Journal of Animal and Feed Sciences, 35, 1, 2026, e10, https://doi.org/10.22358/jafs/205432/2026
The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, Jabłonna

ORIGINAL PAPER

On-farm detection of subclinical ketosis – an investigation on potential indicators

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KEY WORDS: glucose, hyperketonaemia, ketone bodies, NEFA, predictor

Received: 7 February 2025
Revised: 5 May 2025
Accepted: 21 May 2025

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ABSTRACT. In early lactation, dairy cows can develop metabolic imbalances like a significant energy deficit and in consequence subclinical ketosis. First of all β–hydroxybutyrate (BHBA), but also non-esterified fatty acids (NEFA) as well as glucose (Glc) are used to determine the metabolic state of cows in terms of subclinical ketosis (SCK) in blood serum. In this field study in ten commercial dairy farms, ten different non-invasive indicators associated with the risk of developing SCK and obtainable without handling of the cow were investigated in relation to BHBA, NEFA and Glc in early lactating dairy cows. Effects of the indicators were examined using correlation and linear mixed models. Established indicators like fat:protein ratio (FPR) or days in milk showed the largest potential for predicting BHBA and also NEFA and Glc. Parity had no significant effect on the model, except for Glc. In the analysis of covariance, an influence of parity was found between first and 2nd to 4th lactation for NEFA and Glc, however the picture was less clear for BHBA. While time for feeding and/or ruminating showed significant influence for all three metabolites, the direction of influence was not always as expected. The study supports the validity of FPR in the prediction of blood BHBA; the other non-invasive indicators investigated can help to identify cows at risk for developing subclinical ketosis, but under the conditions of practical farming like in the present study they were not perfectly suited to replace established systems requiring some handling of the cow.

Introduction

Subclinical ketosis (SCK) is one of the most important metabolic diseases in dairy cows at the start of lactation. Due to its high incidence and prevalence in dairy herds it has a great impact on animal welfare, involving secondary diseases such as displaced abomasum and fatty liver. In addition, the decrease in milk production and impaired fertility also result in significant economic losses. Earlier studies found the most critical period for SCK to be within the first 65 days postpartum with a peak at 21–25 days in milk (DIM) (Dohoo and Martin, 1984; Andersson, 1988). More recent studies that conducted more frequent testing reported an even earlier peak incidence and prevalence at 5 DIM (McArt et al., 2012b). During this phase an increased energy demand, resulting from the onset of lactation and the inability to meet this demand solely by feed intake, leads to a negative energy balance.

This is characterized by an increase of ketone bodies and non-esterified fatty acids (NEFA) in body fluids as well as a low glucose (Glc) level in blood. To a certain extend this increase is physiological, but high levels of ketone bodies like β–hydroxybutyrate (BHBA), acetone and acetoacetate have a negative effect on the metabolism of the cow. Defined is SCK as an increase in ketone bodies in blood, milk and urine. Different studies defined thresholds of BHBA for SCK, the most common is ≥1.2 mmol/l in the blood serum (McArt et al., 2013). McArt et al. (2012b) found that for 43% of the cows the energy deficit leads to SCK. McArt et al. (2012a) described postpartum incidences of SCK between 26 and 56% within 3 to 16 DIM in 4 US herds. Clinical ketosis was defined as BHBA ≥3.0 mmol/l blood serum (Oetzel, 2004). Berge and Vertenten (2014) reported an average incidence of clinical ketosis of 1.6% in 4 709 cows from 131 herds throughout Western Europe, while Ospina et al. (2010) described an incidence of clinical ketosis of 4.6% for 1 314 cows (1–30 DIM) (US herds).

On-farm indicators for SCK that are used by farmers are commercially available rapid test systems that measure the ketone concentration in blood, milk or urine (Jansen et al., 2021). Most of these test systems require to fixate and/or isolate the cow to take samples. Every treatment performed on the cows has the potential to impose at least some stress on them (Herskin et al., 2004) and includes significant time effort for the person performing it. Therefore, easily quantifiable risk factors and indicators that can be automatically recorded without disrupting routines and with minimal labour investment are likely to benefit the health and performance of early lactating dairy cows. They are also easier to be included in daily routines in high frequency. Milk yield, DIM and parity can be regarded as such potential risk factors; feeding and rumination time as well as rumen fill can be seen as indicators related to feed intake (as the major variable causing an energy deficit at the start of lactation), while fat:protein ratio (FPR) and a change in body condition score can be regarded as indicators of an already established energy deficit.

So, the aim of the study was to explore whether the detection of SCK on farm can be improved by identifying indicators that are easily obtainable, easily implemented in the farm routines and as minimally invasive as possible. To test the reliability of the variables in on-farm conditions, this study was conducted on 10 commercial dairy farms in northwestern Germany.

Material and methods

Animals

The experiment was performed in accordance with the German Animal Welfare Law and approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, Oldenburg, Germany; AZ: 33.9-42502-05-17A106).

Data was collected from April 2017 to March 2018 on 10 commercial dairy farms in northwestern Germany. The herds were kept in loose-housing stalls with resting cubicles. All 10 farms participated in the monthly official milk performance recording with the standard method AS4 which is approved by the International Committee for Animal Recording (ICAR). The cows were milked in conventional milking parlours and had no access to pasture. The study was conducted over three weeks on each farm, consisting of one preparation week (week 0) and 11 days of trial (weeks 1 and 2). Per farm, 10 Holstein Friesian dairy cows were chosen for the trial. They were selected by calving date close to the main sampling period (estimated parturition date within 2–3 weeks before the start). The parities were random due to the first requirement. On every farm one or two heifers were included, if possible. The data collection was integrated into the daily routines of the respective farm.

