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Research article

ENERGY-RELATED HORMONES IN RAW AND RETAIL COW'S MILK AND POSSIBLE RISK FOR CONSUMERS

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The study aimed to determine the concentration of energy-related hormones in cow's milk and to consider them from a public health perspective. Fourteen Holstein cows were subjected to milk and blood sampling on the following days in lactation (DIL): 10, 30, 60, 90, 150, 180, 210, 250 and 280 to determine milk hormones, fat and protein content and blood biochemical parameters. For the same purpose, bulk-tank milk was sampled and samples of retail milk with 1.5% ($CM_{1.5}$) and 3.2% ($CM_{3.2}$) fat was purchased. Milk insulin-like growth factor-1 (IGF-1) values were significantly lower at 90, 150, 180, 210 and 250 and significantly higher at 10, 30 and 60 DIL than lactation average (LA). Milk insulin concentrations were significantly lower at 30, 60 and 90 and higher at 210, 250 and 280 DIL than LA. Free thyroxine (fT_4) level in the milk was higher at 250 DIL, while milk free triiodothyronine (fT_3) concentrations were lower at 30, 60, 90 and 280 DIL, and significantly higher at 10 and 180 DIL than respective LA. Milk cortisol levels were lower at 60 and 280 DIL than LA. All measured milk hormones were significantly lower in $CM_{1.5}$ compared to $CM_{3.2}$, bulk-tank milk and LA. An exception was the LA of IGF-1, which was significantly lower than the IGF-1 content in $CM_{1.5}$. Blood biochemical parameters fluctuated evenly during lactation and were within the reference range. Hormone concentrations in cow's milk fluctuate during lactation, giving milk an important role in the context of public health.

Keywords: cows, milk hormones, consumer health

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INTRODUCTION

Cow's milk is considered as a complete food for human consumption, providing macro and micronutrients including high-quality proteins, fats, carbohydrates (lactose), minerals, vitamins, and water [1]. Additionally, biologically active substances such as immunoglobulins, lactoferrin, enzymes, cytokines, growth factors, and hormones are present in cow's milk [2]. The content of hormones in milk is low, but at higher concentrations it can have a negative impact on consumer health [3]. Thus, although milk provides the consumer with a considerable amount of readily available nutrients, it can be a source of risk factors of chemical and biological origin. Therefore, milk has been the subject of many studies which investigate, not only it's important nutritional role for the human population, but also the relationship between milk's consumption and human health [4]. In addition to an undoubtedly beneficial effect on the health of consumers, there have been some studies on the potential negative impact of certain biologically active components in cow's milk, primarily hormones and growth factors, on human health [5]. Namely, cow's milk contains various hormones whose effects on human health are highly controversial [6]. The main hormones found in cow's milk and other dairy products are prolactin, estrogens, progesterone, corticosteroids, androgens, thyroid hormones, insulin and insulin-like growth factor-1 (IGF-1) [7]. Dairy consumption is associated with a potentially higher cancer risk, particularly cancers of the reproductive system, especially prostate cancer [8,9]. Moreover, there are indications that the risk of colon, pancreatic, endometrial, breast, and prostate tumors might be associated with high levels of IGF-1 or insulin in milk, or both [10]. Harrison et al. [11] concluded that IGF-1 levels in the blood increase with milk consumption and that risk of prostate cancer increases with IGF-1 levels. Also, insulin, which is contained in cow's milk, is a mitogen and increases the bioactivity of IGF-1. Thus, both of these hormones can promote cancer by inhibiting apoptosis and stimulating cell proliferation [12,13]. Thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), are present in cow's milk [14], but, according to our knowledge, their role in the health of consumers is not investigated. The proliferative effect of T_3 has been demonstrated in various types of cancers. In breast cancer cell lines, T_3 can cause tumor proliferation and enhance the effect of cell proliferation by estradiol, so T_3 may play a role in the development and progression of breast cancer [15]. In addition, thyroid hormones appear to have a stimulatory effect on angiogenesis of certain cancers [16]. Cortisol is present in cow's milk in very low concentrations and thus it is not considered as a risk factor for human health [17]. However, Ataallahi et al. [18] demonstrated that in an environment with high temperature and humidity cortisol concentration in retail milk can be significantly increased, while Nolvi et al. [19] found that higher levels of cortisol in human - breast milk can affect infant behavior and brain development. These scientific reports suggest that further studies are needed to investigate the risks and side effects of dairy products for consumers, even when those products have low or very low levels of biological active substances. At the same time, there are no standard quality control procedures for raw and retail cow's milk that include a determination of the concentration of hormones. Therefore, it would be of public health interest to determine the hormone concentrations in cow's milk depending on the stage of lactation, as changes are to be expected since the cows are exposed to various metabolic challenges during lactation [20]. The objective of this study was to determine the concentration of selected hormones (IGF-1, insulin, free T_3, free T_4 and cortisol) in the milk of cows at different stage of lactation, as well as in bulk-tank and retail milk samples and, comparing to available literature data, estimate whether the obtained concentrations may affect the health of consumers.

