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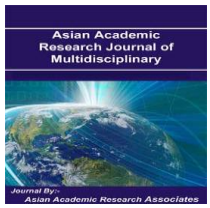

STUDY OF SEASONAL DYNAMICS OF BLOOD METABOLIC PROFILE AND MILK UREA NITROGEN (MUN) LEVEL OFCOWS WITH REPRODUCTIVE DISORDERS

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Abstract

Variations in the components of blood chemistry could be indicative of metabolic or nutritional imbalances in dairy cows. In the recent work an attempt has been made in understanding ofthe seasonal variation of the blood metabolic profile and milk urea nitrogen (MUN) level at individual Holstein Friesian cows with reproductive disorders in one dairy farm in R.Macedonia.Blood and milk samples were collected from 149 dairy cows with reproductive disorders, every monthup to one year between spring /2012and spring /2013.The cows were held in an intensive farm breeding premises and never turned out to graze. Their ration consisted of ordinary hay, silage, haulage and concentrates with energy and protein supplements.The obtained values of sera, total proteins, blood urea nitrogen (BUN), ASAT, ALAT, calcium, phosphorus, potassium, sodium and chlorine, as well as milk urea nitrogen level in different seasons were assessed and also all these values were found in good agreement with the results obtained by other workers in Dairy Science. All the values were determined in term of mean or average values with standard deviation (mean ± S. D), and the aanalysis of Variance (ANOVA) test, was applied to determine the seasonal effects during the experimental period (12 months) and the "P" values of <0.05 were considered statistically significant.

Key words: seasons, blood profile, milk urea nitrogen (MUN), Holstein –Friesian dairy cows.

Introduction

Intensive genetic selection for high milk production increases the risk of metabolic and reproductive problems in dairy breeds of cows in recent years is an important issue in the latest knowledgeof dairy technology, unfortunately there are pure negative genetic links (several times stronger than phenotypic) in between the dairy farming and fertility**(Löf et al., 2007)** [1].The metabolic profile test in farm ruminants is being used to assess the nutritional status, to predict incidentof metabolic diseases and diagnose the diseases, moreover to understand the fertility status of animals **(Ingraham et al., 1998)**[2].Theanimals respondto disturbances of their homeostasis to a set of physiological changes known as acute phase response **(Piňeiro et al. 2003)**[3].The exibition of seasonal physiological functions is reflected in the ability ofendogenous adaptive mechanism to react in advance for the regulation of environmental changes associated with the seasons **(Piccione et al., 2009)**[4].According to the results reported by the authors(**Filipejová et al. 2011)**[5], the metabolic disorders (disturbance in homeostasis of the organism, reduced milk production, lower milk quality, and the premature cows culling in the dairy's outputare caused by the action of the disrupted equilibrium between intake and consumption of dietary matters and metabolites necessary for optimum production and reproduction. During the late lactation appears energy shortages as a result of excessive feeding in the dry period, causing fatty liver degeneration syndrome and reproductive disorders **(Bojkovski et al., 2007**and**Krsmanović, 2010)**[6-7]. Urea as a final product of the protein degradation, it is synthesized in the urea cycle, and is an indicator in nitrogen metabolism as well of liver and renal function **(Balabámová et al., 2009)**[8].Dysfunction of the reproductive system such as ovarian cysts, ovarian hypofunction, decreased or lack of ovulation, etc. occurs as a direct consequence of metabolic disorders in the body. All previous data indicate that high protein content feeding, contributes in increased concentration of urea and ammonia in the bloodstream, from there to the pre-ovulatory follicles, **(Moallem et al., 2011)**[9] as well to the uterine **(Iyathurai, 2012)**[10]and reducing fertility rate in dairy cows **(Tamminga, 2006)** [11]. In high-producing cows, the measurement of the concentration of urea nitrogen in the blood and milk provides additional information relating to the energy and protein requirements of the cows. Milk sampling is far more accessible method than a more complex and invasive method for sampling of blood, and therefore most researchers propose determining the concentration of urea nitrogen in the samples of milk as a routine method for the diagnosis of efficiency of protein nutrition of cows.The milk urea nitrogen assessment should be systematically carried

out in the Republic of Macedonia to establish an optimal scheme of feeding, to determine the energy balance in protein rations in order to improve reproductive and productive traits of dairy cows and reaching of favorable physical, chemical and technological properties of milk in different seasons of the year.