Sampling

Blood samples were collected on days 2, 4, 9 and 11 from the coccygeal vessels into 6 ml tubes with clot activator (BD Vacutainer, Clot Activator Tube/Ref 368815, BD, Becton Dickinson GmbH, Heidelberg, Germany) for analysis of BHBA and NEFA. For the analysis of Glc, blood was collected in 4 ml tubes with NaF (VACUETTE® FE Sodium Fluoride/ K3EDTA, Greiner Bio-One International GmbH, Kremsmünster, Austria) to inhibit glycolysis. The blood samples were left for clotting for approx. 2 h. After that they were centrifuged for 10 min at 2 450 x g, two aliquots of serum from each tube were stored in vials at −18 °C until transportation to the laboratory for analysis. BHBA concentrations were measured with the RANBUT RX MONZA BHBA Kit, No. RB 1007 (Randox, Crumlin, UK). Glucose concentration was determined with the hexokinase-method with the test kit Konelab™ / T series, Glucose (HK) (Thermo Fischer Scientific Oy Clinical Diagnostics Finland, Vantaa, FIN). NEFA was measured with the enzymatic endpoint method (colorimetric) with the test kit LT-FA 0022 (Labor + Technik, LT-SYS©, Eberhard Lehmann GmbH,

Berlin, Germany). With each test kit the respective substance was measured photometrically in the Konelab 20i (Thermo Fisher Scientific, Passau, Germany) in the laboratory of the Institute of Veterinary Medicine at the Georg-August-University Göttingen.

Milk samples were collected on days 1 to 4 and 8 to 11 in the evening and in the morning. The analysed milk parameters of two milk samples (evening and morning) were pooled weighted according to the yield of the samples. On eight farms milk samples were collected by automatic sampling systems integrated in the conventional milking parlours which are calibrated once a year for the use in the monthly official milk performance recording. Two farms had no automatic sampling system, therefore, the milk samples were taken with Trutester (Trutest Ltd., New Zealand), provided by the Landeskontrollverband Weser-Ems. The samplers are officially calibrated yearly as well. After taking the milk samples out of the sampling system the milk was homogenized by pouring it three times from one container into another before it was put into a bottle containing bronopol for conservation. The bottle was shaken to mix the milk with the conservative. Until transport to the laboratory, the milk was stored at room temperature for a maximal time period of 24 h. The analysis with middle-infrared-spectroscopy was conducted with the FOSS MSC FT+. The parameters that are used in this analysis are daily milk yield (milk) and the FPR.

The faecal score (FS) and rumen fill score (RFS) were recorded at the time of the blood sampling in the morning at days 2, 4, 9 and 11 after the milking. The FS was evaluated based on the manure scoring system as reported by Hutjens (2010). The RFS was determined according to Zaaijer and Noordhuizen (2003). The body condition score (BCS) of the cows in the trial was evaluated during the preparation week as well as on day 12 of the trial according to Edmonson et al. (1989). From the two time points, the difference (BCSd) was calculated for each cow as a measure of fat mobilization and energy deficit and it was determined if the difference is larger or equal 0.5. For further analysis, this variable was converted into 0 (difference <0.5) and 1 (difference ≥0.5). All three scores were obtained by two observers that coordinated and harmonized their scoring before the trial and before each period on farm.

All cows in the trial were fitted with a RumiWatch® noseband sensor (ITIN + HOCH GmbH, Liestal, Switzerland) as well as the RumiWatch® pedometer (ITIN + HOCH GmbH, Liestal, Switzerland) during the preparation week to ensure adaptation to the system not only for the cows fitted with it but also the herd to reduce exploring behaviour during the trial. Feeding time and rumination time were recorded continuously over the trial period with the noseband sensor. After each recording period the data was transferred via USB to the computer. In the provided Software RumiWatch® Manager the specific chewing patterns were interpreted, using an algorithm, into different behaviours like time spend on feeding, ruminating and drinking. The pedometer contains a location sensor and an accelerometer. It was attached to one hind leg of the cows. After each recording period the data was transferred as well to the computer via USB. The motion data recorded was interpreted with the Software RumiWatch® Manager into motion patterns like time standing, walking, resting (lying down). The measurements were continuous throughout the two weeks of trial. Both datasets were converted with the RumiWatch® Converter V0.7.4.13 into 1-h resolutions.

Feed analysis

The rations of the herds represent the standard diets used on the farms. In general, they were calculated by a dairy nutritionist and balanced according to the requirements of dairy cows in early lactation (DLG-Arbeitskreis Futter und Fütterung, 2012). Samples of fresh feed were taken on days 1, 3, 8 and 10 immediately after feed distribution at three different locations along the trough (beginning, middle, end). The leftover feed was sampled the following day at three locations as well. The samples were each mixed, vacuumized and stored at −20 °C. The feed was analysed according to VDLUFA Methods (crude protein (XP): VDLUFA 4.1.2; crude fat (XL): VDLUFA 5.1.1; crude fibre (XF): VDLUFA 6.5.1, amylase-treated neutral detergent fibre with ash correction (aNDFom): VDLUFA 6.5.1, sugar: VDLUFA 7.1.3); starch was enzymatically determined according to Brandt et al. (1987). Energy content was calculated according to Boguhn et al. (2003) and GfE (2020). The dry matter intake (DMI) was calculated per group by weighing feed at feeding time and the leftover feed, as well as analysing dry matter of the samples.

Statistical analysis

Statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA) and Microsoft Excel 2016 (Microsoft Corp., Redmond, WA, USA). Descriptive analyses were performed with PROC MEANS in SAS. For further analysis, BHBA

and NEFA were log transformed to obtain normal distribution. In the following BHBA and NEFA log transformed is referred to as BHBA and NEFA. Outliers (identified as more than two standard deviations from the average) in the data were checked for plausibility and removed from the dataset if implausible. Data was compared with other recorded data, such as BHBA measured with handheld devices or the FPR as well as data from the days before and after. Statistical significance level was set at $P \leq 0.05$. Parity was categorized: 1 – first lactation, 2 – second lactation, 3 – third lactation and 4 – fourth lactation and higher.

Analysis of incidence

Incidence of SCK was calculated with PROC FREQ in SAS. The incidence is the rate of newly occurring cases of SCK within a period of time. It was calculated as the proportion of all new cases of SCK within the trial period divided by the number of cows at risk. SCK positive cows were classified based on the BHBA concentration in blood serum. For Glc the incidence was calculated as well. The applied threshold for BHBA was ≥1.2 mmol/l and for Glc ≤2.2 mmol/l (McArt et al., 2013; Dubuc and Buczinski, 2018).

