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Research Article

The Influence of Body and Metabolic Parameters in the Period Before Sexual Maturation in Heifers on the Lameness Score During the First Lactation

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Abstract

The aim of this research was to examine the relation between body and metabolic parameters in 105 young Holstein-Friesian cows in the period before puberty and locomotion score (LS) in the first lactation. During the examination of heifers (6-12 months of age) body condition score (BCS), body measurements and growth rate were determined. The blood to determine metabolic and hematology profile. The LS was evaluated monthly during the first five months after calving. Multivariate analysis and principal component analysis stated that selected body conformation parameters, blood parameters and LS were interrelated. Simple linear trends and/or complex LS prediction models indicated a positive correlation between values of LS and BCS, body growth, bilirubin, aspartate-aminotransferase, glucose, calcium, nonesterified fatty acid, leukocytes, granulocytes and negative correlation with albumin and cholesterol ($P<0.01$). A positive correlation existed between LS in different months of the study ($P<0.001$). Prediction of LS was significantly better ($P<0.0001$) using complex models ($r=0.736$-0.905) that include body measures, blood parameters, and LS in the first month compared to models with individual parameters ($r=0.547$-0.757). Predetermination of cows that will develop lameness problems such as LS>2 or LS>2 using two consecutive measurements are possible based on metabolic parameters. LS and lameness occurrence in the first lactation could be predicted in heifers very early, even before reaching sexual maturity, using blood parameters and body measurements.

Keywords: *Blood parameters, Body parameters, Heifers, Lameness score*

Introduction

Lameness is one of the most common diseases on farms, and in the absence of the reproductive problems and mastitis, the most frequent problem was observed [1-3]. The incidence of lameness has increased since the 1980s, and the prevalence has been considered to range from 6 to 42%. In addition, every animal is periodically or chronically exposed to lameness biennially for various reasons [4,5].

Lameness occurs under the influence of numerous risk factors [6,7]. A more recent meta-analysis and systematic review by Oehm et al.[8] using 53 manuscripts from a database of 1941 manuscripts identified 128 factors that were associated with lameness, and the most significant ones were the body condition score, excessive hoof growth, number of days of lactation, herd size and parity. The period from calving to the onset of lameness is related to prophylactic measures, hoof conformation and the occurrence of infectious or non-infectious lesions [9]. The lactation number and the period of lactation are significant risk factors for lameness, so Kougioumtzis et al.[10] determined that locomotion and lameness problems, during the first lactation, were less pronounced just before and immediately after calving, and that they increased as lactation progressed. Ristevski et al.[11] and Ristevski et al.[12] demonstrated that cows with milk production above 30.9 kg/day showed a higher risk of chronic lameness. Also, a suboptimal body condition score (BCS <2.5 or >3) at the peak of lactation increases the probability of lameness. The same authors showed that metabolic factors such as an increase in BHB, LDH, or lower triglyceride values were also very significant for the occurrence of lameness, and exposure to a greater number of factors exhibited an additional increase in the risk of lameness

The aim of this study was to examine the relationship between the physical and metabolic characteristics of heifers aged 6-12 months and the onset of lameness in the first lactation. Additionally, the study aimed to determine whether the lameness score during the first lactation, in addition to the heifers' physical and metabolic traits, could be used to predict lameness in subsequent lactation months.

Material and Methods

Ethical Statement

The study protocol was approved by the Institutional Ethics Committee of the University of Novi Sad (protocol code IV/2017/02).

Animals and Management

One hundred and five Holstein heifers aged 6-12 months were included in the experiment. The heifers were reared in standard group housing conditions. All the heifers were from the same farm. They were under the constant supervision of a veterinarian. Water was available ad libitum, and feeding was carried out according to the standard for heifers, which ensures a gain of 700-750 g/day. The meal is based on meadow hay, corn silage, corn kernels and sunflower meal with the addition of a complete mixture. A typical meal for heifers aged 6-12 months included 1.5 kg of alfalfa hay, 5 to 10 kg of corn silage (from 6 to 12 months of age), 2.0 kg of sugar beet noodles, and 1.5-2.0 kg of concentrate (from 6 to 12 months of age). The meal contained the following composition: 6-7.5 kg of DM, NEL 40-48 MJ, SP 800-900 g, NDF 25%, NDF from roughage 19%, ADF 19%, and fat around 3%.

