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Ketosis in Dairy Cows during Early Lactation - Detection in Pooled Blood Serum Samples

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ABSTRACT

***Background*:** Ketosis is the most important metabolic disease with prevalence from 15 to 45%. Ketosis is diagnosed using a metabolic profile. Due to the high prevalence, it is necessary to determine a large number of metabolic profiles within farm, which represents an additional cost, so the implementation of pooled serum in assessing the metabolic status of cows was examined. The aim of this study was to validate and evaluate the influence of the relative position (Z-score) of the value of pooled sample metabolic parameters within the known reference value of healthy cows in the detection of ketosis in herd during early lactation.

***Materials, Methods & Results*:** The experiment has been carried out using blood samples collected by puncture of coccygeal vein from 50 ketotic and 50 healthy cows. Laboratory analysis includes determination of beta-hydroxybutyrate-BHB, non-esterified fatty acids-NEFA, cholesterol-CHOL, triglycerides-TGC, glucose-GLU, albumin-ALB, total protein-TPROT, UREA, Ca, P, total bilirubin-TBIL and aspartat aminotransferase-AST. The pooled serum was made from 10 individual samples originating from 10 different cows. A serum aliquot of 0.1 mL was taken from each sample, and a 1 mL volume of pooled serum was finally formed. Three types of serum pools were made: 1) 30 pooled sample were from ketosis; 2) 30 pooled sample were from healthy cows and 3) 60 pooled samples containing mixed sera of healthy cows and cows with ketosis were made as follows: 10 pools contain 10% to 60% of ketotic cows (1/10 to 6/10 samples). Statistical analysis includes: a) difference in metabolite concentration and Z-score in pooled sample and arithmetic mean individual sample in healthy and ketotic cow, b) correlation between Z-score of pooled sample and arithmetic mean of individual sample, c) ability of Z-score of metabolite to divide ketotic from healthy cow, d) correlation between Z-score and % of ketotic cow in pooled sample; and e) calculation of 95%CI of pooled sample Z-scores for each % of ketotic cow in pools. Z-score and all analysis were calculated for each metabolic parameter. The results of the study show that the mean values and Z-scores of the pool and the calculated average value of the individual samples participating in that pool differ significantly in healthy cows and cows in ketosis, except for TPROT and Ca. A higher value and a higher Z-score were found for BHB, NEFA, UREA, TBIL and AST, and a lower value and a lower Z-score for TGC, CHOL, GLU, ALB and P in ketotic cows compared to healthy cows. The value of the Z-score of the pooled sample and the calculated mean values of individual samples participating in the pool are highly correlated with each other (coefficient of determination over 99%). Z-score of metabolites in the pooled sample can be used to distinguish healthy from ketotic cows (ROC AUC= 0.711 to 0.989), except for TPROT and Ca. The Z-score value of the pooled sample shows a linear correlation with the percentage of ketotic cows in the pool and the reference ranges of Z-scores change significantly as a function of the percentage of ketosis cows.

***Discussion*:** Modern research on the metabolic profile in cows requires obtaining a large amount of information from as few samples as possible. The advantages of using the Z-score are reflected in the following: this score does not depend on the absolute value of the metabolite, but on the position within the known population reference value, Z-score of sample and the arithmetic mean of individual samples included in the same pool are almost identical, the Z-score of these 2 groups of results is ideally correlated, the Z-score significantly correlates with the % of ketosis samples in the pooled sample. The use of pooled sample Z-score can be a useful in a herd level assessment of metabolic status and detection of ketosis as most important metabolic disease in dairy cows.

Keywords: dairy cattle, ketosis, metabolic disease, metabolic profile, pooled serum, z-score, diagnostics.

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INTRODUCTION

Ketosis is the most important metabolic disease in cows that occurs as a result of increased homeoresis and negative energy balance in the early lactation, and is characterized by increased lipolysis, ketogenesis, fatty liver, inflammation and insulin resistance [8,10]. The incidence of ketosis in early lactation is from 15-45% and prevalence is from 30-53%, with bed consequence due to associated diseases, reduced reproductive performance and milk production and more frequent culling of cows [3,9,29,32].