Materials and Methodology Used

The present experimental work was carried out on a total of 149 Holstein Friesian cows with an average service period 150.92 ± 79.21 days maintained at the Dairy Farm in Pelagonia region in R. Macedonia. The farm was selected because of proper data recording and relatively good management and cooperation**.**The laboratory analysis was conducted at the "Bitola Laboratory " in Bitola town, Macedonia.

(i) Topography of the Study Area

The present area of dairy farm has 595 meters above sea level and is located in the moderate-continental sub Mediterranean zone, covering an area of over 4,000 km^2,It is surrounded by mountains from the geographical directionsof North, East and West and to the South is open in Greece. The monthly average values of temperature and relative humidity in the region are such as: spring with +10.49°C /51.66%, summerwith +19.99° C /37.23%, fall with +11.66°C /55.06% and the winter with +1.00° C /56.73%.

(ii) Management of Dairy Cows

The cows were kept in the free-boxing system with milking hall type "herringbone" pattern so called in dairy science De Laval. The cows were milked twice a day between 06.00/07.00 hours and 17.00/18.00 hours daily regime (and the cows up to 150 days after calving, were milked three times).

Based on average daily milk yield (DMY) feeding was allocated to the following groups:

•Group 1 – fresh calved cows and heifers with a daily milk yield 30-32 kg of milk.

•Group 2 – fresh calved cows and heifers with a daily milk yield 22-25 kg of milk.

•Third group-cows and heifers with a daily milk yield 15-24 kg of milk.

•Fourth group - cows before drying.

•Fifth group - the dry cows.

As for the diet of dairy cows on the dairy farm feed was given throughout the year as a total mixed ration, and the cows were never turned out to graze. Thefeed rations were formulated before and after the calving, besides also it was in accordance to the National Research Council recomendations (NRC,2001) [12], and the residues of the dietary feed were generally observed in the herd. The rations were homogenized with a special type of machine in our

dairy farm which is fittedon a trailerand called“Mixer” and it were set after the morning milking, as were available to the animals for 24 hours. Their ration (chemical composition) consisted of ordinary hay, silage, haylage and concentrates with energy and protein supplements as it is described in the Table 1.The animals had free access to fresh andclean drinking water all the time. Many of the veterinary aid actions were taken for all dairy cows, which were followed as per farm schedule.

Table 1: Chemical composition of the feed included in the ration of experimental cows (%).

Forage	D.M.	C.P.	C.F.	N.D.F.	Ca	P
Hay	0.90	18.00	30.5	29.2	1.30	0.25
Silage	0.34	8.60	18.3	61.0	0.21	0.15
Haylage	0.32	16.10	32.5	28.7	1.10	0.26
Straw	0.88	3.40	42.5	27.0	0.32	0.21
Concentrate (mixture)	0.89	17.21	61.4	51.1	0.80	0.80

Note:- D.M. – dry matter, C.P.- crude protein, C.F.-crude fiber, N.D.F. -neutral detergent fiber, Ca -Calcium and P-Phosphorus

(iii) Organization of the Reproductive Process

The farm produces artificial insemination of cows and heifers after oestrus detection and determination of the optimal time. Each cow has been noted accompanying veterinary ear tag that is visible at minimum it three meters. By knowing the signs of oestrus, this is an important point in the detection of the animal heat, so workers conducted preliminary training for different phases of the sexual cycle.Animal were confirmed for heat by rectal palpation and inseminated with frozen semen twice at the interval the of 12 hours. The cows, not returning to estrus after 21 days of insemination were examined per rectal palpation of genitalia > 45 days post of artificial insemination(A.I.), for pregnancy confirmation.For artificial insemination was used a cryopreserved semen is imported from thecompanies Swissgenetics, Semex and Alta genetics. Clinical studies of cows were performed by veterinarians on the farm and the animals were registered withreproductive disorders - anoestrus, nymphomania, hypofunction of the ovaries, ovarian cysts and the presence of persisting corpus luteum.

