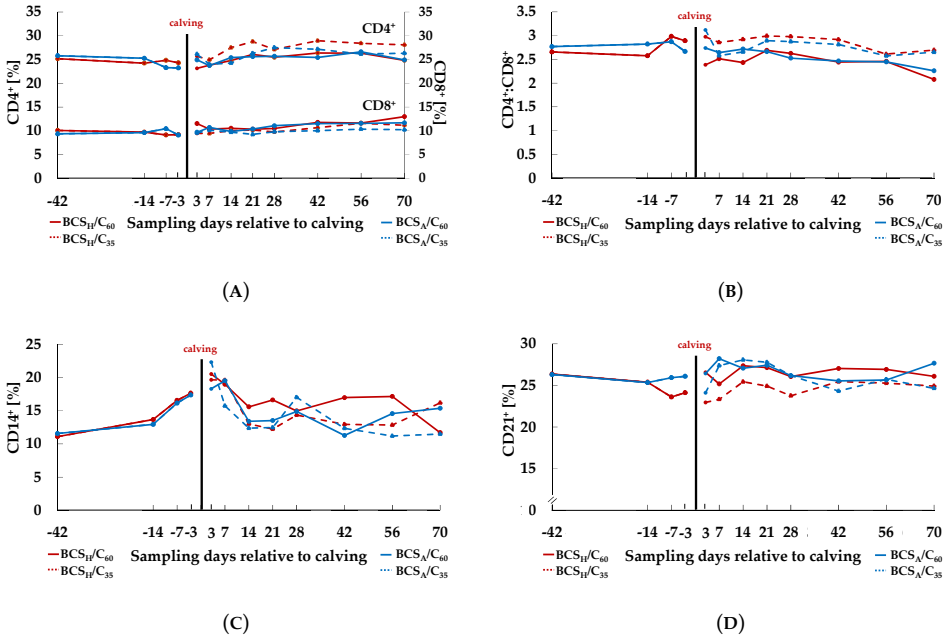

Figure 4. Percentage of $CD4^+$ and $CD8^+$ (**A**), $CD4^+$:$CD8^+$-ratio (**B**), as well as percentage of $CD14^+$ (**C**) and $CD21^+$ (**D**) in the course of the experiment. *Ante partum*, cows were categorized in high body condition score (BCS, BCS_H) and adequate BCS (BCS_A). *Post partum*, the two groups were subdivided again, each into a group with a concentrate proportion of 60% (C_{60}, solid line) in the ration (increasing from 35–60% during the first three weeks after calving) and a group with a concentrate proportion of 35% (C_{35}, dashed line) in the ration. Thus, four groups emerged: BCS_H/C_{60} ($n = 15$), BCS_H/C_{35} ($n = 15$), BCS_A/C_{60} ($n = 15$), BCS_A/C_{35} ($n = 15$).

For the percentage of $CD8^+$ (Figure 4A, Table 3) the same is true, although a time effect was observed after calving only (effect Day *post partum*, $p < 0.001$). After calving the ratio of $CD4^+$:$CD8^+$ (Figure 4B, Table 3) changed over time (effect Day *post partum*, $p < 0.001$). Values ranged between minimum = 2.08 (±0.25) and maximum = 3.12 (±0.25). The percentage of $CD14^+$ (Figure 4C, Table 3) increased before calving (effect Day *ante partum*, $p < 0.001$), whereas *post partum* an initial decrease was noticed for all treatment groups followed by time-dependent fluctuations that were differently influenced by treatments (effect Day × BCS × C *post partum*, $p = 0.005$). We could not detect any significant effects on the percentage of $CD21^+$ (Figure 4D, Table 3).

Table 3. Model corresponding to Figure 4.

	p-Values (before Calving) *						
	Day	**BCS**	**Day × BCS**				
A $CD4^+$	0.013	0.825	0.096				
A $CD8^+$	0.902	0.902	0.585				
B $CD4^+$:$CD8^+$	0.204	0.984	0.313				
C $CD14^+$	<0.001	0.780	0.863				
D $CD21^+$	0.155	0.490	0.298				
	***p*-Values (after Calving) ***						
	Day	**BCS**	**Day × BCS**	**C**	**Day × C**	**BCS × C**	**Day × BCS × C**
A $CD4^+$	<0.001	0.458	0.494	0.090	0.834	0.420	0.885
A $CD8^+$	<0.001	0.509	0.603	0.193	0.834	0.898	0.615
B $CD4^+$:$CD8^+$	<0.001	0.968	0.869	0.128	0.654	0.547	0.939
C $CD14^+$	<0.001	0.250	0.321	0.212	0.074	0.640	0.005
D $CD21^+$	0.314	0.409	0.242	0.250	0.912	0.541	0.934

* before calving: −42 day, −14 day, −7 day, −3 day; after calving: 3 day, 7 day, 14 day, 21 day, 28 day, 42 day, 56 day, 70 day (with tolerated deviation of 2 days).

3.3. Haptoglobin and Serum Metabolites

Hpt (Figure 5A, Table 4) was neither affected by BCS nor C, but increased within the first week after calving and decreased thereafter (effect Day *ante partum*, $p = 0.011$, effect Day *post partum*, $p < 0.001$).

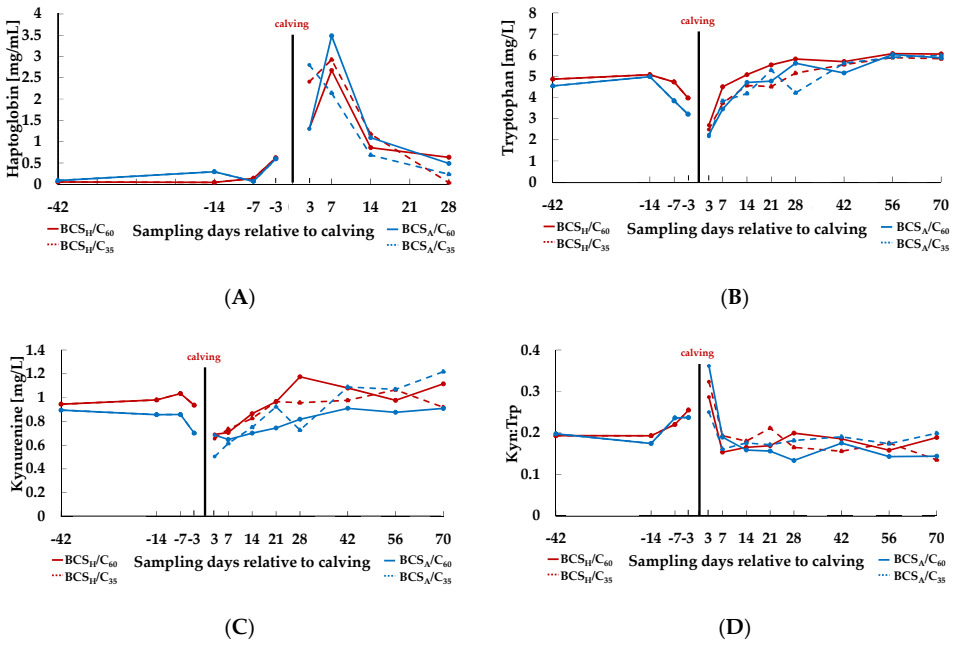

Figure 5. Blood concentrations of haptoglobin (Hpt, (**A**)), Tryptophan (Trp, (**B**)), Kynurenine (Kyn, **C**) and Kynurenine:Tryptophan-ratio (Kyn:Trp, **D**) in the course of the experiment. *Ante partum*, cows were categorized in high body condition score (BCS, BCS_H) and adequate BCS (BCS_A). *Post partum*, the two groups were subdivided again, each into a group with a concentrate proportion of 60% (C_{60}, solid line) in the ration (increasing from 35–60% during the first three weeks after calving) and a group with a concentrate proportion of 35% (C_{35}, dashed line) in the ration. Thus, four groups emerged: BCS_H/C_{60} ($n = 15$), BCS_H/C_{35} ($n = 15$), BCS_A/C_{60} ($n = 15$), BCS_A/C_{35} ($n = 15$).

Table 4. Model corresponding to Figure 5.

	p-Values (before Calving) *						
	Day	**BCS**	**Day × BCS**				
A Hpt	0.011	0.817	0.972				
B Trp	<0.001	0.046	0.357				
C Kyn	0.294	0.046	0.414				
D Kyn:Trp	0.002	0.836	0.742				
	***p*-Values (after Calving) ***						
	Day	**BCS**	**Day × BCS**	**C**	**Day × C**	**BCS × C**	**Day × BCS × C**
A Hpt	<0.001	0.958	0.988	0.884	0.146	0.687	0.642
B Trp	<0.001	0.211	0.887	0.161	0.608	0.329	0.299
C Kyn	<0.001	0.296	0.606	0.675	0.125	0.380	0.664
D Kyn:Trp	<0.001	0.806	0.969	0.503	0.329	0.972	0.040

* before calving: −42 day, −14 day, −7 day, −3 day; after calving: 3 day, 7 day, 14 day, 21 day, 28 day, 42 day, 56 day, 70 day (with tolerated deviation of 2 days). Hpt: Haptoglobin; Trp: Tryptophan; Kyn: Kynurenine; Kyn:Trp: Kynurenine:Tryptophan-ratio

Both, Trp (Figure 5B, Table 4) and its degradation product Kyn (Figure 5C, Table 4) decreased before and increased after calving. For both variables the BCS_H group exhibited higher values *ante partum* (effect BCS *ante partum*, p Trp = 0.046, effect BCS *ante partum*, p Kyn = 0.046). After calving, Trp, as well as Kyn increased (effect Day *post partum*, p Trp < 0.001, effect Day *post partum*, p Kyn < 0.001). The Kyn:Trp-ratio (Figure 5D, Table 4) showed the reverse picture, as it increased towards calving and decreased within 14 days after calving. For Kyn:Trp we detected a time effect (effect Day *ante partum*, p = 0.002) *ante partum*. Time and treatment affected Kyn:Trp in an interactive manner *post partum* (effect Day × BCS × C *post partum*, p = 0.040).

3.4. Correlations

Correlations were performed with the individual BCS of each cow and GR count, $CD4^+$:$CD8^+$-ratio, as well as Kyn:Trp-ratio and Hpt for the first two weeks after calving. None of the performed correlations revealed a significant relation.

