Monitoring the redox status in dairy cows by using plasma dROMs, PAT, and OSI biomarkers

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Received June 23, 2020
Accepted May 26, 2021

Abstract

The aim of this work was to determine the changes of redox indicators such as reactive oxygen metabolites (dROMs), plasma antioxidant test (PAT) and the oxidative stress index (OSI) in dairy cows at different stages of lactation using a diagnostic equipment which is suitable for *in vivo* oxidative stress (OS) monitoring procedures. In total, 628 dairy cows were examined in the pre-parturient period (days in milk [DIM]: -21 to -1 day; n = 117), in the calving and maternity period (DIM: 0 to 7; n = 137), in the early lactation period (DIM: 8 to 30; n = 139), and the in the peak lactation (DIM: 31 to 150; n = 235). The dROMs and OSI values were significantly different ($P < 0.05$) when comparing the 1st and 2nd+ lactation cows in each group. The highest mean value of dROMs was detected at the calving and maternity stage in 1st lactation cows (141 ± 25 U. Carr) and the lowest (103 ± 29 U. Carr) was found in peak lactation. The OSI developed similarly, with the highest value of 5.58 ± 0.94 in the calving and maternity period in the 1st lactation cows and the lowest value of 4.05 ± 1.21 in peak lactation and significant differences were found in many cases. Based on the results, the measurement of dROMs and OSI may be suitable for detecting oxidative stress in different lactation stages.

Oxidative stress, reactive oxygen metabolites, cattle

For the maintenance of health functions of a living organism it is necessary, that the amount of Reactive Oxygen Species (ROS) produced by the body or originated from external sources, should not exceed the protective capacity of the natural antioxidant (AO) defence mechanisms. Once if this balance is upset and ROS cannot be eliminated by the AO defence systems anymore, the oxidative stress (OS) develops leading to cell damage and finally resulting in reduced animal productivity (Sies et al. 1985). Previous studies have revealed the association of OS with diseases and health disorders in cattle, such as retained foetal membranes, metritis, mastitis (Kankofer et al. 1996), udder oedema (Miller et al. 1993; Celi, 2010; Jóźwik et al. 2012; Talukder et al. 2014), disturbances in energy metabolisms including insulin resistance (Pedernera et al. 2010; Xu et al. 2014; Mikulková et al. 2020), infertility, early embryonic death, development of follicular cysts (Miller et al. 1993; Rizzo et al. 2007, 2009; Celi et al. 2012) in cattle. There are several factors that may predispose to the development of OS, such as metabolic stress (Pastorelli et al. 2013), heat stress (Bernabucci et al. 2002; Lacetera et al. 2003; Zimbelman and Collier 2011), consumption of mycotoxin contaminated feeds and unregulated inflammatory processes (Miller et al. 1993). There are some physiological predisposing factors for OS, such as calving and birth (Castillo et al. 2005; Gaál et al. 2006; Albera and Kankofer 2011). However, based on previous experiences, most of the predisposing factors can be reduced by improved housing, nutrition- and animal health management (Dobbelaar et al. 2010). For measuring the effects of these improvements on herd-level prevalence of OS, the OS itself must be recognized as early as possible in time.

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For indirect detection of OS, measurement of efficiency level of AO-defence system through glutathione-peroxidase (GPx), oxidised glutathione (GSSG), glutathione and GSSG ratio (GSH/GSSG), superoxide dismutase (SOD) activity is frequently used in research (Mikulková et al. 2019). The most direct OS biomarkers as malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), F2-isoprostane, Oxygen radical absorbance capacity (ORAC), ferric reducing ability of plasma (FRAP), trolox equivalent antioxidant capacity (TEAC), radical-trapping antioxidant potential (TRAP), measured frequently for the detection of OS are not suitable for routine cow side monitoring purpose, because of relatively high costs and/or require special laboratory background (Celi 2011). There are needs for feasible methods for the evaluation of redox status of cows, that could be applicable in herd level OS monitoring protocols in farm conditions (Píšťková et al. 2019).

