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Evaluation of the metabolic status of Simmental dairy cows in early and mid lactation*

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Fifteen early-lactation cows and 15 mid-lactation cows were chosen for the analysis. Blood samples were collected to measure the beta-hydroxybutyrate (BHB), non-esterified fatty acids (NEFA), triglycerides (TG), glucose (Glu), total protein (TP), albumin (ALB), total bilirubin (TB), urea (U) and the activity of aspartate transaminase (AST) and gamma-glutamyl transferase (GGT). Early-lactation cows showed significantly higher (P<0.05) values of serum BHB and NEFA, and lower of (P<0.05) glycemia levels compared to mid-lactation cows. High lipomobilization (NEFA >0.4 mmol/l) and subclinical ketosis (BHB >1.2 mmol/L) were detected in 6 (40%) and 14 (94.4%) early-lactation cows, respectively, and in none of the mid lactation cows. AST activities above 100 IU/l were detected

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in two early-lactation and none of the mid-lactation cows. TG concentrations below 0.12 mmol/l were found in 7 (44%) early-lactation and 2 (13.3%) mid-lactation cows. Glucose levels were below 2.5 mmol/l in 10 (66.6%) early-lactation and 5 (33.3%) mid-lactation cows. Early-lactation cows showed lower blood serum concentrations of TG (P>0.05), ALB (P>0.05), TP (P<0.05), U (P>0.05) and GTT (P>0.05) activities and higher concentrations of TB (P>0.05) and AST activities (P<0.05), as compared to mid-lactation cows. These metabolic characteristics were correlated with DMI and energy balance (EB). Blood serum values for glucose, TG, BHB, NEFA and AST showed that early-lactation cows suffered from metabolic disturbances, associated with ketosis, and some degree of hepatic lesions, probably due to fat infiltration. These serum parameters may have a key role in evaluating the metabolic status of dairy cows.

KEY WORDS: blood metabolites/ dairy cows/hepatic lipidosis/subclinical ketosis

Production diseases *i.e*. diseases associated with improper nutrition or management are common in dairy cows. They include the fat liver syndrome, ketosis, oxidative stress, *laminitis*, *mastitis*, milk fever, retained placenta, *metritis* and infertility [Oetzel 2004, Jóźwik *et al*. 2012]. Ketosis and fatty liver are closely linked and responsible for severe economic losses in dairy farms due to declining milk yield and reproductive performance, and increasing culling rates. A metabolic profile, as a series of specific blood analytical tests, is routinely used to reveal metabolic problems in dairy cattle. It involves collecting blood samples from 8 to 12 cows at 4 time periods relative to calving (dry, early lactation, peak lactation and mid lactation) and measuring selected blood metabolites [Gross *et al*. 2001, Oetzel 2004, Stengarde *et al*.2008].

Clinical ketosis in dairy cows usually occurs between the second and seventh week of lactation. Nevertheless, most of the cows in this stage of lactation may suffer from subclinical ketosis defined as increased blood ketone bodies, without any other symptoms observed apart from a considerable decrease in milk yield and susceptibility to other diseases [Duffied *et al*. 2007]. The prevalence of subclinical ketosis in high-yielding dairy cows in the Netherlands was estimated in 12 to 47% of the herd [Nielen *et al.*1994]. Such prevalence is considered to be higher during the first month of lactation compared to the second month, with a peak of occurrence in the fourth week [Baird 1982]. Dairy cows suffer from negative energy balance (NEB) during the first week of lactation due to energy expenditure associated with milk production and limited feed intake, resulting in NEB, a high mobilization of lipids from body fat reserve, and hypoglycaemia in early lactation [Veenhuizen *et al.*1991, Djoković *et al.* 2007]. The main blood indicators of lipomobilization in ruminants are beta-hydroxybutyrate (BHB), the most important and abundant ketone body, and non-esterified fatty acids (NEFA) [Oetzel 2004, Civelek *et al.* 2011, Gonzales *et al.* 2011]. NEFA are preferentially and greatly accumulated as TG in the liver, primarily because of a decrease in the very low density lipoproteins (VLDL) synthesis by hepatocytes [Herdt *et al.* 1983, Sevinc *et al*. 2003]. However, when steatosis occurs, endogenous liver synthesis decreases, leading to a reduction in blood glucose, TP, albumins and globulins, cholesterol, TG and urea. Furthermore, the excretory function of hepatocytes is reduced and, accordingly, the blood concentrations of some compounds such as total bilirubin, ammonia and bile acids are generally increased [West, 1990, Veenhuizen

