

## PAPER DETAILS

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## Evaluation of the effect of daily cow's milk production on liver enzyme levels

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## ABSTRACT

**Objective:** This study aimed to determine the possible effect of the total daily amount of skim milk on bilirubin and liver enzymes through regression analysis.

**Materials and Methods:** The study included 63 Holstein-Friesian cows. They were formed in 3 groups, based on the amount of daily milk production. First was the group of lower daily milk production  $n=21$ , the second group of examined cows was the group of medium daily milk production  $n=23$  and third was the group of higher daily milk production  $n=19$ . Peripheral blood was punctured, through which the activities of total bilirubin were analyzed ( $\mu\text{mol/L}$ ), as well as liver enzymes: alanine aminotransferase-ALT (U/L), aspartate aminotransferase-AST (U/L), lactate dehydrogenase-LDH (U/L) and alkaline phosphatase-ALP (U/L), from the blood plasma of the examined blood.

**Results:** The lowest concentration of total bilirubin in blood plasma was recorded in the group of cows that have the lowest daily milk production ( $1.295 \pm 0.255 \mu\text{mol/L}$ ), and highest concentration is in cows that produce the most milk ( $1.855 \pm 0.159 \mu\text{mol/L}$ ), but intergroup differences are not significant. Regression analysis found a statistically significant relationship between the amount of produced daily milk and the concentration of total bilirubin ( $R^2=0.132$ ,  $p=0.005 < 0.05$ ). It is determined that the highest activity of ALT, AST, ALP and LDH was found in cows with the highest amount of daily skimmed milk, but intergroup differences were not statistically significant ( $p > 0.05$ ).

**Conclusion:** The activities of bilirubin and liver enzymes in the examined cows were in physiological balance. This indicates that the cows on the farm are raised in modern and good zootechnical and feeding conditions. In such conditions, dairy cows are able to maintain blood composition and homeostatic integrity within physiological limits and adequate reproductive and productive capacity.

**Keywords:** Liver Enzymes, Bilirubin, Lactating Cows, Milk

## INTRODUCTION

Permanent monitoring of the metabolic profile is a necessary prerequisite for successful management of highly productive dairy cows in farm housing conditions. The metabolic profile is important tool for monitoring health and disease prevention in

these animals (Eşki and Kurt, 2021). All parameters of the metabolic profile are equally important and it is necessary to interpret them correctly, because through them we manage to reach certain pathophysiological deviations, as well as physiologically conditioned variations (Katica et al., 2019; Andjelić et al., 2022). The functional state of

the liver has a particularly significant influence on biochemical indicators (Jašović et al., 2016; Andjelić et al., 2022).

The negative energy balance of cows leads to the mobilization of fatty acids from the body's reserves, and reaches its maximum in the second week of lactation. Free fatty acids that are mobilized in increased amounts from body depots in the peripartum period in high-milk cows are an important predisposing factor in the etiopathogenesis of fatty infiltration and degeneration of liver cells, as well as ketosis of cows (Mukača, 2015).

Increased energy needs during pregnancy and lactation are often not accompanied by adequate energy intake, and as a consequence there is a violation of the functional state of certain organs, most often the liver. Many researchers consider that elevated values of liver enzymes most often occur when acute or chronic disease is suspected (Hadžimusić and Krnić, 2010; Aladrović et al., 2018).

Increased aspartate aminotransferase (AST) activity in dairy cows is mainly associated with fatty liver syndrome, decreased appetite and the occurrence of ketosis in dairy cows during early lactation (Aladrović et al., 2018). Also, lactate dehydrogenase (LDH) together with AST is often used to diagnose cow liver disease, as well as during infections and inflammatory processes (Tedesco et al., 2004; Klein et al., 2020).

Alkaline phosphatase (ALP) originates mainly from the intestinal mucosa, placenta, kidneys and bones, but also from the liver and is mostly found in cell membrane of hepatocytes, and the determination of this liver enzyme can serve as a tumor marker and as an index of liver disease and bone disease (Sato et al., 2005; Valocky et al., 2007; Yenyol and Ricci, 2018; Ogunmoyole et al., 2022; Šaljić et al., 2022; Utari et al., 2022).