In this period, the heifers get used to a coarse meal, so the share of hay and silage increased in 12-month-old heifers compared to 6-month-old heifers.

Lameness Assessment

The Sprecher diagnostic scale of 1 to 5, where a score of 2 or higher indicates different levels of lameness, by descriptive and visual guide was considered for lameness evaluation *(https://open.lib.umn.edu/largeanimalsurgery/chapter/bovine-orthopedics/).*

Body Condition Score and Measurements

The measurement was performed by adspection of heifers and palpation of prominent body parts. For this purposes recommendations from Elanco Animal Health Bulletin Al 8478 (Rev.9/96) were used. Body mass was measured on a farm scale, and growth was determined on a monthly basis.

Laboratory Blood Analysis

Blood was taken by coccygeal puncture. The samples were taken in vacutainers with the addition of EDTA for hematological analysis and in plain tubes for serum separation for biochemical parameters. Hematological parameters included red blood cells (RBC), hemoglobin (HGB), mean cell volume (MCV), hematocrit (HCT), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC). Metabolic parameters in blood associated with metabolic stress were: non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), glucose, total bilirubin (TBIL), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), cholesterol (CHOL) and triglycerides (TGC). An automatic Chemray analyzer (Rayto, China) and standard kits (Biosystem, Spain) were used for biochemical analyses. Hematological analyses were performed with an automatic hematological counter Mek-6550 (Nihon Kohden, Japan).

Statistical Analysis

The relationship between the examined parameters was determined in order to analyze the possibility of establishing certain hypotheses about influence of BCS, body parameters and blood metabolic parameters on LS. This was done using multivariate statistics and principal component analysis. When dependence between the examined parameters was determined, the standard correlation analysis between the examined parameters. For this purpose, Pearson's correlation coefficient was considered. Complex linear models at three levels were carried out: models that included only body measurements, models that included only blood parameters, and models that included body measurements + blood parameters + health status (lameness score in the first month after calving). A linear regression model was applied, and the outcomes are displayed as a partial correlation of each parameter independently, highlighting the significance of each parameter on the LS prediction model as a whole. The difference in model quality was determined by comparing the coefficients of the entire model and using the Fisher transformation method.

Problematic cows - those with average LS above 2 and those with a score above 2 in two consecutive measurements - were identified using a logistic model. Cows that had problems were marked with a value of 1, while cows without such problems had a value of 0. The measure of discriminatory ability was shown by the area under the ROC curve.

The software at the link *https://biit.cs.ut.ee/clustvis/* was used for multivariate analysis, while other statistical analyzes were performed in the statistical program SPSS (IBM, USA).

Results

Multivariate analysis and analysis of principal components was showed the association between BCS and blood

parameters with LS in the first five months of the first lactation. The heat map shows that LS values and the percentage of problematic cows, as well as BCS values and many blood parameters changed in a relatively regular same or opposite direction with LS intensity. Further analyses of the main components showed that the use of selected parameters were enabling the differentiation of cows with different locomotor scores, as well as that discrimination can occur on the basis of BCS, but not on the basis of the age of the heifers when body measurements or blood sampling was considered. The results are shown in *Fig. 1*.

The relationship between the value of the locomotor score per month as well as the average value of the score was established. Tests indicated that there was a statistically significant positive correlation between all LS values from the first to the fifth month, as well as a high correlation of individual LS with the average LSAv. Correlations were of medium to strong intensity, and their value ranged from 0.346-0.793 (P<0.001). BCS generated a positive correlation (P<0.001) with the locomotor score and all problematic categories of heifers, except that it was not related to the category of heifers that had at least once a value of LS=3. Correlation between blood parameters and LS was also established in heifers. ALB showed a negative correlation with LSM4, LSM5, LSMAv, and the proportion of problematic heifers (with average LS more than 2 or LS in two consecutive measurements higher than 2). GLOB showed a positive correlation with LSM3, LSM4, LSM5, LSMAv and with the occurrence of heifers that have lameness problems such as high lameness score at level 4 or 5 and in which two consecutive measurements are higher than 2. TBIL and AST showed a statistically significant positive correlation with almost all LS and with the occurrence of heifers that are had problems with lameness. The correlation coefficient ranged from 0.2 to 0.45, and statistical significance was determined at the P<0.01 level for most connections. GLU shows a positive correlation with all LS (except LSM1) and all categories of problem cows, a correlation level of around 0.25 was reached with a statistical significance of P<0.01 for most connections. Ca showed a positive relationship