Analysis of association between parameters and indicators

To explore the association between the parameters BHBA, NEFA and Glc in blood with possible indicators for SCK, the Pearson correlation coefficients were calculated with PROC CORR in SAS. The interpretation is conducted according to Taylor (1990).

The effects of the indicators on the BHBA, NEFA and Glc in blood serum were examined using linear mixed models (LMM). The analysis was performed using PROC MIXED and PROC GLIMMIX in SAS. In each model the indicators were tested for fixed effects. If the indicator showed $P \leq 0.05$ the effects stayed in the model. Parity class was always left in the model due to its reported important influence on the three investigated factors. Dependent variables are BHBA, NEFA and Glc, independent variables that were tested for significant effects are DIM, parity, milk, FPR, RFS, time feeding, time ruminating, time resting, FS as well as BCSd. Even though there is no significant effect for parity in the models with the predictable variables NEFA and BHBA, parity is reported in literature as one of the most important factors (Seifi et al., 2011; Vanholder et al., 2015). Therefore, the influence of this categorical variable on the dependent variables was tested using an analysis of covariance. Farm and cow were random effects in each model, with cow nested within farm. Backward selection ($P \leq 0.05$) was used to determine significant fixed and interaction effects. Basic model for backward selection:

$$y_i = \mu + \alpha_i + \beta_1 DIM_i + \beta_2 milk_i + \beta_3 feeding_i + \beta_4 ruminating_i + \beta_5 lying_i + \beta_6 FPR_i + \beta_7 RFS_i + \beta_8 fecal\ score_i + \beta_9 BCSd_i + farm + Cow|Farm + e_i,$$

where: y_i – i-th observation of the dependent variable (BHBA, NEFA, Glc) respectively; μ – general means; α – fixed effect of parity class with 4 levels; β_1–β_9 – fixed regression term for the variables *DIM*; milk, feeding, ruminating, resting, *FPR*, *RFS*, *FS* and *BCSd* – fixed effects. Cow is nested within farm, both are considered random effects. To determine the significance of the effects of the different variables on BHBA and NEFA, a log-normal distribution was applied for both parameters, while for Glc a normal distribution was considered in the model.

For BHBA three variables (FPR, milk, time feeding), for NEFA two variables (FPR, DIM) and Glc three variables (DIM, time feeding, milk) with the largest impact in the respective model the least square means (LS-means) were plotted with their respective confidence interval (upper and lower CI 95 %) to visually explore the relationship between them and to the classed variable parity.

Results

Descriptive analysis

The average herd size was 337 cows (lactating and dry) and ranged from 191–584 cows. In 2017 the herds energy-corrected milk (ECM) (based on 4.0% fat, 3.4% protein), calculated according to Kirchgeßner et al. (2014), was 10 124 kg in the mean with a minimum of 9 117 kg and a maximum of 11 606 kg. The mean milk yield per day of life (DOL) was 15.7 kg/DOL with a range of 14.0 to 17.2 kg/DOL. Average life output was 31 764 kg per cow with a minimum of 27 537 kg and a maximum of 40 319 kg per cow (Table 1).

Group sizes ranged from 30.5 to 151.3 cows per group (Table 2). The DMI ranged from 12.5 kg and 20.6 kg per group and day. On average, chemical composition of the diets was 6.8 MJ net energy lactation per kg dry matter (DM), 166.5 g/kg DM crude protein (CP), 37.8 g/kg DM ether extracts (EE), 176.8 g/kg DM crude fibre (CF) and 367.9 g/kg DM aNDFom. Starch showed an average content of 192.1 g/kg DM and sugar of 33.0 g/kg DM.

Table 1. Data from monthly milk performance recordings conducted in 2017 by the Landeskontrollverband Weser-Ems e. V. for the 10 dairy herds in the trial

Items	Unit	Mean	Median	Min	Max
Herd size	n	337	315	191	584
ECM (herd)	kg	10 124	9 845	9 117	11 606
Milk herd	kg	10 002	9 870	9 196	11 084
Milk fat	%	4.08	4.04	3.91	4.28
Milk protein	%	3.42	3.41	3.30	3.61
Milk per DOL	kg/day	15.7	15.8	14.0	17.2
Life output	kg	31 764	30 035	27 537	40 319

ECM – energy-corrected milk yield in kg, calculated according to Kirchgeßner et al. (2014); milk per DOL – milk yield of cull cows per day of life; life output – average lifetime milk production per cull cow

Table 2. Average feeding data of the ten dairy herds

Items	Unit	Mean	Median	Min.	Max.	Standard deviation
Group size	n	99.0	114	30.5	151	42.6
DMI group	kg	19.5	18.7	15.7	26.7	2.8
Energy	MJ NEL/kg DM	6.8	6.9	6.4	7.3	0.3
CP	g/kg DM	166	165	138	198	16.5
EE	g/kg DM	37.8	35.4	31.9	46.1	5.0
CF	g/kg DM	176	176	151	197	12.4
aNDFom	g/kg DM	367	368	324	418	23.2
Starch	g/kg DM	192	182	144	272	40.5
Sugar	g/kg DM	33.0	28.8	20.3	50.8	10.8

n – number of cows in feeding group; DMI group – dry matter intake per individual in the group; CP – crude protein, EE – ether extract; CF – crude fibre; aNDFom – amylase-treated neutral detergent fibre with ash; DM – dry matter

The average parity was 3.12 for the cows, with a minimum of 1 and a maximum of 8 lactations. The classed variable parity (Table 3) shows a mean of 2.76 with a range of 1 to 4. The mean DIM was 18.7 days with a minimum of 5 days and a maximum of 37 days (Table 3).