The activity of alanine aminotransferase (ALT) in blood plasma is influenced by age and muscle activity. The established physiological variations in the activity of this enzyme are related to pregnancy and the beginning of lactation, when the level of ALT activity is reduced (Hadžimusić and Krnić, 2010).

Bilirubin is natural heme degradation product. It binds tightly to albumin and is also rapidly eliminated from the body, mainly by hepatic glucuronidation and elimination by bile (Sane et al., 2014). Hyperbilirubinemia is uncommon in

sick cattle and is mostly commonly associated with the inability of the liver to regulate unconjugated bilirubin (McSherry et al., 1984).

Pathophysiological conditions leading to hyperbilirubinemia are related to: increased production of bile pigments (in hemolytic processes, or in resorption of larger hematomas), decreased uptake of unconjugated bilirubin into hepatocytes, inhibited bilirubin conjugation process, impaired bile duct excretion in biliary canals and obstruction bile in intestines (Mukača, 2015). The level of concentration of total bilirubin in the blood serum of cows is considered as one of the safest indicators of the functional state of the liver. In cows, elevated bilirubin concentration occurs during hemolytic crises (Faixova et al., 2012).

There are reports that indicate a certain correlation between the quality and composition of meals in lactating cows, with the parameters of skimmed milk in correlation with the activity of liver enzymes (Šaljić et al., 2022). On the other hand, another similar study found a correlation between liver enzyme activity depending on the reproductive cycle of dairy cows in the circumstances of lactation, dryness and postpartum period depending on the sampling season (Hadžimusić and Krnić, 2010).

Since the trend of modern intensive breeding of dairy cows is based on constant increase in the daily amount of skimmed milk, the aim of our study was to determining the possible impact of total daily amount of skimmed milk on the level of bilirubin and liver enzymes, by regression analysis.

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## MATERIALS and METHODS

### Ethics Committee Approval

This research was approved by the Ethics Committee of the University of Sarajevo, Veterinary Faculty, under registration number 07-03-764-2/23, Sarajevo, Bosnia and Herzegovina.

### Animal and general experimental procedure and study group

The study included 63 Holstein-Friesian cows, aged between 2 and 9 years and was carried out at the farm in the northern region of Bosnia and Herzegovina placed in modern and very good zoo-technical conditions. The Radio Frequency ID (RFID) technology was used during the breeding and production in this farm, which implies that each cow owns a chip through which the animal's activities related to reproduction, lactation and

history of the diseases is being monitored and recorded.

The study was carried out during the winter period, and it included 63 cows aged 2-9 years (the largest number of cows was between 3 and 5 years old) in different lactation stages (1-8). The largest number of cows, eighteen of them, were in the second, third and fourth lactation stage, while nine cows were in the first lactation stage. Three groups of cows were formed according to the amount of daily milk produced, based on the control of the amount of daily milk production which was performed during the last seven days, prior to the blood sampling (Table 1).

**Table 1.** Classification of cow groups in the study according to daily productivity level and daily milk production amount.

Group	Level of daily productivity	Varying the amount of daily skimmed milk
1 (n=21)	Lower level	20-28 L
2 (n=23)	Middle level	29-34 L
3 (n=19)	Higher level	35-52 L

#### Dietary treatments

Dairy cows were fed with 30 kg of silage, 15 kg of haylage, 10 kg of concentrate mixture containing 18-20% protein. Dry cows were fed with 20 kg of silage, 10 kg of haylage and 3-4 kg of concentrate with 18-20% protein. They consumed water ad libitum.

#### Blood collection and biochemical analysis

Blood samples were taken by the technique of puncturing the cocigaeal vein, in injectors containing heparin á 4-5 ml. The blood was then centrifuged (LC 320, 2000 rpm/10 min) to extract plasma.

We used the spectrophotometer "Beckman DU-64 UV/VIS" and commercial kits manufactured by "Human", Germany, to assess the levels of bilirubin ( $\mu\text{mol/L}$ ) and liver enzymes, including alanine aminotransferase-ALT (U/L), aspartate aminotransferase-AST (U/L), lactate dehydrogenase-LDH (U/L) and alkaline phosphatase-ALP (U/L).

