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Effects of Energy Supply from Roughage and Concentrates and the Occurrence of Subclinical Ketosis on Blood Chemistry and Liver Health in Lactating Dairy Cows during Early Lactation

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Abstract: The objectives of this study were to examine the effects of varying dietary energy supply as well as the impacts of subclinical ketosis (SCK) on blood chemistry and liver health. A total 63 German-Holstein cows were housed from three weeks antepartum until sixteen weeks postpartum. After calving, cows were assigned to one of four treatment groups receiving either moderate or high energy concentrations in roughage and secondly moderate or high amounts of concentrates. Retrospectively, cows were additionally grouped according to their β -hydroxybutyrate concentration (SK: cows with SCK vs. CON: cows without SCK). The different energy supply of treatment groups had little effects on blood and liver variables; greater differences occurred between SK and CON cows. Liver fat content of SK cows was 34% higher compared to CON cows. Also, the activity of aspartate aminotransferase and γ -glutamyl transferase, bilirubin concentration, and percentage of granulocytes were increased in SK cows. The results indicate that cows were able to adjust their metabolism to different dietary energy supplies without having a clearly increased risks for metabolic disorders. However, individual animals of all groups developed a metabolic derailment during the postpartum period resulting in SCK, which is closely connected with impaired liver function, compromised immune-responsiveness, and elevated oxidative stress.

Keywords: nutrition; dietary energy supply; subclinical ketosis; liver enzymes



Citation: Schmitz, R.; Schnabel, K.; Frahm, J.; von Soosten, D.; Meyer, U.; Hüther, L.; Spiekers, H.; Rehage, J.; Sauerwein, H.; Dänicke, S. Effects of Energy Supply from Roughage and Concentrates and the Occurrence of Subclinical Ketosis on Blood Chemistry and Liver Health in Lactating Dairy Cows during Early Lactation. *Dairy* **2021**, *2*, 25–39. <https://doi.org/10.3390/dairy2010003>

Received: 20 November 2020

Accepted: 4 January 2021

Published: 12 January 2021

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1. Introduction

During the transition period, high yielding dairy cows are confronted with massive endocrine, metabolic, and dietary challenges related to parturition and onset of lactation. The increased energy requirements due to onset of lactation are not equivalently accompanied by an increased energy intake, which results in an aggravation of negative energy balance (NEB) [1]. In adaption to this energy gap, cows mobilise body reserves, leading to increased concentrations of non-esterified fatty acids (NEFA) in the blood. However, the capacity to metabolise NEFA is limited, so that excessive lipid mobilisation can lead to an imbalance of the fat to carbohydrate metabolism, with resulting accumulation of triglycerides in the cytoplasm of hepatocytes and elevated ketone bodies in the blood [2]. As a consequence, the metabolic disease complex of subclinical ketosis (SCK) and fatty liver syndrome can occur, which are characterised by an excessive increase of β hydroxybutyrate (BHB) in

blood and an elevated fat content in liver tissue [3]. Therefore, these disorders might impair liver function and damage liver tissue, which is often accompanied by an elevated activity of enzymes that are more or less closely associated with hepatocyte lesions, such as aspartate dehydrogenase (AST), glutamate dehydrogenase (GLDH), and γ -glutamyltransferase (GGT). Hepatic clearance capacity for bilirubin might also be adversely affected, resulting in increased concentrations in peripheral blood

The development of SCK and fatty liver syndrome can compromise the immune competence of dairy cows. This is caused by an inhibiting effect of liver fat and ketone bodies on cell activity of the innate immune system [4,5] and also by an impaired liver function, accompanied by reduced synthesis and degradation capacity [3]. Thus, these conditions might trigger an acute-phase reaction (APR) associated with the release of acute-phase proteins (APP) such as haptoglobin (Hp) as the main APP in cattle [6–8]. Another indicator of an activated immune system is the ratio of the amino acid kynurenine (K) to tryptophan (T), which is the degradation product [9]. In a study of Drong, et al. [10], differences in the kynurenine-to-tryptophan ratio (K:T ratio) were observed in course of the transition period, showing the inflammatory-like processes in this period. Furthermore, infectious diseases and the high metabolic load during early lactation cause the increased production of reactive oxygen and nitrogen species, which can induce cell and tissue damages [11]. Therefore, the antioxidative capacity is generally increased during early lactation, augmenting the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx).

In a recent experiment, we investigated possible ketogenic effects of different energy concentrations in roughage and varying amounts of concentrates in dairy cows during early lactation [12]. On the basis of a threshold of 1.2 mmol BHB/L [13], we found an incidence of 30% SCK in our trial.

Based on these findings, the objectives of the present study were to examine the effects of varying energy concentrations in roughage and different amounts of concentrates on haematological, biochemical, inflammatory, and antioxidative blood variables during early lactation. In view of the individual differences regarding the susceptibility for metabolic derailment during early lactation, we also evaluated the data retrospectively by grouping cows affected with SCK and healthy cows and compared liver health as well as inflammatory and antioxidative blood markers irrespective of dietary treatments.

2. Materials and Methods

2.1. Feeding Experiment

The experiment was carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut (FLI) in Braunschweig, Germany. Experimental work was performed in accordance to the German Animal Welfare Act approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany, No. 33.19-42502-04-15/1858). The cows were housed in free-stall barns and milked two times a day.