The total sample size was reduced due to missing data or outliers. The mean BHBA was over all samples 0.83 mmol/l with a minimum of 0.28 mmol/l and a maximum of 2.26 mmol/l. NEFA showed a mean of 0.37 mmol/l with a range of 0.01 and 1.89 mmol/l. Glc showed a mean of 3.20 mmol/l with a minimum of 1.98 and a maximum of 4.40 mmol/l. The parities of the studied cows were distributed as follows: parity 1: 15%, parity 2: 20%, parity 3: 20%, parity 4: 15%, parity 5: 13%, parity 6: 3%, parity 7: 4% and parity 8: 1%.

The incidence of SCK in the studied cows was 37% during the trial period of 11 days. In total 37 cows crossed at least once the threshold for SCK of ≥1.2 mmol/l during the trial period. However, Glc as a proxy for SCK showed only an incidence of 8% calculated at the threshold ≤2.2 mmol/l.

Correlation analysis for various factors considered in the analysis of subclinical ketosis

Significant correlations between the studied parameters were found in the low to moderate range (Taylor, 1990). Correlation coefficients between FPR and BHBA (0.43), BHBA and Glc (−0.40), milk and Glc (−0.36) and rumination and milk (0.39) indicate the strongest relationships between parameters (Figure 1). Weaker connections were

Table 3. Descriptive analyses of potential indicators for subclinical ketosis, recorded for each farm during the trial period

Indicators	Unit	n	Mean	Min.	Max.	Standard deviation	Median
BHBA	mmol/l	352	0.83	0.28	2.26	0.350	0.75
NEFA	mmol/l	348	0.37	0.01	1.89	0.219	0.33
Glucose	mmol/l	356	3.20	1.98	4.40	0.421	3.19
Parity	class	356	2.8	1.0	4.0	1.09	3.0
DIM	days	356	18.7	5.0	37.0	6.81	18.0
FPR	ratio	324	1.37	0.92	2.29	0.239	1.32
Milk	kg/day	352	37.7	12.3	61.4	8.40	38.2
Time feeding	h/day	340	5.34	1.46	9.04	1.302	5.37
Time ruminating	h/day	341	9.30	2.28	11.94	1.415	9.55
Time resting	h/day	341	10.33	1.38	18.38	2.864	10.53
RFS	score	326	2.8	1.0	4.0	0.87	3.0
FS	score	356	2.5	1.0	4.0	0.87	3.0
BCSd	binary	356	0.5	-	-	0.50	0.0

BHBA – β-hydroxybutyrate in blood serum; NEFA – non-esterified fatty acids in blood serum; DIM – days in milk; milk – milk yield per cow and day in kg; FPR – fat:protein ratio; RFS – rumen fill score; FS – faecal score; BCSd – difference of body condition score between the preparation week (week 0) and the second week of the trial (week 2); n – number of samples

class – parity classes: 1 – first lactation, 2 – second lactation, 3 – third lactation, 4 – fourth lactation and higher; BCSd – difference of BCS between preparation week and second trial week, results were converted into 0 (difference < 0.5) and 1 (difference ≥ 0.5)

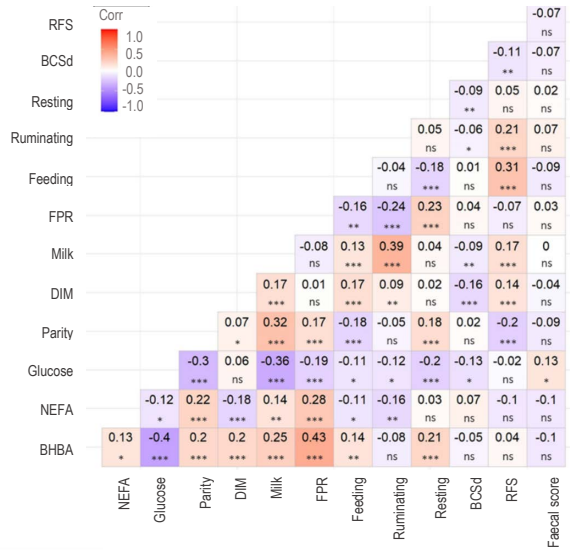

Figure 1. Pearson's correlation between the investigated variables

ns – non significant; *0.05 > *P* ≥ 0.01, **0.01 > *P* ≥ 0.001; *** *P* < 0.001; BHBA – β-hydroxybutyrate in blood serum, log transformed; NEFA – non-esterified fatty acids in blood serum, log transformed; DIM – days in milk; milk – milk yield per cow and day in kg; FPR – fat:protein ratio; BCSd – difference of body condition score between preparation week and second trial week, results were converted into 0 (difference < 0.5) and 1 (difference ≥ 0.5); RFS – rumen fill score

found between parity and milk (0.32) as well as Glc (−0.30) respectively. A correlation of 0.31 was also found between feeding and RFS. The correlation coefficients for NEFA with the other parameters ranged from −0.18 to 0.28, the correlation between NEFA and FPR (0.28) was the highest.

Analysis of factors influencing BHBA, NEFA and Glc

The statistical analysis of the data was performed using a mixed linear model, as described above. Table 4 presents the estimated model parameters using a normally distributed dependent model variable and also the statistical significance level based on a log-normally distributed dependent variable. FPR and milk have the most significant effect ($P < 0.0001$ and $P = 0.0018$) on BHBA. Both factors showed a positive relation to BHBA. The time spend feeding was related positively with BHBA ($P = 0.0091$) as well. There was an interaction of FPR and DIM in their influence on BHBA ($P = 0.0111$). The F-test shows the importance of each explanatory variable in the three models. For predicting BHBA, the FPR has the highest impact with an F-value of 20.03 while milk shows the second highest F-value with 10.07.

In the model predicting NEFA, the effects of DIM and FPR had the highest significance ($P < 0.0001$ and $P = 0.0049$). Increasing DIM had a decreasing effect on NEFA, whereas FPR showed a positive relationship to NEFA. Rumination also showed significant influence ($P = 0.0446$), NEFA decreases with increasing time of rumination. The most important variables predicting NEFA are DIM and FPR with F-values of 18.96 and 8.11 respectively.