et al. 1991, Sevinc *et al.* 1998, 2003, Bobe *et al*. 2004]. Fatty liver infiltration and hepatocyte degeneration involve cell membrane damage and hepatocyte destruction coupled with the release of cytoplasm enzymes (AST, GGT, LDH) and marked increase in the circulating activities [Pechova *et al*.1997, Lubojacka *et al*. 2005, Jóźwik *et al*. 2012]. Diagnosing liver lipidosis and susceptibility to ketosis in dairy cows may include liver biopsy or echography, but a less invasive and more economical analytical method may be the measurement of blood biochemical indicators [Baird 1982, Bobe *et al*. 2004]. Based on blood biochemical indicators, ketosis in cows may be diagnosed when the following values match both the clinical signs (BHB >1.2 mmol/l, glucose <2.5 mmol/l, and TG <0.12 mmol/l) and blood values of NEFA >0.7 mmol/l and AST activity above 100 IU/l, which is indicative of hepatic lipidosis [Sevinc *et al*.1998, Oetzel 2004, Xu *et al*. 2008, Gonzales *et al.* 2011]. The objective of the present study was to evaluate the metabolic status of early and mid-lactation cows through changes in characteristic blood metabolites.

Material and methods

Animals

The study was carried out in January 2012 in a dairy herd (119 Simmental cows) suffering from several metabolic and reproductive disorders (Ćurcić Farm, Mrsać, Kraljevo, Central Serbia). The cows were mid-yielding with a preceding lactation of about 6.500 l (early-lactation cows: 23.5±4 l /day/ cow, mid-lactation cows: 28.5±6 l / day / cow, calculated on the basis of milk yield in the previous lactation). Two groups of clinically healthy cows were chosen from the herd. One group consisted of early lactation cows (n=15) in the first month of lactation (16.1±9.3 days), and the second group included mid-lactation cows (n=15), 3 to 5 month of lactation (114.81±28.5 days). Body condition scores (BCS) were recorded by the same observer using the 1~5 scale according to Ferguson *et al*. [1994], with 1 meaning = too thin and 5 = too fatty. BCS were 3.42±0.55 and 3.27±0.42 in early-lactation and mid-lactation cows, respectively. All the cows were kept in tie-stall barns. Diet and the housing facilities were adapted to research purposes, with diet suited to the energy required by early and mid lactation cows. Early-lactation cows were fed a diet composed of 7 kg lucerne hay, 20 kg maize silage (30% dry matter – DM) and 5 kg concentrate (18% crude protein – CP). Mid-lactation cows received a diet consisting of 5 kg grass hay, 7 kg lucerne hay, 30 kg maize silage (30% DM), 8 kg concentrate (18% CP). Dietary nutrient contents for dairy cows in early and mid lactation are given in Table 1. The chemical analysis of the feed was performed by Weende method. Energy balance was calculated by NRC recommendation [NRC 2001].

Table 1. Nutrient contents in daily ration for early-lactation and mid-lactation dairy cows

Item	Early lactation cows	Mid lactation cows
Dry matter (DM, kg)	16.05	24.82
Net energy of lactation (NEL, MJ)	87.15	130.23
Crude protein (CP, % of DM)	13.58	13.38
Rumen undegradable protein (RUP, % of CP)	35.91	28.33
Fat (% of DM)	3.09	3.14
Fibre (% of DM)	23.26	24.33
EB (MJ/day/cow, mean±SD	-15.21 ±20.37	3.49 ±8.16

Biochemical analysis

Blood samples were collected at 10:00 h a.m. or 4 to 6 hours after milking and feeding, from the jugular vein into sterile disposable test tubes, without anticoagulant. After clotting for 3 hours at 4°C and centrifugation (1500G, 10 min, 4°C), sera were carefully harvested and stored at -20°C until analysis. Blood samples collected on fluoride, immediately centrifuged in the same way and plasmas were assessed for glucose concentration. The following biochemical blood components were measured at Kvarklab Biochemical Laboratory (Kragujevac, Serbia) by different colorimetric techniques using spectrophotometers (Cobas Mira and Gilford Stasar) beta-hydroxybutyrate (BHB) and non-esterified fatty acid (NEFA) levels were measured by Randox (United Kingdom) kit, aspartate transaminase (AST), gamma-glutamyl transferase (GGT), glucose and total bilirubin by Human (Germany) kit, albumin and urea by Biosystem (Spain) kit, and total proteins (TP) and triglyceride (TG) by Elitech (France) kit.