An equipment (FRAS 4 Evolvo; Free Radical Analytical System, H&D s.r.l., Parma, Italy) was developed and has already been involved in several studies on OS in humans (Kanaoka et al. 2010; Serena et al. 2013) and other animal species (Celi 2010; Celi et al. 2010; Po et al. 2013) for research. Tests have also been carried out in cattle herds (Rizzo et al. 2007; Celi et al. 2011, 2012; Golder et al. 2016), but to our best knowledge, ranges of normal values for this species have not been reported yet. In the absence of these data, the usability of the device is limited, so we determined the mean value and standard deviation of primi- and multiparous cows in different lactation stages. These data may provide the basis for definition of reference intervals.

The medical term of reference interval for a test is based on the results that are seen in 95% of the healthy population. Calculation method of reference intervals mainly based on the statistical distribution of data. In case, if data show a normal (Gaussian) distribution, the reference range calculation formula is mean ± 2SD. In case when distribution of data is not normal, a non-parametric statistical method can be applied for estimation of reference intervals according to International Federation of Clinical Chemistry (IFCC) recommendation. In this case, the lowest and highest 2.5% of data are simply excluded and remained data between 2.5 and 97.5% means the reference interval (PetitClerc and Solberg 1987; Bland 2000; Szabó and Vásárhelyi 2015).

Materials and Methods

Data collection

Within the course of regular herd health and metabolic profile monitoring, 40 Hungarian large-scale dairy herds (248–2841/cow/farm) were visited between March 2017 and April 2019 (Plate I, Fig. 1).

Blood samples (n = 628) were collected from dairy cows in the prepartum (PreP; Days in Milk (DIM): -21 to 1 day; n = 117), in the calving and maternity (Calv; DIM: 0 to 7; n = 137), in the early lactation (EarlyL; DIM: 8–30; n = 139) and in the peak lactation (PeakL; DIM: 31 - 150; n = 235) periods.

All of the included animals were clinically healthy and there was no disease or health issue reported in the previous two weeks according to the American Society for Veterinary Clinical Pathology (ASVCP) guideline (Friedrichs et al. 2012). Since some metabolic indicators were also determined, animals whose plasma concentration of β-hydroxybutyrate, non-esterified fatty acids and plasma enzyme activity of aspartate aminotransferase were out of the reference range (> 1.3 mmol/l; > 0.3 mmol/l one to 21 days prepartum or > 0.6 mmol/l postpartum; and >78 U/l, respectively), were excluded from our study (Radostits et al. 2007; Ospina et al. 2010a; Ospina et al. 2010b). Disorders of energy metabolism (ketosis, extended fat mobilisation, or fatty liver) induce metabolic stress which is one of the major predisposing factors for development of OS (Sordillo and Mavangira 2014).

The animals were housed in a loose housing system and were fed *ad libitum* in a similar manner by total mixed rations (TMR) according to National Research Council (NRC) guidelines (Clark 2001).

The sampling took place 3 to 5 h after the morning feeding. The animals were only restrained for the sampling at the treatment stalls for a maximum of 30 min. Blood samples (10 ml) were taken from the milk vein (vena epigastrica superficialis) into heparinized (sodium heparin) glass tubes, using a single-use 15G × 1 ½" needle. Samples were immediately cooled to 4 °C and transported to the laboratory.

Sample analysis

Sample analysis was performed within 12–24 h after sampling according to the manufacturer's guidelines (H&D 2015).

For measurement of reactive oxygen metabolites (d-ROMs), the blood sample was centrifuged by the instrument with 6000 RPM for 90 s. Then, 10 µl of plasma were mixed with chromogen reagent "R2" (iron solution, N, N-diethyl-paraphenylendiamine) for 10 s and then filled into a provided cuvette in which the transition metal ion formed catalysed the hydroperoxide decomposition, generating new radical species, such as hydroperoxyl and alkoxyl radicals according to which it became possible to quantify the amount of hydroperoxides available in the sample by a photometric reading on 505 nm. Then the mixture was inserted into the reader cell of the instrument. The reading process was undertaken for 300 s, then the dROMs result was displayed.