Ketosis is diagnosed using a metabolic profile, when the limit values of BHB in the blood are > 3 mmol/L for clinical and 1.2-2.9 mmol/L for subclinical ketosis, while the value of BHB in milk is 10 times lower [15,23,25,34]. Limit values have also been established for other metabolic profile parameters such as NEFA, glucose or AST [4,31]. Due to the high prevalence, it is necessary to determine a large number of metabolic profiles within the farm, which represents an additional cost, so the possibility of using a pooled serum in assessing the metabolic status of cows was examined. Authors find significant difference between the mean value of pooled sample in healthy and diseased cow [14]. The concentration of metabolites in the pooled sample depends on the percentage of cows whose values are outside the reference values [33].

There are no data on the influence of pathological samples on metabolite values in pooled samples and how pool values are positioned through known reference values. The aim of this study was to validate and evaluate the influence of the relative position (Z-score) of the value of pooled sample metabolic parameters within the known reference value of healthy cows in the diagnosis of ketosis in dairy cows during early lactation.

MATERIALS AND METHODS

Samples and serum pooling

The experiment has been carried out using blood samples collected by puncture of coccygeal vein from 50 ketotic and 50 healthy cows. The cows were a Holstein-Friesian breed. The samples were delivered to the Laboratory of Pathophysiology at the Department of Veterinary Medicine, University of Novi Sad for the purpose of laboratory diagnosis of ketosis or regular metabolic profile of cows. Serum sample volume of 4 mL was obtained from each cow. Part of sample volume of 1 mL was used for determination of metabolic parameters, and 3 mL was divided on 30 portions (0.1mL per portion) - individual samples ready for pooling. Healthy and ketotic samples included in the pool were determined by random selection from basis of 50 ketotic and healthy cows, i.e. 3000 samples. The pooled serum was made from 10 individual samples originating from 10 different cows. A serum aliquot of 0.1 mL was taken from each sample, and a 1 mL volume of pooled serum was finally formed. Three types of serum pools were made: 1) 30 pooled sample were from ketosis, 2) 30 pooled sample were from healthy cows and 3) 60 pooled samples containing mixed sera of healthy cows and cows with ketosis were made as follows: 10 pools contain 10% (1 ketotic and 9 healthy individual sample participate in the pool, 1/10), 10 pools contain 20% (2/10), 10 contains 30% (3/10), 10 contains 40% (4/10), 10 contains 50% (5/10) and 10 contains 60% of ketotic cows (6/10).

Laboratory analysis

Blood samples were in appropriate tubes with clot activator[1]. Biochemical parameters (BHB, NEFA, CHOL, TGC, GLU, ALB, TPROT, UREA, Ca, P, TBIL and AST) were measured by standard reagents[2] at automatic spectrophotometer[3]. Serum was haemolysis-free, transparent and species-appropriate and was processed within the recommended time range to avoid preanalytical variability [17].

Statistical analysis

Difference in metabolite concentration and Z-score in pooled sample and arithmetic mean individual sample included in pool in healthy and ketotic cow were determined. Four groups were formed which include pool healthy, mean healthy, pool ketotic and mean ketotic, and ANOVA analysis was used. The relative positional value of each individual metabolite was determined whether it was determined in a pool or calculated from individual samples that participated in the preparation of that pool. The relative position value is expressed through the Z-score according to the following formula $Z = (x-\mu) / SD$, where Z-position value (Z-score), x - value of the pool or the mean value of individual samples involved in making that pool, μ - mean

population value and SD - standard deviation of the reference population. The mean population and SD population were determined based on the reference values for dairy cows established by our laboratory [1], and included cows in the territories where the farms from which the samples in this study originate are located. In order to determine the possibility of using the Z-score pool as a universal indicator, the correlation between the Z-score of pooled sample and the calculated mean values originating from the same samples that formed the pool using Pearson's correlation coefficient was examined. It was then examined whether the discrimination of healthy and ketotic cows could be performed on the basis of the Z-score value, which was graphically represented as the ROC curve and the area below the ROC curve (ROC AUC) was determined and tested. Finally, the relationship between Z-score and% of cows with ketosis in the pooled sample was examined using Pearson's correlation coefficient and regression lines. At the end, mean value and preliminary 95%CI Z-score reference intervals for each metabolite were calculated as a function of the percentage of ketotic cows in the pooled sample. A standard statistical package[4] was used for statistical processing.