The present research is part of a trial, which is described in detail in Schmitz et al. (2018) [12]. In brief, 63 pluriparous German Holstein cows were examined from three weeks antepartum until sixteen weeks postpartum. Cows were assigned to one of four groups after calving in a 2×2 factorial arrangement, differing in energy concentration of roughage and amounts of concentrates. The groups were equalised in terms of fat-corrected milk yield of previous lactation (6043 ± 1198 kg, 200 d milk yield; mean \pm standard deviation), numbers of lactation (2.0 ± 1.1), body weight (760 ± 88 kg) and body condition score (BCS, 3.45 ± 0.46) at the beginning of the experiment. All cows were fed an identical dry cow ration. After calving, they were assigned to one of four treatment groups that received either a moderate (MR, 6.1 MJ net energy for lactation (NEL)) or a high (HR, 6.5 MJ NEL) energy concentration in roughage. Secondly, they received moderate (MC, 150 g per kg energy corrected milk (ECM)) or high (HC, 250 g per kg ECM) amounts of concentrates on a dry matter (DM) basis. The groups MRMC, MRHC and HRMC consisted of 16 cows, while group HRHC included 15 animals. During the dry period, the ration was offered

as a total mixed ration; the lactating ration was fed as a partial mixed ration (PMR) by self-feeding facilities (type RIC; Insentec B.V., Marknesse, The Netherlands) for ad libitum intake. During the postpartum period the main part of the concentrates were allocated by computerised automatic feeding stations (Insentec, B.V., Marknesse, The Netherlands), and a low amount was mixed into the PMR (on average 11% of DM). The individual water and dry matter intake (DMI) were continuously recorded automatically using electronic weighing troughs mentioned before (Insentec, B.V., Marknesse, The Netherlands) using ear tag detection. More details of DMI and its calculation are described by Schmitz et al. [12]. Two different types of concentrates were prepared to equalise the intake of minerals independent of the quantity of concentrates. The chemical composition of the dry period TMR as well as different PMRs during the trial are provided in Table 1 [12]; a detailed description of ingredients and nutrients of the roughage parts and concentrates was presented in Schmitz, et al. [12].

Table 1. Calculated nutrients and energy level of the dry period ration and the different experimental rations in the postpartum period.

	Dry Period ¹	Postpartum Period ²			
		MRMC	MRHC	HRMC	HRHC
Dry matter [g/kg]	347	547	601	523	580
Nutrient [g/kg DM]					
Crude ash	65	66	65	67	65
Crude protein	118	129	138	135	142
Ether extract	38	37	39	38	40
Crude fiber	189	205	184	188	173
aNeutral detergent fiber om	375	404	366	372	345
Acid detergent fiber om	215	241	221	221	207
Energy [MJ/kg DM]					
Metabolisable energy	11.2	11	11.5	11.3	11.7
Net energy lactation	6.9	6.7	7.1	6.9	7.2

¹ During the dry period (from three weeks antepartum) all groups received the same TMR with a concentrate proportion of 20%; ² during the postpartum period groups received a moderate (MR) or a high (HR) energy concentration in roughage and moderate (MC) or high (HC) amounts of concentrates; Concentrate proportions were 32% in MRMC group, 31% in HRMC group, 43% in MRHC group and 42% in HRHC group on a dry matter (DM) basis.

Retrospectively, cows were grouped according to their BHB concentrations in blood samples of days 7, 14, 28 and 100 after calving. The subclinical ketosis group included cows that exhibited a SCK (SK, BHB > 1.2 mmol/L in at least one of the blood samples; 19 cows), whereas the control group contained cows without SCK (CON, BHB < 1.2 mmol/L in all blood samples; 44 cows). The number of cows exhibiting SCK was not different between the treatment groups and amounted to 5 in MRMC, 4 in MRHC, 3 in HRMC and 7 cows in the HRHC groups [12].

2.2. Sample Collection

On days −50, −14, 8, 28, and 100 relative to parturition, blood was sampled from each cow after the morning milking from a *Vena jugularis externa* and filled into serum and EDTA tubes (Sarstedt, Nümbrecht, Germany). Blood samples were centrifuged (Heraeus Varifuge[®] 3.0R Heraeus, Osterode, Germany; 1900 × g, 15 °C, 10 min) and stored at −20 °C until further analysis. From the samples at d −14 relative to parturition, analyses from nine cows were excluded because samples of these animals were taken earlier than d −20 or later than d −7 relative to parturition (one sample of MRMC, one of MRHC, three of HRMC and four of the HRHC group).

Furthermore, liver biopsy samples were taken from 40 cows (always the same ten cows of each group) on sampling days −14, 8, 28 and 100 relative to parturition by using an automated spring-loaded biopsy instrument (Bard Magnum, Bard, UK) equipped with a 16-gauge needle under local anesthesia (procain hydrochloride; Isocaine 2%, Selectavet,

Weyarn-Holzolling, Germany). In total, approximately 100 mg of liver tissue were sampled on each sampling time and stored at -80°C . Additionally, from these 40 animals, blood samples were taken for measurement of the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in erythrocytes. These samples were put on ice directly after collection and were processed shortly thereafter. Also, the serum concentration of haptoglobin was determined in these specimens.

2.3. Analyses

The concentration of BHB in serum was determined using spectrophotometrical detection with a Cobas Mira analyser (Hoffmann LaRoche, Basel, Switzerland). Haematological analyses were performed immediately after sampling in EDTA full blood via the automatic analyser Celltac- α (MEK 6450; Nihon Kohden, Quinlab Diagnostik, Weichs, Germany). White blood cell profile consisted of total leucocyte count (WBC), lymphocytes, granulocytes (granulocytes exclusive eosinophilic granulocytes), eosinophilic granulocytes and monocytes. The red blood cell profile included red blood cell count (RBC), haematocrit, haemoglobin, and the erythrocyte indices mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH). Blood concentrations of triglycerides, albumin, total protein, cholesterol, urea, alkaline phosphatase (AP), γ -glutamyltransferase (GGT), aspartate aminotransferase (AST) and glutamate dehydrogenase (GLDH) were determined with an automatic analysing system based on photometric measurements (Eurolyser, Type VET CCA, Salzburg, Austria).

