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ANALYSE OF RELATIONSHIPS BETWEEN SOME MILK INDICATORS OF COW ENERGY METABOLISM AND KETOSIS STATE

Oto Hanuš[1], Daniel Falta[2], Marcela Klimešová[1], Eva Samková[3], Jan Říha[4], Gustav Chládek[2], Petr Roubal[1], Růžena Seydlová[1], Radoslava Jedelská[1], Jaroslav Kopecký[1]

[1]Dairy Research Institute, Ltd., Ke Dvoru 12a, 160 00 Prague 6 – Vokovice, Czech Republic
[2]Department of Animal Breeding, Faculty of AgriSciences, Mendel University in Brno, Zemědělská 1, 613 00 Brno, Czech Republic
[3]Agricultural Faculty, University of South Bohemia in České Budějovice, Branišovská 1, 370 05 České Budějovice, Czech Republic
[4]Bentley Czech Ltd., Hlinky 114, 603 00 Brno, Czech Republic

Abstract

HANUŠ OTO, FALTA DANIEL, KLIMEŠOVÁ MARCELA, SAMKOVÁ EVA, ŘÍHA JAN, CHLÁDEK GUSTAV, ROUBAL PETR, SEYDLOVÁ RŮŽENA, JEDELSKÁ RADOSLAVA, KOPECKÝ JAROSLAV. 2017. Analyse of Relationships Between Some Milk Indicators of Cow Energy Metabolism and Ketosis State. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis*, 65(4): 1135–1147.

Good health is important for animal reproduction and support of foodstuff chain quality and safety. Milk is suitable for noninvasive monitoring. Analyses of milk composition and result interpretation along environmental conditions and physiological principles plays important role in health control. Ketosis is a disorder of energy metabolism in dairy cows. It deteriorates milk yield (MY) and quality and animal reproduction and can have a fatal impact. Aim was to analyse relationships between milk energy metabolism indicators as acetone (AC) and component quotients which can serve in health control and estimate their thresholds. AC and fat/lactose (F/L) and fat/crude protein (F/CP) ratio could represent good ketosis indicators. There were 960 individual milk samples from the Holstein (H) and Czech Fleckvieh (CF) dairy cows (1:1). Their whole lactations, first third of lactations (FTL) and summer and winter seasons were analysed. Lactation MY of included herds (7) varied from 5,500 to 10,000 kg. Milk AC levels did not differ ($P > 0.05$) between H and CF breed in whole lactation but differed in FTL (H 3.88 and CF 2.72 $mg.l^{-1}$; $P < 0.05$). This difference 1.16 $mg.l^{-1}$ is equal to 29.9% and this is not too much essential. The F/L ratio was not affected ($P > 0.05$) by parity and season in whole lactation but was influenced ($P < 0.01$ and < 0.05) in this way during FTL. As maximum there can be explained only 17.8% of variability (correlation index 0.42; $P < 0.001$) in log AC values by F/L quotient variability for CF in FTL in summer season. As maximum there can be explained up to 84.4% of variations (correlation index 0.92; $P < 0.001$) in F/CP values by F/L quotient variations for H in FTL in summer season. Equations were used for model calculation of AC and F/L cut-off limits to subclinical ketosis indication in FTL period. These were from 2.05 to 3.29 $mg.l^{-1}$ about AC and from 0.84 to 0.87 about F/L according to breeds (CF and H) and lactation parity (1st and others). The results can be used in methods of cow health problem identification, prevention, treatment and troubleshooting during lactation.

Keywords: cattle breed, season, lactation; health, negative energy balance, acetone, fat/crude protein coefficient, fat/lactose coefficient, diagnostic threshold value

INTRODUCTION

Good health state of farm animals is always more important for support of foodstuff chain safety. There is whole row of components in milk according to which is possible to control the health state of cows via method of noninvasion monitoring (Mottram *et al.*, 2002). There are so called majority components: fat; protein; lactose; somatic cell count. Also minority components as metabolities with direct link to dairy cow nutrition state are important: citric acid; urea; free fatty acids; ketones. The casein from majority milk components is important newly (because of possibility of routine analyses) as well. Milk in contrast to blood or urine offers easy sampling (Mottram *et al.*, 2002), which could be routinely mastered including cold transport into laboratory.

Ketones in milk (acetone and betahydroxybutyrate) are suitable for confident noninvasive monitoring and control of dairy cow nutrition and health state. Ketones are product of fat catabolism in the start of lactation in time of energy malnutrition. It is mostly in early lactation in time during negative energy balance (NEB). They are seen as undesirable metabolities similarly as urea or free fatty acids. NEB, ketosis and ketones in milk and other body liquids (blood, plasma, urine, cervical mucus) reduce milk yield and reproduction performance (Gravert *et al.*, 1991; Vojtíšek *et al.*, 1991; Miettinen, 1995; Gasteiner, 2000; Enjalbert *et al.*, 2001; Duffield *et al.*, 2009; Ducháček *et al.*, 2012; Beran *et al.*, 2012; Januš and Borkowska, 2013) of cows, which can die as well. That is why higher milk urea (Piatkowski *et al.*, 1981; Ropstad and Refsdal, 1987; Butler *et al.*, 1996; Hojman *et al.*, 2004) and acetone concentrations can be connected with aggravated reproduction indicators (Fig. 1 and 2). Higher concentrations of these metabolites can also deteriorate milk technological properties such as fermentationability (Hanuš *et al.*, 1993).