RESULTS

The results of the study show that the mean values of the pool and the mean of the individual samples participating in that pool, as well as the Z-scores of the pool and the calculated mean values differ significantly in healthy cows and cows in ketosis, except for TPROT and Ca, where statistically significant differences between healthy and kerosene cows (Table 1). A higher value and a higher Z-score were found for BHB, NEFA, UREA, TBIL and AST, and a lower value and a lower Z-score for TGC, CHOL, GLU, ALB and P in ketotic cows compared to healthy cows. There is no statistically significant difference between the measured value of the pool and the calculated mean value of the individual as well as their Z-scores within the healthy or ketotic group of cows separately (Table 1).

The value of the Z-score of the pooled sample and the calculated mean values of individual samples participating in the pool are highly correlated with each other (coefficient of determination over 99%) (Figure 1), so the Z-score of the pooled sample can be used to assess metabolic status of herd. By performing logistic regression and analyzing the ROC curve, we conclude that the Z-score of metabolites in the pooled sample can be used to distinguish healthy from ketotic cows (ROC AUC was 0.711 to 0.989), except for TPROT and Ca (ROC AUC was about 0.5) (Figure 2).

The Z-score value of the pooled sample shows a linear correlation with the percentage of ketotic cows in the pool for all examined parameters except for TPROT and Ca (Figure 3). Based on the correlation coefficient (square root of R2), we conclude that there are moderate to very strong correlations between the Z-score and the percentage of ketotic cows in the pooled sample. Based on the calculated mean and 95%CI ranges for Z-score, we found that the reference ranges of Z-scores change significantly as a function of the percentage of ketosis cows participating in the pool, especially when there are 40% or more of such cows within the pool (Table 2).

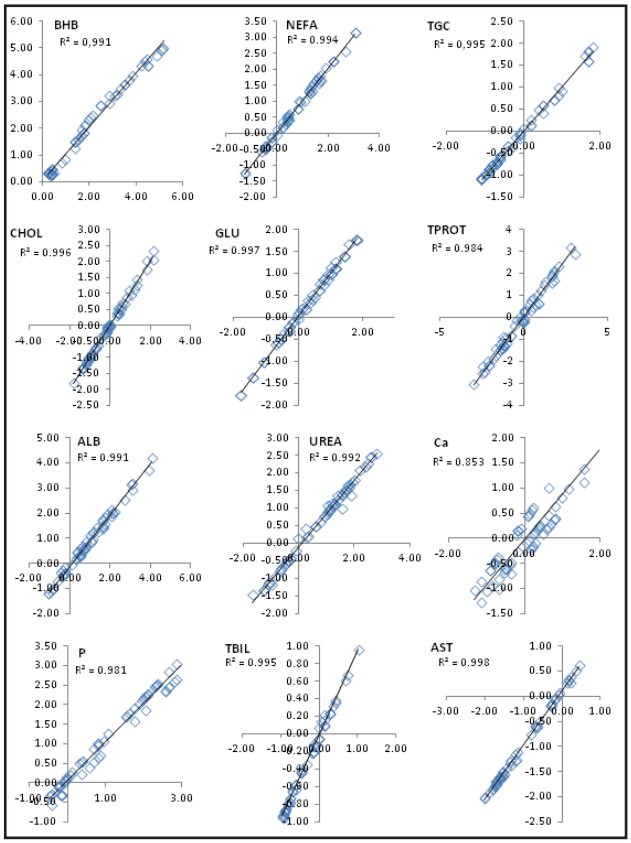

Figure 1. Regression line and correlation between Z-score of pooled sample and calculated mean of individual samples within pool.