4. Discussion

Although the BCS-difference of 0.73 was not great enough to induce significant effects on most of the parameters it was proven to be significant (p = 0.03) at the onset of the experiment. When setting up the experiment we considered the "normal" BCS range of our herd without artificial forcing for extremely low or high BCS animals through strong dietary manipulations. Therefore, we assigned cows from the given BCS range to groups with a higher and an adequate BCS. As these cow groups still differed significantly in mean BCS we hypothesized that BCS variation around a normal BCS would also influence metabolic and health traits significantly.

Cows with a higher BCS at calving are frequently considered being *post partum* more susceptible to metabolic disorders and vulnerable to infectious diseases through a compromised immune-responsiveness compared to cows with an adequate BCS [7]. As cows with higher BCS are less capable *post partum* to increase intake of DM and energy to appropriate levels, they suffer more from the *post partum* NEB than adequately conditioned cows. To investigate the influence of differences in BCS within an overall mean range often observable under practical feeding conditions, we grouped our herd into cows with a higher and an adequte BCS.

4.1. Hematology and Lymphocyte Subsets

The state of pregnancy is characterized by various changes in endocrine and metabolic alterations. Also, the transition from pregnancy to lactation is accompanied with an adaptive change in physiological, metabolic and immune status. The present study describes hematological changes of circulating cell populations during the transition period, reflected by a significant increase of WBC around parturition.

Granulocytes as the major population of leukocytes were impacted markedly compared to lymphocytes which is in line with other studies [12,21]. A possible explanation for the increased GR counts towards calving is given by Burton et al. [3], who assumed an impaired trans-capillary-migration-capacity. Glucocorticoids induce a down-regulation of the expression of adhesion molecules on the surface of neutrophils, which results in a reduced infiltration in affected tissues. In addition, glucocorticoids, such as cortisol, stimulate the release of immature neutrophil granulocytes from the bone marrow [3]. Around calving, cows are exposed to high stress levels, due to exogenous and endogenous changes, such as the adaption from a dry period diet to a lactation diet or the transition from late gestation to lactation [2,21,29]. These marked endocrine changes might explain the observed time course in granulocyte counts in particular and in white blood profile in general. Endo et al. [4] showed a negative correlation between BCS and hair cortisol concentration. If cows with a higher BCS are characterized by a lower cortisol level an increased granulocyte count in blood would be expectable which, however, was not observed in the present experiment. Obviously, the BCS in the BCS_H groups was not high enough to induce significant differences in cortisol levels compared to the BCS_A groups.

Based on this assumption and the known influence of cortisol on release of granulocytes, it seems comprehensible that GR count increased more pronounced in BCS_A groups before calving in the present study. Burton et al. [3] proposed that the surge of cortisol could also be fetus-derived and that it induced adaptive changes of the neutrophil system, which resulted in the favoring of tissue remodeling instead of antibacterial defense. This, in turn, could increase the susceptibility to diseases [2]. Chapwanya et al. [30] demonstrated endometrial infiltration of leukocytes, mainly neutrophil aggregates and elevated expression of pro-inflammatory genes in uteri of *post partum* cows, indicating endometrial inflammatory processes, which explain the *post partum* decrease of GR counts in the present study. The authors described these inflammations as necessary events and beneficial for normal endometrial involution and bacterial clearance.

A higher BCS *ante partum* often leads to complications at calving [7,8] with consequences for *post partum* susceptibility to metabolic disorders and infectious diseases. Even though the BCS groups did not differ concerning WBC and GR count at the start of the experiment (−42 d), the increase of both parameters was less pronounced in the BCS_H groups *ante partum* in the present study without consequences for the dynamics of these cell types *post partum*. Although for lymphocyte counts similar relations were observed *ante partum*, this cell type fluctuated at a lower level when cows with a higher BCS were supplied with more energy (BCS_HC_{60}) *post partum*. In contrast, Eger et al. [31] denied a relation between body condition and lymphocytes, when grouping the experimental cows in lower BCS (mean 2.77) and higher BCS (mean 3.73). The BCS difference of the experimental groups in the present study was even smaller and might not have been large enough to trigger marked effects on lymphocytes.

In accordance with this result, the pre-calving BCS itself did not affect lymphocyte subsets, such as $CD4^+$ and $CD8^+$ in the current investigation. The percentage of $CD4^+$, as well as that of $CD8^+$ increased after calving, which is in accordance with previous reports [21,32].

The $CD4^+$:$CD8^+$-ratio, which was neither affected by treatment, remained within the range indicating a balanced immune homeostasis, as described in previous studies [33,34]. The correlation between BCS and $CD4^+$:$CD8^+$-ratio was performed to examine the relation on an individual basis. The results support the idea of the nonexistent relation between the body condition and the $CD4^+$:$CD8^+$-ratio at least within the rather small depicted BCS range of the present study.

In the present study, $CD14^+$ cells decreased in all groups after calving, independent of experimental treatment. Endometrial inflammatory processes and uterine bacterial infections were observed in *post partum* dairy cows. These infections are associated with LPS, which translocate into the uterus [30,35]. Not related to treatment 33% of the cows in the BCS_H/C_{60} group, 40% of the animals in both the BCS_H/C_{35} and BCS_A/C_{35} group and 53% of the cows in the BCS_A/C_{60} group developed production diseases, such as mastitis and metritis. This results in migration of monocytes, which in turn explains the decrease of peripheral $CD14^+$ cells after calving in the present study. The interaction between

time, BCS and C in the current study suggests that an enhanced C in combination with a higher BCS triggered a higher $CD14^+$ cell proportion.

4.2. Haptoglobin and Kynurenine:Tryptophan-Ratio

The results of this experiment indicate that a BCS effect on mobilization was only detectable in individual fat depots [14]. It was not verifiable that higher conditioned cows have a higher potential to mobilize body fat in general. Moreover, compared to the hepatic production the Hpt synthesis of the subcutaneous and visceral adipose tissue constitutes only 0.02% of the overall synthesis [11]. Based on these facts the unaltered Hpt levels in higher BCS cows of the present study might be explained.

Other studies already indicated that rations differing in C did not influence Hpt and even when drastically increasing C, circulating Hpt remained unaltered [13,36]. Moreover, Drong et al. [22] demonstrated, that Hpt was even unaffected by different BCS in combination with different dietary compositions. Furthermore, the regulation of acute-phase-proteins is described as a function of several parameters, which interact in a complex manner [37]. The individual cow factor explains 22% of the Hpt variation [38].

The Kyn:Trp-ratio provides information about the activity of indoleamine-2,3-dioxygenase, which is activated during inflammations or infections and also suggested to regulate the implantation of the embryo. Due to its degradation via the kynurenine-pathway it leads to a decrease of Trp during gestation. The Trp degradation prevents a maternal immune reaction against the fetus [5,39]. Therefore, the increase of the Kyn:Trp-ratio towards calving observed in the present study is physiological necessary [5,40,41]. Hüther et al. [23] described lower Kyn:Trp-ratios in cows with higher BCS *post partum* compared to cows with adequate BCS. Although we observed a three-way interaction between time, concentrate feed proportion and BCS class *post partum*, a clear effect of BCS was not detected. The relation between BCS and several immune parameters is non-linear and therefore, the impacts are difficult to predict [7,13]. This becomes also apparent in the missing significance for the correlation between BCS and Kyn:Trp-ratio. However, in the afore mentioned study, dry cow nutrition differed concerning C, whereby different diets did not impact the Kyn:Trp-ratio. The mentioned interactions in the present study suggest that different energy levels after calving have greater influence on several parameters than varying dry cow nutrition.

5. Conclusions

All assessed immunological variables changed over time and clearly reflect the transition period as challenging time for dairy cows. It was hypothesized that cows differing in BCS would respond differently to rations varying in concentrate feed proportion *post partum*. Based on the investigated hematological parameters, blood immune cell phenotypes, and inflammatory markers it can be concluded that the given BCS variation in the present study covered a range in which physiological adaptability was not overstretched.

Author Contributions: The experiment's conceptualization was initiated by J.H., S.D., U.M., K.B.; methodology and validation of data was done by J.F., S.K., L.H., H.S., K.B.; Formal analysis was performed by K.B.; Investigation was done by J.F., S.K., L.H., K.B.; Resources were looked up by K.B.; Curation and preparation, visualization of data was done by K.B.; Writing original draft preparation was performed by K.B.; Supervision and writing of the review was done by J.F., S.K., L.H., U.M., S.D., H.S., J.H., A.Z.; Editing was done by K.B.; Project was administrated by J.H., U.M., S.D. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

RBC (Figure A1A, Table A1) increased towards calving in BCS_A and BCS_H groups (effect Day *ante partum*, $p = 0.003$).

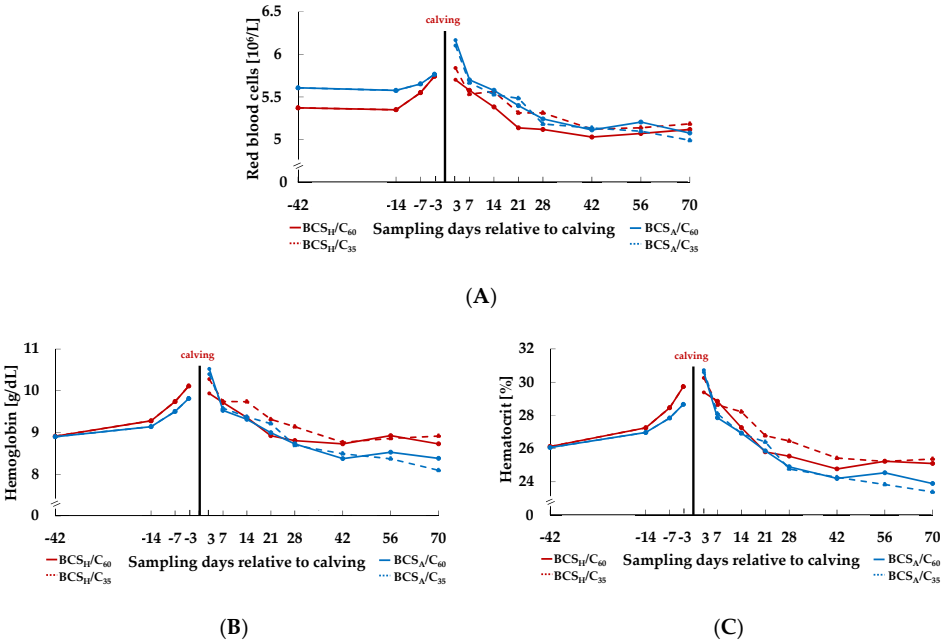

Figure A1. Characteristics of red blood cell counts (**A**), Hemoglobin (**B**) and Hematocrit (**C**) in the course of the experiment. *Ante partum*, cows were categorized in high body condition score (BCS, BCS_H) and adequate BCS (BCS_A). *Post partum*, the two groups were subdivided again, each into a group with a concentrate proportion of 60% (C_{60}, solid line) in the ration (increasing from 35–60% during the first three weeks after calving) and a group with a concentrate proportion of 35% (C_{35}, dashed line) in the ration. Thus, four groups emerged: BCS_H/C_{60} ($n = 15$), BCS_H/C_{35} (n = 15), BCS_A/C_{60} ($n = 15$), BCS_A/C_{35} ($n = 15$). * before calving: −42 day, −14 day, −7 day, −3 day; after calving: 3 day, 7 day, 14 day, 21 day, 28 day, 42 day, 56 day, 70 day (with tolerated deviation of 2 days).