Beside milk paresis also NEB and ketosis play important role in succession of following production disorders such as abomasum displacement, lameness, reproduction disorders (for instance placenta retention and metritis) or mastitis new infection in early lactation during NEB in high yielding dairy cows (Geishauser *et al.*, 1997a, b; Reist *et al.*, 2002; Duffield *et al.*, 2009; Mitev *et al.*, 2011). Ketosis milk yield reduction was observed (Gravert *et al.*, 1991; Gustafsson and Emanuelson, 1996) as well. As mentioned above, ketosis can change also milk composition (Hansen, 1999; Hana *et al.*, 2007; Siebert and Pallauf, 2010). Therefore the predicative value of component quotients as fat/crude protein (F/CP) and fat/lactose (F/L) was studied (Geishauser and Ziebell, 1995; Duffield *et al.*, 1997; Steen *et al.*, 1996; Reist *et al.*, 2002; Eicher, 2004; van Knegsel *et al.*, 2010; Siebert and Pallauf, 2010; Hanuš *et al.*, 2011b; Manzenreiter *et al.*, 2013) in terms of possibilities for NEB (Heuer *et al.*, 2001b) and subclinical and clinical ketosis investigation. Also ketones and their diagnostical and predicative value and objective thresholds as possible health indicators were studied (Gravert *et al.*, 1986; Famigli-Bergamini, 1987; Gustafsson and Emanuelson, 1996; Baticz *et al.*, 2002; de Roos *et al.*, 2007; Duffield *et al.*, 2009; van Knegsel *et al.*, 2010; Hanuš *et al.*, 2011b) in dairy cows.

As it has been introduced, the ketosis is serious animal health, milk quality and economical problem of highyielding dairy herds. Therefore some authors offered methods for ketosis prevention and treatment by successful using of various energy or health additives as glycerol, monensin or *Silybum marianum* in cow feeding rations (Vojtíšek *et al.*, 1991; Miettinen, 1995; Green *et al.*, 1999; Duffield, 2000; Gasteiner, 2003; Tedesco *et al.*, 2004; Hanuš *et al.*, 2011a; Coskun *et al.*, 2012).

Milk indicator testing is possible to carry out during regular milk recording or irregularly according to necessity for advisory service. A regular knowledge about milk ketone concentration can decide about efficiency of correction, preventive or treatment measures in the cow herds (Hanuš *et al.*, 2004, 2011a, b) and about the possibilities of assurance of milk food chain quality. Therefore, it can be effective procedure in the practice, which could support good animal welfare and health, their reproduction performance, milk yield and also herd economical results. That are reasons why various analytical methods for milk (blood, urine) ketones investigation such as gas chromatography, photometry, infrared spectroscopy with Fourier transformation (FT), biosensors or rapid stable tests were tested in terms of result reliability and diagnostical efficiency evaluation (O'Moore, 1949; Vojtíšek, 1986; Geishauser *et al.*, 1997a; Heuer *et al.*, 2001a; Mottram *et al.*, 2002; Baticz *et al.*, 2002; Carrier *et al.*, 2004; de Roos *et al.*, 2007; van Knegsel *et al.*, 2010; Hanuš *et al.*, 2011a; van der Drift *et al.*, 2012).

There is only a few interpretation results about diagnostical reliability of fat/lactose quotient in consideration of ketones and NEB identification. Therefore, the aim of this paper was to analyse and evaluate relationships between milk energy metabolism indicators as acetone and component quotients which can serve in monitoring and estimate thresholds some of them for methodical use at cow health problem identification, prevention, treatment and troubleshooting during lactation.

MATERIALS AND METHODS

Animals, their conditions and milk samples

The individual milk samples (MSs) were taken over three years (n = 960). The milk was sampled regularly in the summer (August and September) and winter (February and March) season. Twelve healthy (clinical and superior subclinical mastitis free) dairy cows with above average for herd milk yield from each herd were included in the one sample set. These represented an average number of lactations in relation to the herd and whole lactation profile in terms of average day in milk. The MSs were taken in the morning and evening milkings by flow samplers (Tru-Test; with milk yield measurement (MY)) similar to the official milk recording system.

Seven dairy herds were included in the investigation and represented two cattle breeds. The Czech Fleckvieh (CF) is a breed with combined milk and beef yield (on the Simmental basis) and Holstein (H) dairy breed. The two breeds were included in approximately two halves in the total set of MSs. The herds presented the whole profile of the nourishment condition scale in the Czech

Republic. Different but typical varieties of dairy cow nourishment, feeding rations and systems were applied in the herds: – alfalfa silage with maize silage in the lowland areas; – clover-grass silage, grass silage with maize silage and grass pasture in the highland areas. The concentrates were fed according to milk yield and nutrition demand standards. The nutritional and feeding systems were well balanced between breeds and keeping areas although the nutrition levels may not have been exactly comparable between breeds and herds. Therefore, the average milk yield varied in range from 5,500 to 10,000 kg per lactation. It was between the mean and high level of milk yield in the country.

Milk sample analyses

The MSs were transported in cold state ($\leq$10 °C) in thermoboxes to milk laboratory. The investigated milk indicators were as follows: milk yield (MY) fat (F) content (in g/100ml = %); crude protein (CP) content (in g/100g = %); lactose (L) content (lactose monohydrate in g/100g = %); milk acetone concentration (AC, in mg.l^{-1}). The F and L contents were determined using the instrument MilkoScan 133B (Foss Electric, Denmark), which was regularly calibrated according to the reference method (standard CSN 57 0536, by Gerber‘s acidobutyrometrical method for fat content and by polarimetric method for lactose content, according to the standard CSN 57 0530). The CP content was determined by the reference Kjeldahl‘s method according to standard CSN 57 0530. The AC content was investigated by photocolorimetry at 485 nm wavelength. The acetone was absorbed into an alkaline solution of KCl with the salicylaldehyde by 24 hours microdiffusion (O’Moore, 1949; Vojtíšek, 1986; Vojtíšek *et al.*, 1991) in special glass vessels (at 20 °C in darkness). The Spekol 11 instrument (Carl Zeiss Jena, Germany) was calibrated by the five points on the standard scale with increased acetone concentration in the calibration samples from 1 to 20 mg.l^{-1}. These method results are reliable however, other ketone and acetone detection methods were and still are under the development (Geishauser *et al.*, 1997; Hansen, 1999; Heuer *et al.*, 2000; Carrier *et al.*, 2004; de Roos *et al.*, 2007) and at first of all the stable tests and FT (Fourier transformation) infrared spectroscopy.