For testing the plasma antioxidant capacity (PAT) concentration, 40 µl of "R2" were added to the cuvette containing the reagent "R1" (thiocyanate derivate pre-dosed solution). Following calibration, 10 µl of plasma were added and mixed. The reading was taken after 60 s at 505 nm.

The results of the measurements were stored in Microsoft Excel datasheets (Microsoft Corporation, Redmond, USA).

Statistical analysis

The data analysis was performed by using R Statistical Program version 3. 3. 1. (R Core Team, 2018). Normal distribution of data was confirmed by Saphiro-Wilk test (Figs 2–4). Following the descriptive statistics, the variance of means and standard deviations (SD) were compared among groups with ANOVA and Tukey *post hoc* tests.

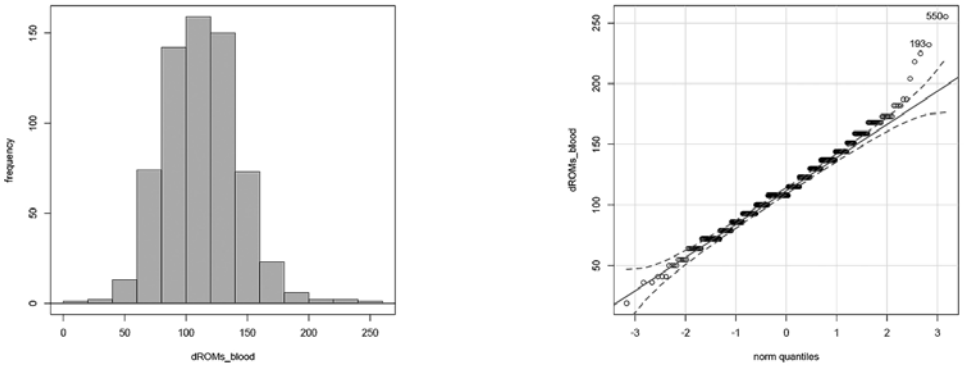

Fig 2. Statistical distributions of reactive oxygen metabolites (d-ROMs) values (Saphiro-Wilk test $P < 0.0001$)

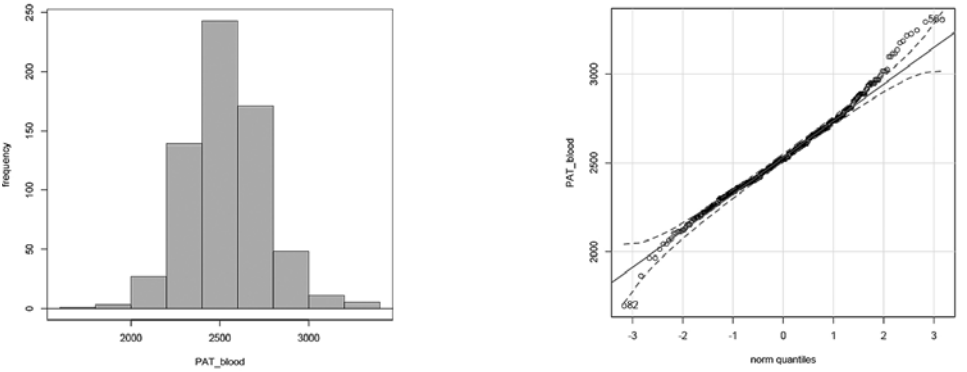

Fig 3. Statistical distributions of plasma antioxidant capacity (PAT) values (Saphiro-Wilk test $P < 0.0001$)

