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The impact of *Fusarium* mycotoxins on macro and micro parameters of dairy cattle

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Abstract: The aim of the present study was to evaluate the impact on macro and micro parameters of dairy cattle when cows consumed total mixed ration (TMR) contaminated with (zearalenone) ZEA and deoxynivalenol (DON) for 60 days. Macro indicators included milk composition and production and micro parameters included blood components, rumen volatile fatty acids (VFAs), and ZEA and DON concentrations in rumen fluid and milk. Twenty dairy cows were fed TMR with naturally contaminated mycotoxins (ZEA 300 – 500 µg/kg; DON 102 – 200 µg/kg). The trial lasted for 60 days. Samples of blood, rumen fluid, and milk were taken three times at intervals of 30 days. The obtained results showed that the combination of ZEA and DON affected liver and kidney enzymes ($p < 0.05$) because, in 60 days, alkaline phosphatase (ALP) increased by 25%, albumin (ALB) decreased by 8.7%, creatinine (CREA) decreased by 40%. Rumen ammoniacal nitrogen (NH_3-N) statistically significantly increased (43%), whereas acetate acid decreased (2.7%). In 60 days, the combination of mycotoxins ZEA and DON statistically significantly reduced the milk yield (20.8%), fats (11.8 %), and proteins (3.5%). After 60 days ZEA in rumen fluid increased (28.6%), DON increased (30%) ($p > 0.05$). In milk samples, ZEA concentration increased from 0.97 µg/L (0th day) to 1.43 µg/L (on the 60th day). Based on the obtained results, it was determined that combination of *Fusarium* mycotoxins occurring in the fodder produced under Lithuanian climatic conditions significantly affected some macro and micro indicators.

Key words: Dairy cows, *Fusarium* mycotoxins, rumen fluid, milk, volatile fatty acids (VFAs)

1. Introduction

Mycotoxins are toxic secondary metabolites produced mainly by molds that infect plants or crops, and by fungi in the genera of *Alternaria, Aspergillus, Fusarium,* and *Penicillium*. *Fusarium* mycotoxins remain stable even under high temperature or during long storage and after reprocessing and may affect animals. Mycotoxins may infer serious risks such as carcinogenic, teratogenic, mutagenic, and immunosuppressive effects on animal health and lead to economic loses. So far, several hundred mycotoxins have been identified. Maize silage is more prone to contamination with multiple mycotoxins compared to grassland products [1,2].

The impact of mycotoxins on animal health and productivity may be variable. Reduced appetite and weight loss are the first signs of negative impact of mycotoxins. Nevertheless, liver is the main target of *Fusarium* toxins [3]. The impact of toxins is reflected in blood parameters. According to Ozer et al. [4], the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) increase due to damaged hepatocytes, whereas the increased concentrations of total bilirubin (TBil) possibly are entailed by damaged liver and gall. In previous investigations, Korosteleva et al. [5] determined that mycotoxins also affected haematological blood components such as white blood cells (WBC), lymphocytes (LYM), neutrophils (NEU), and red blood cells (RBC). They are among blood micro components used by us for the evaluation of toxic impact on the organism.

Previous research has demonstrated that some mycotoxins remain intact in the rumen and that the degradation of mycotoxins is strongly dependent on the rumen conditions. Ruminants are considered to be relatively resistant to *Fusarium* mycotoxins since these compounds are degraded by rumen microorganisms into less toxic metabolites. However, ruminant's detrimental health effects and economic losses in animal production occur, which emphasizes the importance of minimizing

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mycotoxin exposure [3,6-9]. Some other studies have demonstrated that metabolic changes in rumen functions are caused by mycotoxins, including in volatile fatty acids (VFAs) and NH_3-N. Moreover, milk yields have been reported as decreased [10,11].

Due to their importance on animal health and production, and possible health consequences in Lithuania as in the European Union maximum concentration of these mycotoxins in feed is regulated by the European and Lithuania legislations. Maximum limits (ML) in Lithuania for zearalenone (ZEA) in feed for dairy cows is 0.5 mg/kg, deoxynivalenol (DON) – 5 mg/kg [7,12].