Data statistical treatment

F/L and F/CP quotients were calculated for results of individual MSs and evaluated in first third (FTL) and whole lactation. Standard statistical methods were used to evaluate the results (means, standard deviations and coefficient of variation). Differences between groups were tested in terms of breed, lactation number and season using Student‘s t-test. Linear and non linear regression were used for the correlations using relevant regression equations in the same groups using MS Excel (Microsoft, Redmond, USA). The highest correlation and regression coefficients and indexes were the most considered test statistics.

The original AC values were log transformed (Hanuš *et al.*, 2007; Janů *et al.*, 2007; de Roos *et al.*, 2007) as the data were not normally distributed. In this case, the arithmetic mean is not suitable parameter for representation of these data sets in all cases. It made possible the standard statistical testing for work with geometric means as well.

In such way this evaluation means a little bit metaanalytical access to results of our previous paper Hanuš *et al.* (2011b) especially in F/L and AC threshold (cut-off limit) estimation procedure for subclinical ketosis by reciprocal calculation according to linear regression results and literature references to these thresholds.

RESULTS AND DISCUSSION

Statistical comparison of main characteristics of ketosis milk indicators

Milk ketone concentration is influenced also due to various factors as breed, type of herd management, lactation phase or milk yield

I: *Basic statistics for investigated milk indicators*

Data set	n	MY x ± sd	log AC x ± sd xg	F x ± sd	CP x ± sd	L x ± sd	F/CP x ± sd	F/L x ± sd
Whole lactation	960	26.41 ± 7.995	0.2521 ± 0.457 1.79	3.94 ± 0.897	3.32 ± 0.349	4.96 ± 0.217	1.19 ± 0.266	0.80 ± 0.189
First third of lactation	329	28.65 ± 7.862	0.2894 ± 0.46 1.95	3.86 ± 0.973	3.20 ± 0.333	5.01 ± 0.200	1.21 ± 0.308	0.77 ± 0.200
Statistical significance of difference (t values)		4.40 ***	1.27 ns	1.36 ns	5.44 ***	3.68 ***	1.13 ns	2.45 *

Abbreviations: n = number of cases; x = arithmetic mean; sd = standard deviation; xg = geometric mean; MY = milk yield in kg/day; AC = acetone concentration in milk (mg.l^{-1}); F = milk fat content (g.100ml^{-1}, %); CP = milk crude protein content (g.100g^{-1}, %); statistic significance: ns = P>0.05; * = P$\leq$0.05; ** = P$\leq$0.01; *** = P$\leq$0.001; t = t-test value; abbreviations are valid for all tables and figures.

II: *Mean values, variability and test of difference results for acetone content, F/CP and F/L ratio in whole lactation and in first third of lactation according to chosen farmer and lactation factors*

Set	Whole lactation									First third of lactation								
	n	AC	log AC	F/CP	F/L	AC	logAC	F/CP	F/L	n	AC	log AC	F/CP	F/L	AC	logAC	F/CP	F/L
		x ± sd	x ± sd xg	x ± sd	x ± sd	t-value					x ± sd	x ± sd xg	x ± sd	x ± sd	t-value			
CF breed only	477	2.62 ± 2.584	0.2316 ± 0.453 1.70	1.18 ± 0.259	0.82 ± 0.195	1.94 ns	1.38 ns	0.58 ns	4.11 ***	163	2.72 ± 3.058	0.235 ± 0.451 1.72	1.2 ± 0.302	0.79 ± 0.209	1.96 *	2.13 *	0.88 ns	1.36 ns
H breed only	483	3.11 ± 4.882	0.2724 ± 0.461 1.87	1.19 ± 0.274	0.77 ± 0.181					166	3.88 ± 6.911	0.3427 ± 0.464 2.20	1.23 ± 0.314	0.76 ± 0.189				
First lactation only	272	2.96 ± 4.069	0.2488 ± 0.467 1.77	1.16 ± 0.239	0.78 ± 0.184	0.46 ns	0.14 ns	2.10 *	1.48 ns	81	3.41 ± 4.926	0.327 ± 0.403 2.12	1.15 ± 0.282	0.73 ± 0.193	0.19 ns	0.84 ns	2.03 *	2.36 *
Other lactations	688	2.83 ± 3.86	0.2535 ± 0.454 1.79	1.2 ± 0.276	0.80 ± 0.191					248	3.28 ± 5.534	0.2771 ± 0.478 1.89	1.23 ± 0.314	0.79 ± 0.200				
Winter season	480	2.95 ± 3.731	0.238 ± 0.493 1.73	1.2 ± 0.246	0.80 ± 0.176	0.71 ns	0.96 ns	1.16 ns	0.82 ns	158	3.05 ± 4.149	0.2475 ± 0.4800 1.77	1.23 ± 0.267	0.80 ± 0.187	0.84 ns	1.62 ns	0.88 ns	2.74 **
Summer season	480	2.77 ± 4.1	0.2663 ± 0.418 1.85	1.18 ± 0.285	0.79 ± 0.202					171	3.55 ± 6.317	0.3300 ± 0.4390 2.14	1.21 ± 0.342	0.74 ± 0.207				
CF first lactation	130	2.36 ± 2.423	0.1799 ± 0.474 1.51	1.15 ± 0.22	0.80 ± 0.181					30	2.43 ± 3.174	0.2274 ± 0.338 1.69	1.09 ± 0.215	0.72 ± 0.152				
CF other lactations	347	2.71 ± 2.639	0.251 ± 0.444 1.78	1.2 ± 0.271	0.83 ± 0.200					133	2.79 ± 3.04	0.2368 ± 0.474 1.73	1.23 ± 0.314	0.81 ± 0.217				
H first lactation	142	3.5 ± 5.081	0.3119 ± 0.454 2.05	1.17 ± 0.256	0.75 ± 0.184					51	3.98 ± 5.664	0.3855 ± 0.429 2.43	1.19 ± 0.311	0.74 ± 0.215				
H other lactations	341	2.94 ± 4.795	0.2559 ± 0.464 1.80	1.2 ± 0.281	0.78 ± 0.179					115	3.84 ± 7.42	0.3237 ± 0.48 2.11	1.24 ± 0.316	0.76 ± 0.178				
CF winter season	238	2.8 ± 3.033	0.2015 ± 0.521 1.59	1.18 ± 0.251	0.81 ± 0.183					83	2.55 ± 3.148	0.1607 ± 0.506 1.45	1.22 ± 0.291	0.81 ± 0.206				
CF summer season	239	2.43 ± 2.032	0.2617 ± 0.372 1.83	1.19 ± 0.266	0.83 ± 0.205					80	2.9 ± 2.971	0.3121 ± 0.373 2.05	1.19 ± 0.313	0.77 ± 0.211				
H winter season	242	3.11 ± 4.309	0.2739 ± 0.463 1.88	1.22 ± 0.239	0.80 ± 0.169					75	3.6 ± 4.995	0.3435 ± 0.432 2.21	1.25 ± 0.237	0.80 ± 0.165				
H summer season	241	3.11 ± 5.406	0.2709 ± 0.46 1.87	1.16 ± 0.303	0.74 ± 0.187					91	4.11 ± 8.181	0.342 ± 0.492 2.20	1.21 ± 0.366	0.72 ± 0.200				