Table A1. Model corresponding to Figure A1.

	***p*-Values (before Calving) ***						
	Day	**BCS**	**Day × BCS**				
A RBC	0.003	0.209	0.468				
B HGB	<0.001	0.348	0.737				
D HCT	<0.001	0.310	0.642				
	***p*-Values (after Calving) ***						
	Day	**BCS**	**Day × BCS**	**C**	**Day × C**	**BCS × C**	**Day × BCS × C**
A RBC	<0.001	0.571	<0.001	0.520	0.146	0.629	0.797
B HGB	<0.001	0.117	<0.001	0.354	0.608	0.619	0.658
D HCT	<0.001	0.074	<0.001	0.512	0.329	0.681	0.702

* after calving, BCS_A groups developed a higher peak at d 3 (effect Day × BCS *post partum*, $p < 0.001$). RBC: red blood cell counts; HGB: Hemoglobin; HCT: Hematocrit.

Both, HGB (Figure A1B, Table A1) and HCT (Figure A1C, Table A1) increased before calving (effect Day *ante partum*, p HGB < 0.001, effect Day *ante partum*, p HCT < 0.001). After calving, HGB decreased more pronouncedly in BCS_H groups compared to BCS_A groups (effect Day × BCS

post partum, p HGB < 0.001). The same is true for HCT (effect Day × BCS *post partum*, p HCT = 0.001). Both parameters peaked around calving.

MCV (Table A2) increased from day −42 to day −14 and remained relatively constant afterwards in all groups (effect Day *ante partum*, $p < 0.001$). Day and C affected MCV *post partum* in an interactive manner (effect Day × C *post partum*, $p < 0.001$).

The same is true for MCH (Table A2, effect Day *ante partum*, $p < 0.001$, effect Day × C *post partum*, $p = 0.035$). Additionally, the BCS_H groups had *post partum* higher values for MCH compared to BCS_A groups over time (effect Day x BCS *post partum*, $p = 0.029$). For MCHC (Table A2) no effect was found *ante partum*, whereas Day, BCS and C influenced MCHC *post partum* in an interactive manner (effect Day × BCS × C *post partum*, $p < 0.001$). RDW (Table A2) increased *ante partum*, and decreased *post partum*, in all groups (effect Day *ante partum*, $p < 0.001$, effect Day *post partum*, $p < 0.001$).

Table A2. *Ante partum* effects of body condition score (BCS) and Days of experiment (Day), as well as *post partum* effects of BCS, concentrate feed proportion in the diet (C) and Day on parameters of the red cell indices.

Item [+]	Group [§]	Day of Experiment—*ante partum*				SEM [#]	*p*-Value		
		−42	−14	−7	−3		Day	BCS	Day × BCS
MCV [$], fL	BCS_H	48.7	51.2	51.4	51.8	0.7	<0.001	0.055	0.818
	BCS_A	46.6	49.1	49.7	49.8				
MCH [¥], pg	BCS_H	16.6	17.4	17.6	17.6	0.3	<0.001	0.052	0.496
	BCS_A	15.9	16.6	16.9	17.0				
MCHC [‖], g/dL	BCS_H	34.1	34.0	34.3	34.0	0.1	0.528	0.829	0.783
	BCS_A	34.1	33.9	34.1	34.2				
RDW [◊],%	BCS_H	17.54	18.87	18.46	18.38	0.55	<0.001	0.093	0.835
	BCS_A	18.27	20.17	19.66	19.43				

Item [+]	Group [$]	Day of Experiment—*post partum*								SEM [#]	*p*-Value						
		3	7	14	21	28	42	56	70		Day	BCS	Day × BCS	C	Day × C	BCS × C	Day × BCS × C
MCV [$], fL	BCS_H/C_{60}	51.6	51.8	50.7	50.3	50.0	49.3	49.9	49.2	0.9	<0.001	0.018	0.665	0.944	<0.001	0.883	0.454
	BCS_H/C_{35}	52.1	52.1	51.0	50.6	50.1	49.8	49.3	49.1								
	BCS_A/C_{60}	49.5	49.0	48.4	48.0	47.7	47.5	47.2	47.1								
	BCS_A/C_{35}	50.5	49.8	48.8	48.2	48.0	47.4	46.9	47.0								
MCH [¥], pg	BCS_H/C_{60}	17.5	17.5	17.4	17.4	17.2	17.4	17.7	17.1	0.3	<0.001	0.022	0.029	0.879	0.035	0.952	0.174
	BCS_H/C_{35}	17.7	17.7	17.6	17.6	17.3	17.2	17.3	17.3								
	BCS_A/C_{60}	17.0	16.8	16.7	16.7	16.7	16.4	16.4	16.5								
	BCS_A/C_{35}	17.1	16.9	17.0	16.8	16.9	16.6	16.5	16.3								
MCHC [‖var], g/dL	BCS_H/C_{60}	33.8	33.7	34.3	34.6	34.5	35.2	35.3	34.8	0.2	<0.001	0.269	0.073	0.529	0.632	0.576	0.012
	BCS_H/C_{35}	33.9	34.0	34.5	34.8	34.6	34.5	35.1	35.1								
	BCS_A/C_{60}	34.4	34.2	34.5	34.8	35.1	34.6	34.8	35.0								
	BCS_A/C_{35}	33.8	34.1	34.8	34.9	35.1	35.0	35.1	34.6								
RDW [◊], %	BCS_H/C_{60}	19.65	19.35	18.59	18.38	18.28	18.17	17.52	17.33	0.55	<0.001	0.871	0.899	0.290	0.056	0.951	0.834
	BCS_H/C_{35}	18.31	18.33	17.88	17.63	17.70	17.41	17.26	17.08								
	BCS_A/C_{60}	19.53	19.28	18.71	18.73	18.27	17.85	17.56	17.49								
	BCS_A/C_{35}	18.98	18.45	18.15	18.04	17.75	17.36	17.20	17.04								

[§] Before calving cows were assigned to high and adequate body condition score (BCS)-groups (BCS_H, BCS_A). [$] After calving both groups were subdivided again, each into a group with 35% concentrate feed proportion (C_{35}) and a group with 60% concentrate feed proportion (C_{60}) in the ration (increasing from 35–60% during the first three weeks after parturition). Thus, four groups emerged: BCS_H/C_{60} (n = 15), BCS_H/C_{35} (n = 15), BCS_A/C_{60} (n = 15), BCS_A/C_{35} (n = 15). [$] Mean corpuscular volume. [¥] Mean corpuscular hemoglobin. [‖] Mean corpuscular hemoglobin concentration. [◊] Red cell distribution width. [+] Values are presented as LSMeans. [#] Pooled standard error of means.

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7. General Discussion

7.1 Influence of body condition and dietary composition on energy balance and adipose tissue mobilization

The transition period is supposed to be the most interesting time for researchers during the lactation cycle of a dairy cow. The onset of lactation is accompanied with a rapid increase of energy requirements for milk production. However, the energy intake decreases close to parturition and only a relatively slow increase after calving is known. Thus, since the energy and nutrient requirements cannot be met, the energy balance becomes negative (Grummer, 1995, Veerkamp et al, 2003). To compensate the arising energy deficit, dairy cows are able to mobilize energy from adipose tissue depots (Tamminga et al 1997). Additionally, the NEB is influenceable by the composition of the ration. A higher C is known to ease the NEB which results in a less pronounced body fat mobilization (Kleen et al. 2003, Roche et al. 2009, Dänicke et al. 2018). Nevertheless, it has to be considered that lipolysis is a physiological process (Moe et al 1972). Animal health and consequently welfare depends on the extent of lipomobilization which is often accompanied by an increase of circulating ketone bodies with possible impacts on animal health, such as the development of a subclinical ketosis (Kaske et al 2005). BHB-values in blood serum >1.2 mmol/L and NEFA-values >0.4 mmol/L are accepted indicators for a severe mobilization of adipose tissue depots beyond the physiological range (Nielen et al 1994, Oetzel et al 2004). In the present study, higher C decreased the NEB (Paper I), which confirms the assumption of hypotheses 1 concerning the influence of varying C on energy metabolism. However, neither lipolysis nor indicators for subclinical ketosis were influenced by diet composition (Paper I), which rejects another part of the first hypothesis due to a missing triggering C-effect on adipose tissue mobilization. McNamara and Hillers (1986a) described how dietary composition influences lipogenesis, but not lipolysis. The latter is above all controlled by hormones (McNamara and Hillers, 1986b, Smith and McNamara, 1990). Pregnancy and lactation lead to hormonal changes towards the expression of lipolytic genes (Smith and McNamara 1990, Sumner and McNamara 2007). The growth hormone somatotropin enhances the response to lipolytic stimuli by decreasing the insulin resistance in adipose tissue depots. Insulin is necessary for insulin-dependent glucose transporters in fat depots and as long as the insulin resistance remains, the uptake of glucose by adipocytes is inhibited, which stimulates adipose tissue mobilization (Chagas et al. 2009, Roche et al. 2009). Different rates of adipose tissue mobilization due to various genetic constitutions might cover

concentrate effects as well (McNamara and Hillers, 1986a, Roche et al., 2009). Measurements of the responsible hormones could be helpful to determine their influencing role concerning the physiological circumstances. Furthermore, the individuality of each cow has to be considered. Tamminga et al. (1997) described a variation in fat mobilization of cows ranging between 15.5 kg and 41.6 kg during the first weeks after parturition.