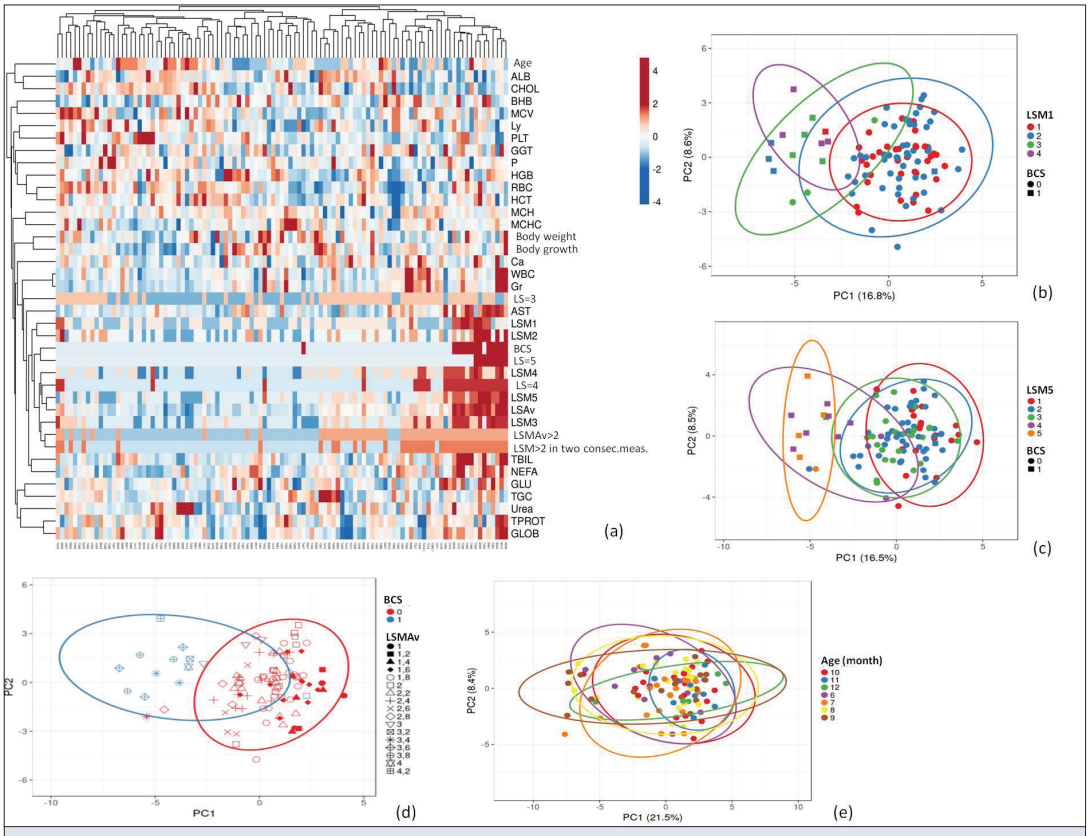

Fig 1. Heat map from multivariate analysis, intensity and direction of changes in locomotor score, body measurements and blood parameters in heifers (a). Principal component analysis and separation of heifers with different LS (b, c), BCS (d) and age (e) based on the values of the multivariate analysis of the investigated parameters. LSM1-5: Locomotion score in month 1 to 5, LSMAv: Average locomotion score in first five month of lactation, BWG: Body weight gain, BCS: Body condition score, TPROT: Total protein, ALB: Albumin, GLOB: Globulin, TBIL: Total bilirubin, AST: Aspartate aminotransferase, GGT: Gamma-glutamyl transferase, GLU: Glucose, Ca: Calcium, P: Inorganic phosphates, CHOL: Cholesterol, TGC: Triglycerides, BHB: Beta-hydroxybutyrate, NEFA: Non-esterified fatty acids, WBC: White blood cells, Ly: Lymphocyte, Gr: Granulocyte, RBC: Red blood cells count, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: platelet