Table 4. The effects of fixed effects und fixed interaction effect on BHBA, NEFA and glucose

Dependent variable	Factors	Regression coefficient	F/ t value	*P*-value
BHBA	Intercept	-0.8328 ± 0.3431	6.35	0.0001
	FPR	0.7922 ± 0.2094	20.03	<.0001
	Milk	0.009923 ± 0.0034	10.07	0.0018
	DIM	0.03485 ± 0.0152	7.09	0.0084
	Time feeding	0.04693 ± 0.0189	6.95	0.0091
	FPR*DIM	0.02409 ± 0.0108	6.58	0.0111
	Parity	Class Variable	1.80	0.1490
NEFA	Intercept	0.5870 ± 0.1584	1.35	0.2114
	FPR	0.2156 ± 0.05754	8.11	0.0049
	DIM	-0.00967 ± 0.002137	18.96	<.0001
	Time ruminating	-0.03079 ± 0.01059	4.09	0.0446
	Parity	Class Variable	1.75	0.1583
Glucose	Intercept	3.6584 ± 0.1684	21.73	<.0001
	Milk	-0.01012 ± 0.00326	9.61	0.0022
	DIM	0.01880 ± 0.00329	32.62	<.0001
	Time feeding	-0.04496 ± 0.01841	5.96	0.0154
	Time ruminating	-0.02854 ± 0.01425	4.01	0.0465
	Parity	Class Variable	9.17	<.0001

BHBA – β-hydroxybutyrate in blood serum; NEFA – non-esterified fatty acids in blood serum; DIM – days in milk; Milk – milk yield per cow and day in kg; FPR – fat:protein ratio; F value – calculated by comparing the variance explained by a fixed effect to the residual variance, it is used to assess the significance of fixed effects within the linear mixed model; t value – calculated as the ratio of the difference between the sample mean and the given number to the standard error of the mean; $P < 0.05$ means that the data is significantly different

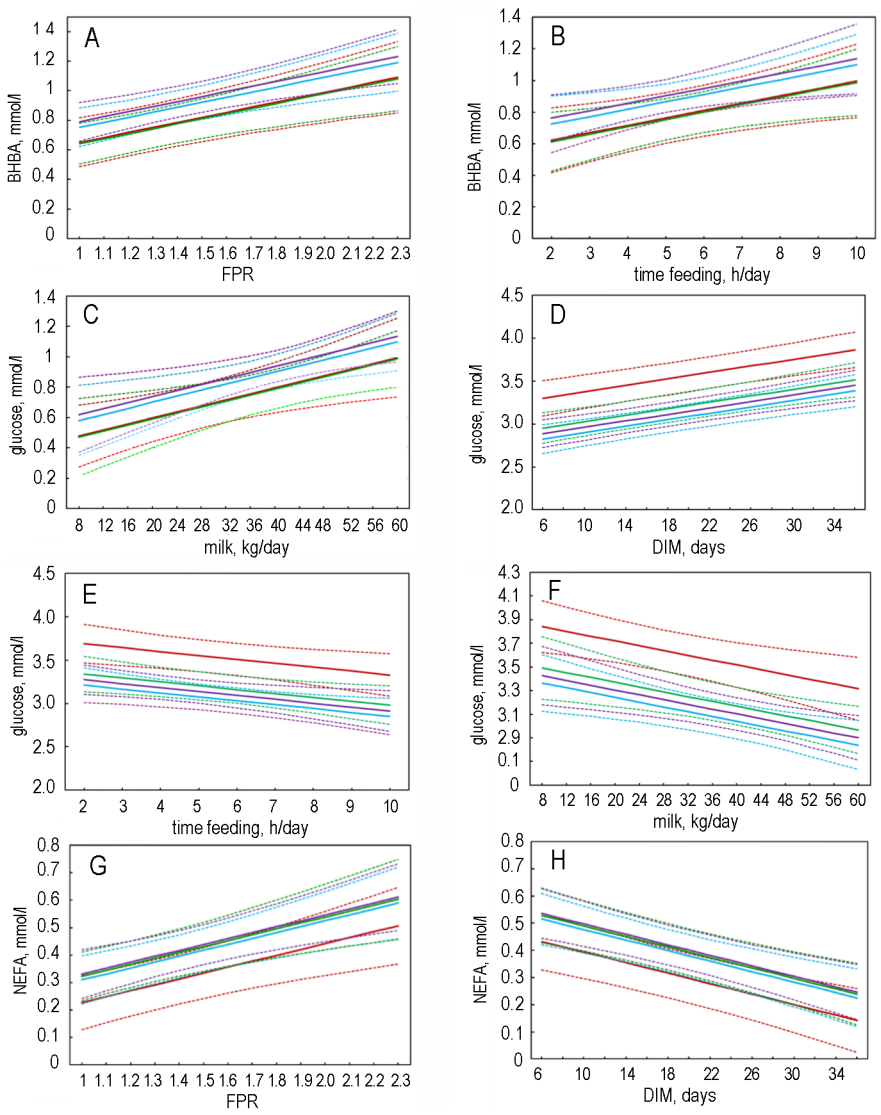

Figure 2. The effect of FPR, DIM and time feeding (duration of feeding) on BHBA (A–C), of DIM, milk and time feeding on glucose (D–F) and of FPR and DIM on NEFA (G, H)

The solid lines show the LS–means at certain levels of the respective independent variables. The dashed lines are the corresponding confidence limits (95%) of LS–means. Red: parity = 1, light blue: parity = 2, green: parity = 3 and purple: parity ≥ 4. FPR – fat:protein ratio; DIM – days in milk; BHBA – β-hydroxybutyrate in blood serum; NEFA – non-esterified fatty acids in blood serum; milk – milk yield per cow and day in kg

For Glc the significant factors in the model were parity ($P < 0.0001$), DIM ($P < 0.0001$), milk ($P = 0.0022$), time feeding ($P = 0.0154$) and time ruminating ($P = 0.0465$). Milk as well as time feeding had a negative effect on Glc whereas DIM and time ruminating showed a positive effect. The effect with the highest F-value in this model is DIM with 32.62.