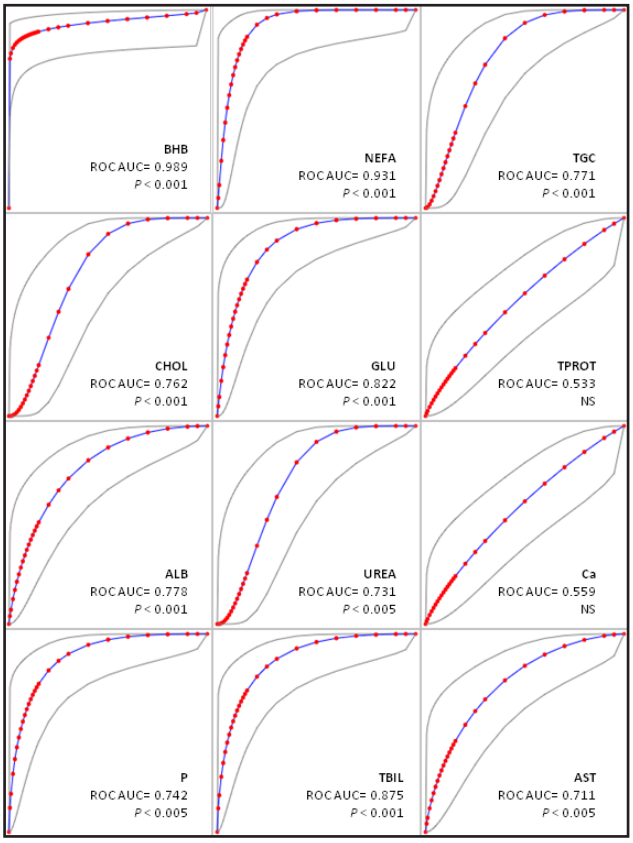

Figure 2. Prediction of ketosis based on Z-scores of metabolites in pooled sample.

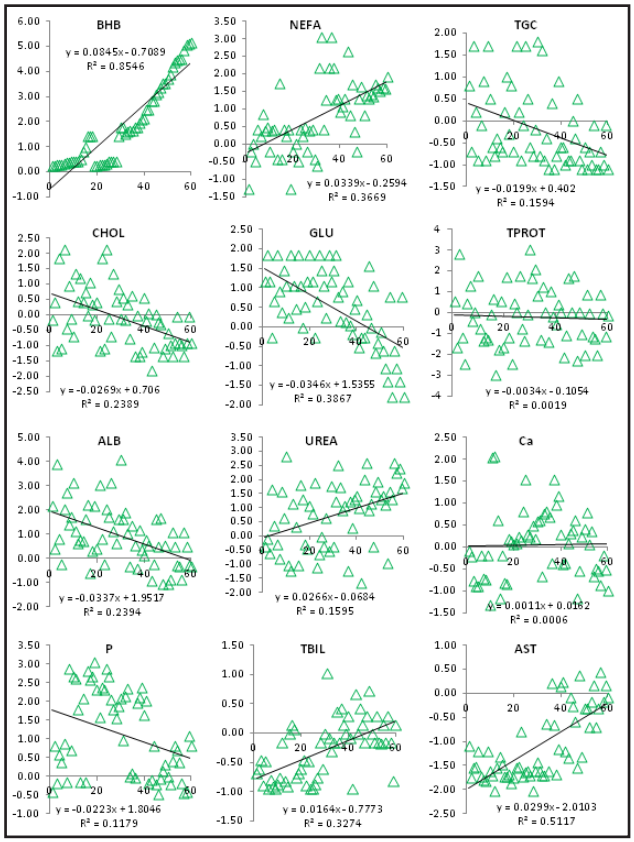

Figure 3. Regressions and correlations between pooled sample Z-score of metabolites and percent of ketotic cows in pool.

Table1. Concentration of metabolites and Z-scores in healthy and ketotic cows.