Abbreviations: CF = Czech Fleckvieh; H = Holstein; AC = milk acetone concentration; F/CP = fat/crude protein ratio; F/L = fat/lactose ratio log = decimal logarithm; n = number of cases; x = arithmetic mean; xg = geometric mean; sd = standard deviation; ns, *, ** and *** = statistic significance = P>0.05, ≤0.05, ≤0.01 and ≤0.001.

(Gravert *et al.*, 1991; Miettinen, 1994; Wood *et al.*, 2004; Hana *et al.*, 2007; Hanuš *et al.*, 2007; Janů *et al.*, 2007) beside cow nutrition and ketosis. Therefore here these factors were taken into consideration in statistical evaluation too.

This paper studies mutual relations among AC, F/L and F/CP while our previous paper (Hanuš *et al.*, 2011b) studied only relations between AC and F/CP. The main statistical results are shown in Tab. I and II. As MSs were split on whole lactation and FTL group there were significant differences for MY, CP, L, and also F/L ($P < 0.001$ and <0.05). The F, log AC and F/CP did not differ significantly ($P > 0.05$; Tab. I) although ketosis is situated in general regularly in FTL (Gasteiner, 2000, 2003; Manzenreiter *et al.*, 2013). In terms of this result the F/L quotient could be more susceptible in ketosis indication than F/CP but both these corresponding stage lactation differences (+0.02 and −0.03) are small in the fact. The mean values of all milk indicators and their standard deviations and relevant stage lactation and other differences (Tab. I, II) are logical in terms of general lactation dynamics and also in accordance with our previous results (Hanuš *et al.*, 2007; Janů *et al.*, 2007). Milk AC levels (log AC) did not differ ($P > 0.05$) between H and CF breed in whole lactation but differed significantly in FTL (H 3.88 and CF 2.72 mg.l^{-1}; $P < 0.05$; Tab. II). This could be promising for effective subclinical ketosis indication as ketosis is more often occuring in H as breed with generally higher MY (Hanuš *et al.*, 2007; Janů *et al.*, 2007). This difference 1.16 mg.l^{-1} is equal to 29.9%. In spite of this fact such difference is not so high and therefore breed is not so essential factor for further evaluation in terms of intepretation of health indicator value to subclinical ketosis. This could be evident after comparison according to AC value scale versus ketosis state. In another words, though the H–CF difference 29.9% is statistically significant regarding ketosis intepretation scale (Hanuš *et al.*, 2001, 2011a), where subclinical ketosis is ≥10, clinical ketosis ≥35 and the highest investigated value 225 mg.l^{-1}, this is not too much essential.

AC (log AC) mean values (Tab. II) were not significantly influenced ($P > 0.05$) by parity (1st versus other lactations) and season (winter and summer). This fact could be promising for subclinical ketosis identification with this indicator. The F/CP ratio was significantly influenced ($P < 0.05$) by parity in whole lactation and FTL period but not ($P > 0.05$) by season. The F/L ratio was not affected ($P > 0.05$) by parity and season in whole lactation but was influenced ($P < 0.01$ and <0.05; Tab. II) in this way in FTL. These combinations for F/CP and F/L ratio are also not so bad in terms of their ability for subclinical ketosis detection.

Relationship analyse of energy milk indicators

The study of log AC × F/L and F/CP × F/L correlations in energy (ketosis) milk indicators is one of the first cases of analysis in terms of their mutual relationship according to accessible scientific literature sources. The relationships among log AC, F/L and F/CP are shown in Tab. III, IV, V and VI. Milk AC values showed high degree of variability (Tab. I and II, high sd, very often over 100% of variability coefficient). This fact can influence AC relations to other indicators (F/L) in negative way. Therefore, as maximum there can be explained only 17.8% of variability in log AC values by F/L quotient variability for CF breed in FTL in summer season (Tab. IV). It means there was also noted the highest correlation index 0.42 ($P < 0.001$; Fig. 3). This summer FTL finding can be caused by usually

III: *The relationships between milk indicators F/L (x) and log AC (y) over whole lactation according to breed, number of lactation and season*