In the Figure 2 the effects of FPR, milk and time feeding on BHBA (A–C); FPR and DIM on NEFA (G–H) as well as DIM, time feeding and milk on Glc (D–F) are shown depending on the fixed effect of parity. The analysis of covariance showed an influence of parity on BHBA, NEFA and Glc depending on the examined parameters. However, the parities 1 and 3 showed in this modelling the lowest BHBA values whereas parities 2 and 4 showed the highest values. BHBA increased with all three investigated factors. Parity showed no significant effect for BHBA in the model, even though differences between parities can be found in the diagrams. NEFA increases with increasing FPR and decreases with DIM. There are also differences seen between parities despite the parameter not showing a significant effect in the model. Parity 1 shows the lowest NEFA-values for both investigated factors, parities 2, 3 and ≥4 show approximately 0.1 mmol/l higher values. There are no large differences between these parities for NEFA depending on FPR. The modelling of the effect of parity on Glc depending on different factors (DIM, milk, feeding) shows a decrease with more time spend feeding, whereas the progression of lactation (DIM) has a positive effect on Glc level.

An increase in milk yield shows a negative effect on Glc as well. Parity 1 shows the highest Glc-values, whereas the Glc levels are lower for parities 2–4 by approximately 0.4 mmol/l.

Discussion

SCK in dairy cows leads to significant health impairments and welfare problems such as fertility disorders as well as secondary diseases, like displaced abomasum or fatty liver. The decrease in feed intake and milk yield also means economic losses. Due to mobilization of body fat as an additional energy source in combination with the competition for metabolites used for gluconeogenesis, ketone body concentration increases in body fluids of early lactating dairy cows. Since the final metabolic problem of SCK is best indicated by ketones (or ketone-associated molecules like BHBA, which is established as the best proxy for SCK; some focus will be on BHBA in the following discussion, therefore), it is clear that further metabolites (NEFA, Glc) are also causally linked in SCK development. The presence of NEFA indicates the use of fat stores by the animal in general (first of all unpathological, but increasing the potential for SCK), while Glc metabolism interacts with fat metabolism in the development of SCK. To capture these interrelations, separate analyses with both of them as dependent variables were included in the present study in addition to BHBA. The relatively high correlation between BHBA and Glc (−0.4; $P < 0.001$) supports the relevance of Glc in the development of SCK, while the considerably lower correlations for NEFA are in line with the general opinion that NEFA is a considerably less direct proxy for SCK. While based on this, Glc could have been involved as independent variable to predict BHBA, we refrained from involving it in the BHBA mixed model since it was the clear intention of the present study to focus on predictors that need no direct handling of the animal and have some potential to be detected in an automated way.

While based on the threshold for BHBA of ≥3.0 mmol/l defined for clinical ketosis by Oetzel (2004) there were no clinically ketotic cows included in this analysis, the incidence of SCK with 37% of the cows highlights the importance of monitoring for this metabolic state in early lactating cows. This finding is supported by different studies which found similarly high incidences for SCK. McArt et al. (2012b) reported that 43.2% of the cows exceed at least once the threshold of ≥1.2 mmol/l in lactation. Kaufman et al. (2016) found incidences between 27–56% (mean = 44%) depending on the herd. Different thresholds to identify SCK cows are suggested, they range between 0.9 and 1.6 mmol/l. Commonly used thresholds range from 1.0 to 1.4 mmol/l and result in higher or lower incidences respectively (McArt et al., 2013). According to McArt et al. (2013) ≥1.2 mmol/l is an adequate compromise due to its balanced sensitivity and specificity.

The impact of high BHBA levels on the individual cow can vary, some individuals showing clinical signs with relatively low BHBA concentrations, whereas others appear to be healthy even with high BHBA concentrations (Oetzel, 2007). Nevertheless, the application of a BHBA-threshold to determine SCK represents a relatively objective approach to identify individual cows that are at least at risk for the mentioned disorders and secondary diseases. Different test systems to diagnose SCK are well evaluated for the use in commercial dairy farms. Due to the increase of ketone bodies in blood, milk and urine, those fluids are used to measure the concentration. With on-farm tests the results can be obtained quickly, with little effort and at low cost for the farmer as well as high accuracy. Jansen et al. (2021) showed for different on-farm tests for blood-BHBA accuracies of 94–97%. Tests for urine and milk are available as well, but not as accurate (Melendez and Serrano, 2024).

Established test systems have in common that the cow must be somehow handled to obtain the sample. Reducing the time effort for the farmer, using indicators that need less handling of the animals have the potential to be evaluated on a very frequent up to an almost continuous level and in an automated way. Herskin et al. (2004) also found a negative impact of stressors like fixation or isolation on the time spent ruminating in dairy cows, which is considered to be an indicator for the wellbeing and health of cows (Cocco et al., 2021). In sum, parameters predicting SCK that can be obtained without human-cow interaction are desirable beyond the available and obviously very sensitive BHBA blood tests.

FPR in milk is established as an indicator for the metabolic status of dairy cows and is used for the detection of SCK. It is an indicator obtainable without handling the cow, apart from routine milking. In this study FPR shows significant correlations with BHBA (0.43), but also NEFA and Glc (0.28 and −0.19). In fact, this was the highest correlation coefficient found for BHBA and the large impact in the model predicting BHBA supports the particular value of FPR for SCK prediction. Buttchereit et al.

(2010) suggests FPR as an indicator of energy balance for cows in early lactation as well. Cabezas-Garcia et al. (2021) found FPR (≥1.5) to be associated with a negative energy balance, higher milk yield, more body condition loss, and also higher NEFA concentrations in blood. They suggest to use FPR on herd level rather than for the individual cow based on significant variations for individual cows. The significant interaction FPR*DIM in our dataset also indicates that milk fat and protein, irrespective of potential metabolic imbalances, follow systematic changes in the course of a lactation, which are particularly pronounced for fat at the beginning of lactation.