Table 2: Distribution of the reproductive disorders in cows during the different seasons of the year.

Seasons	Cows with reproductive disorders		Diagnose		
	No.	%	Corpus luteum (Values in %)	Ovarian cysts (Values in %)	Ovarian hypofunction (Values in %)
Spring	40	27.22	12.50	52.50	35.00
Summer	29	19.73	0.0	48.28	51.72
Fall	31	21.08	6.45	58.06	35.48
Winter	47	31.97	12.77	76.60	10.64

(iv) Laboratory BloodTests

Blood samples for biochemical analysis were taken once a month for one year from the selected groups of cows with reproductive disorders 2-3 hours after the morning feeding. From each animal, about 5 ml blood was performed by puncture of vena coccygea into the screw capped vacuum tubes (CE, ISO 13485). In order to minimize the stress in the animal and to standardize the blood collection procedure, all the dairy cows were restrained with the same technique and the collection was made by the same personnel. The tubes with blood were transported into the rolling fridge to the laboratory for Biochemical analysis "Bitola Laboratory". Blood serum for biochemical analysis was separated after centrifugation of the sample blood at 3000 revolutions / min. for 5 minutes and transferred into a sterilized plastic vial and labelled. Clean glassware, micropipettes of different capacities and analytical grade chemicals were used in this study.The examinations comply with the requirements of the **European Convention for the Protection of Vertebrate Animals of 16.05.1986, from Strasbourg**. Biochemical analysis was performed to determine the following parameters:

(a). Photometric method (CPC) for the determination of calcium (Barnett, R. N. et al., 1973)[13]**:**

The calculation of the concentrations of the calcium were,according to the following formula

$$C = 8 \times \frac{\Delta A sample}{\Delta ASTD} [mg/dl]$$

or

$$C = 2 \times \frac{\Delta Asample}{\Delta ASTD} [mmol/l]$$

where "C" is the concentrations.

The test is linear in calcium concentration of 15 mg / dL or 3.75 mmol / L

(b) Photometric UV method for the determination of phosphorus (Gamst and K. Try1980)[14]**:**

The calculation of the concentrations of the calcium were according to the following formula:

$$C = 10 \times \frac{\Delta Asample}{\Delta ASTD} [mg/dLit]$$

or

$$C = 3.2 \times \frac{\Delta Asample}{\Delta ASTD} [mmol/Lit]$$

Where The test is linear in the phosphorus concentration of 20 mg / dL or 6.4 mmol / L

(c) Photometric turbo dimeric method for determining the potassium (Tietz, 2006)[15]**:**

The calculation of the concentration of potassium was according to the following formula:

C = S x Δ A sample /Δ A std (mmol/Lit)

(d) Colorimetric method for determining the sodium

Henry R.F., et. al., gave a method to determine this in their experimental by the Clinical Chemistry Principles and Technics, 2nd Ed., Harper and Row, Hagerstein, M.D., (1974)[16].

$$\frac{A_{BK} * A_S}{A_{BK} - A_S} \times C_{Standard} = \text{mmol/L (mEq/L)sodium}$$

where, the absorbance of the blank (A_{BK}), the sample (A_S) and the standard (A_{STD}) at 405 n.m.

(e) Colorimetric method for determining the Chloride

For this purpose the Clinical Guide to Laboratory Tests (Tietz. N.W.) were used, 3rd Edition. W.B.Saunders Co. Philadelphia, PA. (1995) [17].

$$\frac{A_{BK} * A_S}{A_{BK} - A_S} \times C_{Standard} = \text{mmol/L (mEq/L)chloride}$$

where, absorbance of the blank (A_{BK}), the sample (A_S) and the standard (A_{STD}) at 470 n.m.

In the above two formulas the(mEq/L) means milliequivalents per Literto measure the normal range for blood sodium levels in calcium and chloride respectively.