Set	Number of samples	Equation form	Coefficient or index of correlation		Coefficient of determination (%)
Whole sample set	960	$y = 0.3351x - 0.0144$	0.14	***	1.9
CF breed only	477	$y = 0.1965x + 0.0704$	0.08	***	0.7
H breed only	483	$y = 0.5387x - 0.1427$	0.21	***	4.5
First lactation only	272	$y = 0.2385 \ln x + 0.31545$	0.12	***	1.5
Other lactations	688	$y = 0.3482x - 0.0261$	0.15	***	2.2
Winter season	480	$y = 0.4133x^2 - 0.3514x + 0.2406$	0.13	***	3.7
Summer season	480	$y = -0.2533x^2 + 0.7578x - 0.163$	0.17	***	2.8
CF first lactation	130	$y = -0.4544x^2 + 1.0091x - 0.3228$	0.1	***	1.1
CF other lactations	347	$y = 0.3812x^2 - 0.4768x + 0.3694$	0.09	***	0.9
H first lactation	142	$y = 0.4524x - 0.0294$	0.18	***	3.4
H other lactations	341	$y = 0.5908x - 0.2034$	0.23	***	5.2
CF winter season	238	$y = 0.2441 \ln x + 0.2865$	0.11	***	1.3
CF summer season	239	$y = 0.1969 \ln x + 0.3395$	0.14	***	1.9
H winter season	242	$y = 1.1715x^2 - 1.5952x + 0.739$	0.17	***	2.9
H summer season	241	$y = -0.4556x^2 + 1.1889x - 0.3917$	0.2	***	4.1

Abbreviations: CF = Czech Fleckvieh; H = Holstein; ln = natural logarithm.

IV: *The relationships between milk indicators F/L (x) and log AC (y) in first third of lactation (FTL) according to breed, number of lactation and season*

Set	Number of samples	Equation form	Coefficient or index of correlation		Coefficient of determination (%)
Whole sample set	329	y = 0.5886x – 0.1655	0.26	***	6.5
CF breed only	163	$y = 0.5298x^2 – 0.4678x + 0.2511$	0.22	***	4.7
H breed only	166	y = 0.8308x – 0.2856	0.34	***	11.5
First lactation only	81	y = 0.6859x – 0.1765	0.33	***	10.8
Other lactations	248	y = 0.5847x – 0.1822	0.25	***	6.0
Winter season	158	y = 0.5284x – 0.1777	0.21	***	4.3
Summer season	171	y = 0.7152x – 0.2036	0.34	***	11.3
CF first lactation	30	$y = –0.5965x^2 + 1.626x – 0.6195$	0.34	***	11.9
CF other lactations	133	$y = 0.7004x^2 – 0.8002x + 0.3942$	0.22	***	4.6
H first lactation	51	y = 0.6365x – 0.0875	0.32	***	10.2
H other lactations	115	y = 0.9699x – 0.4155	0.36	***	12.9
CF winter season	83	$y = –1.2287x^2 + 2.6775x – 1.0309$	0.25	***	6.5
CF summer season	80	$y = 1.2644x^2 – 1.2028x + 0.4865$	0.42	***	17.8
H winter season	75	$y = 2.1166x^2 – 3.2353x + 1.3592$	0.3	***	9.1
H summer season	91	$y = –0.4154x^2 + 1.4167x – 0.4876$	0.32	***	10.1

V: *The relationships between milk indicators F/L (x) and log F/CP (y) over whole lactation according to breed, number of lactation and season*

Set	Number of samples	Equation form	Coefficient or index of correlation		Coefficient of determination (%)
Whole sample set	960	y = 1.1928x + 0.2388	0.85	***	71.8
CF breed only	477	y = 1.1567x + 0.2342	0.87	***	75.9
H breed only	483	y = 1.2864x + 0.2005	0.85	***	71.8
First lactation only	272	$y = –0.218^2 + 1.4659x + 0.1591$	0.85	***	72.2
Other lactations	688	$y = –0.1619^2 + 1.4915x + 0.1118$	0.85	***	72.0
Winter season	480	y = 1.1708x + 0.258	0.84	***	70.2
Summer season	480	y = 1.2089x + 0.2246	0.85	***	73.0
CF first lactation	130	$y = –0.2252x^2 + 1.4651x + 0.1207$	0.90	***	80.2
CF other lactations	347	y = 1.1739x + 0.2269	0.87	***	75.0
H first lactation	142	$y = –0.2705x^2 + 1.6219x + 0.1134$	0.85	***	72.0
H other lactations	341	$y = –0.1923x^2 + 1.6429x + 0.0444$	0.85	***	72.1
CF winter season	238	$y = –0.2769x^2 + 1.5365x + 0.1412$	0.84	***	70.3
CF summer season	239	y = 1.1982x + 0.2358	0.86	***	74.6
H winter season	242	y = 1.2619x + 0.1936	0.84	***	71.2
H summer season	241	$y = –0.329x^2 + 1.7619x + 0.0034$	0.85	***	71.8

higher MY in this period. For comparison, there was noted the highest correlation index between F/CP and log AC 0.48 ($P < 0.001$) for H breed in FTL period and summer season by previous work (Hanuš *et al.*, 2011b). According to factors the correlations varied from 0.08 to 0.23 in whole lactation (Tab. III) and from 0.21 to 0.42 in FTL (Tab. IV). All these were statistically significant ($P < 0.001$). The most important relationship between F/L and log AC regarding probability of subclinical ketosis occurrence in FTL (Gasteiner, 2000, 2003; Manzenreiter *et al.*, 2013) was 0.33 ($P < 0.001$; Tab. IV) which is equal to relevant comparable relation for F/CP and log AC 0.33 ($P < 0.001$; Hanuš *et al.*, 2011b). As all relationships between F/L and log AC were closer in FTL (Tab. IV) than during whole lactation (Tab. III) so this is logically good message for possibility of subclinical ketotis identification by both these milk indicators because of main period of ketosis occurrence in dairy cows.