A higher BCS is known to enhance adipose tissue depot mobilization (Tamminga et al 1997). In our study, animals higher in body condition tended to show enhanced body fat mobilization, which confirms at least parts of the first hypothesis, including different dynamics in adipose tissues triggered by different BCS. Moreover, a significant BCS × period effect was assessed in the subcutaneous adipose tissue, although BCS changes did not indicate such differences (Paper I). As described in the second hypothesis, ultrasonic measurements (USM) of the current study have shown differences in lipolysis, which were not apparent in BCS losses. BHB and NEFA concentrations and therefore the risk for developing a ketosis were neither affected by BCS (Paper I).

However, in our study the mobilized energy from body fat of the experimental animals covered 70% of the NEB altogether (Figure 6). These findings are in line with results of Pfuhl et al. (2007), who showed that 73% of energy is stored in body fat, whereas the remaining 27% are saved in body protein in 18-month-old German Holstein bulls.

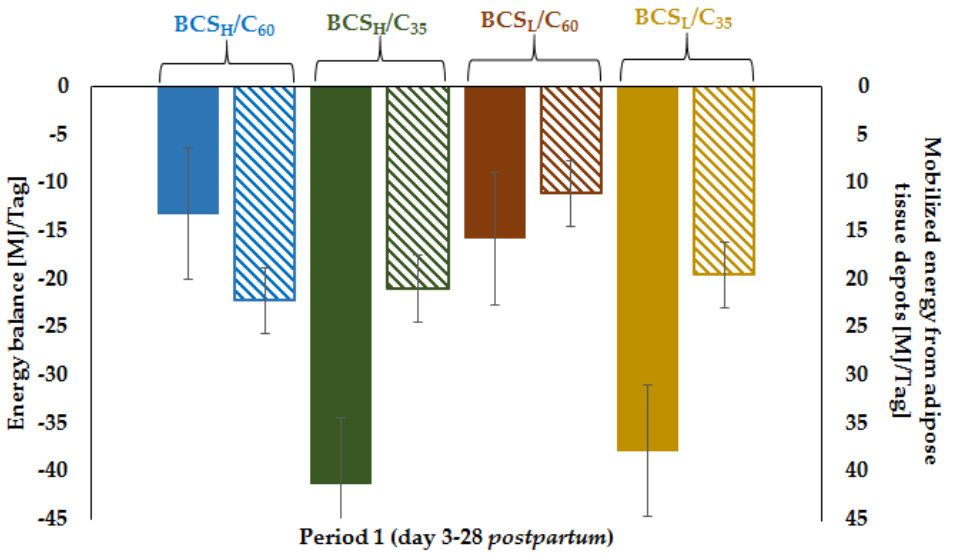

Figure 6. Comparison of energy balance (filled bars) and mobilized energy from adipose tissue depots (striped bars) in period 1 (day 3 – 28 postpartum). Cows were categorized in high BCS (BCS$_H$) and low BCS (BCS$_L$), before calving. After parturition, these two groups were divided again, each into a group with a concentrate proportion of 60% (C$_{60}$) in the ration (increasing from 35% to 60% during the first three weeks after calving) and a group with a concentrate proportion of 35% (C$_{35}$) in the ration. Thus, four groups emerged: BCS$_H$/C$_{60}$ (blue, n = 15), BCS$_H$/C$_{35}$ (green, n = 15), BCS$_L$/C$_{60}$ (red, n = 15), BCS$_L$/C$_{35}$ (yellow, n = 15).

In a study of von Soosten et al. (2012), mobilization of fat and protein of heifers from 1 – 42 days in milk (DIM) were examined in relation to a conjugated linoleic acid (CLA) supplementation. During the first 42 DIM heifers of the control group mobilized on average 0.574 kg body fat and 22.8 MJ NE$_L$ energy from body fat per day and heifers of the CLA group 0.340 kg body fat and 11.5 MJ NE$_L$ energy from body fat per day. In the present study, lipid mobilization as well as mobilization of energy from body fat was within the same area. From 3 – 28 DIM mobilization of body fat ranged from 0.337 – 0.631 kg per day and mobilized energy from body fat varied from 12.6 – 21.1 MJ NE$_L$ per day. However, heifers of von Soosten et al. (2012) exhibited a slightly negative or positive energy balance, whereas NEB in our trial was far more pronounced and markedly negative. Based on these data it was estimated that 0.25 MJ fat energy were mobilized per 1 MJ EB-change in the present study (Paper I).

The high potential for variation regarding the estimation of the energy balance has to be considered, as well. As the energy balance is a calculated size, the accuracy is dependent on the accuracy of the considered variables. Energy excretion with milk can be easily assessed, while energy intake and energy requirement for maintenance involve certain uncertainties. Dänicke et al. (2018) discussed that the estimation of energy intake depends on the used system and its

reliability for varying components of the ration. Estimation of energy requirements for maintenance are variable and cannot be regarded as constant, due to individually different chemical body compositions and metabolic states. Gruber et al. (2007) proposed that the energy maintenance requirements are often underestimated. C-related differences in NEB in the present study indicated the need for higher lipid mobilization in the C_{35} groups, which was not confirmed by USM (Paper I). Therefore, as described in the second hypothesis, a deficit in the precision of NEB determination can be compensated by USM, which visualizes physiological and metabolic relations that remain concealed in simple calculations of values.

7.1.1 Haptoglobin

A higher BCS at calving is regarded to be associated with a more pronounced NEB and therefore with a compromised immune competence and with excessive levels of NEFA, due to excessive lipolysis (Oltenacu and Ekesbo, 1994, Roche et al. 2009, Dänicke et al. 2018). In the present study concentrations of NEFA in blood were significantly higher during the first month after calving. However, although cows differed in BCS and different C artificially induced different NEB, no group differences were found (Paper I). Endocrine and metabolic conditions during the transition period might have been stronger and could have thereby impeded group differences to reach statistical significance (Gross et al 2011). In the present examination Hpt was significantly increased after calving but was neither affected by treatment (Paper III). Saremi et al. (2012) proposed that circulating levels of Hpt depend on parity and parturition rather than on fat depot mass. Internal conditions due to the event of calving might have been dominant compared to external treatment influences in the present study. Thus, Hpt as a marker for typical production diseases, such as mastitis, might have also been influenced by treatment unrelated incidences for mastitis and metritis, which ranged between 33% and 53% in the experimental groups (Paper III).

7.1.2 Relationship between body condition, energy balance and immune competence

In our study higher conditioned cows tended to perform accelerated body fat mobilization. A significant BCS effect was found for the subcutaneous adipose tissue (Paper I). However, a higher predisposition for developing a ketosis of high conditioned cows could not be confirmed. Only the BCS_L/C_{35} group exceeded the threshold of 1.2 mmol/L for BHB, which indicated a subclinical ketosis (Nielen et al. 1994). Furthermore, neither DMI nor NEB were directly influenced by BCS (Paper I). It was expected that a higher BCS would have been associated

with a more pronounced decrease of DMI before calving, leading to a more pronounced NEB (Roche et al. 2009). However, this was not confirmed in the present study which leads to a rejection of hypothesis 1, including the influence of BCS on energy balance. Roche et al. (2009) defined a BCS-range between 3.0 and 3.5 to be optimal for bovine at calving. It might be possible that the BCS difference between high and low conditioned cows was not large enough in the current work, as BCS_L animals only reached a mean BCS of 3.1, whereas higher conditioned cows did not exceed a BCS of 3.8 (Paper I).

In the present study, an explicit BCS effect was neither found for the assessed immunological parameters. Inflammatory markers, such as Hpt or the Kysn:Trp-ratio did not reflect group differences either. Moreover, the $CD4^+$:$CD8^+$-ratio did not indicate a dysregulated homeostasis of $CD4^+$ and $CD8^+$ cells (Paper III). This supports the assumption that all groups, independent of BCS, did have a good immune status (Mehrzad and Zhao 2008). The present study covered a BCS range in which physiological adaptability was not overstrained.

7.2 Influence of dietary composition on short chain fatty acids, pH and rumination behaviour

As stated before, the increase of C is used to ease the NEB and therefore to decrease the risk for ketosis and increase animal welfare, as well as milk production. However, a high C also bears risks. The increase of concentrate intake leads to an increased production of short chain fatty acids (SCFA), which in turn can lower ruminal pH (Kleen et al. 2003, Roche et al. 2009, Bannink et al. 2012). If the production of SCFA exceeds their elimination by absorption through the rumen epithelium as well as their reduction by passage to lower digestive tract or buffer secretions, the risk for SARA increases (Ueda et al. 2003, Dirksen et al. 1984, Allen 1997, Oetzel 2007, Zebeli et al. 2008). SARA involves a large number of negative impacts on the organism. A decrease in ruminal pH decreases feed intake, rumen motility, fibre digestion and microbial efficiency. The reduced feed intake can trigger the development of ketosis (Britton and Stock 1987, Ash 1959, Mould and Ørskov 1983, Hoover 1986). According to Zebeli et al. (2008) a daily mean pH <6.16 and a pH <5.8 for more than 5.24 hours per day classifies a cow as suffering from SARA. Previous studies showed a significant decrease of mean ruminal pH, minimum daily pH and a significant increase of time with a pH <5.8, when feeding rations high in concentrate (Krause et al. 2002, Agle et al. 2010). In the present study, pH parameters of 13 ruminally-cannulated cows were assessed by using a continuous ruminal pH measurement

system. Differences between cows receiving a high C and cows receiving a low C – concerning the thresholds according to Zebeli et al. (2008) – were not detectable in the present study. Nevertheless, both C_{60} as well as C_{35} cows achieved the critical ranges indicating SARA (Zebeli et al. 2008). However, a positive relation between concentrate intake and daily pH variation was found (Paper II). Krause et al. (2002) also described a more pronounced effect of concentrate level on the area below 5.8 than on mean pH and thus emphasized the importance of diurnal variation compared to rather rigid thresholds, when assessing the effects of diet composition on rumen health and animal welfare. Although we detected a relation between proportion of propionic acid and ruminal pH, proportion of acetic acid and pH, as well as between acetate:propionate ratio and pH (Figure 7), we could not observe any differences in SCFA concentrations between groups (Paper II).