MATERIAL AND METHODS

Animals

Fourteen freshly calved Holstein cows (5 to 7 days postpartum) were chosen from the commercial dairy herd and placed in the study. All cows were multiparous and ranged from 4 to 6 years of age. The cows were placed in a free-stall barn. Cows were fed with the same TMR ration twice daily throughout the entire lactation, with pelleted feed and glycerol added during robotic milking (Table 1). The pelleted feed consisted of ground barley grain, soybean meal, molasses, fatty soybean oil, and finely ground corn grain. The amount of pelleted portion of the ration was adjusted to milk production so that cows with high production received a maximum of 7 kg during the first 75 days in lactation, while cows before dry-off received at least 2.5 kg of pelleted feed. On average, cows consumed 3.95 kg of pelleted feed at the milking robot throughout lactation. Cows had a free access to water throughout the study. The diet was initially formulated to either meet or exceed the National Research Council NRC (2001) requirements. Cow health status was monitored daily and the cows did not show any symptoms of illness during the entire study. The animal-related component of the study was approved by the Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade in accordance with the National Regulations on Animal Welfare (approval number 4/2015).

Sampling

Blood and milk samples from each animal in the study were taken at following days in lactation (DIL): 10, 30, 60, 90, 150, 180, 210, 250 and 280. To compare blood variable concentrations without the influence of daily rhythms, blood samples were taken 4 to 6 hours after the morning feeding. Blood samples were collected under aseptic conditions directly from the jugular vein into 10.0-mL Vacutainer tubes (BD Vacutainer, Plymouth, Devon, UK) without anticoagulant and stored on ice until serum was separated by centrifugation at 4°C for 20 minutes at 1000g within 2 hours after collection. Serum samples were stored at -20°C until analysis.

Table 1. Composition and nutritional value of the feed for lactating cows on robotic milking.

Ingredients, kg DM/day	Cows
Corn silage	7.38
Alfalfa haylage	1.70
Rye silage	0.81
Alfalfa hay	2.70
Wheat straw	0.28
Corn grain	1.76
Wheat grain	1.07
Soybean meal	2.07
Sunflower meal	0.74
Mipro HP 600 HR*	0.60
DextroFat Protect*	0.20
DairyFat C16 DE*	0.20
Sodium Bicarbonate	0.18
Magnesium oxide	0.06
KemTRACE Cr 0.4**	0.002
Glycerol (feeding on the robot)	0.26
Pelleted feed (feeding on the robot)	3.95
Total	**23.96**
Nutritional value (% DM)	
Dry matter	49.78
Crude protein	17.95
RUP	6.01
RDP	11.94
ADF	17.56
Forage	53.82
Forage NDF	21.54
peNDF	19.27
Sugar	5.57
Starch	24.66
Total Fat	4.85
Ca	0.93
P	0.41
Mg	0.53
DCAD mEq/kg	240.94
NEL (MJ/kg)	7.20

* – commercial supplements, premixes, and additives, Sano - Modern Animal Nutrition GmbH, Loiching, Germany; ** – Dry Mineral Supplement (0.4% Chromium from Chromium Propionate), KEMIN, Iowa, USA; RUP – rumen undegradable protein; RDP – rumen degradable protein; ADF – acid detergent fiber; NDF – neutral detergent fiber; peNDF – Physically effective neutral detergent fiber; DCAD – Dietary Cation-Anion Difference; NEL – Net Energy of Lactation

Milk samples were taken during morning milking, and were placed in a 50-mL milk bottle at the first milking at 5 a.m., before morning feeding. Bulk-tank milk samples were collected at the same days as milk samples from animals were taken. Bulk-tank was daily fulfilled with milk from approximately 150 lactating cows from a farm which produced approximately 3000 L of milk during each milking. Fourteen milk samples were collected per bulk-tank milk during each sampling period. Milk samples from bulk-tank were collected into 50-mL milk bottles, and samples were transported to the laboratory with transport refrigerator (4°C) and stored at −40°C until analysis. Pasteurized milk samples in original packaging units with 1.5% and 3.2% milk fat, respectively were purchased from a local grocery store (Serbia). Fourteen milk samples 1.5% and 3.2% milk fat, respectively, were aliquoted and stored in milk bottles at −40°C until analysis.