with LSAv as well as with certain categories of problem heifers (those with an average LSAv over 2 and which in two consecutive measurements had LS greater than 2), and these correlations were statistically significant from P<0.05 to P<0.01. CHOL was negatively correlated with LSM5, LSMAv and with heifers that had LS 4 or 5 at least once and that in two consecutive measurements had LS greater than 2), and these correlations were statistically significant from P<0.05 to P<0.01. Blood parameters such as NEFA, WBC and granulocyte (Gr) (which is reflected in the number of neutrophils) showed a positive correlation with all examined parameters of lameness (P<0.01). Hematological parameters of red bloodline showed no significant relationship with lameness in heifers. PLTs

Table 1. *Correlations between the examined parameters and their statistical significance*

Parameters	LSM1	LSM2	LSM3	LSM4	LSM5	LSMAv
LSM1						
LSM2	0.572***					
LSM3	0.346**	0.459***				
LSM4	0.372**	0.415***	0.528***			
LSM5	0.379**	0.432***	0.505***	0.570***		
LSMAv	0.679***	0.750***	0.761***	0.776***	0.793***	
BWG	0.160	0.150	0.163	0.061	0.210*	0.199*
BCS	0.539**	0.436**	0.563**	0.543**	0.629**	0.723**
TPROT	0.021	0.032	0.081	0.092	0.032	0.061
ALB	-0.171	-0.134	-0.192	-0.201*	-0.261**	-0.258**
GLOB	0.151	0.132	0.232*	0.255**	0.232*	0.271**
TBIL	0.427**	0.399**	0.385**	0.398**	0.413**	0.534**
AST	0.232*	0.224*	0.309**	0.261**	0.450**	0.401**
GGT	0.051	0.012	0.082	0.102	0.123	0.101
GLU	0.132	0.246*	0.183	0.265**	0.297**	0.302**
Ca	0.174	0.262**	0.154	0.162	0.191	0.247*
P	-0.14	0.023	-0.082	-0.013	-0.062	-0.074
Urea	0.052	-0.064	0.014	0.101	0.021	0.033
CHOL	-0.152	-0.173	-0.248*	-0.191	-0.237*	-0.265**
TGC	0.093	0.151	0.064	0.012	0.041	0.091
BHB	0.054	0.062	0.024	0.103	0.172	0.112
NEFA	0.343**	0.254**	0.386**	0.345**	0.436**	0.473**
WBC	0.032	0.238*	0.403**	0.260**	0.280**	0.332**
Ly	-0.053	-0.043	0.043	0.013	-0.071	-0.033
Gr	0.052	0.255**	0.392**	0.256**	0.308**	0.345**
RBC	0.153	0.152	0.132	0.103	-0.021	0.132
HGB	0.112	0.033	0.101	-0.042	0.072	0.074
HCT	0.154	0.032	0.172	0.094	0.063	0.134
MCV	-0.013	0.054	0.171	-0.135	-0.171	-0.032
MCH	0.012	0.062	0.09	0.003	0.013	0.054
MCHC	-0.052	0.064	0.122	-0.034	0.084	0.052
PLT	0.236*	0.001	-0.101	-0.052	-0.121	-0.031

LSM1-5: Locomotion score in month 1 to 5, LSMAv: Average locomotion score in first five month of lactation, BWG: Body weight gain, BCS: Body condition score, TPROT: Total protein, ALB: Albumin, GLOB: Globulin, TBIL: Total bilirubin, AST: Aspartate aminotransferase, GGT: Gamma-glutamyl transferase, GLU: Glucose, Ca: Calcium, P: Inorganic phosphates, CHOL: Cholesterol, TGC: Triglycerides, BHB: Beta-hydroxybutyrate, NEFA: Non-esterified fatty acids, WBC: White blood cells, Ly: Lymphocyte, Gr: Granulocyte, RBC: Red blood cells count, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: platelet
** P<0.05; ** P<0.01; *** P<0.001*

showed a positive correlation with LSM1. The results are shown in *Table 1*.