While the predictive potential of FPR proofed to be considerable, further precision in the detection of SCK would still be desirable. As BHBA in blood, FPR can be considered an indicator representing a consequence of an energy deficit. However, besides consequences, also major causes for an energy deficit like feed intake can be considered valid in SCK prediction. To identify farms in the trial with potentially higher risk, the rations of the groups were analysed and weighed. Between farms, the compositions of rations differed considerably, which was an argument for obtaining the DMI of the group in this study. However, due to the wide range in sizes of feed groups as well as the presence of cows that did not take part in the trial and the different stages of lactation, average DMI per cow and group was not included as a factor in the model (a situation reflecting our approach to mimic the situation on a farm). Instead, the time spent feeding was used as a proxy of feed intake itself. De Mol et al. (2016) reported a moderate correlation between feeding time and DMI of 0.53 for totally mixed rations and 0.59 for partially mixed rations. Though, currently there is no adequate algorithm available for the RumiWatch® sensor to deduce the quantitative DMI due to its strong dependence on the individual cow (Leiber et al., 2016) as well as the particle size of the ration (Kronqvist et al., 2021). The concordance with observations of prehension and mastication bites in cows fed in stalls was reported as 0.64 and 0.58, respectively (Norbu et al., 2021). In fact, in the present study feeding time showed a significant effect for predicting BHBA and Glc in blood but not for NEFA which is consistent with the findings of Nielsen et al. (2007) that found no effect of change in energy intake on NEFA in the first weeks postpartum. Jilg et al. (2018) found a correlation between feeding time at the automatic weighing trough and the halter of 0.58 as well, but also reported that the halter overestimated the time feeding by 214 min per day on average. Despite the inaccuracies of the recording of feeding time, RumiWatch® sensors are a useful system to monitor and research feeding and activity behaviour of cattle in commercial farms, especially since the detailed raw data are available in form of pressure curves and 1-min resolution are available as well as different converter versions for different feeding systems (free stall barn, grazing).

However, in the model predicting BHBA, time spent feeding showed an increasing effect, somehow paradoxically. With a longer feeding time, more energy should be ingested by the cow and in consequence decrease BHBA. The opposite result in the data of the present study suggests that while DMI is increasing continuously after parturition, this increase is still not sufficiently steep to narrow the gap between energy intake and demand in early lactation. It closes only slowly, therefore the ketone body concentration in blood increases in the beginning of lactation despite the correspondingly increasing DMI. Metabolic profiles showed the peak for BHBA at four weeks postpartum (Busato et al., 2002). Nielsen et al. (2007) found that even an increase of energy in the ration at the beginning of lactation cannot cover the energy requirements to a sufficient extent to lower BHBA in blood effectively. The incidence of SCK has been reported to be highest at 21–25 DIM on average, which means that our sampling time of 19 DIM on average (range: 5–37 days) was well within the time range where energy requirements and intake are still diverging rather than converging (irrespective of the continuously increasing intake). The reported peak at 4 weeks or even before week 3 postpartum (Busato et al., 2002; Weber et al., 2013) suggests that in this study BHBA goes through an increase as well as decrease during the trial phase which might lead to some of the paradox results. The positive relation of BHBA to feeding behaviour in the present study points to these particularities of the transit phase when testing potential indicators.

Rumination as an indicator could be interpreted as a cause (as it is positively linked to intake) or rather as a consequence of SCK (if rumination is expected to decrease due to impaired welfare by SCK). While the former link should result in a negative correlation with BHBA (the more rumination, the less BHBA), the latter should do the opposite (the higher BHBA, the lower is rumination). Healthy cows have been found to be more active before and after parturition and to spent more time ruminating as well (Stevenson et al., 2020).

Kaufman et al. (2016) suggest that monitoring rumination time during the close-up period and early lactation may assist in finding cows with a higher risk of developing SCK earlier due to the documented decrease in this indicator in cows that developed SCK in comparison to healthy cows. However, in the present study rumination showed no effect on BHBA but only on NEFA and Glc.

While RFS and BCSd did not show relations to BHBA in this study, theoretically they should also reflect intake on a rather short day-to-day basis (RFS) or on a longer timeframe (BCSd). Concerns about the use of such scoring systems involve doubts about being independent of subjective influences by the respective person. Studies indicate that the RFS shows a good repeatability both between different observers as well as for the repeated scoring of one cow by one observer (Burfeind et al., 2010). In BCS evaluation it was noted that when assessing a change of BCS, the accuracy is higher if the same observer scores the cow (Morin et al., 2017). In the present study two observers did the scoring randomly which might have led to less agreement in the assessments despite harmonization of the scoring between observers beforehand. All three scores applied showed low correlation to all three variables. No significant effect on any of the three tested variables could be found. Compared to automatic sensor systems the subjectivity remains higher in such observer based score systems. While faecal consistency (high DM content) has been proposed as predicting factor for clinical ketosis (Duffield, 2000; Berge and Vertenten, 2014), in the present analysis it was not selected as relevant factor in the mixed models, and only a very weak correlation with Glc was found. While faecal consistency can be influenced by a variety of factors, RFS as well as FS are both also highly dependent on the ration, such as composition and the possibility of feed sorting but also on the time of sampling relative to feeding (Burfeind et al., 2010; Melendez and Roy, 2016). Schneider et al. (2022) suggested RFS to be also dependent on individual characteristics of the cow, like rumen capacity depending on body size and rank in the herd.

Factors like milk and also DIM represent obvious predisposing factors to an energy deficit. While its link appears a bit less direct, parity can also be attributed to this group. Multiparous cows have been shown to have higher odds at developing SCK (Berge and Vertenten, 2014). However, in the present study the classed factor parity showed no significant effect for BHBA and NEFA, but only for Glc. It is natural that such indirect predisposing factors must be less predictive than direct indicators like BHBA, since influences can occur on various levels.