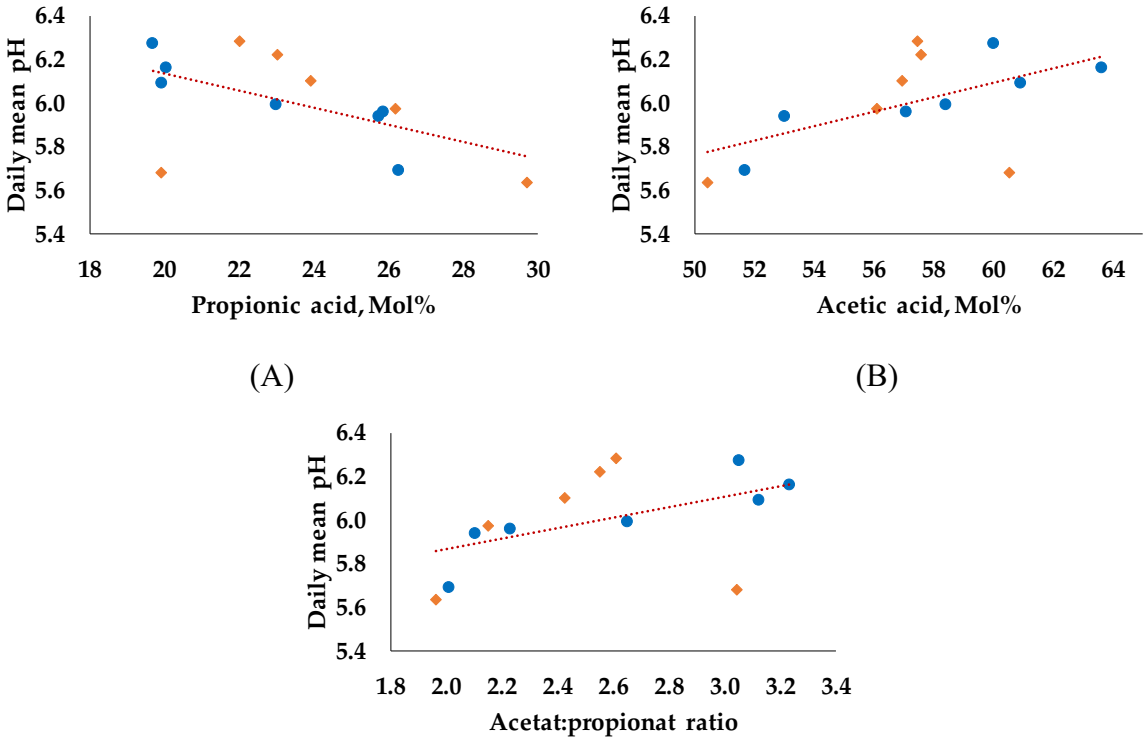

Figure 7. Regression of acetic acid (A, y = 9.632x – 0.568, $r^2 = 0.712$, $p < 0.05$), propionic acid (B, y = −7.964x + 71.271, $r^2 = 0.752$, $p < 0.05$) and acetate:propionate ratio (y = 0.984x − 3.354, $r^2 = 0.740$, $p < 0.05$) on daily mean pH in period 1 (week 1 – 2). After calving cows were assigned to a group with 60% concentrate feed proportion (increasing from 35% to 60% during the first three weeks after parturition, C_{60}, orange, n = 6) and a group with 35% concentrate feed proportion (C_{35}, blue, n = 7) in the ration.

Rations with a high C shift the SCFA ratio towards propionate, which leads to decreasing pH. In contrast, rations higher in fiber are known to support the production of acetate, which results in increasing pH (Bergamnn et al. 1996, López 2005). However, in the present study C did not significantly influence daily mean pH (Paper II). Nevertheless, different SCFA production of individual cows led to different pH values. Both groups showed thereby wide ranges in pH values independent of C. However, although no direct C-effect was found, the present study emphasizes in the context of the rumen condition the importance of individual feeding behavior on the one hand and – following hypothesis 3 – diurnal pH variation on the other hand. It was shown that actual concentrate intake affects diurnal pH variations significantly, independent of experimental grouping (Paper II).

As mentioned before, the ruminal pH is dependent on several factors, such as the functionality of the rumen epithelium and the saliva production during rumination to buffer SCFA. In the present study, rumination behavior was determined by a noseband pressure sensor including a data logger with on-line data analysis and evaluation software (RumiWatch Manager 2, Version 2.2.0.0, RumiWatch Converter, Version 0.7.4.5, RumiWatch, Itin + Hoch GmbH, Liestal, Switzerland). The noseband pressure sensor consists of a silicone tube filled with vegetable oil and lies over the bridge of the cow's nose. The data logger registers the pressure at a constant logging rate of 10 Hz. From week 1 to 10 after calving, the ruminating behavior of each cow was recorded every minute and measured during several consecutive 24-h periods each week (2 ± 1.15, mean ± standard deviation). Additional parameters, such as rumination chews and rumination time were assessed as well.

The missing differences between concentrate feeding groups in daily mean pH and time with pH <5.8 and SCFA concentrations are reflected by the ruminating behaviour (Figure 8).

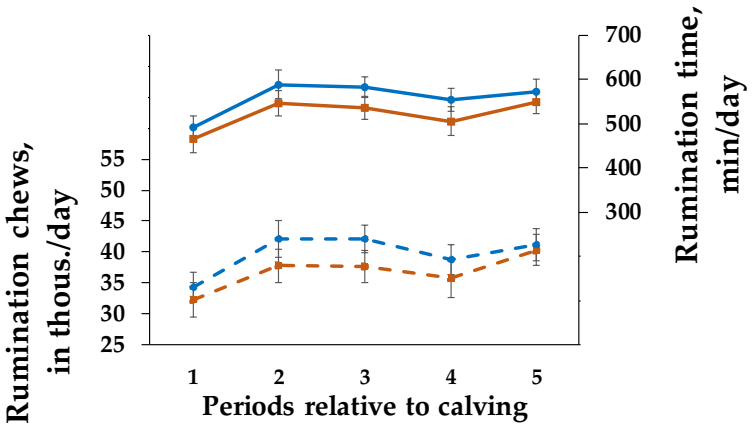

Figure 8. Rumination chews (dashed lines, left ordinate) and rumination time (solid lines, right ordinate) per day during period 1 (weeks 1 – 2 postpartum), period 2 (weeks 3 – 4 postpartum), period 3 (weeks 5 – 6 postpartum), period 4 (weeks 7 – 8 postpartum) and period 5 (weeks 9 – 10 postpartum) comparing the treatment groups. After calving, cows were assigned to a group with 60% concentrate feed proportion (increasing from 35% to 60% during the first three weeks after parturition, C_{60}, orange, n = 6) and a group with 35% concentrate feed proportion (C_{35}, blue, n = 7) in the ration. The MIXED procedure for repeated measures with a compound symmetry structure was used. C and period were applied as fixed effects, as well as the interaction between them. Each cow within treatment was considered to be a random effect. The period of sampling was treated as a repeated measure. The MIXED procedure was considered statistically significant when $p \leq 0.05$ and highly significant when $p \leq 0.01$ while a trend was assumed for $0.05 < p < 0.1$. *p*-values of rumination chews: C = 0.230, period = 0.009, C × period = 0.913, *p*-values of rumination time: C = 0.114, period = 0.005, C × period = 0.981.

Although numerical differences are visible and suggest higher rumination time for the C_{35} group, individual variations between animals impede the values to reach statistical significance (Agle et al. 2010). Rumination and chewing is known to stimulate saliva production (Church 1988). The buffering capacity of saliva bicarbonate neutralizes SCFA from the rumen and therefore influences the ruminal pH (Allen 1997). However, this increase is not apparent, if fibre level of the diet is already adequate. Nevertheless, the incremental output of saliva production with increasing chewing time is not extensive, as increasing salivation during chewing is accompanied with a decreased saliva secretion during resting. Increasing the fiber content of the ration from 30% to 81% increased the eating time by 2.2 hours/day and the ruminating time by 1.2 hours/day. However, the estimated gained saliva increment increased by 8 to 12 L/day, which amounts to approximately 4% only (Beauchemin 1994, Beauchemin 2000). According to Beauchemin and Penner (2009) balancing of ruminal pH is not directly related to chewing or rumination activity, but also to various other factors such as small size of meals, slower eating rate and rumen motility. However, studies propose an average rumination time between 6 and 9 hours per day (Beauchemin and Yang 2005, Leiber et al. 2015) which is supported by the results of the present study (Paper II).

7.2.1 Rumen conditions and its impact on inflammatory processes

SARA might induce inflammatory processes due to tissue lesions of the rumen wall. These inflammations can in turn trigger an acute-phase-response. As mentioned before, Hpt is an acute-phase-protein and the main indicator for inflammations in ruminants (Alsemgest et al. 1994). The lipopolysaccharide-binding-protein (LBP) is another acute-phase-protein, which neutralizes LPS in blood (Sriskandan and Altman 2008). When acidotic conditions in the rumen impact the microorganisms, this can result in lysis of gram-negative bacteria, which in turn release LPS. If the rumen epithelium is additionally stressed and more susceptible for injuries, LPS can translocate into the blood stream, which in turn triggers an acute-phase-response (Nagaraja et al. 1987, Nocek et al. 1984, Khafipour et al. 2009, Kushner et al. 1994).

Gozho et al. (2005) examined the impacts of SARA on acute-phase-responses. SARA was induced in Jersey steers by offering them a ration of chopped alfalfa hay and wheat-barley concentrate. Animals were defined as SARA positive, when pH was <5.6 for more than 3 hours per day. The study confirmed the hypothesis that SARA can cause an acute-phase-response by a significant increase of Hpt. Another study supports these findings and could also register an increase of LBP (Khafipour et al. 2009). In the present study, experimental groups did not differ concerning Hpt, wherefore C did not influence this parameter significantly (Paper III). However, all four groups substantially exceeded the threshold for a mild inflammation in cattle of >0.2 mg/mL and <0.4 mg/mL (Skinner et al. 1991), indicating a high stress level for the cows´ organisms in the current trial. Concerning the link to SARA, we evaluated the Hpt levels only for the fistulated cows as they were equipped with intraruminal probes (Figure 9).