#### Animal care

During the research on dairy cows the usual veterinary diagnostic procedures were used. Blood sampling from the coccigaeal vein was done by an authorized veterinary technician. All procedures on dairy cows during the research were in accordance

with the Law on Protection and Welfare of Animals of Bosnia and Herzegovina (Official Gazette of BiH, no. 25/2009 and 9/2018).

#### Statistical analysis

The results were statistically processed by the method of descriptive statistics. To determine whether there are differences in the arithmetic means of liver enzymes activity levels, a global ANOVA test was used between the groups. Testing of differences in mean values of liver enzyme parameters between the examined groups was performed at the significance level of  $p < 0.05$ . After determining the existence of statistically significant differences, using regression analysis (single linear regression), the possible dependence of liver enzyme parameters on the amount of milk produced was determined. Statistical processing of the results obtained by the research was performed using a software program (IBM Corp. Released, 2016).

#### RESULTS

Comparing the obtained mean values, with Radostits et al. (2000), we notice that all values are within physiological variations. The exception is the mean value for AST, which is slightly lower than the lower physiological limit.

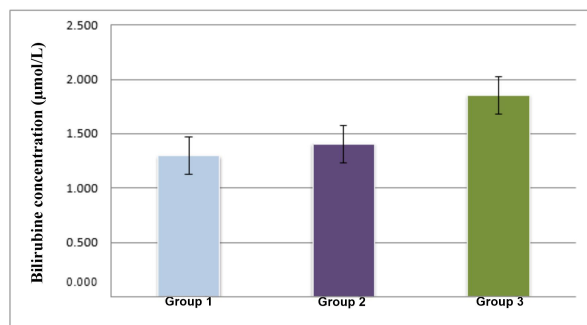
Significance results of differences in bilirubin and liver enzymes between groups of cows with different amounts of daily skimmed milk.

The average concentration of total bilirubin in blood samples of all examined cows on the farm ( $n=63$ ) was  $1.513 \pm 0.117 \mu\text{mol/L}$ , and individual values varied within the range  $0.21-3.42 \mu\text{mol/L}$  (Table 2) with the distribution of individual values as is shown in Figure 1. The lowest concentration of total bilirubin in blood plasma was recorded in the group of cows that have the lowest daily milk production ( $1.295 \pm 0.255 \mu\text{mol/L}$ ), and the highest in cows that produce the most milk ( $1.855 \pm 0.159 \mu\text{mol/L}$ ), but intergroup differences are not significant (Figure 1).

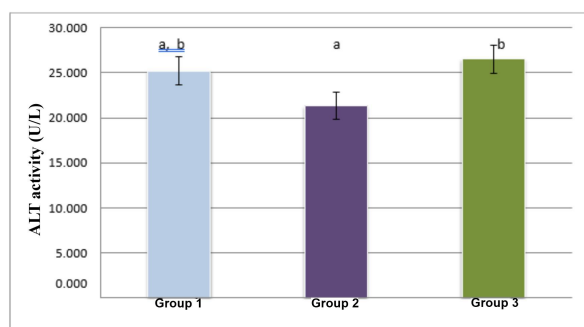
Although there are differences in daily milk productivity between cows in ALT activity (Figure 2), they statistically significantly differ only between cows of middle and higher levels of daily milk production ( $F = 3.659$ ,  $p = 0.032 < 0.05$ ).