During the investigation we hypothesized that *Fusarium* toxins (ZEA – 300 – 500 µg/kg and DON – 102 – 200 µg/kg) may affect micro parameters, i.e., morphological blood and serum biochemical parameters, rumen volatile fatty acids (VFAs), ZEA, and DON concentration in rumen fluid and milk, as well as macro parameters, i.e., milk composition and production.

The aim of the current study was to determine the impact macro-indicators (milk composition and production) and micro-indicators (blood morphological and biochemical parameters, rumen volatile fatty acids (VFAs), ZEA and DON concentration in rumen fluid and milk) in dairy cows when feeding total mixed ration (TMR) with naturally contaminated *Fusarium* fungi mycotoxins, when the concentration of ZEA is the maximum limit (ML) allowed in Lithuania.

2. Materials and Methods

2.1. Animal care

The study was performed according to the provisions of the Law of the Republic of Lithuania No. 1 – 2271 on Protection, Keeping and Use of Animals, dated 03/10/2012 the Official Gazette No. 122 – 6126 dated 20/10/2012) and of the by-laws, education and training purposes of animals used in storage, maintenance, and conditions of use No. B1 – 866, dated 31/10/2012 (The Official Gazette) No. 130 – 6595 dated 10/11/2012). Also, in order to carry out the research, a permit to test animals was received from Lithuania's State Food and Veterinary Service (SFVS) (permit No. G2 – 102).

2.2. Location, animals, and experimental design

The study was carried out during the period from 01/10/2019 – 01/12/2019. The experiment took place on a dairy farm in the Kaunas region of Lithuania. A total of 20 Lithuanian Black and White dairy cows (1 – 30 days *postpartum*) having had second or more lactations (on average 2.70 ± 0.40 lactations) and being clinically healthy were included in the study. The average body weight (BW) of all cows was 552.13 ± 15.92 kg, 35 kg/day of milk throughout the study period, and the average level of milk somatic cell count (SCC) was 102 ± 12 thousand/mL. The cows were housed in a ventilated tie-stall barn on a stall (180 × 130 cm) and were allowed to go outside for exercise two times per day. All selected cows were tested 60 days and fed total mixed ration (TMR) with naturally contaminated mycotoxins. Samples of feed, blood, rumen fluid, and milk were selected 3 times in a 30-day period. The feed ration was balanced to fit the energy and nutrient requirements of a 550 – 650 kg Holstein cow producing, on average. TMR for cows composed of 45% grass silage, 20% corn silage, 15% grass hay, 15% grain concentrate mash (50% barely and 50% wheat), and 5% of mineral mixture. Composition of the ration – dry matter (%) – 49.2; neutral detergent fiber (% of DM) – 26.2; Crude protein (% of DM) – 16.2; Crude ash (% of DM) – 19.6; Net energy for lactation (Mcal/kg) – 1.4. Water consumption was not limited (*ad libitum*).

2.3. Determination of mycotoxins concentrations

Mycotoxins deoxynivalenol (DON) and zearalenone (ZEA) concentrations in TMR samples were tested by high-performance liquid chromatography (HPLC) with MS/MS detection or fluorescent detector (FLD) and ultraviolet detector (UVD) described by CEN/TC 327 for ZEA and DON followed the method of MacDonald et al (2005) instructions.

A total of 1 kg TMR samples were collected 3 times from different localities places for cows feeding and stored in the dark at −20 °C until the date of analysis. Feed samples were dried at 70 ℃ for 24 h in a ventilated oven; 100 g feed sample was ground to pass a 1 mm screen and then stored for subsequent analysis. For mycotoxins analysis, a total of 5 g sample was used.

Acetonitrile (ACN), methanol (MeOH) (both HPLC grade), sodium chloride (NaCl, ACS grade) were purchased from R-Biopharm (AG, Germany). Deionized water (H_2O) was obtained using a Simplicity UV water purification system (Millipore, USA). Mycotoxin standards. ZEA, DON – 100 µg/mL in ACN (Romer Labs Diagnostics GmbH (Tulln, Austria) was used.