In liver tissue samples, the total lipid content (TL) was determined by a gravimetric method [14]. Approximately 35 mg of liver tissue were homogenised and TL were extracted with hexane:isopropanol solution (mixing ratio 3:2, continuous agitation for 24 h, 20°C); values are expressed in mg/g fresh liver weight. SOD and GPx in erythrocyte lysate were determined in duplicates using commercially available enzyme kits (for analysis of SOD Randox SD125 and for analysis of GPx Randox RS505, Randox Laboratories, Crumlin, UK). For this, lysate of erythrocytes was prepared in 2 mL EDTA blood by adding 10 mL cold distilled water and followed by centrifugation (10 min, $10,000 \times g$, 4°C). The enzyme assays were conducted according to the manufacturer's protocols and adjusted in accordance with Bühler, et al. [15]. A coefficient of variation was calculated as measurement of precision for the enzyme assays and was considered acceptable $<10\%$. In those cows selected for liver biopsies the antioxidative capacity of plasma was measured by ferric-reducing ability of plasma (FRAP) assay according to methods of Benzie and Strain [16]. Hereby, the conversion of ferric tripyridyltriazine (Fe^{3+} -TPTZ) to ferrous tripyridyltriazine (Fe^{2+} -TPTZ) iron by extracellular antioxidants is measured, which results in the formation of a blue colored complex. The measurement of absorbance was conducted kinetically at 593 nm and 37°C , whereby the end-point value was taken at 15 min. Each sample was analysed in triplicate and the FRAP values were calculated in $\mu\text{mol/L Fe}^{2+}$ by use of a calibration curve of Fe^{2+} . For quantification of the serum concentrations of haptoglobin a specific Enzyme-linked Immunosorbent Assay method was conducted in those 40 cows according to Hiss, et al. [17].

Moreover, the serum concentrations of tryptophan and kynurenine were measured in a subset of 20 cows chosen for liver biopsy (5 of each group) at sampling times -14 , 8, 28 and 100 relative to calving via high-performance liquid chromatography (Shimadzu, Kyoto, Japan) after fat extraction with n-hexane and protein precipitation using cold ethanol according to methods described by Hüther, et al. [18].

2.4. Statistics

For statistical analyses, the MIXED procedure of SAS software package (SAS 9.4, SAS Inst. Inc., Cary, NC, USA [19]) for repeated measures was used. For evaluation of the influences of the feeding, the different energy concentrations of roughage (R), the varying allocations of concentrates (C), the time (experimental week (W) relative to parturition), and the interactions between these factors were used as fixed effects. Hereby, the values of

lactation period in which the different experimental rations were provided were used in statistical calculations, whereas the basal values of d -50 relative to calving were regarded as covariates in the statistical model.

Additionally, to investigate the effects of SCK on liver health and selected blood variables, cows were retrospectively subdivided into a SK group and a CON group irrespective of the original treatment assignment. Hereby, the statistical model included the presence of SCK (SCK; SK or CON groups) and time (T; experimental day relative to parturition) as well as the interactions between these factors as fixed effects. For this evaluation, all sampling times, including the antepartum period were included into statistics to investigate cow-individual effects. For both statistical models, the frequent measurements during the experiment for each cow were considered as repeated measurements and the individual cow as a random effect.

To define the best covariance structure, the lowest Akaike information criterion between unstructured, autoregressive, and compound symmetry was identified. Differences were considered to be significant at $p < 0.05$, and a tendency was defined for $0.05 \leq p \leq 0.10$. All variables are presented as least square means and standard error of the mean.

3. Results

3.1. Effects of Lactation Stage and Postpartum Energy Supply from Roughage and Concentrates

The haematological variables of the feeding groups during the postpartum period are illustrated in Table 2. The RBC count showed a $R \times C$ and a $C \times W$ interaction, which mainly occurred due to high differences between MRMC and MRHC groups (6.15 ± 0.12 vs. $5.72 \pm 0.12 \times 10^6$ cells/ μL), whereas the other groups exhibited a quite similar RBC. This $R \times C$ interaction was detected for the MCV and MCH ($p = 0.026$ and $p = 0.019$, respectively) as well. These variables also differed in groups receiving different proportions of concentrates, so that cows of the HC group showed an increased MCV and MCH. Furthermore, there was a tendency for a $C \times W$ interaction for haematocrit and haemoglobin, resulting from lower values at d 28 and d 100 in the MC groups compared to the HC groups. All variables of red blood cells showed a time effect during early lactation ($p < 0.001$). The red blood cell count, haematocrit and haemoglobin were elevated values during the peripartal period with highest values on d 8 after calving and decreasing levels until the end of the trial. MCV decreased during early lactation, whereas MCH showed an elevation in the course of lactation.

The WBC did not indicate any difference between the treatment groups (Table 2). We observed an $R \times C$ interaction for the percentage of granulocytes ($p = 0.034$) and a tendency for an $R \times C$ interaction for the percentage of lymphocytes in blood ($p = 0.070$). Hereby, the greatest differences were found between the MRMC and the MRHC group, so that the ratio of granulocytes to lymphocytes was 0.86 in MRMC group and 0.67 in HRHC group. The corresponding values of HRMC and HRHC groups ranged between them (0.71 and 0.77, respectively). Also, the absolute numbers of granulocytes and lymphocytes differed between MRMC and MRHC groups, leading to an $R \times C$ interaction for lymphocytes ($p = 0.046$) and for granulocytes, a tendency for an interaction ($p = 0.093$). Few effects of time were apparent on white blood counts; the WBC showed elevated values during the peripartal period with a peak at d 8 after parturition, which led to a tendency for lowering of WBC in the course of early lactation ($p = 0.069$). Furthermore, the absolute number of eosinophils decreased during early lactation ($p = 0.011$) and also their proportion in white blood cell count tended to be lower ($p = 0.064$).

Table 2. Effect of a moderate (MR) or a higher (HR) energy concentration in roughage and moderate (MC) or higher (HC) amounts of concentrates on haematological variables during the postpartum period.