(f)GLUCOSE MR - Enzymatic colorimetric method

Also for this purpose the same Clinical Guide to Laboratory Tests (Tietz. N.W.) [17] were used and the calculation of the concentration of potassium is according to the following formula:

$$\frac{A_{Sample}}{A_{Standard}} \times C_{Standard} = \text{mg/dL glucose}$$

(g) Photometric method for the determination of total protein (Weichselbaum, 1946)[18]**:**

Calculation of protein concentration has been determined by followings:

(i) with factor:- C = 19 x Δ A (gr/dl) or C = 190 x Δ A (gr/Lit)

(ii) with standard:- C = 8 x Δ A sample / Δ A std (gr/dLit)

or

C = 80 x Δ A sample / Δ A std (gr/Lit)

(h) Photometric enzymatic method for the determination of urea (modified reaction Berthelot, 1859)[19]**:**

The calculation of the concentration of urea and BUN is calculated by the following formula:

$$C = \frac{\Delta A \text{ sample}}{\Delta A \text{ STD}} \text{ x Factor}$$

Conversion factors for the calculation of urea and BUN:

C (BUN) = 0.466 x C (Urea)

C(Urea) = 2.14 x C (BUN)

(i) Method for the determination of ASPARTAT-AMINOTRANSFERASE -ASAT (Thefeld, 1974)[20]**:**

The calculation of the concentration of ASAT is determined according to the following formula:

$$\frac{\Delta A}{\min} \times \frac{V_t * 10^6}{\varepsilon * Lit * V_s} = \text{U/L}$$

(j) Method for the determination ofALANINE AMINOTRANSFERASE – ALAT (Thefeld, 1974) [20]:

The calculation of the concentration of ALAT is calculated according to the following formula:

$$\frac{\Delta A}{\min} \times \frac{V_t * 10^6}{\varepsilon * Lit * V_s} = \text{U/L}$$

(v) Testing of Milk in Laboratory

Milk samples for analysis, were taken from whole milk obtained at the morning milking cows in accordance with the rules for milk sampling by applying the sterile plastic cup of the individual collector from milking system. Milk examinations included samples collected exclusively from animals with a healthy udder, namely with somatic cell count in milk not exceeding 400,000 SC in 1 ml of milk according to the "Rules and regulations designed for safe milk production"(Official Gazette 151.2007) [21]. Individual milk samples, (without added preservatives) were placed in movable refrigerator and were transported to the independent laboratory 'Mlekokontrol - Pelagonija' - Bitola. Testing of milk was performed within 3 hours after milking.

(vi) The Examination of raw milk

Photometric method for the determination of urea in milk (Merck KGaA, 64271 Darmstadt, Germany)was practiced for this analytical work and the concentration of urea- Pharo 300 Spectroquant Merck, Germany.

(a) Principle of the method

The addition of urease to the solution containing urea causes the following reaction - CO (NH_2) 2 H2O → 2NH_3 CO_2. This reaction is limited and the speed depends on the amount of urease added. Its half-life varies between 2 and 20 seconds (10-100 U / mL urease) and producesammonia. The resulting ammonia establishes by Nessler reagent with yellow coloring and is photometering at a wavelength of 420 nm.

The calculation of the concentration of urea in milk is calculated according to the following formula:

$$C = \frac{\Delta A\ (sp)}{\Delta A\ (st)} * C\ (st)$$

where:C- urea concentration, ΔA (sp)-absorbance of the sample, ΔA (st)-absorbance of urea standard and C (st)-concentration of urea standard.

(b) Statistical Analysis

All values of measured parameters were calculated in the form of mean or average values with standard deviations (means ±S.D.) and the "p" values <0.05 was considered significant using analysis of variance and were analyzed statistically using Statistic 6.0 software. The means were compared by Least Square Difference (LSD) test.