Values for alanine aminotransferase (ALT) activity in 63 cows included in our research ranged from  $10.79-44.11 \text{ U/L}$  ( $24.208 \pm 0.853$ ) (Table 2 and Figure 2). The highest value of ALT activity was in cows with the highest daily milk production ( $26.539 \pm 1.846$

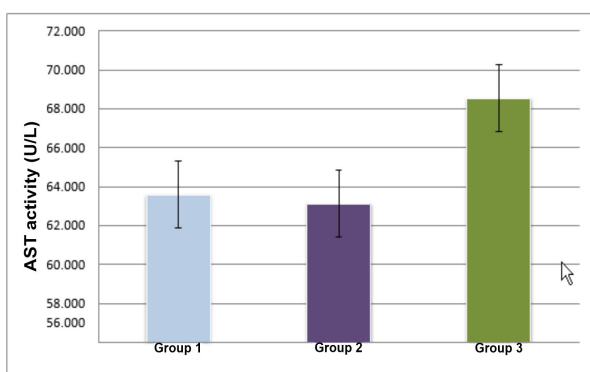
U/L), and the lowest in plasma in cows with daily milk production of 29-34 L of milk ( $21.371 \pm 1.149$  U/L) and this difference is statistically significant ( $F=3.659$ ,  $p=0.032 < 0.05$ ) (Figure 2).



**Figure 1.** The concentration of bilirubin ( $\mu\text{mol/L}$ ) in blood plasma of cows with different amounts of daily skimmed milk (Group 1=20-28 L; Group 2=29-34 L; Group 3=35-52 L of milk). All values are represented as  $\bar{x} \pm S\bar{x}$ .



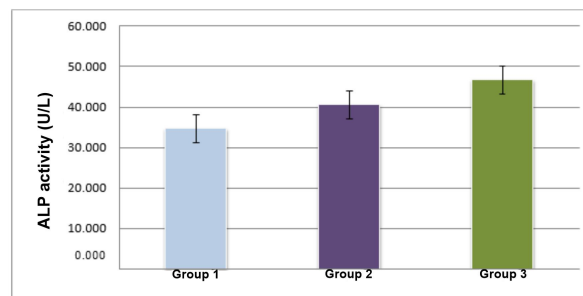
**Figure 2.** The activity of ALT (U/L) in blood plasma of cows with different amount of daily skimmed milk (Group 1=20-28 L; Group 2=29-34 L; Group 3=35-52 L of milk). All values are represented as  $\bar{x} \pm S\bar{x}$ . a, b = values that have a different letter are statistically significantly different ( $p < 0.05$ ).



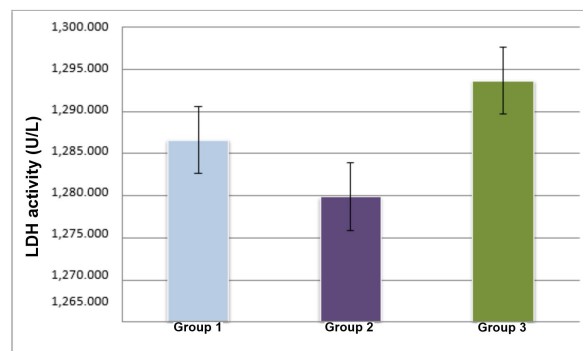
**Figure 3.** The activity of AST (U/L) in blood plasma of cows with different amount of daily skimmed milk (Group 1=20-28 L; Group 2=29-34 L; Group 3=35-52 L of milk). All values are represented as  $\bar{x} \pm S\bar{x}$ .

We also found that AST activity varied in range 25.40-94.60 U/L ( $64.831 \pm 2.906$ ; Table 2). The lowest AST activity was recorded in the group of cows with daily milk production in quantity of 29-34 L, and

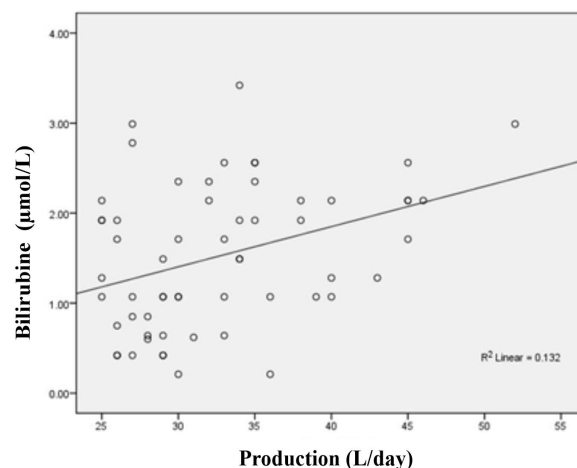
highest in cows with the highest amount of daily skimmed milk. However, the differences between the three examined groups of cows are not significant ( $p > 0.05$ ), (Figure 3).