Blood analyses

Each blood sample was analysed for: insulin (mIU/L), glucose (mmol/L), albumin (g/L), total proteins (g/L), total bilirubin (µmol/L), blood urea nitrogen (BUN; mmol/L), aspartate aminotransferase (AST; U/L), alanine transaminase (ALT; U/L), calcium (Ca; mmol/L), phosphorus (P; mmol/L), and magnesium (Mg; mmol/L). Insulin concentration (mIU/L) were determined using the RIA method, while biochemical metabolites were analysed using the appropriate methods/kits: total protein (biuret reaction); albumin (bromcresol green method), BUN (urease/glutamate dehydrogenase method); total bilirubin (diazotized sulfanilic acid method); ALT (IFCC method), AST (IFCC method). The analyses were performed automatically with spectrophotometer (A15; BioSystems S.A., Barcelona, Spain). Glucose was measured immediately after blood collection in a drop of whole blood using commercial test strips (Abbott Diabetes CareLtd., Oxon, UK).

Milk analyses

Each milk sample was analyzed for: IGF-1 (ng/mL), insulin (mIU/L), fT_4 (ng/dL), fT_3 (ng/dL) and cortisol (ng/dL). Determination of IGF-1 concentration in milk serum was carried out by ELISA commercial kit for IGF-1 manufactured by MyBioSource (San Diego, CA) according to the manufacturer's instructions. Determination of insulin concentration in sample was performed using the RIA method. Determination of fT_4, fT_3, and cortisol concentrations was carried on an automated immunoassay analyzer (AIA-360, Tosoh Bioscience, Japan) using original commercial tests from the same producer.

Statistical Analysis

The data were statistically processed using the software STATISTICA, v. 8.0 (StatSoft, Inc., Tulsa, OK, USA) and the results are presented as mean ± standard error of the mean (SEM). The normality of data distribution was tested using Shapiro-Wilk test and all data showed the normal distribution ($p>0.05$). The significance of the differences in the observed blood and milk parameters of the examined cows between different stages of lactation was analysed using a dependent Student's t-test. On the other hand, the significance of the differences in hormone concentration between the milk of the examined cows, bulk-tank milk on the farm and retail milk was analysed using an independent Student's t-test. Significance was declared at $p<0.05$.

RESULTS

The concentrations of the milk hormones, as well as fat and protein concentrations, are shown in Table 2 and these results are given for each examined period as well as their average marked as the lactation average. The lactation average of the IGF-1 concentration in cow's milk was significantly different from the values determined in all test periods, with the exception of the value determined on day 280 of lactation. Compared to lactation average values, milk IGF-1 concentrations were significantly lower on days 90, 150, 180, 210 and 250, and significantly higher on days 10, 30 and 60 of lactation. The average insulin concentration in the milk differed significantly from the values obtained in all tested periods, except for the values obtained on days 10, 150, and 180 of lactation. Compared to the average milk insulin concentration, values determined on days 30, 60 and 90 of lactation were significantly lower while concentrations determined on days 210, 250 and 280 of lactation were significantly higher. On the contrary, the average fT_4 concentration in the cow's milk did not significantly differ from the values determined in all examined periods, except on day 250, when the fT_4 concentration was significantly higher. The average milk fT_3 concentration was significantly different from the values determined in all test periods, except for days 150, 210, and 250 of lactation. The fT_3 concentrations in milk measured on days 30, 60, 90 and 280 of lactation were significantly lower, and those obtained on days 10 and 180 of lactation were significantly higher than the lactation average value. Compared to average milk cortisol levels, concentration determined on days 60 and 280 of lactation were significantly lower and those determined at day 250 in lactation were significantly higher. There was no significant difference between average milk cortisol levels and concentrations obtained in other tested days in lactation. Milk fat concentrations ranged from 34.11 ± 0.76 g/L (day 30) to 40.42 ± 1.1 g/L (day 210) and the average milk fat concentration was significantly different from the values determined in all investigated periods, except for days 10, 150, and 250 of lactation. Concentrations of milk fat obtained on days 30, 60, and 90 of lactation were significantly lower and those obtained on days 180, 210, and 280 of lactation were significantly higher than average value. Milk protein concentrations were significantly different from the values determined in all examined periods, except for day 180 of lactation. Concentrations of milk protein obtained on days 30, 60, 90 and 150 of lactation were significantly lower and those obtained on days 210, 250, and 280 of lactation were significantly higher than average value.

The concentrations of milk hormones, fat and protein in three different groups of milk samples, including bulk-tank milk, retail milk (fat content of 1.5% and 3.2%) and lactation average of examined cows are presented in Table 3.

Table 2. Dynamic changes in energy-related hormones, fat and protein concentrations (mean±SEM) in cow's milk at different stages of lactation.