In some studies (Buttchereit et al., 2010; Siebert and Pallauf, 2010), a distinct separation of level of FPR between 2nd and 3rd lactation was found; if generally present, such differences would need consideration and therefore any potential effect of parity on the relation of FPR and BHBA should be checked. The analysis of covariance indicated differences between parities for BHBA, NEFA and Glc in our dataset, depending on the examined factor. Since FPR was found to increase with the energy deficit of cows with increasing parity number (Buttchereit et al., 2010), a corresponding effect could have been expected in the present study regarding BHBA, NEFA and Glc. However, the differences between parities were not completely found as expected. For BHBA, parity 1 and 3 showed the lowest estimated LS-Means, whereas parity 2 shows values almost as high as parities 4 and higher in the present study. For the other two blood metabolites, a distinction between parity class 1 and classes 2–4 was noticeable, with lower NEFA and higher Glc levels for parity class 1. It might be relevant for the interpretation that the distribution between the parity classes was uneven due to the primary selection criterion calving date and limited selection options in the herds (15, 29, 20 and 39 cows in the respective parity classes). Distribution between farms varied as well, with a range of 2.6 to 4.3 lactations on average. Ebert et al. (2017) suggest that due to lower DMI (primiparous cows ousted from the trough by higher ranking cows) and not fully developed udder tissue, primiparous cows show lower milk yields than multiparous cows. They found the difference between first and following lactations to be 11 kg on average. Metabolic differences between primiparous and multiparous cows, such as differences in nutrient partitioning with bias to either growth or milk yield (Wathes et al., 2007) may be another reason for the results not meeting expectations as well as impaired energy metabolism of multiparous cows which showed a greater dependence on lipid utilization according to Lean et al. (2023). The in parts unclear results of this study in relation to parity suggest that the metabolic differences between parities 2, 3, 4 and higher are not as pronounced or clear as between 1 and higher parities. For following investigations, a distinction between parity 1 and higher parities may show clearer results.

In this study factors known or suspected to be associated with SCK were investigated to test how reliably they could be applied on commercial farms to predict the concentration of BHBA, NEFA and Glc in blood. It was the particular approach to investigate the relations of potential predictors to blood values (in particular BHBA as gold proxy) not in a research environment but under the varying conditions of non-standardized practical farming (in which the indicators have to work finally). Data collection was conducted for two weeks on each farm and was integrated into daily farm routines. Potential factors associated with SCK were closely monitored. The internal structures of the farms varied in e.g., herd and group sizes, processes, diet components, etc. Such farm-associated factors must be considered to influence the outcome of research on commercial dairy farms. For example, the composition of the ration of the respective farm was not considered due to the lack of individual feed intake data in the present study (despite its influence on SCK). Additionally, the human factor is critical as well, either in relation to the work of the herdsmen with the research team, but even more so in relation to the cows and technology used on farm. General management practices like the feeding process, health monitoring and health management are dependent on it, too. The farms in this study were visited consecutively over the course of one year; in consequence, the season or rather weather differed for each farm visit to some extent. In regards to heat stress (milk yield, DMI, etc.) it can influence the results for single farms. In conclusion, such farm-specific factors that are hard to be controlled completely pose particular challenges to studies on farm. However, due to the number of farms and cows per farm, the impact of the individual farm should be reduced.

Although BHBA is considered the distinctively best predictor and a strong proxy for SCK, direct metabolic interactions justify some separate considerations on Glc, in addition to the discussion so far. Glucose decreases with increasing milk yield per day. As mentioned, this can be attributed to the onset of lactation and therefore higher demand for Glc as energy fuel and lactose component. Fitting with the unexpected result on BHBA, a longer feeding time is associated with decreasing Glc levels in blood in early lactation. Metabolic studies show that with onset of lactation the DMI increases and Glc decreases during the first weeks. The length of this period depends on the energy density and glucogenic potential of the ration as well as DMI (Van Knegsel et al., 2007; Nielsen et al., 2007). In the present study, Glc levels increase with progressing lactation, the increase is low with a regression coefficient of 0.02. This indicates that the lowest level of Glc in blood occurs in the studied cows at an early date and the energy supply in the rations, relative to milk yield, is sufficient to make Glc levels reach the turning point on average within the first month of lactation (5–37 DIM). The threshold for identifying SCK using Glc with ≤2.2 mmol/l was defined by Dubuc and Buczinski (2018). In the present study, cows showed values higher than the mentioned threshold on average and an incidence of 8% during the trial.

In the present study, factors already established like FPR, DIM or milk can be found in all three predictions as indicators with significant impact, which shows their validity under the particular conditions of on-farm investigations. Milk yield per day, time ruminating and feeding were also identified as factors that support the correct identification of cows with some risk of a metabolic imbalance (although the validity of feeding and rumination time can be questioned due to the sometimes paradox direction of the effect). On the other hand, observer based parameters like RFS, FS and BCS were not applicable in this study.

Conclusions

In this study, indicators that are potentially associated with the risk of developing subclinical ketosis (SCK) in early lactating dairy cows and that can be obtained without handling the animals were investigated in relation to the blood metabolites β–hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA) and glucose (Glc) on commercial dairy farms. Established indicators for the prediction of SCK could be found to be useful predictors under these conditions. Scoring systems like rumen fill score, feacal score and body condition score were insignificant factors in these models as well as resting. The fat:protein ratio showed the largest impact for predicting BHBA, whereas in the model predicting NEFA, days in milk (DIM) was the factor with the highest impact. Glc was influenced most by DIM as well. For parity, no significant influence beside Glc could be found. In the analysis of covariance an influence of parity could be found, with some distinction of first lactation vs. 2^{nd}–4^{th} lactation for NEFA and Glc.

Acknowledgements

This study was conducted in the project 'Evaluation of animal welfare in dairy farming – Indicators for metabolism and feeding' (IndiKuh, grant number: 2817905815), supported by the Federal Ministry of Food and Agriculture (BMEL) by decision of the German Bundestag. Project sponsor is the Federal Office for Agriculture and Food (BLE). The authors thank the owners of the dairy farms for contributing to our project by giving us access to their farms, herds, and data, the basis of this research. We acknowledge support by the Open Access Publication Funds of the Göttingen University.

Conflict of interest

The Authors declare that there is no conflict of interest.

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