Models based on body measurements include the use of parameters such as breast girth, body length, weight, gain and BCS in the prediction of LS in heifers in the first five months after calving (LSM1-5) and LSMAv. The use of these parameters enables a statistically significant prediction of the locomotor score in each month as well as the average LSMAv with a correlation level of r=0.519-0.757 (P<0.001). The BCS value had the largest partial correlation and a dominant role in the model. Additionally, LSM5 and LSMAv positively correlate with body weight gain. The results are shown in *Fig.2*.

The blood parameter-based model enables statistically significant predictions of both monthly LS and LSAv, with a correlation level of r=0.604-0.787 (P<0.01 to P<0.001). Depending on the month of LS determination, blood parameters such AST, GLU, Ca, TBIL, NEFA, BHB, WBC, and Gr were statistically significant in the model. The results are shown in *Fig.2*.

Models based on body measurements + blood parameters + locomotor score in the first month include the use of all measured parameters in the prediction of the locomotory score in heifers from the second to the fifth month after calving, as well as the average score. The use of these parameters enables a statistically significant prediction of the locomotor score in each month as well as the average for all five examined months with a correlation level of 0.736-0.905 and a statistical significance of P<0.001. BCS plays a dominant role in the model, and parameters such as TBIL, GLU, Ca, NEFA, WBC, MCV, MCH or HCT have statistical significance, which depends on the month of the test, while LSM1 significantly correlates with LSM2 and LSAv. The results are shown in *Fig.2*.

The model with the lowest correlation with LS was the one that just included body measures. Models with blood parameters and body measurements also showed a stronger association, and models with blood parameters, body measurements, and health (LSM1) showed the strongest correlation with LS. When body measurements, blood parameters, and LSM1 were included in complex