Further, the F/L and F/CP quotients have common the F indicator. Therefore, as maximum there can be explained up to 84.4% of variations in F/CP values by F/L quotient variations for H breed in FTL in summer season (Tab. VI). It means there was also noted the highest correlation index 0.92

VI: *The relationships between milk indicators F/L (x) and F/CP (y) in first third of lactation (FTL) according to breed, number of lactation and season*

Set	Number of samples	Equation form	Coefficient or index of correlation		Coefficient of determination (%)
Whole sample set	329	$y = -0.2428x^2 + 1.7676x + 0.0025$	0.89	***	78.6
CF breed only	163	$y = 1.3104x + 0.1669$	0.91	***	82.0
H breed only	166	$y = -0.2933x^2 + 1.9319x - 0.0569$	0.88	***	78.2
First lactation only	81	$y = -0.4521x^2 + 1.9908x - 0.0469$	0.87	***	76.1
Other lactations	248	$y = -0.2075x^2 + 1.7404x + 0.0026$	0.89	***	79.2
Winter season	158	$y = -0.22x^2 + 1.6042x + 0.0907$	0.86	***	74.5
Summer season	171	$y = -0.2392x^2 + 1.893x - 0.0673$	0.91	***	83.1
CF first lactation	30	$y = 1.286x + 0.1703$	0.91	***	82.6
CF other lactations	133	$y = 1.307x + 0.1729$	0.9	***	81.4
H first lactation	51	$y = -0.6668x^2 + 2.3433x - 0.1536$	0.87	***	75.8
H other lactations	115	$y = 0.4403x^2 + 0.9179x + 0.273$	0.9	***	81.6
CF winter season	83	$y = 0.8477 \ln x + 1.4252$	0.83	***	69.5
CF summer season	80	$y = 0.2473x^2 + 1.1536x + 0.1836$	0.9	***	81.7
H winter season	75	$y = 1.3294x + 0.1696$	0.89	***	79.2
H summer season	91	$y = -0.4144x^2 + 2.1771x - 0.164$	0.92	***	84.4

VII: *The relationships between milk indicators log AC (x) and F/L (y) and F/L (x) and log AC (y) in first third of lactation according to breed and number of lactation*

Data set	Equation form	
	log AC × F/L	**F/L × log AC**
CF first lactation	$y = 0.1534x + 0.6834$	$y = 0.757x - 0.3164$
H first lactation	$y = 0.1598x + 0.6815$	$y = 0.6365x - 0.0875$
CF other lactations	$y = 0.0856x + 0.7855$	$y = 0.4098x - 0.0934$
H other lactations	$y = 0.1329x + 0.7191$	$y = 0.9699x - 0.4155$

VIII: *The results of reciprocal estimation of cut–off values for milk indicators such as aceton content and F/L ratio for the purpose of diagnosis of dairy cow subclinical ketosis in first third of lactation*

Milk indicator	Previous cut–off limit	Specification	Estimated improved cut–off limit
AC	10 $mg.l^{-1}$	CF first lactation	0.84 F/L
	10 $mg.l^{-1}$	H first lactation	0.84 F/L
	10 $mg.l^{-1}$	CF other lactations	0.87 F/L
	10 $mg.l^{-1}$	H other lactations	0.85 F/L
F/L	0.99	CF first lactation	2.71 $mg.l^{-1}$ AC
	0.95	H first lactation	3.29 $mg.l^{-1}$ AC
	0.99	CF other lactations	2.05 $mg.l^{-1}$ AC
	0.95	H other lactations	3.21 $mg.l^{-1}$ AC

According to equations in Tab. VII.

IX: *The estimations of milk indicator cut–off limits for cow subclinical ketosis indication in first third of lactation according to frequency distribution law (x + sd × 1.64 for unilateral range, from Tab. II)*

Set	AC	log AC	F/CP	F/L
Unit	**$mg.l^{-1}$**	**$mg.l^{-1}$**	-	-
CF first lactation	7.64	6.07	1.44	0.99
CF other lactations	7.78	10.33	1.74	1.17
H first lactation	13.27	12.28	1.7	1.09
H other lactations	15.71	12.91	1.76	1.05

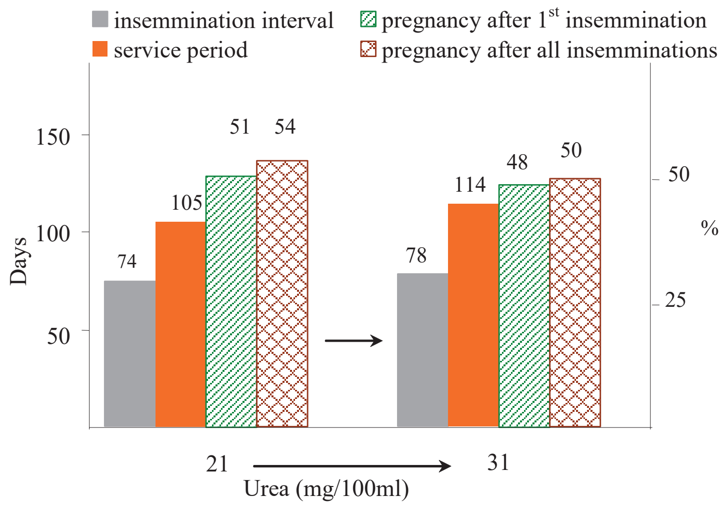
($P < 0.001$; Fig. 4). This summer FTL finding can bee also caused due to usually higher MY in this period. According to factors the correlations varied from 0.84 to 0.9 in whole lactation (Tab. V) and from 0.83 to 0.92 in FTL (Tab. VI). All correlations were statistically significant ($P<0.001$). The most essential relation between F/L and F/CP regarding probability of subclinical ketosis occurrence in FTL (Gasteiner, 2000, 2003; Manzenreiter *et al.*, 2013) was 0.87 ($P < 0.001$; Tab. VI). As all relationships between F/L and F/CP were closer in FTL (Tab. VI) than during whole lactation (Tab. V) so this is also good result for possibility of subclinical ketotis identification by both these milk indicators because of main period of ketosis occurrence. Ketosis mainly (80%) occurs during the first 50 days of lactation and about 35% of the positive diagnoses were made during the first 10 days of lactation (Manzenreiter *et al.*, 2013). Therefore, the practical utilization of information from regular milk recording (monthly sampling) is limited by this fact that for 49% of the positively diagnosed dairy cows no milk recording (sampling) was conducted within a relevant time period before ketosis was diagnosed. Manzenreiter *et al.* (2013) estimated in this way that only 30% of ketosis occurrence can be successfuly investigated in practice because of missing of milk recording results in the important time of lactation beginning (only regular monthly sampling and no sampling in colostrum period during first 5 lactation days) although there are good abilities of quotients (from main milk composition) for subclinical ketosis identification and prediction.