Results and Discussions

The data analysis revealed significant seasonal variations of some blood metabolites, minerals concentrations and milk urea nitrogen level at dairy cows with reproductive disorders.The Table 3 shows the mean values of the studied parameters, expressed in their conventional units of measurement with standard deviation (±SD)and statistical significances of the total proteins (g/Lit), blood urea nitrogen, BUN (mmol/L), ASAT and ALAT (U/L) and milk urea nitrogen, MUN (mmol/L), during different seasons. The serum levels of total proteins were significantly higherin summer (91.72± 15.78), with ($p<0.05$)and above the reference values (65- 85 g/L). Related conclusions have been recorded by **(Bonev et al., 2012)** [22].In addition, specified total protein levelsfor other seasons were: fall (83.97 ± 15.08), spring (83.50 ± 10.67), and winter (83.31 ± 18.09), all these values are with ($p<0.05$).Blood urea nitrogen (BUN) concentrations significantly increased in winter (8.46 ± 2.10)and fall (7.14 ± 1.55),with ($p<0.05$), being above the normal values (2.0-6.6 mmol/Lit**)** in comparison to seasons spring (5.84 ± 2.56) and summer (6.28 ± 1.80). Such an increase values above the normal, established authors **(Herak et al., 2000)** [23]**.**Blood plasma enzymes ASAT and ALAT showed a significant variation ($p<0.05$) between the seasons and their concentrations were above the reference values **[**24-25**]**. Similar to our results were reported by **(Bonev et al., 2012;**[22]and **Sangsritavong et al., 2002)** [26]**.** ASAT values were significantly higher in spring and fall (118.63 ± 22.66 and 115.00 ± 20.38). ALAT concentrations significantly increased in winter (51.73 ± 15.57) and fall (46.43 ± 14.11).Significantly higher MUN concentration was measured in summer (9.74 ± 1.55) ($p<0.05$), respect to the fall and winter (7.73 ± 3.24 and 7.64 ± 1.58), as well as to the spring season (6.32 ± 1.91), with ($p<0.05$).Some other workers in the same field (**Kapela K., Guliński P, 2008**) [27] also demonstrated the effect of the different seasons on milk urea concentration, which was higher in summer season rather than the winter season, as well as some of them emphasized that the high MUN values are often associated with the reproductive performance (**Calamari, 2005 and Kamoun, 2012**) [28-29].

Table 3. Content of urea in the blood and milk, total protein, ASAT, ALAT, and concentration of glucose during the different seasons of the year.

Indicators	Spring		Summer		Fall		Winter		Referent
	No.	Mean±S.D.	No.	Mean±S.D.	No.	Mean±S.D.	No.	Mean±S.D.	values
Level of total protein in the blood. (g/Lit.)	53	83.50 ± 10.67^{A}	29	91.72±15.78^{ABC}	34	83.97± 15.08^{B}	32	83.31± 18.09^{C}	65-85 g/Lit
Content of urea in the blood. (BUN) in (m-mol/Lit.)	53	5.84 ± 2.56^{AB}	29	6.28 ± 1.80 ^{C}	34	7.14 ± 1.55^{AD}	32	8.46 ± 2.10^{BCD}	2.0-6.6 m-mol/Lit
Level of ASAT. in (U/Lit.)	51	118.63±22.66 ^{AB}	29	102.16±26.40^{AC}	33	115.00±20.38^{CD}	32	97.64±16.61^{BD}	48-100 U/Lit
Level of ALAT. in (U/Lit.)	53	45.27 ± 12.01 ^{AB}	29	36.45±8.93^{ACD}	34	46.43 ± 14.11^{C}	32	51.73±15.57^{BD}	17-37 U/Lit
Content of urea in milk, in (m-mol/Lit.)	53	6.32 ± 1.91^{ABC}	29	9.74 ± 1.55^{ADE}	34	7.73 ± 3.24^{BD}	32	7.64 ± 1.58^{CE}	3.0-6.6 m-mol/Lit

Note: - In the above table, within a row, the different superscripts^{ABCDE} denote the significance($P < 0.05$) differences between the seasons, and the referent values have been taken from references [24-25].

where, the meaning of abbreviations in theabove tables are such as: T.P.- total proteins, BUN - blood urea nitrogen, ASAT - aspartate aminotranferase, and ALAT - alanine aminotransferase and also the Referentvalues mean -(Physiological concentration limits). The referent values have been taken from references [24-25].

