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Oral supplementation with organically modified clinoptilolite during prepartum period influences the redox status of peripheral blood and colostrum of primiparous dairy cows

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ABSTRACT

Redox imbalance in peripartum period influences health of dairy cows and their foetus and, through the colostrum, health of new-born calves. Oxidative stress in cattle can be suppressed by dietary supplementation with natural minerals, and we investigated the effect of supplementation with organically modified clinoptilolite on redox status parameters in healthy pregnant primiparous dairy cows. Holstein cows were randomly assigned to receive daily oral drenching, with 1 L of water containing either 0 g/L ($n=14$; control group) or 150 g/L of clinoptilolite ($n=17$; supplemented group). Treatment lasted from 24 ± 4 days prior to parturition until 2 days postpartum (pp). Blood samples were collected on days 24 ± 4 (-24 D) and 4 ± 2 (-4 D) prior to parturition and on days 1 ($+1$ D), 2 ($+2$ D), and 7 ($+7$ D) pp, and colostrum were collected at 2, 12, 24 and 36 h pp. Total antioxidant capacity, lipid peroxides, and advanced oxidation protein products (AOPP) levels were determined in peripheral blood plasma, erythrocytes, and colostrum whey. The concentration of antioxidants in the peripheral blood of supplemented cows was increased by 41% and 19% on ($+2$ D) and ($+7$ D), respectively, while the concentration of lipid peroxides on ($+7$ D) was lowered by 57% compared with the control group. In addition, this supplementation increased erythrocyte AOPP level on (-4 D) 61% and colostrum lipid peroxides level (90%) at 24 h pp. The results of this study showed that applied short-term supplementation with clinoptilolite influences redox homeostasis and may contribute to effective adaptation of primiparous cows to redox imbalance in the peripartum period.

HIGHLIGHTS

- Short-term dietary supplementation with clinoptilolite in the prepartum period modulates redox homeostasis of the dairy cows' blood plasma.
- Short-term dietary supplementation with clinoptilolite contributes to adaptation of dairy cows to redox imbalance in the peripartum period.
- Short-term dietary supplementation with clinoptilolite increase the level of lipid peroxides in colostrum of dairy cows.

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Introduction

Various environmental, physiological, and dietary conditions can contribute to the exacerbation of oxidative stress in high-producing dairy cows, already intrinsically susceptible to oxidative stress (Vázquez-Añón et al. 2008). Among known physiological factors, the periparturient or transition period (defined

as the period from 3 weeks before calving until 3 weeks after calving) is considered critical for dairy cows' health (Gitto et al. 2002, Sordillo and Aitken, 2009; Sharma et al. 2011; Abuelo et al. 2019). In this period, dairy cows are more prone to infection and metabolic diseases (mastitis, metritis, ketosis, digestive disorders, displaced abomasum, lameness) than in the period of peak or late lactation (Sordillo et al.

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 Supplemental data for this article can be accessed [here](#).

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2007; Sordillo and Aitken, 2009), due to impaired redox balance, or the so-called 'Oxygen Paradox' (Gitto et al. 2002; Sordillo and Aitken, 2009; Sharma et al. 2011; Abuelo et al. 2019). Namely, during this transition phase cows have substantial energy requirements due to foetal growth, rapid differentiation of secretory parenchyma and mammary gland growth, and colostrum and milk synthesis and secretion (Gitto et al. 2002). To meet the energy demands, cows mobilise reserves predominately from adipose tissues, and the increased lipid mobilisation increases the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Gitto et al. 2002). ROS and RNS are essential signalling molecules involved in the regulation of normal, physiological, immune response. However, an imbalance in their productions might change antioxidative capacity and allow pro-oxidative mediators to induce an inflammatory reaction, tissue damage, and immune dysfunctions responsible for numerous diseases occurring within 1 month after calving (Abuelo et al. 2019).

The calves experiencing oxidative stress *in utero* are shown to have compromised immune response and higher level of basal inflammation (Abuelo et al. 2019). Calves also produce ROS intrinsically, and even before the ingestion of colostrum, their serum ROS level is higher than in their dams (birth-associated oxidative stress phenomenon; Gaál et al, 2006; Abuelo et al. 2014). The colostrum contains numerous enzymatic and non-enzymatic antioxidants (Przybylska et al. 2007), and the colostrum antioxidant capacity, lipid peroxidation, and protein SH-group level increase in time (Kankofer and Lipko-Przybylska, 2008; Albera and Kankofer, 2011). The colostrum antioxidant activity correlated negatively with ROS level, but a positive correlation is found with serum IgG in new-born calves 2h after colostrum ingestion (Abuelo et al. 2014). Besides antioxidants, colostrum also contains prooxidants. Namely, colostrum ROS is an essential component of anti-bacterial defence in the intestine of calves (Przybylska et al. 2007). However, in some pathological conditions, a high ROS concentration (ROS originating from colostrum as well as intrinsic calves' ROS) outbalance calves antioxidant capacity leading to oxidative stress, immune dysfunctions, and onset of diarrhoea and pneumonia (Abuelo 2019).