Statistical

Data were subjected to statistical analysis using the GLM model and t-test (Statgraphic Centurion, Statpoint Technologies Inc.Warrenton, Virginia, USA). The model included lactation period and metabolite values. Pearson's test was performed to evaluate significant correlations between milk yield, dry mater intake (DMI), energy balance (EB) and biochemical indicators separately. The intensity of change in metabolic profile during negative energy balance in early and mid lactation stages was analysed by comparison of b parameters in linear equations (t-test). Finally, correlation between metabolic parameters was evaluated. Differences were considered significant at P values below 0.05 or 0.01.

Results and discussion

Compared were metabolic parameters of early-lactation with mid-lactation cows. Blood parameters estimated for both groups of cows are shown in Table 2. BHB

Table 2. Blood metabolites in early and mid-lactation dairy cows. Results are expressed as means±SD

Parameter	Early lactation cows (n=15)	Mid lactation cows (n=15)	P
Glucose (mmol/l)	2.29±0.48	2.76±0.43	<0.05
BHB(mmol/l)	1.59±0.25	0.91±0.16	<0.05
NEFA(mmol/l)	0.38±0.29	0.13±0.04	<0.05
TG(mmol/l)	0.12±0.02	0.15±0.04	NS
TP(g/l)	75.27± 4.49	78.89±4.52	<0.05
Albumin(g/l)	34.61±3.56	37.57±3.15	NS
Urea (mmol/l)	5.29±1.32	5.33±0.95	NS
Total bilirubin (μmol/l)	3.91±2.85	3.22±1.05	NS
AST (IU/l)	69.46±27.54	39.31±18.90	<0.05
GGT (IU/l)	20.61±4.16	23.03±9.94	NS

NS – non significant.

and AST concentrations were higher (P<0.05) in early-lactation cows than in mid-lactation cows. Blood glucose and total protein levels were lower (P<0.05)**.**

These metabolic changes did not correlate with the intensity of milk production during lactation, but a significant correlation was observed between metabolite values and DMI and EB (Tab. 3). Lower DMI could explain lower concentrations of glucose, total protein, albumin and triglycerides and higher concentrations of NEFA, BHB and AST. Negative energy balance could explain lower glucose and albumin concentrations and higher NEFA, BHB and AST levels. Insufficient DMI in early lactation is known to lead to negative energy balance with the above mentioned changes occurring in the metabolic profile. Metabolic changes during early lactation were more intensive as a function of EB, compared to mid lactation (Tab. 4). These changes are due to both lipolysis and ketogenesis occurring during homeorhetic processes and metabolic adaptation in the liver during early lactation (Tab. 3 and 4).

Table 3. Correlation coefficients between metabolites and milk production, DMI and EB. Bolded coefficients are significant (P<0.05)

Metabolites	Milk (L/day)	DMI (/cow/day)	EB (MJ/day)
Glucose	*r* = 0.18	***r* = 0.43**	***r* = 0.45**
NEFA	*r* = -0.21	***r* = -0.5**	***r* = -0.49**
BHB	*r* =-0.25	***r* = -0.38**	***r* = -0.53**
TG	*r* = -0.11	***r* = 0.42**	*r* = 0.18
TP	*r* = 0.09	***r* = 0.43**	*r* = 0.29
Albumin	*r* = 0.08	***r* = 0.44**	***r* = 0.45**
Urea	*r* = -0.15	*r* = 0.02	*r* = 0.29
Bilirubin	*r* = -0.14	*r* = -0.16	*r* = 0.11
AST	*r* = 0.07	***r* = -0.49**	***r* = -0.41**
GGT	*r* = 0.05	*r* = 0.14	*r* = 0.09

Table 4. Change in metabolite level as a function of EB in early and mid lactation (comparison of b parameters from linear equation)

Metabolite	b parameters (relation EB to metabolite)		P
	early lactation	mid lactation	
Glucose	0.08	0.05	<0.05
NEFA	-0.07	-0.03	<0.01
BHB	-0.09	-0.05	<0.01
TG	0.002	0.003	NS
TP	0.68	0.66	NS
Albumin	0.34	0.19	<0.05
Urea	0.05	0.07	NS
Bilirubin	-0.16	-0.07	<0.01
AST	-4.35	-2.28	<0.01

Homeorhesis induces intense lipid mobilization and ketogenesis, and the liver is adapted to metabolic changes in dairy cows. Correlations among the biochemical metabolites calculated for all 30 cows in this study are given in Table 5.