The results of serum macroelements studied in this experimental work are presented in Table 4. The total calcium and inorganic phosphorus levels in cowsblood plasma quoted by various authors **[**24,25**]** fluctuate within a broad range. In our survey, the mean content of calcium and inorganic phosphor in the case of studyingdairy cows reached a bottom limit of normative values **[**24**].**

During the fall season, significant increased levels of calcium (Ca) was observed (3.12 ± 0.37), incomparison with the spring (2.61± 0.47), summer (2.88 ± 0.37) and winter (2.91 ± 0.32), with ($p<0.05$).The mean values of sodium, and chlorine in the cows blood plasma also remained within the limits of values recognized as normative ones **[**24**].**

These minerals showed a significant variation between the seasons ($p<0.05$). The sodium concentrations significantly increased in summer (160.76 ± 6.74) compared to the spring (147.60 ± 3.22), fall (144.06 ± 7.38) and winter (148.13 ± 4.00). Blood values for chlorine were significantly higher in winter (118.06 ± 1.96), than in spring (116.66 ± 3.79), fall (108.32 ± 18.47) and summer (106.06 ± 3.90). The results obtainedare not indicative of the occurrence of electrolyte metabolism disorders in the examined cows.Blood potassium levels were in agreement with referent values quoted by some authors **[**24, 25**],**and there were significant differences betweenspring (3.86 ± 0.68), to summer (4.15 ± 0.44) and winter (4.14 ± 0.53), with ($p<0.05$).

Table 4. Content of macroelements in the blood plasma of the cows during the different seasons of the year (mean ± SD).

Indicators	Spring		Summer		Fall		Winter		Referent values
	No.	Mean ± S.D.	No.	Mean ± S.D.	No.	Mean ± S.D.	No.	Mean ± S.D.	
Calcium (Ca) in (m-mol/Lit)	53	2.61±0.47^{ABC}	29	2.88±0.37^{AD}	34	3.12±0.37^{BDE}	32	2.91±0.32^{CE}	2.2-3.1
Chlorine (Cl) in (m-mol/Lit)	15	116.66±3.79^{ABC}	29	106.06±3.90^{A}	34	108.32±8.47^{B}	32	118.06±1.96^{C}	95-110
Potasium (K) in (m-mol/Lit)	53	3.86 ±0.68^{AB}	29	4.15±0.44^{A}	34	3.97 ± 0.36	32	4.14±0.53^{B}	4.1-5.6
Sodium (Na) in (m-mol/Lit)	15	147.60 ±3.22^{A}	29	160.76±6.74^{ABC}	34	144.06±7.38^{BD}	32	148.13±4.00^{CD}	135-155
Phosphorous (P) in (m-mol/Lit)	53	1.82 ±0.49 ^{AB}	29	1.73±0.44 ^{C}	34	1.53±0.25 ^{A}	32	1.48±0.34 ^{BC}	1.5-2.9

Note: - In the above table, within a row, the different superscripts^{ABCDE} denote the significance($P < 0.05$) differences between the seasons, and the referent values have been taken from references [24-25].

Conclusions and Outcomes

Having spent of one year monitoring, we identified significantly higheraverage concentration of total proteins in summer (91.72 ± 15.78) with the “p-values” ($p<0.05$);blood urea nitrogen (BUN) concentrations significantly increased in winter (8.46 ± 2.10); significantly higher MUN concentration was measured in summer (9.74 ± 1.55) ($p<0.05$). The obtained results for blood minerals are not indicative of the occurrence of electrolyte metabolism disorders in the examined cows,besides beingalso in accordance to the various authors reported in their experimental work.

These findings indicate seasonal variations of blood biochemical components and milk urea nitrogen level in dairy cows with registered reproductive problems. Further investigation of energy - protein balance of the feeding rations during the seasons, should confirm its influence of blood metabolic profile and lead to different predispositions for the development of metabolic diseases, low reproductive performances and culling risk.

Based on the present experimentalwork, one can say that such types of study are worth mentioning for the advancement of recent dairy technology. The produced results show a lot of good agreement with the results obtained by various authors.

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