**Figure 4.** The activity of ALP (U/L) in blood plasma of cows with different amount of daily skimmed milk (Group 1=20-28 L; Group 2=29-34 L; Group 3=35-52 L of milk). All values are represented as  $\bar{x} \pm S\bar{x}$ .



**Figure 5.** The activity of LDH (U/L) in blood plasma of cows with different amount of daily skimmed milk (Group 1=20-28 L; Group 2=29-34 L; Group 3=35-52 L of milk). All values are represented as  $\bar{x} \pm S\bar{x}$ .



**Figure 6.** Dependence of bilirubin concentration ( $\mu\text{mol/L}$ ) on the amount of daily skimmed milk.

The average value of ALP activity in blood samples of all examined cows on the farm ( $n=63$ ) was  $40.515 \pm 2.244$  U/L, and individual values varied within the range of 5.51-78.12 U/L (Table 2). The lowest activity of ALP in blood plasma was recorded



in the group of cows that have the lowest daily milk production ( $34.680 \pm 3.331$  U/L), and the highest is in cows with the highest daily milk production ( $46.665 \pm 3.593$  U/L), but intergroup differences are not significant (Figure 4).

The values of lactate dehydrogenase (LDH) activity in our research vary in the range of 299.52-2075.02 U/L ( $1286.365 \pm 53.575$  U/L; Table 2). In the case of LDH activity, the highest activity was recorded in

cows with the highest daily skimmed milk (Figure 5), and intergroup differences are not significant ( $p > 0.05$ ).

Results of the dependence of bilirubin and liver enzymes on the amount of daily skimmed milk

The results of the regression analysis for all examined parameters are shown in Table 3.

**Table 2.** Results of descriptive statistics for total bilirubin, ALT, AST, ALP and LDH from the blood of the entire sample

Parameters	NVO	Mean	SE	Med.	SD	Var.	SDA	Min.	Max.
Tot. Bilirubin ( $\mu\text{mol/L}$ )	63	1.51	0.11	1.50	0.90	0.82	-0.17	0.21	3.42
ALT (U/L)	63	24.21	0.85	24.11	6.77	45.86	0.27	10.80	44.11
AST (U/L)	63	64.83	2.9	63.31	14.69	215.80	0.16	25.40	94.60
ALP (U/L)	63	40.51	2.24	36.99	17.09	292.00	0.60	5.51	78.12
LDH (U/L)	63	1286.36	53.57	1249.32	421.85	17796.52	-0.06	299.52	2075.02

NVO: Number of valid observations, SE: Standard error, Med: Median, SD: Standard deviation, Var: Variance, SDA: Skewness distribution asymmetry. Min: Minimum, Max: Maximum.

**Table 3.** Results of regression analysis to determine the dependence of the values of the examined parameters on the amount of daily skimmed milk.

Regression parameters (in summary)	Model		ANOVA		Non-standardized coefficients (B)	
	R	R <sup>2</sup>	F	Sig.	Constant	Production (L/day)
Bilirubin( $\mu\text{mol/L}$ ) *	0.36	0.13	8.65	0.00	0.06	0.04
Alanine aminotransferase (U/L)	0.14	0.02	1.25	0.27	19.44	0.15
Aspartate aminotransferase (U/L)	0.17	0.03	1.68	0.20	51.40	0.42
Alkaline phosphatase (U/L)	0.10	0.01	0.57	0.45	31.91	0.27
Lactate dehydrogenase (U/L)	0.06	0.00	0.23	0.63	1427.69	-3.95

\* significantly at the level of 5% ( $p < 0.05$ )

Using regression analysis (single linear regression), a statistically significant effect of the amount of daily produced milk on concentration of bilirubin was determined the same indicates a statistically significant relationship between the amount of daily skimmed milk and the concentration of total bilirubin ( $R^2=0.132$ ,  $p=0.005 < 0.05$ ; Table 3 and Figure 6).