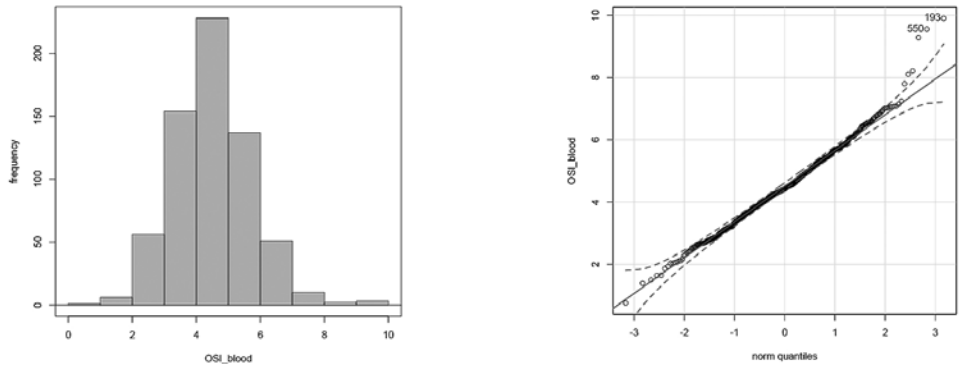

Fig 4. Statistical distributions of the oxidative stress index (OSI) values (Saphiro-Wilk test $P < 0.0001$)

Results

The distribution of dROMs, PAT, and OSI data was tested for normality by Saphiro-Wilk test and showed normal distribution ($P < 0.0001$) (Figs 2–4). The mean ± SD of dROMs, PAT, and OSI values are shown in Table 1. Data were also analysed according to the parity. The values of cows in the first lactation (n = 195) and in the second and further lactations (n = 433) were significantly different ($P < 0.05$) (Tables 2 and 3). Therefore, further comparisons of the groups were performed in both age groups. There was a significant difference ($P < 0.05$) between the dROMs and OSI mean values for each group, whereas the difference in PAT values was not supported by statistical tests in any of the cases (Table 4).

The study period covered all four seasons, so we compared the summer data with the combined data for spring, autumn, and winter (Table 5), but we did not find any significant associations or differences.

Reference intervals for different groups were calculated using the formula mean ± 2SD due to the fact that they showed a normal statistical distribution (Figs 2–4) (PetitClerc and Solberg 1987; Bland 2000; Szabó and Vásárhelyi 2015). The calculated ranges of each observed group are shown in Table 6.

Table 1. The mean and standard deviation (SD) of redox indicators in each group.

Grops/OS markers	PreP (n = 117)	Calv (n = 137)	EarlyL (n = 139)	PeakL (n = 235)
dROMs (U. Carr) mean ± SD	112 ± 23	133 ± 31	114 ± 28	104 ± 28
PAT (U. Cor) mean ± SD	2533 ± 256	2520 ± 205	2550 ± 240	2538 ± 187
OSI mean ± SD	4.44 ± 0.89	5.30 ± 1.23	4.48 ± 1.16	4.10 ± 1.11

PreP - preparturient cows; Calv - cows in calving and maternity; EarlyL - cows in early lactation; PeakL - cows in peak lactation; dROMs - reactive oxygen metabolites; PAT - plasma antioxidant capacity; OSI - oxidative stress index

Table 2. The mean and standard deviation (SD) of redox indicators in different age classes.

OS markers/parity	First lactation (n = 195)	Second+ lactation (n = 433)	*P* value
dROMs (U. Carr) mean ± SD	119 ± 30	112 ± 29	0.0184
PAT (U. Cor) mean ± SD	2539 ± 197	2541 ± 219	0.99
OSI mean ± SD	4.71 ± 1.24	4.42 ± 1.16	0.0132

dROMs - reactive oxygen metabolites; PAT - plasma antioxidant capacity; OSI - oxidative stress index

Table 3. Comparison of differences of the mean values (mean ± standard deviation; SD) of redox indicators in each group.