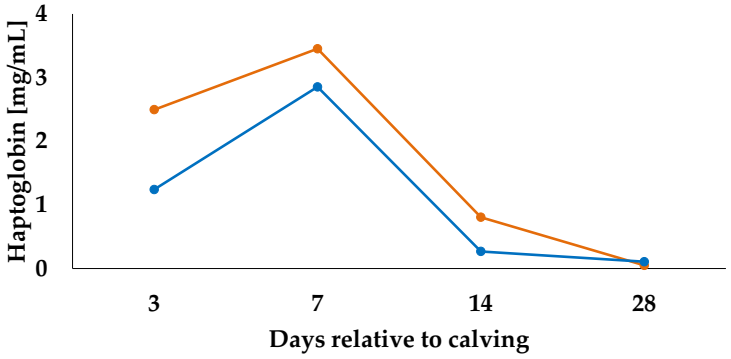

Figure 9. Development of haptoglobin from day 3 until day 28 after calving. After calving, cows were assigned to a group with 60% concentrate feed proportion (increasing from 35% to 60% during the first three weeks after parturition, C_{60}, orange, n = 6) and a group with 35% concentrate feed proportion (C_{35}, blue, n = 7) in the ration. The MIXED procedure for repeated measures with a compound symmetry structure was used. C and sampling day were applied as fixed effects, as well as the interaction between them. Each cow within treatment was considered to be a random effect. The sampling day was treated as a repeated measure. The MIXED procedure was considered statistically significant when $p \leq 0.05$ and highly significant when $p \leq 0.01$ while a trend was assumed for $0.05 < p < 0.1$. *p*-values: Day = 0.003, C = 0.436, Day × C = 0.895.

However, we failed to observe a C-effect. This is in line with the SARA-defining parameters, which did neither exhibit a difference of feeding groups (Paper II). The Hpt levels indicate that both groups of the fistulated animals, independent of C, were exposed to inflammatory stimuli due to the event of calving. This includes tissue lesions, endometrial inflammatory reactions, uterine bacterial infections as well as the ration change from dry cow nutrition to lactation diet (Nordlund et al. 1995, Hachenberg et al. 2007, Magata et al. 2015). The findings mentioned above lead to the rejection of the assumed C-effect on immunological parameters of hypothesis 6 in case of Hpt.

As LBP was not examined in the current study, we aimed to have a closer look at $CD14^+$, which is part of the LPS receptor complex as well (da Silva et al. 2001). Before calving, $CD14^+$ was influenced by time only, whereas after calving it was affected by BCS, C and time in an interactive manner (Paper III). Viewing the fistulated cows separately, the impact of C on percentage of $CD14^+$ was close to significance ($p_C = 0.051$), whereby C_{60} cows exhibited higher values (data not shown). This would underline the before discussed hypothesis, that higher C on the one hand would impair rumen conditions and consequently microorganisms in a way that would lead to lysis of gram-negative bacteria and release of LPS. On the other hand, C would affect the rumen mucosa, which would lead to translocation of LPS into the blood stream and in turn result in an increased percentage of $CD14^+$ as part of the LPS receptor complex.

However, it needs to be considered that a decrease in other leucocyte counts would also increase the proportion of $CD14^+$. Since Hpt was not significantly increased in the C_{60} cows, the processes do not appear to have triggered an acute-phase-response. However, Hpt is known to be subjected to a high individual variation, even after drastical increase of C (Gozho et al. 2005, Dänicke et al. 2018). Jacobsen et al. (2004) discovered that the individual cow as an explanatory variable explains 22% of the variation in Hpt. Another explanation for the increasing $CD14^+$ concentration before calving is an inflammation associated event, which is also reflected in the parallel enhancement of granulocytes and Kyn:Trp-ratio as accepted inflammatory markers (Bauman and Gauldie 1994, Murata et al. 2004). The decrease of $CD14^+$ concentration in the systemic circulation after parturition might be the result of an endometrial bacterial infection and consecutive inflammations. This leads to a local LPS accumulation and a migration of leukocytes, including $CD14^+$ cells to the site of inflammation (Chapwanya et al. 2012, Magata et al. 2015).

7.2.2 Relation between ruminal pH and milk fat synthesis

Assessing the rumen conditions of non-fistulated animals is difficult. It is assumed, that milk fat synthesis might provide information. However, the initial low-fat percentage mostly occurring in early lactation is also related to the degree of postpartum lipolysis (Zebeli and Ametaj 2009).

Nevertheless, feeding rations high in concentrate and low in forage is known to cause milk fat depression, as shown in the present study (Paper I). A severe decline in ruminal pH can impact microorganisms in a way that leads to an increase of ruminal LPS. LPS affect milk fat synthesis indirectly by inducing mediators that inhibit fatty acid synthesis. Thereby, key enzymes such as fatty acid synthetase and acetyl-CoA-carboxylase are particularly affected (Pekala et al. 1983, Lopez-Soriano and Williamson 1994). Zebeli and Ametaj (2009) were able to show that milk fat variables were negatively correlated to plasma C-reactive protein (CRP), another acute-phase-protein, while associations to Hpt and LBP were rather weak. Moreover, the authors calculated the milk energy efficiency (MEE), expressed as grams of milk fat produced per kilogram of DMI, to examine the effect of dietary treatment on energy efficiency of the animals. The authors concluded that feeding high-grain diets lowered efficiency for cows of the experiment. Although milk yield increased, MEE decreased, due to a drop of milk fat content in that study. Thereby, MEE is reduced by 26% for animals receiving a diet with 45% of barley

in comparison to animals offered a barley free diet. In conclusion, Zebeli and Ametaj (2009) proposed, that assuming animals with a higher milk yield to be more efficient is not applicable for their animal model. The same circumstances apply for the present study, where higher C led to lower milk fat content (Paper I). Consequently, C_{35} animals exhibited a MEE of 73 g of milk fat content/kg of DMI, which was 17% higher than the MEE of cows of the C_{60} group, which was 61 g of milk fat content/kg of DMI (data not shown). This is also in line with other results of our study, as lower DMI and equal milk yield led to an improved efficiency in C_{35} cows (Paper I). Therefore, although C and BCS did not influence milk yield, other performance related parameters, such as milk fat and resulting efficiency parameters were affected by experimental treatment, as assumed in hypothesis 1.

Nevertheless, a high individual variability among cows regarding responses to high grain diets was found in the study of Zebeli and Ametaj (2009). It was apparent in variables related to inflammatory responses, such as rumen LPS, as well as in milk and milk fat production (Zebeli and Ametaj 2009). A high individual influence was also visible for CRP, which suggests an importance of genotype in production of CRP during inflammation as well as an interindividual variation in rumen LPS-mediated inflammatory reactions (Danik and Ridker 2007, Zebeli and Ametaj 2009). Our study supports these findings, as milk fat content was only influenced by C during the weeks 5 – 17 after calving (Paper I). We would have expected to observe a significant impact immediately after parturition, when C was initially increased. However, this finding is in line with the described SARA thresholds (Paper II), as well as with the Hpt levels (Paper III) of the present study, which were neither affected by C. Enemark et al. (2002) explained that milk fat content is a proper indicator of fermentation conditions in the rumen. However, they declared that on herd level the initial low-fat percentage, which is generally observed during the onset of lactation, is also dependent on the degree of postpartum adipose tissue depot mobilization in the herd. Thus, the drop in milk fat percentage shortly after calving is inadequate to properly assess the conditions in the rumen. These hypotheses are reflected in the BCS × period effect in milk fat percentage in the present study, as a higher BCS led to an increased milk fat content in period 1 (Paper I).

7.3 Dietary composition and its impact on microbial efficiency

As mentioned before, a higher C in the ration is supposed to impact microbial efficiency negatively by deteriorating the rumen conditions. On the one hand, a drop in ruminal pH can

support lysis of bacteria. On the other hand, unfavourable ruminal environmental conditions would lead to energy spilling, because energy is primarily needed for non-growth processes, such as maintaining the intracellular pH on an optimum level, instead of being used for cell growth. As a result, fibre digestion and consequently microbial efficiency decreases (Strobel and Russell 1986, Oba and Allen 2003). Another study explained the decrease of microbial efficiency with an increased heat production resulting from accumulation of reserve carbohydrates (Hackmann and Firkins 2015). However, Clark et al. (1992) observed an increased microbial growth rate due to an increased intake of fermentable carbohydrates and therefore an enhanced availability of nutrients for microorganisms. At the same time, an increased passage rate was observed. The same relation was seen in the present study, as dry matter flow (DMF) and organic matter flow (OMF) were positively associated with microbial protein synthesis (Paper II). Higher passage rates are known to reduce microbial lysis and limit the use of energy for non-growth processes (Oba and Allen 2003, Dijkstra et al. 2005).

In the present study, C had neither a negative nor a positive effect on microbial efficiency (Paper II). The before mentioned hypothesis 4 of a drop in ruminal pH due to higher C which would impact microorganisms was not confirmed, as C_{60} and C_{35} groups did not differ in the SARA defining thresholds. A possible explanation for the missing positive C-effect on microbial efficiency might lie in the lack of amino acids and peptides in the C_{60} diet. Microorganisms fermenting rapidly fermentable carbohydrates can use around 2/3 of the needed N from amino acids and peptides (Russell et al. 1983). Fibrolytic bacteria, by contrast, can only use ammonia-N. As rations high in starch change the microbial population towards amolytic bacteria, the availability of amino acids and peptides might have been limited for the C_{60} animals in the current study (van Kessel and Russell 1996). However, to test this hypothesis more closely, the microbiome needs to be examined more intensively.

7.3.1 Ruminal-nitrogen-balance and microbial efficiency

Riemeier (2004) investigated the influence of different RNBs on microbial efficiency. An RNB of −0.3 g/MJ ME is defined as uncritical by the Society of Nutrition Physiology (Gesellschaft für Ernährungsphysiologie, GfE, 2001). Riemeier (2004) aimed to test RNB levels lower and higher than that regarded as optimum, thereby comparing the RNBs of −0.6, −0.3, 0 and +0.3. For the slightly negative and the balanced RNB, a more efficient NH_3 utilization was observed. This was reflected in an increased N flow at the duodenum. The N flow was 28% higher than

the N intake via food. In our study, an RNB of 0.6 was calculated for the C_{60} cows, whereas the C_{35} groups achieved an RNB of 0.1. Although the difference was not significant, the N flow at the duodenum of the C_{35} cows was by 8% higher than the N intake via food, whereas for the C_{60} cows the N flow was 18% below the N intake. Even though not significant, the C_{35} group showed a higher microbial protein synthesis compared to the C_{60} cows. Therefore, the present study confirmed the findings of Riemeier (2004). The same is true for the classification into more and less efficient, which we additionally chose in the present study. To gain more information from another perspective, cows were classified (retrospectively) according to their individual microbial efficiency, independent of initial experimental grouping according to C during the week of duodenal chyme sampling (week 13 postpartum ±16 days). For this purpose the average microbial efficiency of 156 g synthesized microbial crude protein (mCP)/kg fermentable organic matter (fOM) determined by the GfE (2001) was used as threshold for dividing the cows into more or less efficient. For less efficient animals an RNB of 0.7 was observed, whereas the RNB of more efficient animals was only slightly positive (0.1). The N flow at the duodenum of these animals was 7% higher than the N intake via food, whereas the N flow of the less efficient group was 23% below the N intake. (The above described results are summarized in Table 1).