Qualified estimation of suitable and efficient F/L and AC tresholds (cut-off limits)

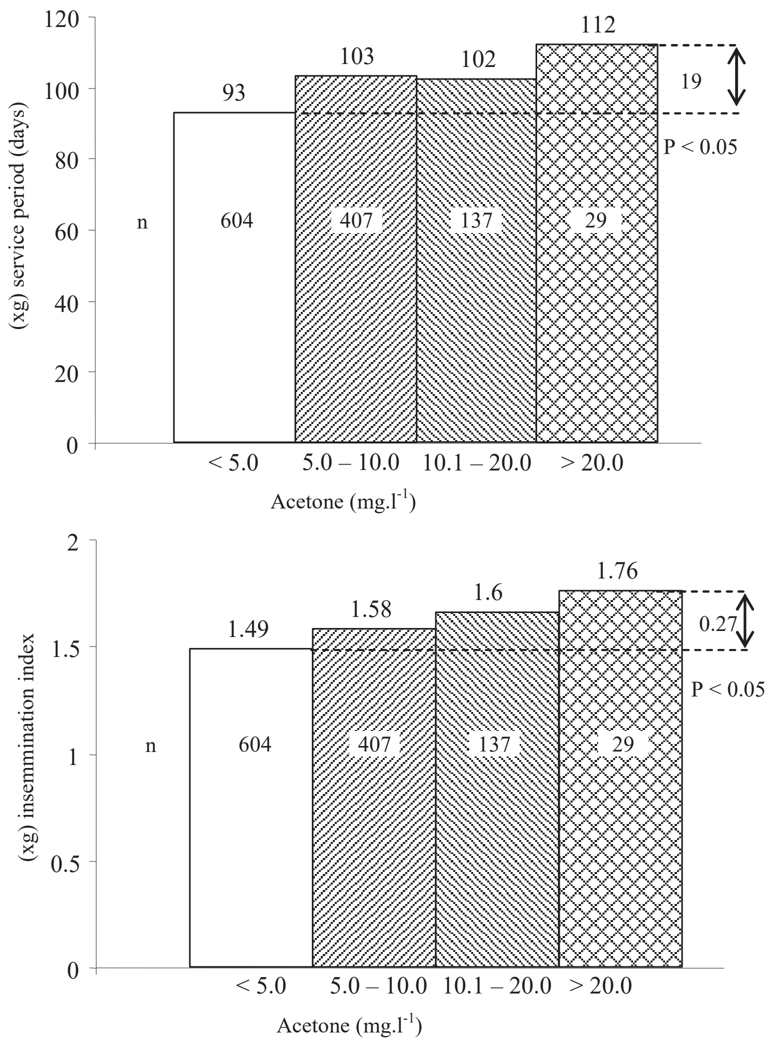
Siebert and Pallauf (2010) recommended to reduce F/CP threshold from 1.5 to 1.33. Under these conditions Manzenreiter *et al.* (2013) found 60.9% of right ketosis diagnose (F/CP over 1.33 in ketosis group – subclinical and clinical state defined as ketosis/acetonemia by authors) and in control group there was 38.5% with false positive findings (group without ketosis with F/CP value over 1.33 (Fleckvieh cows)). The diagnostical reliability was better for F/CP value 1.33 as compared to 1.5. Also our previous results (Hanuš *et al.*, 2011b) confirmed F/CP threshold value 1.27 and 1.32 (FTL, first lactation) and 1.52 and 1.42 (FTL, other lactations, CF and H dairy cows) at different way of statistical estimation according to milk acetone concentration. Steen *et al.* (1996) and Reist *et al.* (2002) found the reliability of F/L value for ketosis identification (subclinical and clinical, with threshold 0.9) as better in comparison to F/CP value. The same result was concluded also by Manzenreiter *et al.* (2013). They found 66.7% of right diagnose in ketosis group and in control group 37.5% of F/L values were over 0.9 as false positive findings. Considering ketosis diagnose it is too late at clinical signs and in terms of effective prevention and treatment the diagnose of subclinical state is very important (Duffield *et al.*, 1997; Duffield, 2000; Gasteiner, 2000; Hanuš *et al.*, 2011a, b) because of practical purposes. A part of ketosis occurence could be indetified in its subclinical period for its efficient prevention. In practice it is possible to remove subclinical mastitis from milk delivery according to somatic cell count result but it is problem to eliminate milk for ketosis. Subclinical ketosis can threaten milk quality. That is why ketosis identification methodical improvement is important. The cut-off value (threshold) 1.5 at F/CP quotient for subclinical ketosis was used also by van Knegsel *et al.* (2010) although Duffield *et al.* (1997) reported 1.3. Under such conditions the sensitivity to diagnose of hyperketonemic dairy cows was 66% which means lower as compared to milk beta-hydroxybutyrate and acetone (80%) by FT infrared spectroscopy with relevant cut-off values 23 and 70 $\mu mol.l^{-1}$. Unglaub (1983) studied milk acetone concentration in German dairy cow population. He stated acetone physiological concentration and also its risk subclinical ketosis threshold 7 $mg.l^{-1}$ for first 10 weeks of lactation. There is historical variability about this limit which can range from 2 to 41 $mg.l^{-1}$ (from 0.03 to 0.7 $mmol.l^{-1}$), Gustafsson and Emanuelson (1996). Their high cut-off limit was derived in dependence on relevant milk losses. Nevertheless, most of studies described cut-off value from 7 to 23 $mg.l^{-1}$ (from 0.12 to 0.4 $mmol.l^{-1}$), Gravert *et al.* (1986), Miettinen (1995) and Gasteiner (2000). In our previous papers (Hanuš *et al.*, 2011a, b) we used winter and summer cut-off limit 10 and 7 $mg.l^{-1}$.