	PreP 1st (n = 42)	PreP 2nd+ L (n = 75)	Calv 1st L (n = 47)	Calv 2nd+ L (n = 90)	EarlyL 1st L (n = 48)	EarlyL 2nd+ L (n = 91)	PeakL 1st (n = 58)	PeakL 2nd+ L (n = 177)
dROMs mean ± SD (U. Carr)	114 (22)	112 (23)	141 (25)	129 (33)	121 (31)	109 (27)	103 (29)	104 (27)
P value	0.844		0.0357		0.0358		0.768	
PAT mean ± SD (U. Cor)	2514 (202)	2554 (275)	2523 (194)	2522 (214)	2542 (229)	2567 (243)	2549 (188)	2531 (184)
P value	0.869		0.983		0.581		0.536	
OSI mean ± SD	4.57 (0.88)	4.43 (0.90)	5.58 (0.94)	5.14 (1.31)	4.79 (1.29)	4.28 (1.08)	4.05 (1.21)	4.10 (1.06)
P value	0.756		0.0414		0.0232		0.764	

- preparturient cows; Calv - cows in calving and maternity; EarlyL - cows in early lactation; PeakL - cows in peak lactation; L - lactation; dROMs - reactive oxygen metabolites; PAT - plasma antioxidant capacity; OSI - oxidative stress index

Table 4. Differences in the values of redox indicators between the groups.

	P value					
	dROM		PAT		OSI	
	1st L	2nd+ L	1st L	2nd+ L	1st L	2nd+ L
PreP vs Calv	< 0.01	< 0.001	0.979	0.794	< 0.001	< 0.001
PreP vs EarlyL	0.74561	0.908	1.000	0.984	0.72477	0.845
PreP vs PeakL	0.13320	0.112	0.997	0.878	0.15873	0.136
Calv vs EarlyL	0.00264	< 0.001	0.965	0.574	0.00334	< 0.001
Calv vs PeakL	< 0.001	< 0.001	0.913	0.989	< 0.001	< 0.001
EarlyL vs PeakL	0.00424	0.489	0.998	0.654	0.00517	0.655

PreP - preparturient cows; Calv - cows in calving and maternity; EarlyL - cows in early lactation; PeakL - cows in peak lactation; L - lactation; dROMs - reactive oxygen metabolites; PAT - plasma antioxidant capacity; OSI - oxidative stress index

Table 6. Estimated reference intervals of the values of redox indicators (mean ± standard deviation; SD).

	PreP (n = 117)	Calv 1st L (n = 47)	Calv 2nd+ L (n = 90)	EarlyL 1st L (n = 48)	EarlyL 2nd+ L (n = 91)	PeakL (n = 235)
dROMs (mean ± SD; U. Carr)	112 (23)	141 (25)	129 (33)	121 (31)	109 (27)	104 (28)
Reference interval	68–158	91–191	63–195	59–183	55–163	48–160
OSI (mean ± SD)	4.44 (0.89)	5.58 (0.94)	5.14 (1.31)	4.79 (1.29)	4.28 (1.08)	4.10 (1.11)
Reference interval	2.64–6.22	3.70–7.46	2.52–7.76	2.21–7.37	2.12–6.44	1.88–6.32
PAT (mean ± SD; U. Cor)	2533 (256)	2523 (194)	2522 214)	2542 (229)	2567 (243)	2538 (187)
Reference interval	2021– 3045	2135– 2911	2094–2950	2084–3000	2081–3053	2164– 2912

PreP - preparturient cows; Calv - cows in calving and maternity; EarlyL - cows in early lactation; PeakL - cows in peak lactation; L - lactation; dROMs - reactive oxygen metabolites; PAT - plasma antioxidant capacity; OSI - oxidative stress index

Table 5. Comparison of the values of redox indicators (mean ± standard deviation; SD) measured in the summer and combined spring-autumn-winter periods.