The ZEA was extracted using 25 mL of ACN/H_2O (75/15 v/v), containing NaCl (5 g per 100 mL) for 2 h in an orbital shaker (RS – OS 10/20, Phoenix Instrument GmbH, Germany) and purified using an immuno-affinity column (EASI-EXTRACT ZEARALENONE, R–Biopharm, Germany). ZEA were analyzed by HPLC–FLD (Model LCMS – 8060, Shimadzu Corporation, Kyoto, Japan). Chromatographic conditions for ZEA: HPLC column: 250 – 4 RP – 18e (5 µm), fluorescent detector: PMT Gain 10, mobile faze: H_2O/ACN/MeOH (46/46/8 v/v/v), flow rate mL/min: 1 mL/min, column temperature: 30 ℃, injection volume: 100 µL. Method limit of detection (LOD) – 3 µg/kg.

The DON was extracted with 1 g PEG (polyethylene glycol) and 40 mL of H_2O mixture for 1 h in an orbital

shaker and purified using an immune – affinity column (DONPREP, R-Biopharm, Germany) and analyzed by HPLC – MS (Model Sciex API 5000, McKinley Scientific, USA). Chromatographic conditions for DON were as follows: HPLC column: 250 – 4.6, RP –18-300 (5 µm), mobile faze: H_2O/MeOH/ACN (94/3/3, v/v/v), flow rate mL/min: 1 mL/min, column temperature: 30 ℃, injection volume: 100 µL. LOD – 20 µg/kg.

The concentrations of ZEA and DON in rumen fluid were determined using a HPLC with MS/MS detection. Rumen fluid samples preparation and chromatographic conditions same as in feed.

The mycotoxin zearalenone (ZEA) in milk was analyzed by enzyme – linked immunosorbent assay (ELISA) method. The RIDASCREEN Zearalenon (Art. No.: R1401) quantitative test kit (R–Biopharm AG, Darmstadt Germany) was used for the analysis. Zearalenone extraction was performed according to manufacturer's instruction. Milk samples were centrifuged (3,000 x, 15 min) at 4 ℃. The upper cream layers were removed. 20 µL of glucoronidase/arylsulphatase (Helix pomatia, Merck, Darmstadt, Germany) were added to 1 mL of a sample and incubated for 3 h at 37 ℃. After incubation, 0.1 mL of methanol was added to 0.9 mL of hydrolyzed and defatted milk and 50 µL of the resulting solution was used in the assay. Standards were prepared freshly each test day in skim milk containing 10% of methanol. The ELISA procedure was carried out based on the manufacturer's instructions. Absorbance was determined using the microtiter plate spectrophotometer (Bio-tek Synergy HT, USA) at 450 nm. LOD approx. 60 ng/L.

2.4. Blood, rumen fluid and milk samples collection and parameters measured

Blood samples were collected from the left jugular vein using vacutainer tubes (with and without anticoagulant) (BD Vacutainer, Great Britain) after milking on the 0th, 30th and 60th day. The collected blood samples were stored in an ice bath until all samples were taken. The serum samples were analyzed at National Food and Veterinary Risk Assessment Institute (NFVRAI) (Lithuania). Automatic biochemical analyser (COBAS INTEGRA® 400 plus, Tegimenta Ltd, Roche, Switzerland) was used. The samples were analyzed to estimate alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), creatinine (CREA), total bilirubin (Tbil), glucose (GLUC), total protein (TB), gamma-glutamyl transferase (GGT), calcium (Ca), lactate dehydrogenase (LDH), magnesium (Mg), and urea (UREA) parameters. The morphological blood parameters, collected samples were delivered in 2 h in the Lithuanian University of Health Sciences Veterinary Academy's Large Animal Clinic laboratory (Lithuania). The samples were examined for criteria of leukocytes (WBC), lymphocytes (LYM), monocytes (MON), neutrophils (NEU), erythrocytes (RBC), hemoglobin (HGB), and thrombocytes (PLT). Morphological blood testing was accomplished with an automatic analyzer (Abacus Junior Vet, Diatro Messtechnik GmbH, Austria).