The results of the regression analysis did not show a statistically significant influence of the amount of daily produced milk on the examined parameters: ALT, AST, ALP and LDH (Table 3).

## DISCUSSION

Increasing the intensity of milk production i.e. at the maximum utilization of genetically conditioned high milk yield, is aimed with the modern method of breeding. In its full activity, the mammary gland represents the greatest metabolic load for the organism of high-milk cows (Katica et al., 2019).

The activities of the examined enzymes indicate that they represent a morphological and functional hemostatic integrity of the liver, which achieves metabolic activity in extremely demanding conditions of high milk production. Intensive

metabolic efforts of the liver imply that all necessary neurohumoral regulatory mechanisms involved in the control of glycomobilization, lipomobilization and gluconeogenesis, with adequate nutritional conditions, function purposefully to provide metabolic precursors for synthetic processes in the mammary gland (Andjelić et al., 2022).

The period of early lactation of cows is characterized by a negative energy balance, i.e. a condition when the energy intake in the body is less than its needs for milk production and the ability to take food. Until a balance is established between the amount of energy ingested and the amount of milk produced (between the sixth and tenth week of lactation), the difference is compensated by the mobilization of body reserves, first glycogen reserves, then fat, and then protein (Mukača, 2015).

However, in conditions of negative energy balance due to intensification of the lipomobilization process, in cows that accumulated higher fat reserves in the last phase of lactation during the previous pregnancy and during the drought period, an uncontrolled process of mobilization and deposition of non-esterified fatty acids in liver can occur. The consequence of such condition is the appearance of a fatty liver, infiltrative or degenerative in nature (Akgül et al., 2017).

Determination of the activity of enzymes originating from liver cells is used in the examination of the functional state of the liver. If their activity in the blood is elevated, they can be a good indicator of functional liver damage (Jašović et al., 2016). Damage to the liver parenchyma can be caused by various inflammatory agents, drugs, toxins, metabolic disorders and autoimmune diseases. The values for ALT activity obtained in our research correspond to the results (Krnić et al., 2003; Hadžimusić, 2010). The value of ALT activity is significantly higher during the winter, compared to the summer period. Higher values of ALT activity in the plasma of cows with the highest daily milk yield can be explained by the high variability of liver enzymes, which may indicate the intensification of metabolic processes in high milk production (Reynolds et al., 1991).

Examination of the value of aspartate aminotransferase (AST) activity is considered a relevant indicator in the assessment of liver function (Krnić et al., 2003; Andjelić et al., 2022). Increased activity of the AST enzyme is found in acute hepatitis, liver cirrhosis, toxic liver necrosis,

but also in muscle injury, acute hemolytic anemia, muscular dystrophy, etc.

Like ALT, higher values of AST activity in the plasma of cows with the highest daily milk production may indicate an intensification of metabolic processes at high milk production. Such differences can be explained by the high variability of enzymes, which results in their instability in blood plasma (Reynolds et al., 1991).

Lactation has a great influence on the intensity of metabolism and metabolic parameters in the blood and this agrees with the results of Filipejova and Kovačik (2009), who found that AST in dairy cows at the beginning of lactation was significantly higher compared to the dry period (Ghada, 2014).

The increase in AST activity after birth could be explained by the degradation of muscle cells caused by the mobilization of body reserves. Furthermore, increased AST values may often be due to mobilization of muscle proteins for gluconeogenesis (Ghada, 2014.).

Single linear regression analysis showed that the amount of daily milk production did not have a significant effect on activity of AST in blood plasma.

In this study ALP is a very widespread enzyme in the body and hydrolyzes phosphoric acid esters. The level of activity of this enzyme in the blood changes in various physiological conditions, as well as in many diseases, especially when a metabolic bone and liver disease is suspected. In younger individuals, the activity in blood plasma mainly originates from bone, and in adults from hepatic ALP. The regression model did not determine a statistically significant effect of the amount of daily skimmed milk on the activity of ALP in blood plasma.