Table 5. Correlation coefficients for the biochemical metabolites calculated for all cows used in this study (significant coefficients (P<0.05) are bolded)

Item	NEFA	BHB	TG	TP	Albumin	Urea	Bilirubin	AST	GGT
Glucose	***r* = -0.35**	***r* = -0.47**	***r* = 0.65**	*r* = 0.01	***r* = 0.47**	***r* = 0.43**	*r* = -0.03	*r* = -0.23	***r* = -0.32**
NEFA		***r* = 0.39**	*r* = -0.21	***r* = -0.34**	*r* = -0.26	***r* = -0.45**	***r* = 0.63**	***r* = 0.34**	*r* = -0.17
BHB			***r* = -0.36**	*r* = 0.06	*r* = -0.23	*r* = -0.27	*r* = 0.13	*r* = 0.15	*r* = 0.06
TG				*r* = 0.05	***r* = 0.63**	***r* = -0.61**	*r* = -0.28	*r* = -0.04	*r* = 0.24
TP					*r* = 0.11	*r* = -0.29	*r* = 0.24	*r* = 0.30	*r* = 0.07
Albumin						***r* = -0.46**	*r* = -0.28	*r* = -0.29	***r* = -0.35**
Urea							*r* = -0.07	***r* = -0.33**	*r* = -0.14
Bilirubin								*r* = 0.16	*r* = 0.01
AST									*r* = 0.22

Intensive postpartal lipid mobilization and ketogenesis are sufficient for a series of compensatory metabolic processes with changes in blood metabolic profile during early-lactation in healthy cows [Cincović *et al.* 2012]. In early-lactation cows, NEFA and BHB values were significantly higher (P<0.05) than in mid-lactation cows. NEFA concentrations > 0.40 mmol/l indicate problems with energy balance and subsequent intensive lipomobilization [Oetzel 2004]. According to this report, 6 out of 15 early lactating cows (40%) and none out of 15 mid-lactation cows showed evidence of high lipomobilization (NEFA >0.40 mmol/l) in the present study. Given the fact that serum NEFA concentrations >0.70 mmol/l are associated with ketosis [Oetzel 2004], 2 early-lactation cows (13.3%) and none of mid-lactation cows in the present study had NEFA concentrations above the value indicative of subclinical ketosis. Subclinical ketosis also may be diagnosed when serum BHB concentrations are above 1.2 mmol/l, while clinical ketosis is associated with BHB concentrations above 2.6 mmol/l [Duffied

2000, Oetzel 2004]. In the present study, 14 early-lactation cows (94.4%) and none of mid-lactation cows had serum BHB concentration above 1.2 mmol/l. These data suggest that serum NEFA could be less efficient indicator of subclinical ketosis (13.3%) than serum BHB (94.4%) in dairy cows during early lactation. In other words, high lipomobilization (high NEFA values) does not necessarily indicate that the cow is afflicted with subclinical ketosis. The data presented here show that serum NEFA may be used for detecting high lipomobilization, but not subclinical ketosis. This is in accordance with Duffield [2000], who stated that the use of NEFA is a better indicator of energy imbalance in prepartum animals than BHB, but BHB is more useful postpartum. In the present study, a significant positive correlation (r = 0.39, P<0.05) was established between NEFA and BHB in the sera, suggesting that both parameters are helpful indicators of energy balance during lactation. Additionally, the relationship between BHB and NEFA may be inferred from the significant correlations between BHB and glucose (r = -0.47; P<0.05), and BHB and TG values (r = -0.36; P<0.05). Blood glucose values in mid-lactation cows were within the physiological range (2.5-4.2 mmol/l) [Radostis *et al.* 2000], whereas hypoglycemia was detected in early-lactation cows. In the present study, 10 (66.6%) early-lactation and 5 (33.3%) mid-lactation cows had blood glucose concentrations below 2.5 mmol/l. According to the serum parameters, ketosis in cows may be diagnosed when the following values match the clinical signs: BHB >1.2 mmol/l, glucose <2.5 mmol/l and TG <0.12 mmol/l [Sevinc *et al.*1998, Oetzel 2004, Xu *et al*. 2008, Gonzales *et al*. 2011]. Taking this criterion into account, 7 (44%) early-lactation cows and no mid-lactation cow had indicative values, but did not display any clinical manifestations, suggesting that they are in a typical subclinical condition. In the study by Gonzales *et al*. [2011] a typical subclinical condition was detected in 6 (22%) high-yielding cows in early lactation.