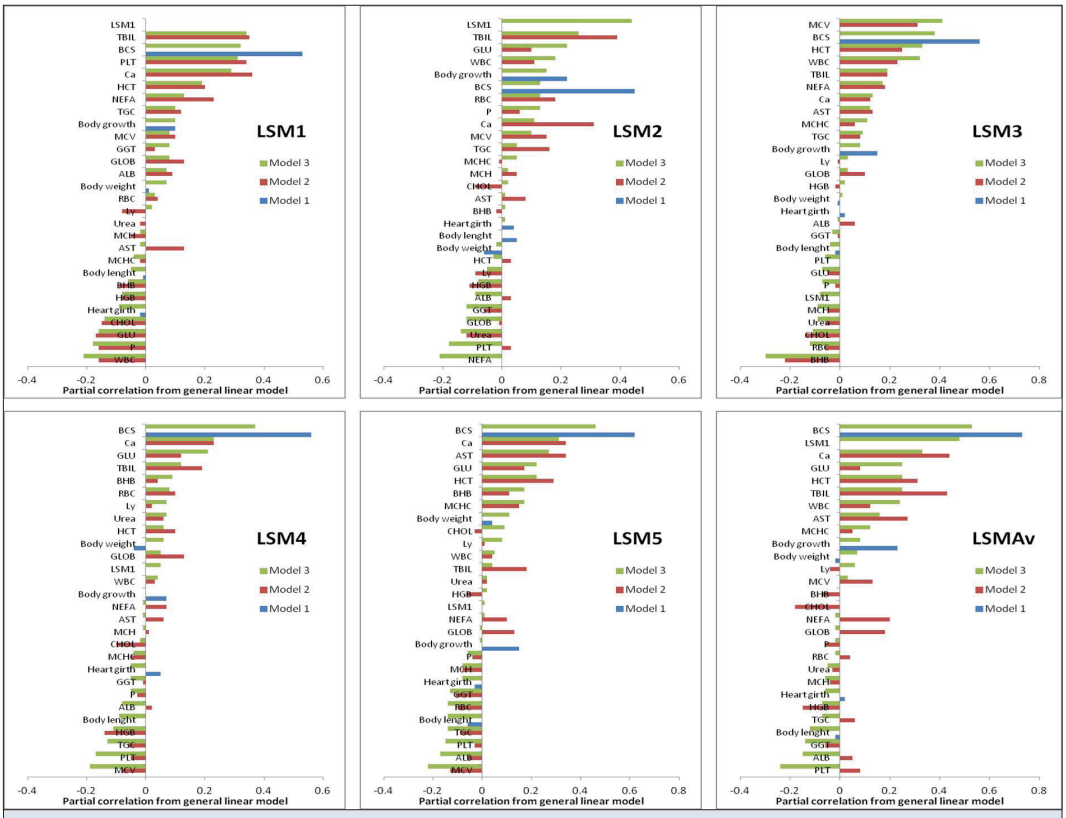

Fig 2. Partial correlations of parameters with LS obtained from GLM models including body measurements (Model 1), blood parameters (Model 2), body measurements + blood parameters + LSM1 (Model 3). (**Partial correlation** r>|0.15|, P<0.01). LSM1-5: Locomotion score in month 1 to 5, LSMAv: Average locomotion score in first five month of lactation, BWG: Body weight gain, BCS: Body condition score, TPROT: Total protein, ALB: Albumin, GLOB: Globulin, TBIL: Total bilirubin, AST: Aspartate aminotransferase, GGT: Gamma-glutamyl transferase, GLU: Glucose, Ca: Calcium, P: Inorganic phosphates, CHOL: Cholesterol, TGC: Triglycerides, BHB: Beta-hydroxybutyrate, NEFA: Non-esterified fatty acids, WBC: White blood cells, Ly: Lymphocyte, Gr: Granulocyte, RBC: Red blood cells count, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, PLT: platelet

Table 2. *Efficacy of models by comparing their correlation coefficient between the predictor and the outcome variable via Fisher's r-to-z transformation*

Outcomes	Correlations in Model 1 (Body Measurements)	Correlations in Model 2 (Blood Parameters)	Correlations in Model 3 (Body Measurements + Blood Parameters + LSM1)	Significance of Difference		
				Model 1: Model 2	Model 1: Model 3	Model 2: Model 3
LSM1	0.547	0.681	0.736	NS	<0.001	NS
LSM2	0.519	0.633	0.758	NS	<0.001	NS
LSM3	0.600	0.741	0.794	NS	<0.001	NS
LSM4	0.593	0.604	0.706	NS	<0.001	NS
LSM5	0.647	0.699	0.793	NS	<0.001	NS
LSMAv	0.757	0.787	0.905	NS	<0.001	<0.001