Variable	Days in lactation									Lactation average
	10	30	60	90	150	180	210	250	280	
IGF-1 (ng/mL)	9.23±0.7^{A}	8.09±0.64^{AB}	6.53±0.52^{D}	3.70±0.28^{E}	3.31±0.25^{CE}	3.16±0.22^{CE}	2.80±0.22^{C}	6.52±0.4^{BD}	4.71±0.4^{F}	5.34±0.22^{F}
Insulin (mIU/L)	16.36±1.7^{ABC}	13.80±0.95^{A}	15.94±0.96^{C}	15.24±1.12^{AC}	20.07±1.46^{BD}	16.23±1.02^{ACE}	20.17±1.01^{D}	20.33±1.1^{D}	20.66±1.5^{D}	17.59±0.6^{BE}
fT_4 (ng/dL)	0.217±0.004^{A}	0.212±0.007^{AB}	0.203±0.005^{AC}	0.218±0.007^{AD}	0.209±0.01^{AE}	0.216±0.007^{AE}	0.216±0.012^{AEG}	0.239±0.01^{G}	0.198±0.01^{BCDE}	0.214±0.002^{AC}
fT_3 (ng/dL)	6.56 ± 0.44^{AC}	3.87±0.21^{B}	3.38±0.26^{BE}	3.93±0.31^{BFI}	5.33±0.42^{G}	6.61±0.34^{C}	5.21±0.31^{DHG}	4.54±0.2^{HIG}	3.14±0.22^{EF}	4.73 ± 0.14^{G}
Cortisol (ng/dL)	0.336±0.015^{BDE}	0.321±0.018^{BDE}	0.259±0.013^{ACF}	0.315±0.015^{BDE}	0.301±0.02^{BCDE}	0.298±0.02^{BCE}	0.307±0.02^{BCDE}	0.342±0.01^{D}	0.259±0.014^{A}	0.304±0.01^{E}
Fat (g/L)	39.74±0.93^{AGF}	34.11±0.76^{B}	36.61±1.13^{C}	36.97±0.79^{CD}	39.37±0.95^{AEGI}	40.4±0.96^{AEG}	40.42±1.1^{AH}	39.4±1.0^{EF}	41.66±1.3^{GH}	38.74±0.8^{FI}
Protein (g/L)	32.04±0.69^{ABCDEFG}	30.94±0.64^{ABC}	31.41±062^{ACD}	31.46±0.64^{ACD}	31.63±0.64^{D}	32.14±0.71^{GF}	33.02±0.64^{E}	32.9±0.8^{EF}	33.37±0.7^{E}	32.09±0.6^{G}

IGF-1 – insulin-like factor 1; fT_4 – free thyroxine; fT_3 – free triiodothyronine; Lactation average - the average value of the respective variable for all examined days in lactation (10, 30, 60, 90, 150, 180, 210, 250 and 280); ^{AB} – different uppercase letters in the superscript indicate the significant differences ($p<0.05$) between individual examined days, including lactation average.

Table 3. Concentration of hormones, fat and protein (mean±SEM) in bulk-tank and retail milk and lactation average of the examined cows.

Variable	Bulk-tank milk	Retail milk		Lactation average
		Fat content (1.5%)	Fat content (3.2%)	
IGF-1 (ng/mL)	8.33±0.54^{A}	5.95±0.18^{C}	8.82±0.27^{A}	5.34±0.22^{B}
Insulin (mIU/L)	16.45 ± 0.48^{A}	13.41 ± 1.03^{B}	18.77 ± 0.90^{A}	17.59±0.6^{A}
fT$_4$ (ng/dl)	0.233±0.007^{A}	0.139±0.006^{B}	0.191±0.003^{C}	0.214±0.002^{D}
fT$_3$ (ng/dl)	4.77±0.31^{A}	3.79±0.24^{B}	6.09±0.23^{C}	4.73 ± 0.14^{A}
Cortisol (ng/dL)	0.357±0.016^{CD}	0.229±0.011^{B}	0.342±0.016^{AD}	0.304±0.01^{A}
Fat (g/L)	37.12±0.49^{B}	15.00±0.00^{A}	32.00±0.00^{B}	38.74±0.8^{B}
Protein (g/L)	33.15±0.15^{B}	35.00±0.00^{AC}	33.00±0.00^{BC}	32.09±0.6^{B}

IGF-1 – insulin-like factor 1; fT4 – free thyroxine; fT3 – free triiodothyronine; Lactation average - the average value of the respective variable for all examined days in lactation (10, 30, 60, 90, 150, 180, 210, 250 and 280); ^{AB} – different uppercase letters in the superscript indicate the significant differences ($p<0.05$) between individual examined days, including lactation average.