Rumen fluid was collected using an oral stomach tube (GDZ-1) about 2 h after the morning feeding. The first 50 – 100 mL of flowing liquid was discarded to avoid contamination with saliva, and the oral stomach tube was washed twice using fresh water before the next sampling. Preparation of rumen fluid sample was performed following Hu et al [13]. The rumen fluid was examined outright using pH-meter (Laqua Twin, Spectrum Technologies, Inc., Illinois, USA). Buffer solutions with pH value of 5 and 7 were used for calibration of the device. After calibration, from 10 to 20 drops of rumen fluid were placed on the sensor and the displayed value recorded. For each sample three measurements were performed. The concentrations of rumen VFAs were determined using gas chromatographer (GC – 2010 Plus, Shimadzu corp., Kyoto, Japan) with mass spectrometric detector (GCMS – QP2010. Shimadzu corp., Kyoto, Japan). For acid extraction Stabilwax-DA column (30 m, inner diameter 0.25 mm, thickness 0.2 µm) (Restek, Bellefonte, PA, USA) was used. Helium was used as the carrier gas. The injection temperature was 200 ℃ and ion source temperature 230 ℃. The column temperature was raised from 100 ℃ to 240 ℃. The test took place for 21 min. To determine the content of acids, the analytical standard (Volatile free acid mix, Merck KGaA, Darmstadt, Germany) was used. The content was measured using (GC solution, Shimadzu corp., Kyoto, Japan) software. Fluid samples from rumen were analyzed for acetate, propionate, isobutyrate, butyrate, isovalerate, valerate, hexanoate, and N hexanoate acids. Rumen ammoniacal nitrogen (NH_3-N) was analyzed with an ultraviolet visible spectrophotometer (UV – 2000, Unico Instrument Co. Ltd, Shanghai, China).

Individual milk yield was recorded at every milking and summed for each day. About 50 mL samples of a thoroughly mixed composite of milk (morning and afternoon – 60/40 v/v) of individual cows were taken weekly for determination of milk composition. The milk samples were analyzed for fat, protein, lactose, urea, and milk fat/protein ratio using the mid-infrared analyser (LactoScope FTIR, Delta Instruments, Netherlands).

2.5. Data analysis and statistics

The data were analyzed using the IBM SPSS Statistics v25.0 for Windows. The distributions of the evaluated traits were used to carry out the assessment according to the Kolmogorov–Smirnov test with Lilliefors Significance Correction. The mean value and standard error of the mean were calculated. To compare in time the blood parameters, volatile fatty acids (VFAs), ammoniacal

nitrogen (NH_3–N), pH, milk yield, milk composition, and mycotoxins concentration on TMR, rumen fluid, and milk was used a paired Student's t-test. The relationships between ZEA in rumen fluid and milk yield, protein in milk, and ZEA in the milk of a daily cow were assessed using the Pearson correlation. The results were considered to be significant at $p < 0.05$.

3. Results

3.1. Biochemical and haematology indicators

The detected concentration of ZEA is the maximum content of mycotoxin in feeds of cows as regulated by the Lithuania legislation. The measured concentrations of ZEA and DON in the TMR were 300 – 500 µg/kg and 102 – 200 µg/kg, respectively (Table 4).

There were no significant differences in most of the blood parameters (Table 1). However, we found that mycotoxins significantly increased ($p = 0.030$) the ALP in the 60th day but not in the 30th day, decreased ($p = 0.010$) the ALB in the 60th day but not in the 30th day, decreased ($p = 0.003$) the CREA in the 30th day and the 60th day, significantly increased ($p = 0.003$) the TP in the 60th day, significantly decreased ($p = 0.030$) the Ca in the 30th day but not in the 60th day, the LDH significantly increased in

Table 1. Effects of feed contaminated with mycotoxins[1] on biochemical and haematology indicators of dairy cows.