	PreP		Calv		EarlyL		PeakL	
	1st L	2nd+ L	1st L	2nd+ L	1st L	2nd+ L	1st L	2nd+ L
				dROMs				
Summer (mean ± SD)	112 (21) n = 16	126 (19) n = 18	153 (16) n= 6	135 (33) n = 19	131 (24) n = 17	116 (29) n = 41	101 (29) n = 33	103 (27) n = 105
Spring -autumn-winter (mean ± SD)	116 (22) n = 26	110 (24) n = 57	139 (25) n = 41	128 (31) n = 71	115 (32) n = 31	102 (22) n = 50	105 (30) n = 26	105 (28) n = 72
				PAT				
Summer (mean ± SD)	2541 (130)	2498 (137)	2537 (192)	2492 (162)	2429 (125)	2490 (154)	2502 (167)	2507 (153)
Spring -autumn-winter (mean ± SD)	2497 (237)	2492 (235)	2521 (196)	2497 (192)	2594 (253)	2501 (203)	2604 (198)	2537 (192)
				OSI				
Summer (mean ± SD)	4.41 (0.88)	5.04 (0.82)	6.03 (0.51)	5.43 (1.30)	5.43 (1.12)	4.66 (1.21)	4.07 (1.19)	4.11 (1.07)
Spring -autumn-winter (mean ± SD)	4.67 (0.89)	4.41 (0.88)	5.51 (0.97)	5.12 (1.23)	4.43 (1.24)	4.12 (0.97)	4.08 (1.25)	4.16 (1.07)

PreP - preparturient cows; Calv - cows in calving and maternity; EarlyL - cows in early lactation; PeakL - cows in peak lactation; L - lactation; dROMs - reactive oxygen metabolites; PAT - plasma antioxidant capacity; OSI - oxidative stress index; n = number of cows

Discussion

The periparturient period is very challengeful for dairy cows and as a result, most of the metabolic disorders and also infectious diseases may occur within the first few weeks of lactation (Goff and Horst 1997). Strong evidence has been reported on the role that OS plays in the initiation, progression, and maintenance of these pathological cases (Abuelo et al. 2013).

Once the redox-homeostasis of the body is disturbed, malicious cell-damaging processes may develop and jeopardize the general health and productivity (Abuelo et al. 2015). This challenge frequently occurs in the early lactation in dairy cows, when the energy balance is negative (Sordillo and Aitken 2009). For compensation, the energy-producing metabolism is becoming more intensive, finally resulting in higher ROS formation. However, based on the results, this does not seem to be the riskiest period in terms of OS. Even though slight elevation of dROMs was detected (121 ± 31 and 109 ± 27 U. Carr) in the first lactation and multiparous early lactation cow groups, respectively, they did not differ significantly ($P > 0.05$) from the data of the preparturient period (114 ± 22 and 112 ± 23 U. Carr) in the first lactation and multiparous cows, respectively (Table 1). The dROMs data recorded in fresh cow groups were significantly different ($P < 0.01$) from data in the calving and maternity period, and from data in the peak yielding cows ($P < 0.01$).

We found the period near around calving to be the most endangered by OS, when the

highest dROMs and OSI values were recorded at this time in both primi- and multiparous cows (dROMs: 141 ± 25 and 129 ± 33 U. Carr, respectively; OSI: 5.58 ± 0.94 and 5.14 ± 1.31, respectively). These results are similar to Píšťková et al. (2019) who also found very apparent signs of OS in 2–3 days postpartum in dairy cows. In this period, they recorded a significant decrease in the total antioxidant status, vitamin A and E concentration and β-carotene, while the GPx and SOD activity were increased. The pattern of dROMs values in the near to calving period we found are also similar to others' findings (Abuelo et al. 2013) who reported 153 U. Carr in the group until 1 month after calving and also detected a decline in the peak lactation stage (145.1 U. Carr). However, they found the highest dROMs levels in the prepartum (-1 month to calving) period, whereas we found low values at that stage. Their results are generally higher than ours in all observed lactation stages. They studied fewer (n = 22) healthy cows, but the main difference was in the methodology, in that they measured dROMs from serum, whereas we did that from heparinized plasma which may explain the difference.