The concentrations of all measured milk hormones in retail milk with 1.5% fat were significantly lower than in retail milk with 3.2% fat and bulk-tank milk, respectively. Average of milk IGF-1 concentrations was significantly lower than in bulk-tank as well as retail milk with 1.5% and 3.2% of fat, while averages of insulin and cortisol concentrations were significantly higher than in retail milk with 1.5% of fat. Average milk cortisol concentration was also significantly lower than in bulk-tank milk. Average fT$_4$ concentration was significantly higher than in retail milk with 1.5% and with 3.2% of fat, respectively, and significantly lower than in bulk-tank milk. The average fT$_3$ concentration was significantly higher than in retail milk with 1.5% of fat and significantly lower than in retail milk with 3.2% of fat. The average of fat milk concentration was significantly higher than in retail milk with 1.5% of fat. Average protein milk concentration was significantly lower than in retail milk with 1.5 % of fat.

The concentrations of insulin and biochemical parameters in the blood are listed in Table 4. The results are shown for each examined period as well as their average marked as the lactation average.

Insulin concentration continuously increased from day 10 to day 280 of lactation with a significant increase between days 30 and 60. Glucose concentration fluctuated between days 10 and 280 of lactation between 2.85±0.22 mmol/L (minimum value at day 30) and 3.94±0.09 mmol/L (maximum value at day 280). Blood total protein and total bilirubin concentrations alternately increased and decreased from day 10 to day 280 between 69.40±2.04 mmol/L (minimum value at day 10) and 85.61±1.51 mmol/L (maximum value at day 280) for total protein concentrations and between 5.58±0.23 µmol/L (minimum value at day 280) and 8.42±0.28 µmol/L (maximum value day 10) for total bilirubin concentration. Albumin concentration alternated between days

Table 4. Concentration (mean±SEM) of insulin and blood biochemical parameters in the blood of the examined cows.

Variable	Days in lactation									Lactation average
	10	30	60	90	150	180	210	250	280	
Insulin (mIU/L)	17.41 ± 0.99^{A}	18.29 ± 0.68^{A}	21.63 ± 1.41^{B}	21.92 ± 0.53^{B}	22.94 ± 1.39^{B}	23.20 ± 0.90^{BC}	24.48 ± 1.35^{BC}	25.36 ± 1.99^{BC}	27.67 ± 1.98^{C}	22.55 ± 0.62^{B}
Glucose (mmol/L)	3.12 ± 0.20^{A}	2.85 ± 0.22^{A}	3.43 ± 0.16^{ACE}	3.69 ± 0.10^{BCDE}	3.78 ± 0.17^{BCDE}	3.83 ± 0.14^{BCD}	3.86 ± 0.12^{BCD}	3.68 ± 0.08^{BCE}	3.94 ± 0.09^{D}	3.58 ± 0.08^{E}
T-Proteins (g/L)	69.40 ± 2.04^{A}	78.97 ± 1.55^{BH}	78.66 ± 1.29^{B}	80.33 ± 1.53^{BC}	89.19 ± 1.86^{BDG}	81.90 ± 1.26^{DCEH}	81.43 ± 1.18^{BEF}	83.05 ± 1.26^{CFGH}	85.61 ± 1.51^{D}	80.06 ± 0.82^{BE}
Albumin (g/L)	32.39 ± 1.95^{A}	36.38 ± 1.65^{B}	38.69 ± 0.78^{BDG}	39.89 ± 1.08^{BEG}	41.33 ± 0.94^{CE}	41.53 ± 0.83^{CE}	41.08 ± 1.23^{BEFG}	41.61 ± 1.13^{CDE}	42.19 ± 0.64^{CF}	39.45 ± 0.49^{G}
Urea (mmol/L)	4.17 ± 0.16^{A}	4.38 ± 0.15^{B}	5.23 ± 0.22^{C}	5.90 ± 0.25^{DG}	6.43 ± 0.29^{DE}	6.56 ± 0.25^{E}	6.79 ± 0.30^{F}	6.90 ± 0.31^{F}	6.88 ± 0.30^{F}	5.92 ± 0.19^{G}
T-Bilirubin (µmol/L)	8.42 ± 0.28^{A}	7.54 ± 0.13^{BE}	7.71 ± 0.08^{BD}	7.47 ± 0.09^{EF}	7.49 ± 0.08^{BDF}	6.20 ± 0.32^{CG}	5.86 ± 0.26^{CG}	5.89 ± 0.28^{G}	5.58 ± 0.23^{C}	6.91 ± 0.07^{H}
ALT (U/L)	36.40 ± 2.07^{A}	43.43 ± 0.99^{B}	46.51 ± 0.97^{CFG}	46.51 ± 0.79^{CFG}	48.27 ± 0.94^{CEF}	48.53 ± 0.82^{CF}	47.84 ± 0.91^{C}	48.74 ± 0.81^{F}	49.63 ± 0.81^{DE}	46.20 ± 0.59^{G}
AST (U/L)	82.62 ± 5.57^{A}	83.10 ± 4.72^{A}	100.23 ± 10.14^{AC}	107.75 ± 9.20^{BC}	109.96 ± 8.08^{BC}	112.53 ± 7.26^{BC}	113.73 ± 7.52^{B}	114.55 ± 7.63^{B}	117.32 ± 7.23^{B}	104.64 ± 5.18^{BC}
Calcium (mmol/L)	2.22 ± 0.05^{A}	2.29 ± 0.05^{A}	2.55 ± 0.05^{B}	2.58 ± 0.07^{B}	2.64 ± 0.08^{B}	2.65 ± 0.07^{B}	2.66 ± 0.07^{B}	2.69 ± 0.09^{B}	2.71 ± 0.09^{B}	2.56 ± 0.03^{B}
Phosphorus (mmol/L)	1.64 ± 0.12^{A}	1.84 ± 0.18^{BD}	1.91 ± 0.06^{B}	1.97 ± 0.07^{BD}	2.25 ± 0.11^{CD}	2.25 ± 0.12^{CD}	2.30 ± 0.12^{C}	2.33 ± 0.08^{C}	2.39 ± 0.09^{C}	2.09 ± 0.05^{D}
Magnesium (mmol/L)	1.08 ± 0.06^{A}	1.18 ± 0.0^{ABC}	1.21 ± 0.06^{ABC}	1.28 ± 0.08^{ABC}	1.32 ± 0.08^{B}	1.37 ± 0.07^{B}	1.36 ± 0.08^{B}	1.36 ± 0.06^{B}	1.35 ± 0.06^{B}	1.28 ± 0.03^{BC}