Indicator	Time			SEM	*p*-value
	0th day	30th day	60th day		
Biochemical indicators					
ALP (U/L)	44.05[a]	46.37[ab]	58.90[b]	7.300	0.030
ALT (U/L)	30.67	34.38	32.87	7.506	0.443
AST (U/L)	111.12	134.03	136.65	29.113	0.472
ALB (*g*/L)	40.58[a]	39.29[ab]	37.03[b]	2.004	0.010
GGT (U/L)	36.13	32.18	44.05	10.324	0.111
TBil (µmol/L)	1.53[ab]	2.33[a]	1.17[b]	0.424	0.034
TP (*g*/L)	66.75[ab]	72.83[a]	77.30[b]	9.757	0.030
LDH (U/L)	1340.3[ab]	1165.0[a]	1318.5[b]	219.749	0.011
CREA (µmol/L)	113.0[a]	76.33[b]	67.50[b]	9.678	0.003
GLUC (mmol/L)	2.57	2.29	2.13	0.309	0.235
Urea (mmol/L)	5.81	6.03	5.95	0.694	0.561
Mg (mmol/L)	0.82[ab]	1.11[a]	0.89[b]	0.153	0.006
Ca (mmol/L)	2.67[a]	2.51[b]	2.61[ab]	0.073	<0.001
Hematological indicators					
WBC (x 10^9 *cells/L)*	8.27	10.53	10.55	2.268	0.292
LYM (x 10^9 *cells/L)*	4.44[a]	5.20[b]	5.15[ab]	1.187	0.049
MON (x 10^9 *cells/L)*	0.57	0.86	0.97	0.277	0.184
NEU (x 10^9 *cells/L)*	3.27	4.47	3.82	1.433	0.502
RBC (x 10^{12} *cells/L)*	6.62[ab]	6.77[a]	7.32[b]	0.827	0.032
HGB (*g/L)*	101.33[ab]	102.83[a]	106.33[b]	7.198	0.044
PLT (x 10^9 *cells/L)*	253.33	276.17	256.67	93.329	0.649

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, albumin; CREA, creatinine; TBil, total bilirubin; GLUC, glucose; TP, total protein; Ca, calcium; LDH, Lactate dehydrogenase; GGT, γ-glutamyl transpeptidase; Mg, magnesium; MON, monocytes; WBC, white blood cells; LYM, lymphocytes; NEU, neutrophils; RBC, red blood cells; HGB, haemoglobin; PLT, thrombocytes; [1] Total mixed ration was naturally contaminated *Fusarium* mycotoxins (ZEA – 300 – 500 µg/kg, DON – 102 – 200 µg/kg); [a,b] – different letters indicate significant differences between the means at the same row ($p < 0.05$); SEM – standard error of the difference between the means.

the 60th day, the Mg significantly decreased (p = 0.006) in the 60th day. Hematology indicators (Table 1) significantly increased (p = 0.049) the LYM in the 30th day but not in the 60th day, significantly increased (p = 0.032) the RBC in the 60th day and significantly increased (p = 0.044) the HGB in the 60th day.

3.2. Concentration of volatile fatty acids (VFAs), ammoniacal nitrogen (NH_3–N) and pH amount of rumen fluid

The rumen concentrations of VFAs, NH_3–N and pH were used as indicators of rumen fermentation. We found that significantly (p < 0.05) increased the rumen NH_3–N concentration during all trial periods (Table 2).

3.3. Milk samples parameters

The study data on milk yield and milk composition are shown in Table 3. Milk yield we found that significantly (p < 0.05) decreased by 20.8% after the 60th day of trial. Fat also significantly (p < 0.05) decreased after the 30th day of trial respectively 5.5%. However, there were no differences in lactose and urea in the time during the whole study.

3.4. Mycotoxin's concentration in samples

During the investigation, the concentrations of ZEA and DON in the rumen fluid and in feed increased: ZEA by 22% and DON by 23%. Yet the increase was not statistically significant. During the study, we determined that on the 60th day the concentration of ZEA in milk accounted for 0.29% of ZEA concentration in the fodder (500 µg/kg in DM).