The elevated ROS values are clearly associated with calving and they can trigger the development of OS in cows (Castillo et al. 2005; Gaál et al. 2006; Albera and Kankofer 2011). Increased activity of mitochondria is detectable in human females during pregnancy being linked with increased metabolism, which may lead to ROS overproduction (Wisdom et al. 1991; Albera and Kankofer 2011). This process may be similar in other species as well. It has been reported that BCS correlates with these indicators and may influence the level of oxidative processes in cows during the periparturient period (Mikulková et al. 2020). This finding supports the assumption that peripartum energy disbalance may predispose to the development of OS. The physical stress related to the delivery of foetus may contribute to metabolic reactions resulting in overproduction of ROS (Miller et al. 1993; Albera and Kankofer 2011). The lipid-peroxidation is growing more intensive around parturition in the cows (Castillo et al. 2005), and the start of respiration may result in elevated ROS formation in new born calves (Covarrubias et al. 2008; Halliwell and Gutteridge 2015).

The intensive development of the mammary gland and the onset of colostrum and milk secretion may overwhelm the female body. The cow uses a remarkable amount of AOs for colostrum production (Goff and Horst 1997). As a consequence, the decreased concentration of major AO such as vitamins A and E has also been observed at parturition (Goff and Stabel 1990). Consequently, the AO defence capacity may be lower than ROS production.

All of these actions may predispose the body for development of OS in the periparturient period and may explain why the highest dROMs and OSI values were detectable in the first few days of lactation.

The lowest dROMs (103 ± 29 and 104 ± 27 U. Carr in first-parity and multiparous cows, respectively) and OSI values (4.05 ± 1.21 and 4.10 ± 1.06 in first-parity and multiparous cows, respectively) were in the peak production stage of the lactation. Our finding is in accordance with earlier results, and the metabolic status seems to be more stable as manifested in a better AO status when the animal reaches peak lactation (Castillo et al. 2006; Konvičná et al. 2015).

Heat stress may elevate ROS production (Sordillo and Raphael 2013). We did not find any significantly consistent trend when comparing the data originated from the summer and other seasons (Table 5). However, our current study was not designed to investigate the effects of heat stress on OS.

Since statistically detectable differences in dROMs and OSI values were found for each lactation period during comparison of calving and maternity periods with others, it was considered appropriate to define reference ranges accordingly. This is to draw attention to the fact that when interpreting the measured values, attention must be paid to the physiological state and lactation.

The PAT biomarker did not show any significant difference among the observed lactation stages in our study. We did not research the question why PAT did not show a reasonable difference in the observed groups; however, it is an exciting and motivating point for further research.

Based on the mean values of significant differences shown in the dROMs and OSI values we established the reference intervals for these indices (Table 6). Although there are publications available directed to determine the reference ranges of biochemical indices, in which the requirement for inclusion of animals was being clinically healthy (Lumsden et al. 1980; Kusano et al. 2016), since we had the opportunity to investigate some relevant indicators of energy metabolism (BHB, NEFA, and AST) from the same samples, we decided to exclude from the study those animals whose values were out of the normal range because metabolic stress is one of the major predisposing factors in development OS.

We calculated the reference intervals for all groups that showed significant differences, hence our data might be overdetailed from a clinical point of view, however, it is reasonable to show the details from scientific aspects.

Acknowledgements

The project was supported by the European Union and co-financed by the European Social Fund (grant agreement number: EFOP-3.6.2-16-2017-00012, project title: Development of a Product Chain Model for Functional, Healthy and Safe Foods from Farm to Fork Based on a Thematic Research Network; and grant agreement number: EFOP-3.6.1-16-2016-00024, project title: Institutional Developments for Intelligent Specialization in co-operation of the University of Veterinary Medicine and the Faculty of Agricultural and Food Sciences, Széchenyi István University).

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Fig 1. Locations of involved dairy farms