T-Proteins – total proteins; T-Bilirubin – total bilirubin; ALT – alanine aminotransferase; AST – aspartate aminotransferase; Lactation average - the average value of the respective variable for all examined days in lactation (10, 30, 60, 90, 150, 180, 210, 250 and 280); ^{AB} – different upercase letter in the superscript indicate the significant differences ($p<0.05$) between individual examined days, including lactation average.

10 and 280 of lactation between 32.39±1.95 mmol/L (minimum value at day 10) and 42.19±0.64 mmol/L (maximum value at day 280). Blood urea concentration continuously increased from day 10 to day 250 of lactation with a significant increase between days 30 and 60. ALT activity increased from days 10 to 180, after which its concentration fluctuated between 47.84±0.91 U/L (day 210) and 49.63±0.81 U/L (day 280). AST activity constantly increased from day 10 to day 280. Calcium and phosphorus concentrations gradually increased from day 10 to day 280 with significant increase between days 30 and 60 for Ca and days 10 and 30 for P concentration. Magnesium concentration fluctuated between days 10 and 280 of lactation among 1.08±0.06 mmol/L (minimum value at day 10) and 1.37±0.07 (maximum value at day 180) mmol/L.

DISCUSSION

In our study, the concentration of hormones in milk was measured throughout the lactation period in order to register fluctuations in hormone concentrations which depend on lactation phase [6]. Also, hormone concentrations were measured in retail milk with different fat level in order to investigate whether fat content has an influence on hormone concentrations in milk. In aim to provide background information on protein biosynthesis, utilization, and excretion, as well as kidney failure, liver damage, and nutritional health, blood biochemical parameters were obtained in our research [21]. Biochemical parameters as well as insulin measured in our study were within the reference range suggested by Cozzi et al. [22] and Moretti et al. [23], indicating that animals included in our study were healthy.