	MR		HR		SEM ²	R	C	p-Value ¹		
	MC (n = 16)	HC (n = 16)	MC (n = 16)	HC (n = 15)				W ³	R × C	C × W
White blood cell count										
White blood cells [10 ³ /μL]	7.89	7.54	8.37	7.80	0.34	0.277	0.174	0.069	0.751	0.995
Granulocytes ⁴ [%]	48.6	56.0	54.4	53.0	2.0	0.491	0.135	0.862	0.034	0.237
Granulocytes ⁴ [10 ³ /μL]	3.87	4.30	4.78	4.16	0.31	0.204	0.759	0.213	0.093	0.718
Lymphocytes [%]	41.9	37.7	38.4	40.6	1.7	0.859	0.554	0.448	0.070	0.327
Lymphocytes [10 ³ /μL]	3.28	2.78	3.05	3.16	0.15	0.614	0.196	0.837	0.046	0.261
Eosinophils [%]	8.49	5.94	6.23	6.24	0.90	0.268	0.156	0.064	0.153	0.099
Eosinophils [10 ³ /μL]	0.61	0.47	0.50	0.47	0.06	0.379	0.140	0.011	0.371	0.099
Monocytes [%]	0.86	0.64	0.59	0.53	0.10	0.169	0.311	0.849	0.560	0.732
Monocytes [10 ³ /μL]	0.07	0.04	0.04	0.03	0.01	0.124	0.147	0.830	0.384	0.313
Red blood cell count										
Haematocrit [%]	28.3	28.3	28.1	28.5	0.5	0.936	0.664	<0.001	0.702	0.087
Haemoglobin [mmol/L]	5.54	5.63	5.51	5.63	0.09	0.908	0.282	<0.001	0.875	0.079
Red blood cells [10 ⁶ /μL]	6.15	5.72	5.85	5.93	0.12	0.714	0.163	<0.001	0.042	0.027
Mean corpuscular volume [fL]	46.2	49.7	48.0	48.1	0.7	0.845	0.015	<0.001	0.026	0.697
Mean corpuscular haemoglobin, [pg]	14.6	16.1	15.3	15.4	0.3	0.973	0.006	<0.001	0.019	0.915

Values are presented as LS-means; ¹ Effects of energy concentration in roughage (R), amounts of concentrates (C) and week of lactation (W) as well as the interactions R × C and C × W, p-values for “R × W” and “R × C × W” interactions were >0.05 for all variables; ² Standard error of the mean; ³ weeks 1–16 in lactation; ⁴ including neutrophil and basophil granulocytes.

The effects of treatment on biochemical blood variables are displayed in Table 3. The total protein content in serum was with 71.5 ± 0.5 g/L higher in HC groups compared to MC cows with 69.7 ± 0.5 g/L during the lactation period ($p = 0.018$). Cows receiving roughage containing the lower energy concentration tended to have less blood cholesterol (3.75 ± 0.1 mmol/L in LR vs. 4.09 ± 0.1 mmol/L in HR groups, $p = 0.051$). The other biochemical blood variables were not affected by treatments, but all variables displayed significant changes during early lactation with the exception of GLDH activity. The concentrations of total protein, albumin, urea, triglycerides, and cholesterol in blood were lowered during the periparturient period and showed ascending values throughout early lactation ($p < 0.001$). In contrast, AST activity and total bilirubin content exhibited a peak on d 8 postpartum ($p < 0.001$ and $p = 0.002$, respectively) and GGT activity had elevated values on days 28 and 100 ($p < 0.001$). The AP activity was reduced during early lactation with a nadir at d 28 postpartum ($p < 0.001$).

Table 3. Effect of a moderate (MR) or a higher (HR) energy concentration in roughage and moderate (MC) or higher (HC) amounts of concentrates on biochemical metabolites and liver enzymes during the postpartum period.

	MR		HR		SEM ²	<i>p</i> -Value ¹		
	MC (n = 16)	HC (n = 16)	MC (n = 16)	HC (n = 15)		R	C	W ³
Albumin [g/L]	36.0	35.9	36.5	36.0	0.5	0.545	0.614	<0.001
Cholesterol [mmol/L]	3.72	3.79	4.06	4.10	0.16	0.051	0.738	<0.001
Total protein [g/L]	68.5	71.5	70.8	71.4	0.7	0.143	0.018	<0.001
Total bilirubin [μ mol/L]	5.88	6.03	6.49	6.30	0.34	0.186	0.947	<0.001
AST ⁴ [μ kat/L]	1.57	1.52	1.57	1.52	0.10	0.973	0.622	0.002
GLDH ⁵ [μ kat/L]	0.45	0.43	0.32	0.43	0.12	0.578	0.701	0.373
AP ⁶ [μ kat/L]	0.76	0.72	0.70	0.74	0.05	0.641	0.960	<0.001
GGT ⁷ [μ kat/L]	0.54	0.58	0.61	0.56	0.04	0.516	0.830	<0.001
Triglycerides [mmol/L]	0.139	0.139	0.142	0.139	0.008	0.837	0.849	0.001
Urea [mmol/L]	3.50	3.29	3.15	3.16	0.15	0.128	0.543	<0.001
Uric acid [μ mol/L]	71.92	70.30	72.44	71.69	2.98	0.755	0.701	<0.001

Values are presented as LS-means; ¹ Effects of energy concentration in roughage (R), amounts of concentrates (C) and week of lactation (W); *p*-values for "R \times C", "R \times W", "C \times W" and "R \times C \times W" interactions were >0.05 for all variables; ² Standard error of the mean; ³ weeks 1–16 in lactation; ⁴ AST = aspartate aminotransferase; ⁵ GLDH = glutamate dehydrogenase; ⁶ AP = alkaline phosphatase; ⁷ GGT = γ -glutamyltransferase.

In Table 4, the liver fat content as well as the indicators for antioxidative capacity and inflammation during early lactation are presented. The liver fat content did not differ between the treatment groups but displayed elevated values after calving compared to antepartum period with a peak amounting to 120 ± 7 mg/g on d 8 postpartum ($p < 0.001$). The activities of the antioxidative enzymes GPx and SOD in erythrocyte lysates and the FRAP were not affected by treatment, but values of all variables increased throughout early lactation ($p < 0.001$). Furthermore, the K:T ratio and the blood concentration of haptoglobin were not affected by treatments but by time. Haptoglobin was increased over the course of early lactation, exhibiting with 1146 ± 284 μ g/mL a peak on d 8 after calving ($p = 0.002$). The concentrations of kynurenine and tryptophan increased in the course of early lactation and the K:T ratio tended to be elevated on d 28 postpartum ($p = 0.063$).