Based on the data presented in Table 3 and 4, were determined correlation between ZEA in rumen fluid with milk yield and protein in milk (Figure 1). We found not statistically significant (p > 0.05) negative correlation of milk yield with ZEA concentration in rumen fluid (r = – 0.173) and statistically significant (p = 0.002) positive correlation coefficients with ZEA concentration in milk (r = 0.685) and also statistically significant (p = 0.015) positive correlation coefficients with protein in milk (r = 0.561)

4. Discussion

The goal of the present study was to determine how small doses of toxins consumed for a long time affect cattle health and productivity. The determined ZEA concentration was the maximum permissible concentration in dairy cattle according to the Lithuanian legal acts. As feed may naturally contain a few mycotoxins it was important to determine the impact of combination of mycotoxins on animal health and productivity.

The majority of biochemical blood parameters may define the physiological functions of cattle or reveal their immune or antioxidant activity. The liver function may be evaluated according to blood parameters such as GGT, AST, ALT, and ALP [11,14]. We determined that the increase of ALP enzyme and decrease of ALB on the 60th day were statistically significant and indicative of damaged liver function due to permanent consumption of ZEA and

Table 2. Effects of feed contaminated with mycotoxins[1] on the concentration of volatile fatty acids (VFAs), ammoniacal nitrogen (NH_3–N) and pH amount of rumen fluid from dairy cows.

Indicator	Time			SEM	*p*-value
	0th day	30th day	60th day		
Acetate (mmol/L)	43.46	56.87	42.29	7.205	0.099
Propionate (mmol/L)	10.62	12.19	9.94	0.953	0.125
Isobutyrate (mmol/L)	3.12	3.08	3.06	0.084	0.576
Butyrate (mmol/L)	6.64	7.09	5.88	1.008	0.358
Isovalerate (mmol/L)	2.24	2.17	2.15	0.093	0.519
Valerate (mmol/L)	2.82	2.82	2.75	0.183	0.624
Hexanoate (mmol/L)	0.14	0.13	0.35	0.107	0.144
N hexanoate (mmol/L)	2.63	2.64	2.63	0.030	0.835
Acetate/ Propionate	4.05	4.64	4.19	0.383	0.087
Total VFAs (mmol/L)	75.71	91.63	73.24	9.554	0.139
NH_3–N (mg/100 mL)	20.19^{a}	26.60^{b}	29.02^{c}	0.879	0.001
pH	6.03	6.01	5.99	0.112	0.789

[1] Total mixed ration was naturally contaminated *Fusarium* mycotoxins (ZEA – 300 – 500 µg/kg, DON – 102 – 200 µg/kg); ^{a,b,c} – different letters indicate significant differences between the means at the same row (p < 0.05); SEM – standard error of the difference between the means.

Table 3. Effects of feed contaminated with mycotoxins[1] on milk yield and milk composition.

Indicator	Time			SEM	*p*-value
	0th day	30th day	60th day		
Milk yield, kg/head day	35.02[a]	35.70[a]	27.72[b]	2.080	<0.01
Fat, %	3.79 [ab]	3.58[a]	4.06[b]	0.346	0.050
Protein, %	3.34[a]	3.46[b]	3.46[b]	0.191	0.029
Lactose, %	4.57	4.56	4.54	0.137	0.687
Urea, mg/100 mL	34.67	25.50	28.50	4.813	0.111
fat-protein ratio	1.14[a]	1.04[b]	1.18[a]	0.106	0.013

[1] Total mixed ration was naturally contaminated *Fusarium* mycotoxins (ZEA – 300 – 500 µg/kg, DON – 102 – 200 µg/kg); [a,b] – different letters indicate significant differences between the means at the same row ($p < 0.05$); SEM – standard error of the difference between the means.

Table 4. Mycotoxins concentration on TMR, rumen fluid, and milk.

Indicator	Time			SEM	*p*-value
	0th day	30th day	60th day		
ZEA in TMR, µg/kg in DM	300–500				
ZEA in rumen fluid, µg/kg in DM	291.67	358.33	375.00	84.052	0.388
ZEA in milk, µg/L	0.97	1.00	1.43	0.686	0.186
DON in TMR, µg/kg in DM	102–200				
DON in rumen fluid, µg/kg in DM	95.83	129.17	125.00	29.736	0.220
DON in milk, µg/L	NT	NT	NT		

SEM – standard error of the difference between the means; DM – Dry material; NT – not tested.