Table 1. Effects of concentrate feed proportion in the ration (C) and microbial efficiency (synthesized microbial crude protein/fermented organic matter) on nitrogen (N)-flow at the duodenum, ruminal-nitrogen-balance (RNB) in g/day and g/MJ metabolizable energy (ME).

Item[+]	Grouping model 1[$]		SD[#]	*p*-value	Grouping model 2[§]		SD[#]	*p*-value
	C_{60} n = 5	C_{35} n = 4			More efficient n = 5	Less efficient n = 4		
N-flow at the duodenum, % of N-intake	82	108	17	0.052	107	77	12	**0.014**
RNB, g/d	176.0	33.5	84.5	0.059	38.5	204.7	66.8	**0.018**
RNB/ME, g/MJ ME	0.6	0.1	0.3	0.053	0.1	0.7	0.3	**0.021**

[+]Intergroup comparison (simple t-test) for the week of duodenal chyme sampling (on average week 13 postpartum ± 16 days) for both assignments (**C_{60}** vs. **C_{35}**, **more** vs. **less efficient**, respectively). [$]After calving cows were assigned to a group with 60% (increasing from 35% to 60% during the first three weeks after parturition, **C_{60}**) in the ration and a group with 35% concentrate feed proportion (**C_{35}**). [§]For the week of duodenal chyme sampling cows were additionally grouped into a **more** (206 ± 17 g/kg, n = 5) and a **less efficient** (122 ± 17 g/kg, n = 4) group according to the mean microbial efficiency of 156 g/kg, defined as synthesized microbial crude protein/fermented organic matter in compliance with GfE (2001). The t-test was considered statistically significant when $p \leq 0.05$ and highly significant when $p \leq 0.01$ while a trend was assumed for $0.05 < p < 0.1$. Values are presented as means, [#]Pooled standard deviation (SD).

McAllan et al. (1987) and Riemeier (2004) proposed a more efficient N utilization, if N deficiency occurs. Simon (2008) considered a NH_3 concentration of 10 – 25 mg/100 mL rumen fluid as sufficient for microbial protein synthesis. None of the groups in the present study achieved such values, indicating an undersupply of N on the one hand, but consequently a more efficient utilization on the other hand.

Although according to N flow at the duodenum and therefore NH_3 utilization a C-effect can be assumed, neither dry matter flow nor organic matter flow were affected by dietary treatment in the present study (Paper II). In the same manner, C_{60} and C_{35} cows did not differ significantly in digestibility values. However, following hypothesis 5, a significant difference was seen between more and less efficient animals. The more efficient group exposed higher dry matter flow as well as higher organic matter flow values, which in turn led to a decreased digestibility, as shown in Firkins et al. (2007).

8. Conclusion

The present study confirmed the advantage of a higher C to reduce the NEB. Due to a less pronounced NEB in C_{60} animals, a decreased need of lipid mobilization was expected. However, this could neither be demonstrated by group differences in BHB and NEFA concentrations nor in the used USM. On the contrary, the USM visualized a more pronounced mobilization of single adipose tissue depots in the BCS_H groups which were not apparent in BCS losses. Nutritional status and metabolic situation of the transit cow might be more reliably evaluated by USM than by NEB or BCS. The current study showed that 70% of the NEB was covered from mobilization of body fat. The rumination behaviour tended to be affected by C. However, it had been assumed that rumination behaviour would have produced insufficient saliva output to influence pH values. Fistulated cows of the C_{60} group tended to exhibit higher values of percentage of $CD14^+$. As $CD14^+$ is part of the LPS receptor complex, it had been suspected that C influenced the rumen mucosa as well as the rumen microbes in a way that led to lysis of gram-negative bacteria and a release of LPS into the blood stream. However, as Hpt was not affected by dietary treatment, it was concluded, that no acute-phase-reaction was triggered. The number of lymphocytes was influenced by BCS and C; however, the functionality was not compromised. It was concluded that the physiological adaptability was not overstretched within the given BCS range of the present study.

Diurnal pH fluctuations were likewise rather dependent on individual concentrate intake than on diet composition. Even though C did neither affect microbial efficiency, more efficient animals exhibited a higher DMF as well as a higher OMF, which were related to ruminal pH values in duodenally-cannulated animals. Therefore, microbial efficiency was indirectly influenced by rumen conditions. Moreover, the higher microbial efficiency was attributed to a more efficient N utilization, reflected in an increased N flow at the duodenum.

9. Summary

During the past decade, science has increasingly focused on animal welfare. The agreement between the economic requirements on the one hand and animal welfare and health on the other hand is often difficult. In this context, the transition period is considered as the most critical time. With the event of calving and the change from late gestation to lactation the energy requirements of a transition cow suddenly increase, which results in a negative energy balance. Dairy cows are able to mobilize energy, stored in body fat depots, to balance that energy deficit. However, if the lipolysis exceeds the physiological range, this can lead to an accumulation of ketone bodies which may result in metabolic disorders, such as ketosis. A high body condition before calving might push this development additionally. In practice, concentrate proportions of the rations are often increased to ease the energy deficit. However, an enhanced concentrate feed proportion also bears risks. Due to the degradation of rapid fermentable carbohydrates, the rumen of the cow might become acidotic. If the described relations are not synchronized properly, further health problems next to ketosis and acidosis can evolve.

With regard to the explained relations, a feeding study was performed including 60 pluriparous German Holstein cows. The animals compromised 13 ruminally-fistulated as well as 10 additionally duodenally-cannulated animals. The aim was to investigate the effect of the body condition score (BCS) and of varying concentrate feed proportions on performance parameters, mobilization of energy stored in body fat, rumen fermentation patterns and microbial efficiency, as well as various health parameters. For this, the animals were allocated 42 days before the calculated calving according to their BCS into a high BCS (BCS_H) or low BCS (BCS_L) group. Until parturition, all cows received the same ration with a concentrate proportion of 20%. After calving, both groups were further subdivided by supplying different diet compositions. Groups with a low concentrate amount received a ration with a concentrate feed proportion of 35% (C_{35}), whereas groups with a high concentrate amount received a ration with a concentrate feed proportion of 60% (C_{60}), thereby increasing it from 35% to 60% during the first three weeks after calving. To assess rumen fermentation patterns and microbial efficiency, only the fistulated cows were involved. Due to the resulting small number of animals, the grouping according BCS was abandoned, and cows were only classified according to the concentrate feed proportion of the respective ration. To gain further information, the fistulated cows were additionally (retrospectively) assigned according to their microbial efficiency into a more

efficient and a less efficient group concerning the week of duodenal chyme sampling (week 13 postpartum ±16 days). Besides the collection of feed and milk samples and measurements of feed intake and milk yield, ultrasonic data of the body fat depots were recorded (Paper I). Moreover, ruminally-fistulated animals were equipped with continuous ruminal pH measurement devices as well as with rumination halters. Duodenal chyme of the duodenally-cannulated cows was collected over one week to calculate microbial efficiency, nutrient flows and digestibilities (Paper II). To assess immunological processes and inflammatory markers, blood samples were taken from all animals (Paper III).

The results of the present study confirm that an increased concentrate feed proportion leads to an enhanced dry matter intake and therefore to a less pronounced negative energy balance. However, milk yield was not positively affected. Animals receiving a lower concentrate feed proportion even developed higher efficiency values. These results were attributed to a milk fat depression in the C_{60} groups. The hypothesis of a reduced lipolysis due to a less pronounced energy deficit could neither been proved, as USM did not demonstrate differences between concentrate groups. The increased ketone body concentration accompanied with an accelerated body fat mobilization was neither affected by different feeding. The BCS difference between groups stayed relatively constant over the whole trial and did not allow to assume a higher mobilization for BCS_H groups. This was however disproved by ultrasonic data, as mobilization of single fat depots revealed a BCS effect. In the current study, 70% of the negative energy balance was covered by mobilized energy stored in body fat. Thereby, 0.25 MJ fat energy were mobilized per 1 MJ energy change.

Also the varying concentrate feed proportions did not influence the assessed rumen pH-parameter of the fistulated cows. However, it was provable that independent of the allocated ration, the individual concentrate intake affected diurnal pH fluctuations. Both groups exhibited wide pH ranges and included animals with both high and low daily mean pH values. The ruminal pH did neither demonstrate a direct relation to the microbial efficiency, as more and less efficient groups did not differ in this regard. However, a correlation was demonstrated between the average pH over 24 hours and the dry matter flow. An increasing dry matter flow increased the microbial crude protein synthesis, which was attributed to a decreased lysis of bacteria. The higher dry matter flow of the more efficient animals also explains the lower digestibility of this group. As the assumption that animals with a higher BCS and a lower

concentrate feed proportion are predisposed to develop metabolic disorders, such as ketosis, was not verifiable, the health status of the animals was examined more closely. Haptoglobin, as main inflammatory marker in cows, did not reveal group differences. The presumption to find a BCS effect, as haptoglobin is also considered as adipokine and BCS_H groups showed a higher mobilization, could neither be confirmed. Instead, haptoglobin was documented to be highly variable at an individual level. The $CD4^+$:$CD8^+$-ratio confirmed an uncritical immune status for all groups. The higher $CD14^+$ values of fistulated cows of the C_{60} group was explained by LPS transfer into the blood stream, due to lysis of gram-negative bacteria and tissue lesions of the rumen wall. However, as haptoglobin was not significantly increased, it was assumed that no acute-phase reaction was triggered. Even though the number of lymphocytes was influenced by BCS and concentrate feed proportion, the functionality was not impaired. Therefore, it was concluded that the given BCS range in the present study covered an area in which physiological adaptability was not overstretched.

The current study could indicate that the term of efficiency has to be viewed critically, as a high efficiency is often associated with a highly negative energy balance. Moreover, it became clear that USM can present differences in body fat mobilization that cannot be captured by sole BCS determination. The calculation of the negative energy balance on the contrary can lead to an overestimation of the necessary mobilization. Furthermore, this study could demonstrate that ration composition alone does not influence rumen conditions significantly. It rather became clear that the individual feeding behavior and the actual concentrate intake play an important role and that particular attention should be payed to diurnal pH fluctuation. Although no direct relation was detectable between ruminal pH and microbial efficiency, an indirect influence via the dry matter flow was demonstrable.