IGF-1 concentration in milk of examined cows was highest at the beginning of the lactation (9.23±0.71 ng/mL) and then decreased during lactation. The lowest concentration was registered at day 210 of lactation and was 2.80±0.22 ng/mL. Our results are in accordance with results of Sutariya et al. [10] who determined IGF-1 levels in cow's milk from 0.3 to 15 ng/mL as well as with findings of JECFA [24]. Milk IGF-1 concentrations are higher in the milk of cows in early lactation due to the negative energy balance that cows experience during that period [25]. As milk obtained from cattle in the early stages of lactation contains high levels of IGF-1, it may promote active cell growth, which is undesirable for oncology patients. In contrast, milk in the later stages of lactation is more suitable for consumption in its natural form. Additionally, we have investigated IGF-1 concentrations in retail milk with different fat content and revealed that retail milk with 1.5% fat had significantly lower concentration on IGF-1 (5.95±0.18 ng/mL) than retail milk with 3.2% fat (8.82±0.27 ng/mL) and that those concentrations were significantly higher than in samples of bulk tank milk (8.33±0.54 ng/mL) and lactation average (5.34±0.22 ng/mL). All of those values were higher than those published by Vicini et al. [26], who determined 3.12±0.059 ng/mL of IGF-1 in retail milk. Marín-Quiroga et al. [27] recorded highest IGF-1 in whole milk, followed by low-fat milk, suggesting that the milk fat fraction contributes to the total IGF-1 content of milk. On the other hand, heat treatment of milk such as pasteurisation had no effect on the concentrations and distribution of the IGF-1 and insulin in different fractions of pasteurized and non-pasteurized milk [28]. It is accepted that consumption of milk and dairy products with high IGF-1 content might be a causative factor in the development of neoplastic processes which may provoke increased risk of cancer in humans [29]. Furthermore, Simonov et al. [30] pointed out that milk consumption promotes proliferative processes in humans. The lower level of this hormone in milk is combined with the lower risk of cancer [31, 32]. Fraser et al. [33] found a significantly increased risk of breast cancer in US women consuming ~160 mL of retail milk daily, regardless of the fat content of the milk. For final conclusion related to impact of milk IGF-1 concentrations on human health, further investigations that include not only concentrations but also a volume of consumption are necessary.

In our study, the average insulin concentration during the lactation period was 17.59±0.56 mIU/L, while the insulin concentration in retail milk was 13.41±1.03 mIU/L (1.5% milk fat), and 18.77±0.90 mIU/L (3.5% milk fat). These values are within the range reported by Jouan et al. [34], who found milk insulin concentration to be 5–40 ng/mL. Paronen et al. [35] observed that oral exposure to bovine insulin in cow's milk triggered insulin-specific T-cell and antibody responses in infants at increased risk for type 1 diabetes. Infants who later develop type 1 diabetes mellitus or autoimmunity to beta cells were found to have impaired oral tolerance, not only to dietary insulin but also to cow's milk proteins in general [36]. Insulin peptides in whey-based formulas can potentially trigger deleterious T-cell responses, similar to gliadin peptides from wheat in celiac disease [37]. Accordingly, bovine insulin peptides in cow whey may act as environmental mimics of autoantigenic peptides and induce non-tolerogenic T cells to activate autoreactivity against the primary autoantigen, i.e., human insulin expressed in islet cells [38]. There are no data in the literature on insulin levels posing a risk to human health, but the authors mentioned above state that consumption of cow's milk may cause type 1 diabetes in children. However, this topic needs further research.

The concentrations of fT_4 in cow's milk in our study varied between 0.198±0.009 ng/dL and 0.239±0.007 ng/dL. These values are lower than those observed by Pezzi et al. [39] who detected a very low concentration of fT_4 in the colostrum (0.70 ± 0.1 ng/mL), with a decreasing trend in subsequent samples being below the detection limit of the method. The fT_3 concentration in cow's milk in our study fluctuated between 3.14±0.22 and 6.61±0.44 ng/dL. Our results for fT_3 at early lactation are in accordance with results obtained by Pezzi et al. [39], since they reported that milk fT_3 concentrations showed a downward trend as early lactation progressed: highest values were determined in colostrum on day 0 of lactation (3.54 ± 0.81 ng/mL), and those values were significantly reduced on day 5 of lactation and steadily decreased over the two months. Pan'kiv et al. [3] found that the concentration of T_3 and T_4 were 4.92 and 4.75 nmol/L three days after calving, and dropped to values of 2.15

and 2.35 nmol/L two weeks after calving, and were 1.77 and 1.56 nmol/L between 30 and 40 days. According to Pan'kiv et al. [3], the concentration of triiodothyronine and thyroxine in the colostrum is highest and can be explained by the need to stimulate metabolic processes in neonatal calves, since their endocrine system is not yet mature. The decrease in thyroid hormones in cow's milk in the later stages of lactation may be related to changes in cows' metabolism that reduce the need for exogenous hormones in calves [3]. Pezzi et al. [39] confirm that dairy cows are in a hypothyroid state in early lactation, but the question remains whether the hypothyroid state in lactating animals is the cause or the consequence of decreased deiodinase activity in the liver. One of the possible explanations for hypothyroidism in lactating cows is the secretion of thyroid hormones into the milk at low concentrations. In our study, the concentration of thyroid hormones in retail milk with 1.5% fat were 0.139±0.006 ng/dL and 3.79± 0.24 ng/dL for fT_4 and fT_3, respectively, and 0.191±0.003 ng/dL and 6.09± 0.23 ng/dL, respectively, for milk with 3.5% milk fat. There are no data in the literature related to fT_4 and fT_3 in retail cow's milk and the levels that pose a risk to human health, so this topic needs further investigation.