Table 4. Effect of a moderate (MR) or a higher (HR) energy concentration in roughage and moderate (MC) or higher (HC) amounts of concentrates on liver fat content and inflammatory variables during the postpartum period.

	MR		HR		SEM ²	<i>p</i> -Value ¹		
	MC	HC	MC	HC		R	C	W ³
Number of cows	10	10	10	10				
Liver fat content [mg/g]	92.9	85.6	72.4	94.6	11.5	0.616	0.522	<0.001
GPx ⁴ [mU/mL haemoglobin]	2349	2264	2257	2122	129	0.367	0.406	<0.001
SOD ⁵ [mU/mL haemoglobin]	64.6	59.0	55.9	59.3	4.1	0.314	0.792	<0.001
FRAP ⁶ [μmol/L]	297	303	320	310	11	0.157	0.856	<0.001
Haptoglobin [μg/mL]	705	1044	536	450	326	0.251	0.701	0.009
Number of cows	5	5	5	5				
Kynurenine (K) [mg/L]	1.02	0.82	1.06	1.05	0.12	0.242	0.384	<0.001
Tryptophan (T) [mg/L]	5.16	5.23	5.75	5.46	0.37	0.280	0.772	<0.001
K:T ratio ⁷	0.17	0.18	0.18	0.18	0.02	0.685	0.964	0.063

Values are presented as LS-means; ¹ Effects of energy concentration in roughage (R), amounts of concentrates (C) and week of lactation (W), *p*-values for "R × C", "R × W", "C × W" and "R × C × W" interactions were >0.05 for all variables; ² Standard error of the mean; ³ Weeks 1–16 in lactation; ⁴ GPx = glutathione peroxidase; ⁵ SOD = superoxide dismutase; ⁶ FRAP = ferric-reducing ability of plasma; ⁷ K:T ratio = kynurenine to tryptophan ratio.

3.2. Influence of Subclinical Ketosis

In addition to investigating the treatment effects, we retrospectively analysed the effects of SCK on liver health as well as on inflammatory and antioxidative blood variables (Table 5). Throughout the trial the BHB concentration in blood was higher in the SK group compared to the CON group (0.87 ± 0.03 mmol/L vs. 0.59 ± 0.02 mmol/L; $p < 0.001$). The SK group had a 34% higher liver fat content over the course of the trial compared to the CON group, leading to a SCK × W interaction ($p < 0.001$, Figure 1). The greatest difference between SK and CON groups was observed on d 28 after parturition, when the liver fat content of the SK group was almost twice as high as in the CON group. Also, the fat-to-protein ratio in milk was higher in the SK group compared to the CON group (1.40 ± 0.02 vs. 1.29 ± 0.02 ; $p < 0.001$). We also detected significantly increased activities of the liver enzyme activities of AST and GGT in cows with SCK, whereby AST activity peaked at 8 days, and GGT at 28 days after calving (Figure 2). These differences resulted in SCK × W interactions ($p < 0.001$ and $p = 0.024$, respectively). Furthermore, on d 8 postpartum, the level of total bilirubin in blood of SK cows was elevated compared to CON cows, leading to an effect of SCK ($p = 0.005$) and a SCK × W interaction ($p < 0.001$).

In white blood count, the percentage of granulocytes differed between SK and CON cows ($p = 0.026$), whereby the cows of the SK group showed with $54.2 \pm 1.3\%$ a higher proportion of granulocytes compared to CON group in which the percentage was $50.7 \pm 0.9\%$. The other constituents of white blood count were not affected by SCK. Also, the haematological variables of red blood cell count showed only marginal differences between the SK and the CON group in our trial (data not shown). The activity of GPx tended to be increased in SK group ($p = 0.088$), whereas the activity of SOD, the FRAP, and the haptoglobin concentration in blood were not significantly different between SK and CON cows. Also, the blood concentrations of kynurenine and tryptophan as well as the K:T ratio did not diverge between the treatment groups.

Table 5. Influences of subclinical ketosis (SCK) on liver fat content and selected blood variables during the periparturient period and early lactation.

	Incidence of SCK ¹		SCK	p-Value	
	Control Group	SK Group		Week	SCK × Week
Number of cows	44	19			
β-hydroxybutyrate (mmol/L)	0.59 ± 0.2	0.87 ± 0.3	<0.001	<0.001	<0.001
White blood cells [10 ³ /μL]	7.79 ± 0.2	7.68 ± 0.3	0.780	<0.001	0.853
Lymphocytes [%]	40.46 ± 0.9	37.81 ± 1.4	0.119	0.107	0.568
Eosinophiles [%]	7.82 ± 0.43	7.16 ± 0.64	0.396	0.003	0.596
Monocytes [%]	0.97 ± 0.08	0.80 ± 0.11	0.236	0.004	0.513
Granulocytes ² [%]	50.7 ± 0.9	54.2 ± 1.3	0.026	<0.001	0.978
Total bilirubin [μmol/L]	5.64 ± 0.17	6.33 ± 0.17	0.005	<0.001	<0.001
AST ³ [μkat/L]	1.42 ± 0.07	1.47 ± 0.1	0.662	<0.001	<0.001
GLDH ⁴ [μkat/L]	0.32 ± 0.04	0.40 ± 0.06	0.315	0.001	0.330
AP ⁵ [μkat/L]	0.82 ± 0.03	0.76 ± 0.04	0.219	<0.001	0.226
GGT ⁶ [μkat/L]	0.54 ± 0.02	0.56 ± 0.03	0.500	<0.001	0.024
Triglycerides [mmol/L]	0.203 ± 0.001	0.199 ± 0.008	0.726	<0.001	0.047
Number of cows	26	14			
Liver fat content [mg/g]	63.4 ± 4.2	96.3 ± 5.7	0.616	0.522	<0.001
GPx ⁷ [mU/mL HG]	2039 ± 77	2269 ± 106	0.088	<0.001	0.961
SOD ⁸ [mU/mL HG]	54.8 ± 2.3	61.3 ± 3.2	0.105	<0.001	0.201
FRAP ⁹ [μmol/L]	287 ± 6	285 ± 8	0.878	<0.001	0.702
Haptoglobin [μg/mL]	420 ± 128	728 ± 174	0.155	0.002	0.265
Number of cows	12	8			
Kynurenine (K) [mg/L]	0.96 ± 0.07	1.02 ± 0.08	0.599	<0.001	0.133
Tryptophan (T) [mg/L]	5.44 ± 0.18	5.31 ± 0.20	0.623	<0.001	0.277
K:T ratio ¹⁰	0.17 ± 0.01	0.19 ± 0.01	0.296	0.157	0.798