Serum TG concentration was significantly lower (P<0.01) in ketotic cows compared to healthy cows [Djoković *et al*. 2007]. This suggests that TG accumulate in the liver cells of ketotic cows and cause their blood values to decrease. The present study showed that 7 early-lactation (44%) and 2 mid-lactation cows (13.3%) had TG concentration less than 0.12 mmol/l. In addition, no significant correlation (r = -0.21, P>0.05) was found between TG and NEFA, indicating that TG values may not be considered as an adequate indicator of lipomobilization in dairy cows. However, all cows suffered from subclinical ketosis (BHB >0.12mmol/l) according to the criterion cited above [Sevinc *et al.*1998, Oetzel 2004, Xu *et al*. 2008, Gonzales *et al*. 2011]. Six cows or 40% of early lactation cows (NEFA >0.40 mmol/l) and none of mid lactation cows were found to have TG values less than 0.12 mmol/l and glycemia below 2.5 mmol/l.

Hepatic lipidosis is generally preceded by an increase in the concentration of ketone bodies in serum and urine. During the first month of lactation, 5 to 10% of high-yielding dairy cows suffer from severe hepatic lipidosis and 30 to 40% have mild hepatic lipidosis [Bobe *et al.* 2004]. This means that nearly 50% of cows are at risk of metabolic disorders. When fat infiltrates the liver, a lesion appears in hepatic tissues and the levels of enzymes that indicate liver injury (AST, GGT, and LDH)

are generally augmented [Pechova *et al.*1997, Lubojacka *et al.* 2005, Jóźwik *et al*. 2012]. AST values in the present study were statistically higher (P<0.05) in early-lactation cows than in mid-lactation cows, and no significant difference (P>0.05) was observed between GGT activities in the two groups of cows. If AST activity higher than 100 IU/L is indicative of hepatic lesions [Gonzales *et al*. 2011], 2 (13.3%) out of 15 early- lactation cows in our study suffered from some degree of hepatic lesions, probably due to fat infiltration. These animals included 2 out of 7 cows considered to be ketotic according to our criteria, with blood NEFA values above 0.70 mmol/l. Meanwhile, none of 15 mid-lactation cows had AST values higher than 100 IU/L. Also, a positive correlation was observed between AST activity and lipomobilization, *i.e*. NEFA values (r = 0.34, P<0.05). In the present study, all data concerning serum AST activities suggested that the process of lipomobilization was sufficient to cause liver lesions in 13.3% of the early-lactating cows. In the study by Jóźwik *et al*. (2012), AST activities in the blood serum were lower in mid-yielding than in high-yielding cows, with no differences, however, observed within these groups in both aminotransferase activities between points of lactation. The authors showed that the values of aminotransferase activities were within the reference intervals, suggesting that the liver functions of high yielding cows were not disturbed.

Fat infiltration into the liver may also affects the concentration of some blood components. Glucose levels and TP, albumin, urea and total bilirubin concentrations may be diminished [West 1990, Veenhuizen *et al*.1991, Sevinc *et al*. 2003]. In the present study, glycemia and TP were significantly lower (P<0.05) in early-lactation than in mid-lactation cows. No significant difference (P>0.05) was identified in serum values for albumin, urea, TG and total bilirubin between the two groups of cows. Serum levels of glucose, TP, albumin and urea are indicators of hepatic functionality [Bobe *et al*. 2004] and decrease in their concentration may suggest fat infiltration into the liver. In fact, significant correlation was observed between NEFA values and glucose r = -0.35; P<0.05), TP (r = -0.34; P<0.05), urea (r = -0.45; P<0.05) and total bilirubin (r = 0.63; P<0.05) values. Possible changes in the liver function may have deleterious effects on the metabolism of these animals, and may affect milk production or reproduction.

Biochemical estimation suggested that early-lactation cows suffered from metabolic disturbances, which were associated with ketosis, and some degree of hepatic lesions, probably due to fat infiltration. Serum concentrations of BHB, NEFA, TG, glucose and AST may have a key role in evaluating the metabolic status of dairy cows.

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