Study of Yokus and Cakir (2006) showed different mean ALP values depending on the production and reproduction status of the animas, and lactating cows had a mean ALP value that were consistent with the results of our research. The results closest to our established values of ALP activity are the results of Hadžimusić (2010) during the winter lactation period. It is possible that these differences are related to the variability of nutrient composition related to climate, nutrient type, soil type and climatic conditions defined by different altitudes Belic et al. (2018), and Yokus and Cakir (2006) state that ALP activity varies depending on the physiological condition, but also due to seasonal variations.

The values obtained for LDH in our research ranged within physiological limits (Radostits et al., 2000; Kaneko, 2008). In a research conducted by Sako et al., (2007) recorded slightly LDH values in moving cows, in the range of 1230-2074 U/L. The research conducted by Hodžić et al., (2007) also showed high mean values of this enzyme in clinically healthy cows.

Such wide variations in LDH activity are often due to the different physiological and production statuses of the cows (Yokus and Cakir, 2006). Increased activity of this enzyme can occur during infectious, inflammatory, toxic or metabolic liver damage. It shows the greatest activity in the kidneys, heart, skeletal muscles, pancreas, spleen, liver, lungs and placenta. By damaging the cells, it easily passes into the blood. In chronic liver disease or slower-progressing liver disease, the activity of this enzyme may fall below the lower reference value if a small number of hepatocytes are damaged and the hepatocellular mass is significantly reduced (Lechtenberg and Nagaraja, 1991). The amount of daily produced milk has no statistically significant effect on LDH activities.

When assessing the activity of liver enzymes in lactating cows, the sampling season, the age of animal and the energy status should be taken into account. All of the above can to a greater or lesser extent affect the values of liver enzymes (Stojević et al., 2005).

Increased concentration of bilirubin in the blood plasma is mainly with clinically manifest signs of icteric skin and other visible mucous membranes. Occurrence of hyperbilirubinemia over 8.55  $\mu\text{mol/L}$  (Radostits et al., 2000), is considered a pathophysiological condition and is usually due to impaired morphological and functional integrity of liver cells (Faixova et al., 2012). Significantly higher concentration of total bilirubin was found in cows with ketosis compared to blood values in healthy cows (Jovanović et al., 1991; Đoković, 1998; Bugarski, 2002). Total bilirubin in the blood of cows is therefore an indispensable test for examining the functional state of the liver (Reid et al., 1982).

Differences in the value of total bilirubin concentration are evident even for individual stages of cow production in farm breeding. According to Jovanović et al. (1997), values for total bilirubin in the blood of cows 10-15 days before calving is 4.7  $\mu\text{mol/L}$ , 10 days after calving is 5.4  $\mu\text{mol/L}$ , in the second month of lactation is 4.0  $\mu\text{mol/L}$ , while in the fifth month of lactation is about 3.9  $\mu\text{mol/L}$ .

The bilirubin concentration values in our research were within physiological limits, (Radostits et al., 2000), and which is in collision with the research of Krnić et al., (2003). According to Krnić et al., (2003), established hyperbilirubinemia in lactating cows of 9.67  $\mu\text{mol/L}$ , link to the present problem of nutrition in a longer period before blood sampling on the farm, and state that the finding indicates a violation of liver function or increased hemolytic processes. In our research, the cows used in the research were bred in modern and very good zootechnical conditions, and were fed balanced meals that could ensure normal liver function within physiological balance. However, single linear regression values indicate a statistically significant relationship between the amount of daily skimmed milk and the total bilirubin concentration in blood plasma. This indicates that when the amount of daily milk produced increases, the concentration of total bilirubin also increases. The stated positive correlation between the amount of daily milk production and the concentration of bilirubin indicates that the liver with its function can meet the needs of the organism in conditions of high milk productions.

## CONCLUSION

The activities of bilirubin and the activities of the enzymes ALT, AST, LDH and ALP in the examined cows were in physiological balance. This indicates that high milk cows are kept and bred on the examined farm under modern and very good zootechnical and feeding conditions. In such conditions, dairy cows are able to maintain blood composition and homeostatic integrity within physiological limits and adequate reproductive and productive capacity.

A positive correlation between the amount of daily milk production and the concentration of bilirubin indicates that the liver with its function can meet the needs of the organism in conditions of high milk production.

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