¹ cows of the SK group were classified by BHB values > 1.2 mmol/L in at least one of the blood samples collected postpartum; ² including neutrophil and basophil granulocytes; ³ AST = aspartate aminotransferase; ⁴ GLDH = glutamate dehydrogenase; ⁵ AP = alkaline phosphatase; ⁶ GGT = γ-glutamyltransferase; ⁷ GPx = glutathione peroxidase; ⁸ SOD = superoxide dismutase; ⁹ FRAP = ferric-reducing ability of plasma; ¹⁰ K:T ratio = kynurenine to tryptophan ratio.

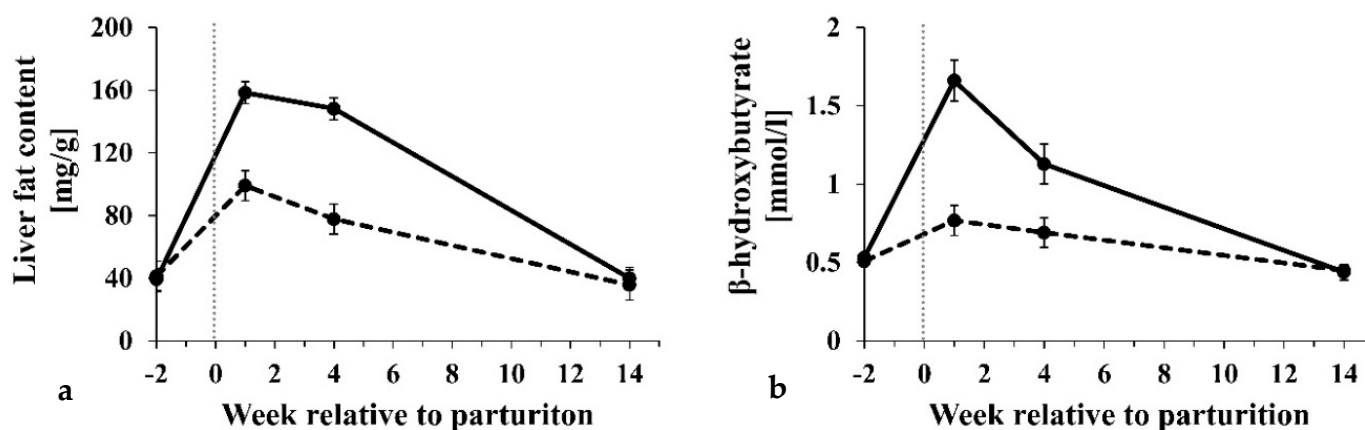


Figure 1. Time course of liver fat content (a) and serum concentrations of β-hydroxybutyrate (b) in cows of subclinical ketosis group (—) and control group (---) in course of the trial (LS-means with standard error of the mean); significance of subclinical ketosis (SCK) and time for liver fat content: SCK: $p < 0.001$, week: $p < 0.001$, SCK × week: $p < 0.001$; significance of SCK and time for concentrations of β-hydroxybutyrate: SCK: $p < 0.001$, week: $p < 0.001$, SCK × week: $p < 0.001$.