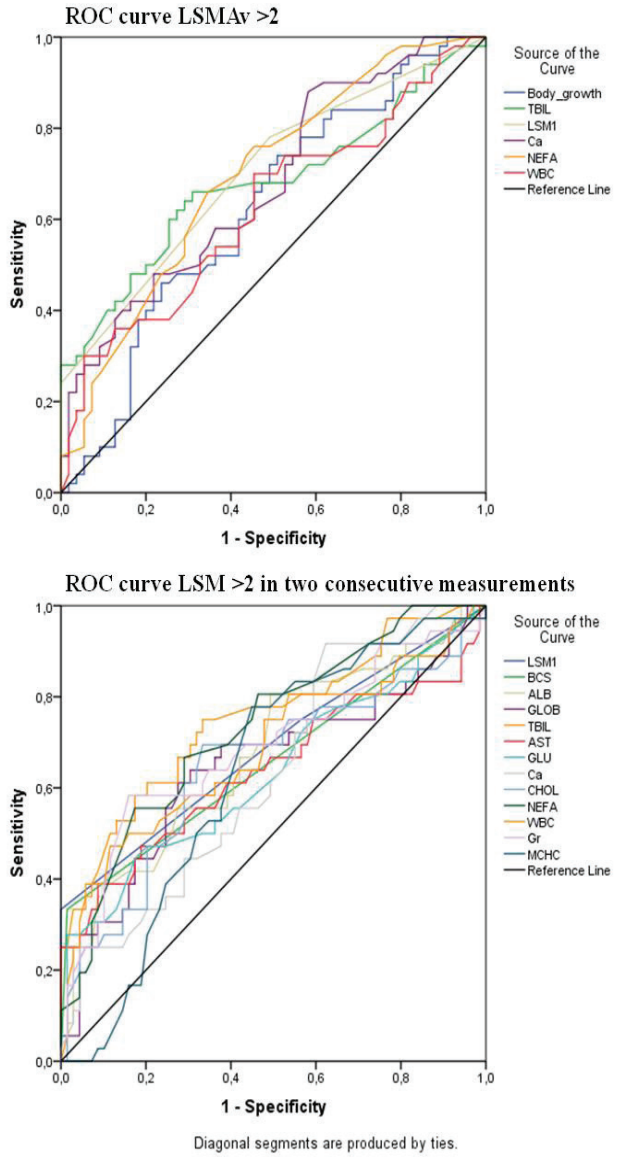

Fig 3. The ability of the tested parameters to discriminate cows that will not have problems with lameness and that will have the following problems: LSMAv >2 and LSM>2 in two consecutive measurements. LSM1: Locomotion score in month 1, BCS: Body condition score, ALB: Albumin, GLOB: Globulin, TBIL: Total bilirubin, AST: Aspartate aminotransferase, GLU: Glucose, Ca: Calcium, CHOL: Cholesterol, NEFA: Non-esterified fatty acids, WBC: White blood cells, Gr: Granulocyte, MCHC: Mean corpuscular hemoglobin concentration

models (r=0.736-0.905) versus individual parameter models (r=0.547-0.757), the prediction of LS was much greater (P<0.0001). The results are shown in *Table 2*.

Body and blood parameters assessed before puberty can aid in the early detection of heifers with substantial lameness issues, such as LSMAv >2 or LSM >2 in two consecutive monthly measurements. Heifers with an average LSMAv >2 can be identified and differentiated from those with lower average values using LSM1, body gain, TBIL, Ca, NEFA, WBC, and Gr. The ROC AUC value ranged from 0.62-0.7 (P<0.05 to P<0.01). The parameters LSM1, BCS, ALB, GLOB, TBIL, AST, GLU, Ca, CHOL, NEFA, WBC, Gr, and MCHC can be used to distinguish heifers with lameness problems from those without. ROC AUC values varied from 0.63-0.75 (P<0.05 to P<0.01). The results are shown in *Fig.3*.

Discussion

LS were examined during our experiment on a monthly basis for the first five months of the first lactation of heifers and a positive correlation was demonstrated. The percentage of different LS grades in our sample was: 19% for LS=1.48% for LS=2.25% for LS=3.9% for LS=4 and 5% for LS=5. Our results roughly match the results obtained by Matson et al.[5]. The author [11] used a similar research model to ours and found that the average lameness score (LS) was 2.9±1.1. LS increased in the first and second months of lactation, decreased in the third and fourth months, and increased in the fifth and sixth months.

In addition, a positive link was observed between the intensity of lameness from the first to the fifth month of lactation. Our findings agree with those obtained by the cited author. Based on research conducted on Danish dairy cows, there was a substantial relative risk that lameness from the previous lactation could occur in the subsequent one [13]. There has also been shown a correlation between several forms of hoof disease; for example, hoof ulcers and pre-existing interdigital dermatitis, and solar ulcers and interdigital dermatitis in the future [14-16]. Large-scale investigations done in France

on dairy cows have established the link between lameness and lactation [17]. Compared to non-lame cows, previously lame cows had a two- to three-fold higher risk for all types of lameness, according to a sizable database compiled by French researchers [18].

Complex models determined that cows that exhibited lameness in the week immediately before calving were often lame in weeks 2 and 8 after calving as well. Daros et al.[19] conducted a prospective longitudinal study involving hundreds of cows during the dry season. Randall et al.[20] presented their results from an eight-year study that included complete longitudinal data for over 1500 cows. The results indicated that 70-90% of lameness in cows was actually a repeated episode that had been previously present. Between 9 and 21% of lameness cases could be attributed to previous lameness that occurred >16 weeks before the risk period.