Metabolites	Ref. value	Ketotic				Healthy			
		Pooled conc.	Mean conc.	Zscore pool	Zscore mean	Pooled conc.	Mean conc.	Zscore pool	Zscore mean
BHB* (mmol/L)	0.68 ± 0.29	1.59 ± 0.35^{A}	1.64 ± 0.32^{A}	3.17 ± 1.22^{a}	3.3 ± 1.1^{a}	0.85 ± 0.13^{B}	0.85 ± 0.13^{B}	0.57 ± 0.45^{b}	0.57 ± 0.45^{b}
NEFA (mmol/L)	0.5 ± 0.23	0.84 ± 0.16^{A}	0.85 ± 0.17^{A}	1.49 ± 0.72^{a}	1.52 ± 0.75^{a}	0.51 ± 0.14^{B}	0.52 ± 0.14^{B}	0.06 ± 0.6^{b}	0.08 ± 0.61^{b}
TGC (mmol/L)	0.21 ± 0.1	0.15 ± 0.07^{A}	0.16 ± 0.07^{A}	-0.56 ± 0.66^{a}	-0.52 ± 0.69^{a}	0.22 ± 0.09^{B}	0.22 ± 0.09^{B}	0.14 ± 0.92^{b}	0.13 ± 0.93^{b}
CHOL (mmol/L)	4.26 ± 1.67	3.27 ± 1.12^{A}	3.28 ± 1.14^{A}	-0.59 ± 0.67^{a}	-0.60 ± 0.68^{a}	4.87 ± 1.63^{B}	4.88 ± 1.64^{B}	0.36 ± 0.97^{b}	0.37 ± 0.99^{b}
GLU (mmol/L)	2.61 ± 0.76	2.56 ± 0.72^{A}	2.61 ± 0.72^{A}	-0.06 ± 0.95^{a}	0.002 ± 0.95^{a}	3.38 ± 0.49^{B}	3.35 ± 0.49^{B}	1.02 ± 0.65^{b}	0.97 ± 0.64^{b}
TPROT (g/L)	69.87 ± 6.84	68.7 ± 8.6^{A}	68.9 ± 9.2^{A}	-0.17 ± 1.3^{a}	-0.13 ± 1.4^{a}	68.2 ± 10.5^{A}	68.4 ± 8.6^{A}	-0.21 ± 1.6^{a}	-0.21 ± 1.5^{a}
ALB (g/L)	32.36 ± 5.12	34.1 ± 4.5^{A}	33.5 ± 4.5^{A}	0.33 ± 0.88^{a}	0.22 ± 0.92^{a}	40.1 ± 6.2^{B}	39.8 ± 6.2^{B}	1.51 ± 1.2^{b}	1.45 ± 1.21^{b}
Urea (mmol/L)	4.62 ± 1.38	6.28 ± 1.48^{A}	6.1 ± 1.43^{A}	1.2 ± 1.1^{a}	1.04 ± 1.05^{a}	5.1 ± 1.49^{B}	4.82 ± 1.39^{B}	0.28 ± 1.1^{b}	0.15 ± 1.04^{b}
Ca (mmol/L)	2.26 ± 0.31	2.26 ± 0.26^{A}	2.23 ± 0.21^{A}	0.08 ± 0.69^{a}	-0.11 ± 0.65^{a}	2.28 ± 0.21^{A}	2.31 ± 0.24^{A}	0.02 ± 0.84^{a}	0.17 ± 0.82^{a}
P (mmol/L)	2.07 ± 0.4	2.31 ± 0.37^{A}	2.37 ± 0.37^{A}	0.62 ± 0.93^{a}	0.76 ± 0.93^{a}	2.72 ± 0.44^{B}	2.69 ± 0.47^{B}	1.63 ± 1.1^{b}	1.57 ± 1.17^{b}
TBil (µmol/L)	9.11 ± 6.7	9.48 ± 2.7^{A}	9.23 ± 2.61^{A}	0.054 ± 0.4^{a}	0.02 ± 0.39^{a}	5.01 ± 2.26^{B}	5.2 ± 2.33^{B}	-0.61 ± 0.35^{b}	-0.59 ± 0.35^{b}
AST (IU/L)	113.5 ± 48.25	84.4 ± 34.4^{A}	88.42 ± 36.5^{A}	-0.61 ± 0.72^{a}	-0.52 ± 0.76^{a}	36.8 ± 12.7^{B}	35.5 ± 12.6^{B}	-1.59 ± 0.26^{b}	-1.62 ± 0.26^{b}

*The unit of measure refers to the mean value of the pool and the calculated mean value of the individual samples. The Z-score has no units of value; ^{A,B}Different superscript indicates differences in metabolite concentration; ^{a,b}Different superscript indicates differences in Z-scores.