10. Zusammenfassung

In den letzten zehn Jahren hat sich die Wissenschaft vermehrt mit dem Tierwohl beschäftigt. Die Vereinbarung von ökonomischen Anforderungen auf der einen Seite und Tierwohl und Tiergesundheit auf der anderen Seite gestaltet sich dabei oftmals diffizil. Als besonders kritische Zeit gilt in diesem Zusammenhang die Transitphase. Mit dem Ereignis der Abkalbung und dem Wechsel von Trächtigkeit zu Laktation erhöht sich der Energiebedarf der Transitkuh abrupt, was in einer negativen Energiebilanz resultiert. Die Milchkuh ist in der Lage, Energie, die in Form von Körperfett gespeichert ist, zu mobilisieren, um das Energiedefizit auszugleichen. Übersteigt die Lipolyse jedoch den physiologischen Bereich, kommt es zur Anreicherung von Ketonkörpern und Stoffwechselerkrankungen wie Ketose können entstehen. Dabei kann eine zu hohe Körperkondition vor der Kalbung diese Problematik zusätzlich forcieren. In der Praxis werden zudem oftmals höhere Kraftfutteranteile gefüttert, um das Energiedefizit abzuschwächen. Eine Erhöhung des Kraftfutters birgt aber auch Risiken. Durch den Abbau der schnell fermentierbaren Kohlenhydrate zu kurzkettigen Fettsäuren, kann der Pansen der Kuh azidotisch werden. Stehen all diese Zusammenhänge nicht im Einklang, können sich neben der Entstehung von Ketose und Azidose weitere gesundheitliche Probleme entwickeln.

In Bezug auf die dargestellten Zusammenhänge wurde ein Fütterungsversuch mit 60 pluriparen Deutsche Holstein Kühen durchgeführt. Zu den Tieren zählten 13 pansenfistulierte sowie 10 zusätzlich duodenalfistulierte Kühe. Dabei war das Ziel, den Einfluss der Körperkondition (Body Condition Score = BCS), sowie den Effekt unterschiedlich hoher Kraftfutteranteile in der Ration auf die Leistungsparameter, die Mobilisierung von in Körperfett gespeicherter Energie, die Pansenfermentation und die mikrobielle Effizienz, sowie verschiedene Gesundheitsparameter zu untersuchen. Hierzu wurden die Tiere 42 Tage vor der errechneten Kalbung entsprechend ihrer Körperkondition in eine Hoch-BCS-Gruppe (BCS_H) oder in eine Niedrig-BCS-Gruppe (BCS_L) eingeteilt. Bis zur Abkalbung erhielten alle Tiere die gleiche Ration mit einem Kraftfutteranteil von 20 %. Nach der Geburt wurden die beiden BCS-Gruppen durch die Zuteilung unterschiedlicher Rationen weiter aufgeteilt. Dabei erhielten die Gruppen mit einem niedrigen Kraftfutteranteil 35 % Konzentrat (C_{35}) und die Gruppen mit einem hohen Kraftfutteranteil 60 % Konzentrat (C_{60}), wobei dieser innerhalb der ersten drei Wochen nach der Kalbung von 35 % auf 60 % gesteigert wurde. Zur Untersuchung der Pansenfermentation

und der mikrobiellen Effizienz wurden nur die fistulierten Kühe herangezogen. Durch die dadurch reduzierte Tierzahl wurde auf die Einteilung der Tiere nach BCS verzichtet und die Kühe wurden nur nach dem Kraftfutteranteil der entsprechenden Ration klassifiziert. Um zusätzliche Informationen zu erhalten, wurden die fistulierten Kühe außerdem unabhängig der Kraftfutterzuordnung während der Woche der Duodenalchymussammlung (Woche 13 postpartum ± 16 Tage) (retrospektiv) nach der mikrobiellen Effizienz in eine hoch effiziente (more efficient) und eine weniger effiziente (less efficient) Gruppe eingeteilt. Neben der Sammlung von Futter- und Milchproben und der Messung der Futteraufnahme sowie der Milchleistung wurden Ultraschalldaten der Körperfettdepots erhoben (Paper I). Des Weiteren wurden die pansenfistulierten Tiere mit kontinuierlich-messenden ruminalen pH-Messgeräten, sowie mit Widerkauhalftern ausgestattet. Bei den duodenalfistulierten Kühen wurden eine Woche lang Duodenalchymusproben genommen, um die mikrobielle Effizienz, Nährstoffflüsse und Verdaulichkeiten zu berechnen (Paper II). Zur Bewertung von immunologischen Vorgängen und Entzündungsmarkern wurden allen Tieren Blutproben entnommen (Paper II).

Die Ergebnisse dieser Studie bestätigen, dass eine erhöhte Kraftfuttergabe zu einer erhöhten Trockenmasseaufnahme und damit zu einer weniger starken negativen Energiebilanz führt. Die Milchleistung wurde dadurch jedoch nicht positiv beeinflusst. Tiere, die einen geringeren Kraftfutteranteil bekamen, erzielten sogar höhere Effizienzwerte. Dies wurde auf eine Milchfettdepression in den C_{60}-Gruppen zurückgeführt. Die These einer reduzierten Lipolyse durch ein weniger starkes Energiedefizit konnte ebenfalls nicht bestätigt werden, da die Ultraschalldaten diesbezüglich keinen Unterschied zwischen den Kraftfuttergruppen aufzeigten. Auch die mit einer forcierten Fettmobilisierung einhergehenden erhöhten Ketonkörperkonzentrationen waren von der unterschiedlichen Fütterung nicht beeinflusst. Die BCS-Differenz zwischen den Gruppen blieb über den gesamten Versuchsverlauf relativ konstant und ließ keine erhöhte Mobilisierung der BCS_H-Gruppen vermuten. Dies wurde jedoch durch die Ultraschalldaten widerlegt, da die Mobilisierung einzelner Fettdepots einen BCS-Effekt aufwies. In der vorliegenden Studie konnten 70 % der negativen Energiebilanz durch die Mobilisierung der in den Körperfettdepots gespeicherten Energie gedeckt werden. Dabei wurden 0.25 MJ Fettenergie pro 1 MJ Energieveränderung verbraucht.

Die unterschiedlichen Kraftfutteranteile hatten auch keinen Einfluss auf die erhobenen Pansen-pH-Werte der Fistelkühe. Allerdings konnte nachgewiesen werden, dass die individuelle

Kraftfutteraufnahme unabhängig von der zugeteilten Ration die täglichen pH-Schwankungen beeinflusste. Beide Gruppen zeigten einen weiten pH-Bereich und enthielten sowohl Tiere mit einem eher niedrigen mittleren Tages-pH-Wert als auch Tiere mit einem eher hohen mittleren Tages-pH-Wert. Der Pansen-pH-Wert zeigte auch keinen direkten Zusammenhang zur mikrobiellen Effizienz, da sich die hoch effiziente und die weniger effiziente Gruppe diesbezüglich nicht unterschieden. Allerdings konnte ein Zusammenhang zwischen dem mittleren pH-Wert über 24 Stunden und dem Trockenmassefluss nachgewiesen werden. Je stärker der Trockenmassefluss anstieg, desto höhere war auch die Konzentration des mikrobiellen Rohproteins, was auf die Reduktion der Bakterienlyse zurückgeführt wurde. Der höhere Trockenmassefluss der hoch effizienten Tiere erklärt außerdem die niedrigeren Verdaulichkeitswerte in dieser Gruppe. Da die Annahme, dass Tiere mit einem höheren BCS und einem geringeren Kraftfutteranteil in der Ration eine höhere Prädisposition zur Entwicklung von Stoffwechselkrankheiten wie Ketose haben, nicht eindeutig bestätigt werden konnte, wurde der Gesundheitszustand der Tiere genauer betrachtet. Für Haptoglobin als Hauptentzündungsmarker für Kühe konnte kein Gruppenunterschied festgestellt werden. Die Vermutung eines BCS-Effekts durch die Zuordnung als Adipokin und der höheren Mobilisierung der BCS_H-Gruppen konnte auch nicht bestätigt werden. Stattdessen wurde Haptoglobin eine hohe individuelle Varianz zugesprochen. Das $CD4^+$:$CD8^+$-Verhältnis bestätigte für alle Gruppen einen unkritischen Immunstatus. Die höheren $CD14^+$ Werte der fistulierten Kühe der C_{60} Gruppe können auf die Lyse gram-negativer Bakterien und gelichzeitig auf Läsionen der Pansenwand schließen lassen, was wiederum den Transfer von LPS in den Blutkreislauf ermöglichte. Ein interaktiver Effekt von BCS und Kraftfutteranteil konnte, wenn auch nicht für die Lymphozytenfunktionalität, so doch für die Lymphozytenanzahl demonstriert werden. Daraus lässt sich schließen, dass die erfasste BCS Spanne der vorliegenden Studie, einen Bereich einschließt, in dem die Fähigkeit der physiologischen Adaptation noch nicht ausgereizt ist.

Die vorliegende Studie konnte zeigen, dass der Effizienzbegriff kritisch betrachtet werden muss, da eine hohe Effizienz meist mit einer stark negativen Energiebilanz assoziiert ist. Des Weiteren wurde deutlich, dass Ultraschallmessungen Unterschiede in der Mobilisierung von Körperfett darstellen können, die durch die alleinige BCS-Bestimmung nicht erfasst werden. Die Berechnung der negativen Energiebilanz hingegen, kann zu einer Überschätzung der notwendigen Mobilisierung führen. Auch konnte in dieser Studie gezeigt werden, dass die

Rationszusammensetzung allein keinen signifikanten Einfluss auf den Pansen nimmt. Vielmehr wurde deutlich, dass das individuelle Fressverhalten und die tatsächliche Kraftfutteraufnahme eine wichtige Rolle spielen, und dass vor allem den täglichen pH-Verläufen Aufmerksamkeit geschenkt werden sollte. Obwohl kein direkter Zusammenhang zwischen Pansen-pH und mikrobieller Effizienz nachweisbar war, so konnte doch ein indirekter Einfluss über den Trockenmassefluss aufgezeigt werden.

11. References

(accounts for chapters: 1. Introduction, 2. Background and 7. General Discussion)

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12. Appendix

Curriculum vitae

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Eidesstattliche Erklärung / Declaration under Oath

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

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