Obtained significant linear relationships between F/L and log AC were included in Tab. VII. These were used for reciprocal model calculation of AC and F/L cut-off limits to subclinical ketosis indication in FTL period (Tab. VIII) according to above mentioned recommended and corresponded literature (previous) cut-off limits. These were from 2.05 to 3.29 $mg.l^{-1}$ about AC and from 0.84 to 0.87 about F/L according to breeds (CF and H) and lactation parity (1^{st} and others). This estimations could be validated also in easy way by conventional calculation using 95% confidence interval of probability (Tab. IX; $x + sd \times 1.64$ for unilateral range, from Tab. II) as frequency occurrence of subclinical ketosis is approximately considered. However, these cut-off limits (Tab. IX) show higher values than previous (Tab. VIII) estimations and probably are less efficient.

Januš and Borkowska (2013) monitored higher levels of ketone bodies in the urine of cows (Black-and-White Polish Holstein-Friesian) at higher milk yield in first six months (from 26.8 to 20.0 versus 24.3 and 18.8 kg per day) after calving but especially in first third of lactation. Duffield *et al.* (2009) found significant impact of hyperketonemia in early lactation of dairy cows on their subsequent abomasum displacement and metritis occurrence and also on their greater fat percentage and less protein percentage on first test day in milk recording. A similar impact on milk

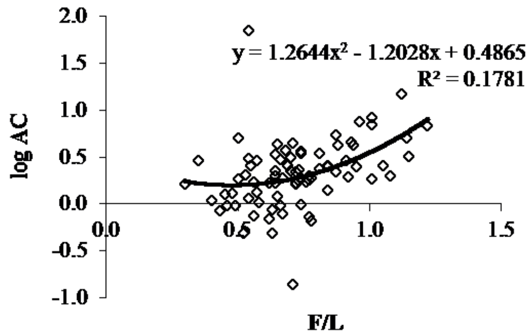


1: *The relationships between long term bulk milk urea level increase and means for reproduction performance indicators of dairy cow herds (the correlation (P < 0.01) of metioned relationships moves from 0.15 to 0.22, Říha and Hanuš, 1999 a)*



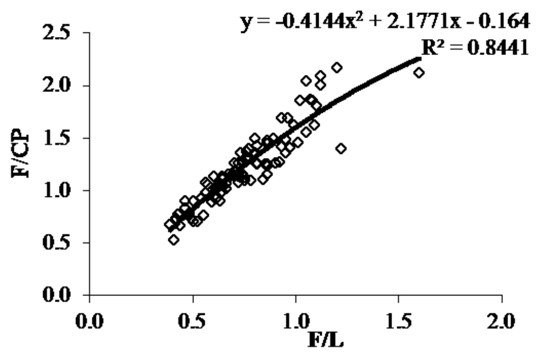
n = number of cases; xg = geometric mean

2: *The relation of acetone levels in dairy cows (individual milk samples) to some reproduction indicators in the first third of lactation (Říha and Hanuš, 1999b; Hanuš et al., 2004)* composition in terms of fat/protein ratio during ketosis observed previously also Geishauser and Ziebell (1995). The calving interval increased by 4.56 days for each unit increase in the natural logharithm to the concentration of acetone measured at first artificial insemination. This implies that cows with the highest values of acetone experienced a calving interval prolonged by 20.3 days as compared to cows with the lowest measured for acetone at first artificial insemination (Waldmann *et al.*, 2003). Heuer *et al.* (2000) carried out the evaluation of prediction precision at multiple regression model for estimation of energy balance of high yielding dairy herd from second to twelwth lactation week. The control of milk yield, dairy cow body condition score (similarly as Ducháček *et al.* (2012) as well), ketone test, fat, protein and lactose content from test day of milk recording and fat/ptotein ratio were included into this model. The information from milk recording test day without ketone level test and body condition score is sufficient for estimation of herd mean energy balance, but herd size limits the precision of prediction, as it was concluded by these authors.



(n = 80; r = 0.422; P < 0.001)

3: *The relationship between F/L ratio (fat/lactose monohydrate) and log AC (acetone in mg.l⁻¹) in individual milk samples for Czech Fleckvieh cattle breed and first third of lactation in summer season*



(n = 91; r = 0.9187; P < 0.001)

4: *The relation between F/L ratio (fat/lactose monohydrate) and F/CP ratio (fat/crude protein) in individual milk samples for Holstein cattle breed and first third of lactation in summer season*

CONCLUSION

Milk AC level and also F/CP and F/L quotient are good indicators for monitoring and prevention checks of subclinical ketosis in dairy cows (by threshold or cut-off values) owing to the close correlations between them, especially in FTL period. Milk AC levels did not differ between H and CF in whole lactation but differed significantly in FTL. In practice interpretation the AC breed (H–CF) difference (1.16 mg.l^{-1} = 29.9%; P < 0.05) in FTL is not so essential. Cut-off values of F/L and AC according to relevant milk AC and F/L threshold for subclinical ketosis were predicted by modelling. Now, the real time analysis of main milk components (fat, protein, lactose, solids non fat) and somatic cell count are implemented in milking parlours. Dairy cow breeders can know milk composition, calculate milk energy quotients, investigate and identified subclinical ketosis in this way every day. It could improve the ketosis prevention and reduce economical losses for farmers. Higher operation possibility of this system is clear. The estimations of thresholds of studied milk indicators (AC and F/L) in early lactation for subclinical ketosis (from 2.05 to 3.29 mg.l^{-1} and from 0.84 to 0.87) can be used at above mentioned technological innovation in animal husbandry.

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Contact information

Oto Hanuš: hanus.oto@seznam.cz
Daniel Falta: daniel.falta@mendelu.cz
Marcela Klimešová: marcela.vyletelova@seznam.cz
Eva Samková: samkova@zf.jcu.cz
Jan Říha: jan@bentleyczech.cz
Gustav Chládek: chladek@mendelu.cz
Petr Roubal: roubal@milcom-as.cz
Růžena Seydlová: seydlova@milcom-as.cz
Radoslava Jedelská: radka.jedelska@seznam.cz
Jaroslav Kopecký: jaroslav.kopecky@email.cz