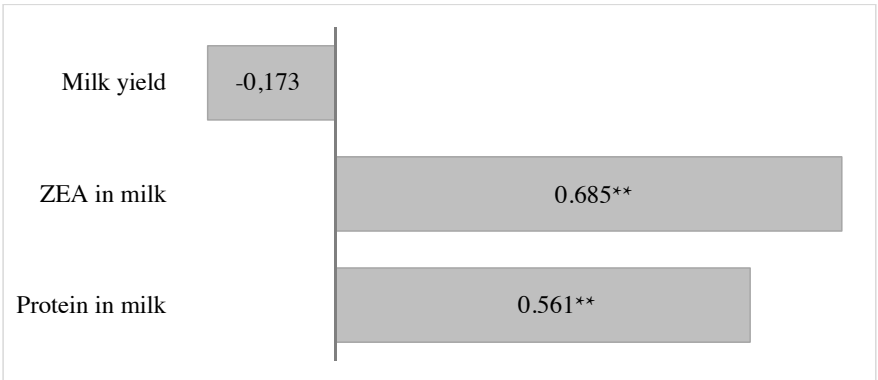

Figure 1. Pearson correlation coefficient between ZEA in rumen fluid with milk yield, protein in milk and ZEA in milk (* - $p < 0.05$; ** - $p < 0.01$).

DON concentrations. Also, ALP increase illustrates the liver cell membrane permeability is increased. Huang et al. [15] also determined that ZEA (500 µg/kg) together with other mycotoxins statistically significantly affected liver enzymes in ruminants. According to Wang et al. [2,14], mycotoxins may affect liver functions, yet the impact depends on the dose and duration of exposure. We determined a statistically significant decrease of CREA which shows that permanent consumption of ZEA and DON concentrations affected kidney function. Also, CREA

decrease illustrate the glomerular filtration disorders. Wang et al. [14] investigated dairy cows and found no impact on kidney function when combination of mycotoxins was ZEA – 85.13 μg/kg and AFB_1 – 20.08 μg/kg. Therefore, we may conclude that disorders of kidney function may occur at higher ZEA concentrations. Total enzyme is indirectly indicative of resistance of the organism and of the link between the resistance and health status. During our study, we determined that on the 60th day the TP concentration was statistically significantly higher indicating that the combination of ZEA and DON mycotoxins might have affected hepatocytes or permeability of cell membranes. The study showed that the changes of ALT, AST, GGT, TBil, GLUC, and Urea on the 60th day were statistically insignificant. Summarized the biochemical blood parameters it was observed that liver or kidney function damage are not acute, but chronic. Kiyothong et al [16] showed that combination of mycotoxins (DON – 720 μg/kg; ZEA – 541 μg/kg; ochratoxin A (OTA) – 501 μg/kg and fumonisin B_1 (FB_1) – 701 μg/kg) statistically significantly affected many morphological blood components. According to our results, the combination of ZEA and DON mycotoxins statistically significantly affected LYM, RBC, and HGB concentrations. This can be attributed to immunosuppressive effect of mycotoxins combination. However, the investigation carried out by Korosteleva et al. [5] did not show any significant morphological changes.