Metabolic factors were associated with the onset of lameness. In an interesting study [21], the use of a complete metabolic profile in predicting lameness and non-infectious hoof diseases was evaluated, and it was discovered that serum albumin and proteins significantly decreased prior to the occurrence of sole ulcers and sole bleeding, AST significantly increased in cows with bleeding and sole ulceration, and NEFA increased significantly prior to hemorrhage and/or sole ulceration. Additionally, lame cows had reduced levels of BCS, cholesterol, glucose, albumin, urea, Ca, and Mg [22].

Metabolic parameters such as NEFA, GLU, AST, or BCS were related to LS in our study as well, so our results match the ones obtained by the previously mentioned authors. The importance of metabolites such as NEFA and BHB was also demonstrated in primiparous cows that developed hoof lesions during mid-lactation, and blood was sampled retrospectively in the first weeks after calving [23]. The authors found that NEFA and BHB concentrations were significantly higher in the first weeks of lactation in cows that developed signs of lameness, and cows exhibited a more pronounced loss of body condition. Higher glycemia and an increased NEFA value indicated insulin resistance. During the period of positive energy balance, it is accompanied by a high insulin value, which all together reduces the production of keratin and disrupts the anatomy of the hooves, which increases the risk of lameness [24]. The greater the sensitivity to insulin and positive energy balance in the period before calving, the greater the negative energy balance, lipolysis and loss of fat depot after calving [25], which also reduces hoof fat pads and increases lameness. Our results demonstrate that higher body condition, higher glycemia and higher value of NEFA and BHB in the prepubertal period cause a higher lameness score in early lactation. Higher availability of sugar components even long before lactation can lead to the onset of lameness during lactation. It had been noticed that farms that used corn silage had more lameness cases than those not based on silage, and the oligofructose overload model proved to be extremely efficient for studying laminitis [26,27]. When heifers are being fed abundantly to achieve growth greater than 700 g/day, lameness occurs without clinically or morphologically visible causes [28]. All of the above is in accordance with our findings, which demonstrate that increased body growth and higher body condition in heifers before puberty mean higher LS after parturition. Blood calcium concentration is positively associated with LS. It is known that high levels of calcium in the blood weaken the bones due to the action of the parathyroid gland, and this effect is especially visible after calving and during lactation [29].

Models for evaluating the onset of lameness or evaluating the LS include the use of various indicators, such as body condition, metabolic parameters of milk and photographic parameters [30-32]. These models gave estimates that were most often at the level of 57-62%, and that percentage increased to a level of over 75% when there was a dichotomous classification of lameness or when a longitudinal assessment of lameness was performed. Our results coincided with the stated results of the researchers, so our model could be considered quite efficient in assessing the occurrence of lameness in heifers at the beginning of the first lactation, although we used parameters obtained from a time distant point in relation to the first calving, which was the period before puberty maturation and during intensive growth. Future research (to fully evaluate and upgrade the model) should include a larger number of farms with different zoohygiene conditions, different feeding methods, with heifers of different breeds, through different seasons, with heifers of different health status after calving, and so on, because all of these factors can have an impact on the model's quality and lameness predictions. Metabolic diseases should be studied in depth because of their relationship to body condition and metabolic parameters [33-35], as this allows us to precisely quantify the influence of body condition, disease, and metabolic variability on the onset of lameness.

The test results demonstrate that based on body condition and blood parameters in the period before puberty as well as the locomotion score in the first month of the first lactation, the value of LS in the first five months of lactation as well as the average value of LS can be predicted. Metabolic parameters and assessed body condition exhibit a significant correlation with LS values, and can indicate the ability to classify cows into those that will and will not have problems with lameness in the first lactation. These results confirm that in the early development of heifers, before sexual maturity, using

their body and metabolic characteristics, the value of LS can be predicted during the first five months of the first lactation.

Declarations

Availability of Data and Materials: Data will be available at https://www.open.uns.ac.rs/ after dissertation defense of first author (N. Zahirović).

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Author Contributions: Conceptualization, N.Z. and B.T.; methodology, N.Z. and B.T.; software, M.C.; validation, O.S.; formal analysis, N.Z. and M.C.; investigation, N.Z., B.T., M.C., O.S.; data curation, N.Z., M.C.; writing-original draft preparation, N.Z.; writing-review and editing, M.C., B.T., O.S.; visualization, M.C.. All authors have read and agreed to the published version of the manuscript

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