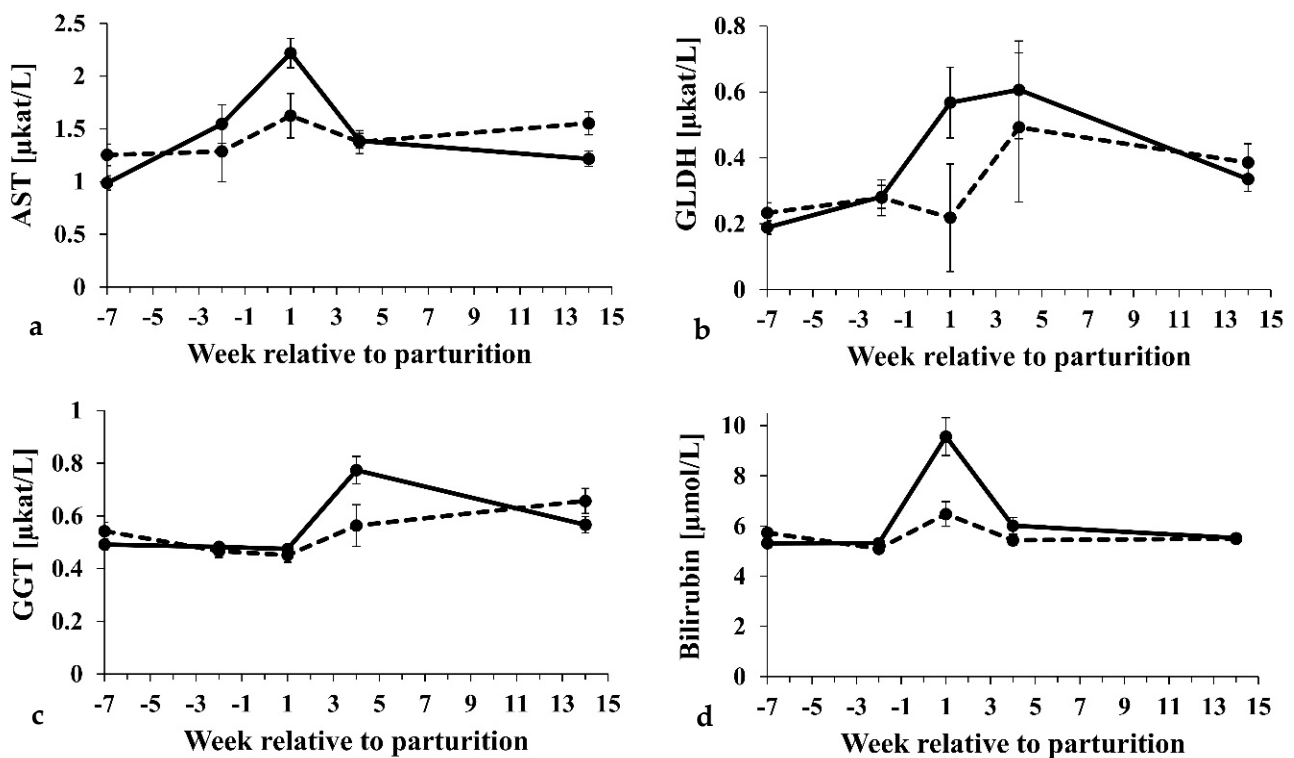


Figure 2. Time course of blood concentrations of aspartate aminotransferase (AST, (a)), glutamate dehydrogenase (GLDH, (b)), γ -glutamyltransferase (GGT, (c)) and bilirubin (d) in subclinical ketosis group (—) and control group (- - -) during the trial (LS-means with standard error of the mean); significance of subclinical ketosis (SCK) and time for AST: SCK: $p = 0.662$, week: $p < 0.001$, SCK \times week: $p < 0.001$; significance of SCK and time for GLDH: SCK: 0.315, week: $p = 0.001$, SCK \times week: $p < 0.330$; significance for GGT: SCK: $p = 0.315$, week: $p < 0.001$, SCK \times week: $p = 0.024$; significance for bilirubin: SCK: $p = 0.005$, week: $p < 0.001$, SCK \times week: $p < 0.001$.

4. Discussion

The purpose of the present study was to investigate the effects of a varying energy supply from roughage and concentrates on haematological, biochemical, inflammatory and antioxidative blood variables and, furthermore, to determine how these variables are altered in cows with SCK. Data of performance, energy balance, energy efficiency and metabolic variables of this experiment were presented previously [12]. Briefly, the higher allocation of concentrates in HC cows resulted on average in a 1.8 kg/d higher DMI and consequently in higher energy intakes. This also led to enhanced performance, but the increased energy concentration in roughage could largely counterbalance reduced intake of concentrates without considerable losses in milk yield. In addition, the energy balance was improved in HC groups, but this increased energy balance did not affect animal health or metabolic blood variables such as BHB, NEFA, glucose, insulin, insulin-like growth factor I and adiponectin, but resulted in a decline of energy efficiency.

In the present study we observed just minor effects of the treatments on blood chemistry and liver health, whereas most of the variables showed alterations in course of the peripartal period. The haematological variables were in all treatment groups in the physiological range according to Kraft [20], and levels as well as changes throughout the peripartal period were similar to Drong et al. [21]. During early lactation, the proportion of granulocytes was increased in all groups, which is a common observation during that period [22,23]. An increased release of granulocytes could be an indicator for inflammatory processes; but also exercise and excitement, leading to adrenaline release, could cause a mobilisation of granulocytes [23]. It was noticeable that the differences in haematological variables were more pronounced between groups receiving roughage with lower energy concentration. This observation indicates that especially the limited energy supply from

roughage could be a nutritional challenge for the cows. Hereby, it is interesting that cows of MRMC group showed a lower proportion of granulocytes and a slightly higher proportion of lymphocytes compared to MRHC cows, which was most pronounced during the peripartal period. The low concentration of granulocytes in these animals may indicate that the low energy consumption of this group did not trigger acute inflammatory events, which concurs with the absence of significant influences of energy supply on health reported in Schmitz et al. [12]. In this group also, the RBC was increased combined with lowered MCV and MCH values and slightly reduced bilirubin concentration in blood, which could be signs of a reduced turnover rate of erythrocytes [24]. Also, most variables of biochemical blood variables remained on physiological levels in all treatment groups without clear signs of treatment effects. The decrease in total protein in MC groups was probably mainly caused by low protein levels in MRMC cows, reflecting the reduced milk protein production of this group, which was observed previously in Schmitz, et al. [12]. The drop of cholesterol after calving is a common phenomenon at the onset of lactation, even though the synthesis of cholesterol is maximised at that time [25]. This drop occurs due to an altered lipoprotein synthesis from hepatocytes, because high density lipoproteins are primarily produced at the expense of low-density lipoproteins, which are sharply reduced and contain the greatest amount of cholesterol [26]. The higher energy concentration of roughage increased the cholesterol production of HR cows compared to MR cows. Cholesterol is closely connected to liver metabolism and transport of fatty acids via very low-density lipoproteins [25], so that the enhanced blood concentration might indicate an improved liver health. However, we did not see any effects of treatment on liver fat content or liver enzymes. Furthermore, also inflammatory and antioxidative blood variables were unaffected by treatment. These findings are in accordance with the outcome of the underlying feeding experiment, because the differences in postpartum energy supply merely led to slight differences in blood and liver variables. This outcome indicates that cows were able to cope with the different experimental rations without necessarily exhibiting metabolic disorders.