Table 2. Mean value (95%CI) of pooled sample Z-score with different percent of ketotic cow within pool.

Metabolites	% of ketotic cow in pooled sample					
	10%	20%	30%	40%	50%	60%
BHB	0.32 (0.27-0.36)	0.78 (0.48-1.09)	0.6 (0.27-0.93)	1.79 (1.67-1.91)	3.09 (2.82-3.36)	4.61 (4.34-4.88)
NEFA	0.02 (-0.35-0.39)	0.13 (-0.36-0.62)	0.02 (-0.25-0.29)	1.78 (1.23-2.33)	1.15 (0.66-1.64)	1.54 (1.43-1.65)
TGC	0.32 (-0.28-0.91)	-0.21 (-0.19-0.61)	0.33 (-0.33-0.99)	-0.28 (-0.86-0.3)	-0.59 (-0.9-0.3)	-0.8 (-1.05-0.55)
CHOL	0.17 (-0.54-0.88)	0.58 (0.27-0.89)	0.34 (-0.39-1.07)	-0.28 (-0.79-0.23)	-0.66 (-1.1-0.3)	-0.85 (-1.13-0.57)
GLU	1.01 (0.61-1.41)	0.99 (0.56-1.42)	1.05 (0.64-1.47)	0.59 (0.21-0.97)	0.06 (-0.39-0.5)	-0.82 (-1.4-0.24)
TPROT	0.1 (-0.9-1.1)	-1.1 (-1.7-0.4)	0.12 (-0.9-1.16)	0.35 (-0.39-1.1)	-0.26 (-1.1-0.6)	-0.61 (-1.27-0.05)
ALB	1.8 (1.1-2.6)	0.94 (0.5-1.5)	1.8 (0.98-2.62)	0.73 (0.23-1.23)	0.24 (-0.4-0.9)	0.02 (-0.42-0.46)
Urea	0.49 (-0.24-1.22)	0.16 (-0.53-0.85)	0.2 (-0.43-0.83)	1.04 (0.43-1.65)	1.04 (0.27-1.8)	1.53 (0.91-2.15)
Ca	-0.61 (-0.86-0.36)	0.21 (-0.84-0.9)	0.46 (0.19-0.73)	0.58 (0.17-0.99)	-0.04 (-0.4-0.3)	-0.31 (-0.66-0.04)
P	0.82 (0.12-1.52)	1.96 (1.23-2.69)	2.11 (1.83-2.39)	1.35 (0.72-1.98)	0.22 (-0.2-0.67)	0.29 (-00.6-0.64)
TBIL	-0.76 (-0.88-0.64)	-0.46 (-0.71-0.2)	-0.62 (-0.83-0.4)	0.15 (-0.1-0.4)	0.1 (-0.2-0.4)	-0.08 (-0.28-0.12)
AST	-1.53 (-1.7-1.36)	-1.68 (-1.8-1.56)	-1.56 (-1.75-1.3)	-1.04 (-1.5-0.57)	-0.61 (-1.02-0.18)	-0.16 (-0.24-0.24)

DISCUSSION

Modern research on the metabolic profile in cows requires obtaining as much information as possible from as few samples as possible or as few metabolic parameters as possible that are determined in biological material such as blood or milk.

The goal is to obtain one central result from a large number of results in the form of an integral data that would indicate a metabolic imbalance. Today, numerous technological methods are used, from sensors to various models of neural networks in order to maximize the value of routine data obtained in the diagnosis of ketosis, and the use of Z-scores is of great importance in normalizing the network [19,27,30].

In order to obtain integral data, intraindividual variability and subject-based reference values were determined [18]. Also, the classification of cows according to the values of anabolic and catabolic indicators in periparturient period enables the prediction of the values of metabolic parameters in the first eight weeks after calving [2,6,16]. Determination of the main components and reduction of the number of metabolic parameters in ketosis cows shows which parameters are higher and which are less important in diagnosis [7], and centering and normalization of these parameters using Z-score is also of great importance.