The concentration of cortisol in our study varied between 0.259 ±0.014 ng/dL on day 60 and 0.342 ±0.008 ng/dL day 250 of lactation. Gellrich et al. [40] found the highest concentrations in milk during the first week of lactation (5.8 ± 0.48 nmol/L), while weekly values were between 3.6 and 6.2 nmol/L. The concentration of cortisol in milk is lower than that in blood, ranging from 500 pg/mL to 10 ng/mL [41]. In our study, the concentration of cortisol in retail milk with 1.5% milk fat was 0.229±0.011 ng/dL, and in the milk with 3.5% milk fat was 0.342±0.016 ng/dl. Malekinejad et al. [6] reported that cortisol is still detectable in commercial dairy products after sterilization and pasteurization processes, and its concentration and structure are not significantly affected during heat treatment. Xu et al. [42] found no significant differences in cortisol content between bovine and human colostrum. Based on our results and results obtained by other authors, it can be assumed that cortisol present in cow's milk has no adverse effects on children, as the presence of cortisol residues in commercial dairy products is not a health concern for consumers, as the levels are usually very low and harmless [17]. Further studies are recommended to investigate the risks and side effects of higher consumption of cow's milk by other categories of consumers, although they are exposed to very low levels of cortisol residues in milk.

CONCLUSION

This study has shown that the energy-related hormones were present in the milk of the lactating cows in a detectable amount and that their presence varied according to the period of lactation. Furthermore, hormones were found in both bulk-tank milk and retail milk, with the concentrations of these hormones being lowest in low-fat milk. This underlines that in addition to the nutritional aspect, the health dimension of milk consumption in societies should also be considered, as hormones in milk can have potentially harmful effects on consumers' health. However, further studies are still needed to determine the critical concentrations or threshold levels of hormones in milk that may have an impact on consumers' health, as well as the recommended daily amounts for milk consumption, especially in certain social groups with an increased risk or genetic predisposition to developing various cancers and endocrinological disorders.

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Authors' contributions

DK, IV, ŽS and DK contributed to the design and conception of the study. SN and IV conducted the field experiment. DB, SD and SN performed biochemical analyses of blood serum samples. MP and MS measured hormone concentration in the milk samples. OV, ŽS and DK participated in data analyses. DK, DB, SD and DK were involved in draft manuscript preparation. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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HORMONI U SIROVOM I MALOPRODAJNOM MLEKU KRAVA I POTENCIJALNI RIZIK PO ZDRAVLJE POTROŠAČA

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Istraživanje je imalo za cilj određivanje koncentracije hormona u mleku krava u različitim fazama laktacije, mleku iz laktofriza i maloprodajnom mleku. Uzorci mleka i krvi prikupljeni su 10., 30., 60., 90., 150., 180., 210., 250. i 280 dana laktacije od četrnaest krava holštajn rase radi određivanja koncentracije hormona, masti i proteina u mleku i biohemijskih parametara krvi. U iste svrhe uzorkovano je mleko iz laktofriza i nabavljeno je komercijalno mleko sa 1,5% ($CM_{1,5}$) i 3,2% ($CM_{3,2}$) mlečne masti. Koncentracije insulinu-sličnog faktora rasta-1 (IGF-1) u mleku bile su značajno niže 90., 150., 180., 210. i 250. dana i značajno više 10., 30. i 60. dana laktacije poredeći sa laktacionim prosekom (LP). Insulin je imao značajno niže koncentracije u mleku 30., 60. i 90. dana i značajno više koncentracije 210., 250. i 280. dana laktacije poredeći sa LP. Koncentracija slobodnog tiroksina (fT_4) je bila viša 250. dana, dok je nivo slobodnog trijodotironina (fT_3) bio značajno niži 30., 60., 90. i 280. dana i značajno viši 10. i 180. dana u poređenju sa odgovarajućim LP. Nivo kortizola u mleku je bio niži 60. i 280. dana laktacije poredeći sa LP. Svi hormoni su imali značajno niže koncentracije u $CM_{1,5}$ nego u $CM_{3,2}$, mleku iz laktofriza i LP. Izuzetak je LP za IGF-1, koji je bio značajno niži nego nivo IGF-1 u $CM_{1,5}$. Biohemijski parametri krvi su blago fluktuirali tokom laktacije, ali su ostali u referentnom opsegu. Koncentracije hormona u kravljem mleku fluktuiraju tokom laktacije, što daje značaj mleku u kontekstu javnog zdravlja.