However, in all groups, some individual animals were not able to adjust their metabolism to the high energy demands of early lactation so that they developed a metabolic derailment, resulting in SCK. Therefore, we retrospectively grouped cows that showed increased BHB values greater than 1.2 mmol/L in at least one postpartum blood sample so that they were expected to suffer from SCK and then compared their data with the inconspicuous CON group. The increased lipomobilisation of SK group was reflected in an increased fat-to-protein ratio in milk, which is an appropriate indicator for ketosis and the extent of NEB [27,28]. As expected, SCK was associated with an increase of liver fat content, showing the close relationship between the occurrence of ketosis and fatty liver syndrome [29,30]. The accumulation of fat in liver cells can result in damages, which explains the more pronounced postpartum increase of the liver enzyme activities in the SK group. Hereby, the enzyme activity of AST showed an earlier response at day 8 postpartum as compared to GGT, which only showed a difference after 4 weeks. These observations concur with findings of Kessel, et al. [31], who observed higher activities for AST in week 2–4 postpartum and for GGT in week 3–8 postpartum. Furthermore, the increased liver fat content can result in a reduced liver function, so that cows with SCK exhibited increased concentration of bilirubin in blood. According to Bobe, Young, and Beitz [3] the elevated bilirubin level could be caused by a decreased bile flow in fatty liver. In addition, the increased susceptibility to inflammation during the peripartal period contributes to impaired liver function, which is also related to reductions in performance [32]. On the other hand, metabolically compromised liver function also contributes to an impaired immune competence due to reduced protein synthesis of the liver tissue. The inflammatory conditions must not necessarily be obvious as clinical disease, but may become manifest in abnormalities of blood count. The increased concentrations of leucocytes and especially granulocytes in the peripartal period indicate intensified immune reactions, whereby only the percentage of granulocytes showed differences in cows of the SK compared to the CON group. SCK and fatty liver syndrome also affect the functionality of neutrophil granulocytes, so

that these metabolic disorders impair immunity of cows and increase the susceptibility for metritis [33].

The increased blood concentration of BHB and the elevated liver fat content alter the protein synthesis in hepatocytes. Generally, during the peripartal period the concentrations of positive acute-phase proteins like haptoglobin and serum amyloid A are increased, while negative acute-phase proteins like albumin are decreased [32,34]. The incidence of ketosis and fatty liver syndrome lead to a shift in gene network expression, so that protein synthesis, de novo fatty acid synthesis and cholesterol synthesis as energy-intensive processes are reduced. In a study of Looor, et al. [35], a nutritionally induced ketosis led to upregulation of some inflammation-related genes and acute-phase proteins, whereas other proinflammatory genes were unaffected. However, it seems to be unclear if the changes in expression of these genes are an effect of liver lipidosis and ketosis or, more likely a generalised reaction of immune and liver cells on disturbed homeostasis.

The antioxidative enzymes GPx and SOD show an elevated activity in course of lactation, which is necessary to compensate the increased oxidative stress at this time. The mobilisation of body fat, associated with release of NEFA and the increased metabolic efforts to ensure high milk production, lead to elevated production of oxygen radicals [36,37]. In our study, the cows of the SK group tended to have a higher activity of GPx compared to the CON group and also the activity of SOD was numerically higher in this group. The increased oxidative stress in cows suffering by SCK was also observed by Xu et al. [36]. Presumably, also increased inflammatory conditions associated with SCK could contribute to a higher production of oxygen radicals. In a recent study, Urh et al. [38] observed differences in the pro-oxidative and antioxidative balance between cows of two different farms during early lactation. Cows at the farm with increased oxidative status exhibited increased BHB concentrations in blood and showed a greater loss in body condition, which might be associated with increased metabolic or health problems of animals in this farm. We assume that there is a close interrelation between SCK, impaired liver function, compromised immune-responsiveness and oxidative stress, which is primarily caused by a more pronounced NEB around calving. In this context, it was also conceivable that the occurrence of SCK could influence the K:T ratio in serum; under systemic inflammatory conditions, the enzyme indoleamine-2,3-dioxygenase has an increased activity, which promotes the metabolism of tryptophan to kynurenine [9,10]. In our study, both the kynurenine and the tryptophan level in blood dropped at day 8 postpartum, but we did not observe any influence of treatment on these variables or the kynurenine-to-tryptophan ratio. This indicates that ketotic conditions may not necessarily influence the activity of the enzyme indoleamine 2,3-dioxygenase. This result is corroborated by studies in which peripartal inflammatory diseases like mastitis and metritis were not affecting the kynurenine-to-tryptophan ratio [39,40].

5. Conclusions

The results of the present study illustrate the important challenges for the immune system and metabolism which have to be overcome by dairy cows during peripartal period and early lactation. The cows were able to adjust their metabolism to different energy supplies from the rations provided without necessarily developing metabolic disorders, so that only slight differences in haematological variables and liver fat content occurred between the treatment groups. In general, feeding with different proportions of concentrates without obvious strain on the metabolism is possible if high-quality roughage is provided. However, our results emphasise that individual cows are at high risk for developing a metabolic derailment in this period that is often associated with subclinical ketosis and fatty liver syndrome. These diseases are closely connected with impaired liver function, compromised immune-responsiveness and elevated oxidative stress with adverse consequences on health and productivity of dairy cows.

Author Contributions: Conceptualization: S.D., U.M., H.S. (Hubert Spiekers), R.S.; Methodology: J.F., L.H., H.S. (Helga Sauerwein); Statistical analyses: R.S., S.D., J.F., D.v.S.; Sample collection: R.S., D.v.S., K.S.; Writing—Original Draft Preparation: R.S.; Writing—Review & Editing: U.M., D.v.S., L.H., J.R., H.S. (Hubert Spiekers), H.S. (Helga Sauerwein), K.S., J.F., S.D.; Supervision: S.D., H.S. (Hubert Spiekers), J.R.; Project Administration: H.S. (Hubert Spiekers), U.M.; Funding Acquisition: H.S. (Hubert Spiekers), S.D., U.M. All authors have read and agreed to the published version of the manuscript.

Funding: The project is part of the optiKuh programme and was supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office of Agriculture and Food (BLE) under the innovation support programme.

Institutional Review Board Statement: Experimental work was performed in accordance to the German Animal Welfare Act approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany, No. 33.19-42502-04-15/1858).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors thank the staff of the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut (FLI) for their great support in taking care of animals, sample collection and data collection. Furthermore, we thank the laboratory staff of the Institute of Animal Nutrition in Brunswick, the University of Veterinary Medicine in Hanover and the University of Bonn for their assistance in performing analyses.

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

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