Mavrommatis et al. [17] maintains that in ruminants the impact of mycotoxins is not as strong as in monogastric animals because microbiota of rumen is able to metabolize and biologically transform some toxins. Rumen microorganisms convert carbohydrates into volatile fatty acids (VFAs), which through the digestive system are absorbed into the circulatory system and spread in the organism. For this reason, VFAs are considered important source of energy in ruminants [14,18]. The determined rumen concentrations of VFAs and NH_3–N were used for evaluation of rumen fermentation. We determined that duration of exposure and concentrations of mycotoxins affected acetate but had no effect on propionate, isobutyrate, butyrate, isovalerate, valerate, hexanoate, and N hexanoate acids and total VFAs (Table 2). An investigation by Hall et al. [18] carried out six years ago showed that total VFAs are inapplicable for evaluation of rumen fermentation and composition of microbiota because in the course of the day, the content of rumen and its composition changes considerably. This opinion was supported by Xiong et al [11]. Yet, the statistically not significant data obtained during 60 days about the differences of acetate, propionate, and other VFAs concentration may be indicative of the impact of the combination of ZEA and DON mycotoxins on fermentation taking place in the rumen. It is still not clear why the concentration changes of other VFAs were statistically insignificant. In order to find out the link between mycotoxins and VFAs concentration, it is necessary to prolong the time of investigation and to run tests with different combinations and concentrations of mycotoxins. According to Wang et al [14], the NH_3–N concentration in the rumen is important due to nitrogen metabolism. We determined that the combination of *Fusarium* mycotoxins in 60 days significantly increased the NH_3–N concentration and exceeded the best, according to Wanapat and Pimpa [19], NH_3–N concentration in ruminants, which ranges between 5 and 28 mg/100 mL, which is indicative of possible imbalance of enzyme decomposition.

Kemboi et al [20] and Whitlow et al [21] assume that the presence of *Fusarium* mycotoxins in feed is responsible for milk yield reduction. This opinion does not concur with our finding showing that after feeding dairy cows with feed containing *Fusarium* mycotoxins for 60 days milk yield reduction was not statistically significant. But the aforementioned opinion concurs with the findings of Korosteleva et al. [5], which maintains that natural contamination of fodder with *Fusarium* mycotoxins has no effect on milk yield and composition. This conclusion was possibly predetermined by the fact that the researcher used small concentrations of ZEA and DON; 240 μg/kg and 320 μg/kg respectively. We also determined that the combination of ZEA and DON statistically significantly decreased the content of milk fats and proteins proving that ZEA concentration affected milk composition. Kiyothong et al. [16] also determined that mycotoxins are able to significantly affect milk proteins. Fat-protein ratio (FPR) commonly is regarded as indicator of energy deficiency or subclinical ketosis [22,23]. Zschiesche et al [24] maintains that FPR < 1.0 is the limit and is indicative of Subacute rumen acidosis (SARA). This assumption is supported by other researchers [25,26]. We determined that on the 30th day the FPR was 1.04, which proves that the combination of ZEA and DON may affect SARA.

During our investigation, we also determined that ZEA and DON concentration in rumen remains stable due to the rumen microbiome. We found out that longer exposition of cows to TRM with ZEA and DON combination increased the concentration of untransformed ZEA and DON in rumen. We suppose that this was predetermined by permanent consumption of toxins disturbing rumen microbiome activity. Also, accumulation of mycotoxins in the rumen is possible. Researchers [8] maintain that ZEA is not fully decomposed in rumen and some untransformed ZEA reach the intestine. On the 0th day of investigation, milk contained 0.19% and on the 60th day, 0.29%. ($p > 0.05$) of untransformed ZEA. This proves that long exposition of cows to TMR naturally contaminated with ZEA and DON increase their concentrations in milk.

We think that the correlation may have possibly had caused by decreased pH value and also increased NH_3–N

in rumen. The increased NH_3–N concentration in cow`s organism affects cow`s metabolism, and that might result in a lower milk yield. Decreased pH value in rumen also changed the different microorganism ratio, which is partly responsible for mycotoxin degradation in the rumen. That causes increased higher concentrations of mycotoxins into the intestines where it is later absorbed into the blood then liver and kidneys. The outcome of this process is the mycotoxins are converted to mycotoxin derivatives and reach the milk with blood. That's why we believe that it caused a positive correlation between ZEA in rumen and ZEA in milk.

5. Conclusion

According to the results of our study, we can conclude that natural feed contaminants ZEA and DON in 60 days affected liver (ALP, ALB) and kidney (CREA) enzymes and morphological blood parameters (LYM, RBC and HGB) (all $p < 0.05$). The changes of NH_3–N concentration were determined indicating the combination of mycotoxins affected rumen function. Also, a positive correlation ($p < 0.05$) between ZEA concentration in rumen fluid and protein and ZEA content in milk was determined. After 30 days of exposition to ZEA and DON, the index FPR approached 1.00, which is indicative of SARA.

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