In our paper, we examined the possibility of reducing the number of samples by pulling individual samples, and the Z-score was chosen as a universal indicator of position within the known frequency distribution of reference values used in our region. The frequency distribution of the pools relative to the normal distribution expressed through the QQ plot shows a sigmoid curve (non-shown results) which implies a tendency for the pools to be uniform and more stable than individual samples. Pulling of samples of different biological materials and determination of Z-score is of great importance in the application of modern omix methods when maintaining variability within the examined group [20,21]. All of the above speaks in favor of efforts to find a way to obtain the most valid diagnostic or prognostic information from as few samples and/or as few blood parameters as possible, where pooling and the use of Z-scores are of great importance.

The concentrations of metabolites in the sample pool are unique values for the herd participating in the pool. There is an almost ideal correlation between the Z-score of the pool and individual values,

which means that the Z-score of the pool is suitable for herd-level assessment of metabolic status. Our results agree with previously published results [28,33]. The value of the Z-score does not show signs of systemic deviation in the pool in relation to the Z-score of the calculated average, as well as in relation to the known reference value (Z-score deviation <2 for most parameters, except for those that change drastically in ketosis). This finding confirms the possibility of using the Z-score, because the system deviation values of the pooled sample in relation to the average of individual samples reduces the possibility of using the pool in decision making [12]. Z-scores of blood metabolites showed ability for discrimination between healthy and ketotic cow. In general, an ROC AUC of 0.7 to 0.8 is considered acceptable, 0.8 to 0.9 is considered excellent, and more than 0.9 is considered outstanding [13].

All selected metabolites and their Z-scores (except TPROT and Ca) showed a significant difference between healthy and ketotic cows. Hussein *et al*. [14] showed that the lactation period significantly affects the values of metabolites in the pooled sample, and there is a statistically significant difference between a healthy herd and a herd that was burdened with metabolic diseases such as ketosis or milk fever. Differences in metabolic values between different health categories were most pronounced in early lactation, which is consistent with our results. The same author found statistically significant differences for BHB, NEFA, TBIL, AST, Ca, P, which coincides with our results, but did not find a statistically significant difference for CHOL. In our experiment, the concentration of BHB is significantly higher in ketosis cows and increases linearly with the percentage of ketosis samples in the pooled sample. BHB is important in the diagnosis of subclinical and clinical ketosis, and a large number of different tests have been developed for its determination, which confirm that this metabolite is of primary importance in the diagnosis of ketosis [11]. In addition to the above, cut-off values were found for NEFA, TBIL, GLU and AST [4,8,22,31] important for the diagnosis of ketosis, which confirms the diagnostic or prognostic significance of these metabolites. Lower concentrations of ALB, CHOL and TGC are signs of hepatocyte fatty infiltration that often accompanies ketosis, as it occurs as a result of subcellular rearrangement of hepatocytes due to their overload of fatty acids and ketones in early lactation [5,24,26].

Z-score values significantly correlated with the percentage of ketotic cows in the pool, which agrees with previous results in which a correlation was found between standard deviation and the percentage of cows with metabolic parameters outside the reference values [33]. In order to evaluate the practical application of the Z-score of the pulled sample in the detection of ketosis cows, we calculated their potential reference range and examined the degree of overlap of these ranks between pools with different percentages of ketosis cows. The results show that 95% of the CI reference ranges of the Z-score overlap less (i.e., differ more) if there are 40-60% of ketosis cows in the pool compared to pools in which there are 10-30% of ketosis cows. Considering that the prevalence of ketosis in cows ranges from 30 to over 50% [3], this further confirms the potential of the Z-score of the pooled sample in detecting the presence of ketosis in the herd.

CONCLUSIONS

The measured values of metabolites in the pooled sample and the arithmetic mean of the values of individual samples included in the same pool are almost identical, and the Z-score of these 2 groups of results is ideally correlated, which confirms the possibility of using the pooled sample in assessing metabolic status. The Z-score of the metabolite of the pooled samples can be used to distinguish ketotic from healthy cows. The Z-score significantly correlates with the % of ketosis samples in the pooled sample. The use of a Z-score of a pooled sample can be a useful and economical way to assess ketosis in a herd of cows during early lactation.

MANUFACTURERS

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***Ethical approval*.** The Ethics Committee on animal use at University of Novi Sad (number IV-2017-02) approved this study.

***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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