According to EU Regulation No 651/2013 (2013) natural zeolite, clinoptilolite has been authorised for the use as an additive in feedstuffs for farm animals. Clinoptilolite improves production performance, acting as ammonia-, heavy metal-, and mycotoxin-

binding adsorbent, as an antioxidant, and anti-diarrheic and growth-promoting agent (Papaioannou et al. 2004; Katsoulos et al. 2006; Daković et al. 2007; Safameher, 2008; Pourliotis et al. 2012; Karatzia et al. 2013; Wu et al. 2015). There are also data indicating its role in the prevention of metabolic diseases in dairy cows (milk fever, ketosis) and positive effects on general health (Papaioannou et al. 2005; Katsoulos et al. 2006) and milk production (Karatzia et al. 2013). Although zeolites might act as antioxidants, limited and contradictory data are available on the effect of zeolites on the redox status in calves. Yarovan (2008) measured the concentration of malondialdehyde (MDA), conjugated dienes, keto dienes and ceruloplasmin (CP) in the plasma of dairy cows and showed that natural zeolite (Khotynets) has a positive effect on the oxidant status. However, Ipek et al. (2012) showed that although supplementation with natural zeolite (Nat Min 9000[®]) in the period from 2 to 4 months after calving decreased the lipid hydroperoxides concentration, it did not further strengthen the antioxidant defence system in healthy dairy cows. Besides, Kerwin et al. (2019) showed that 3-week-long prepartum supplementation of cows in the second and greater lactation with synthetic zeolite A (X-Zelit) did not modulate the concentration of reactive oxygen and nitrogen species and antioxidant capacity of peripheral blood plasma. Although all these studies showed that zeolite/clinoptilolite influence cows' redox status, the results are difficult to compare directly because of differences in (1) zeolite/clinoptilolite forms and concentrations, (2) age of cows (3) timing and duration of treatment, (4) analysed biomarkers, and (5) applied analytical methods.

We have already shown that supplementation with organically modified clinoptilolite (patent of Milosevic and Tomasevic-Canovic 2003, 2009) improved the quality of colostrum of primiparous dairy cows by increasing the percentage of fat and proteins and concentration and mass of IgG, with no adverse effects on the cows' energy status, protein, lipid, and mineral metabolism (Stojić et al. 2020). Since the colostrum redox balance affects passive immune transfer, Abuelo et al. (2014) proposed an examination of the oxidative profile of colostrum as a parameter of colostrum quality.

This study assessed whether the supplementation affects the redox status of primiparous cows' peripheral blood and colostrum by measuring total antioxidant capacity, lipid peroxidation levels, and advanced oxidation protein products.

Material and methods

Experimental design

The total number of 31 healthy pregnant Holstein primiparous dairy cows, grown at a tie stall barn farm (Padinska Skela, AL Dahra, Belgrade, Serbia), were randomly selected 30 days before the expected calving term and placed in a separate facility in which they were kept under the same hygienic and dietetic conditions. According to the National Regulation on Animal Welfare, the Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade, approved the study on animals (the ethical approval code number: 01-19/10). Investigations on cows were enrolled at the same season, from September to November.

On the farm where the study was performed cows were artificially synchronised and inseminated. Deworming program was performed at least 60 days before calving, and vaccination against clostridial diseases with Bravoxin (MSD Animal Health, EU) was also performed. All cows were, on regular basis, underwent a general clinical examination (determination of body temperature, pulse rate, respiration, and rumen contraction) by an authorised veterinarian on the farm. Before the start of the experiment, 25 days before the expected calving term, cows included in the study were randomly selected from the farm 'pool' of healthy primiparous cows, and then placed in a separate facility where they kept under the same hygienic and dietetic conditions. These cows were in good general health condition without any obvious clinical signs of disease, in optimal body condition score, and aged 23 ± 2 months. During the experiment it was performed regular clinical examination and taking the blood samples for determination the parameters of metabolic profile. The results are given as [Supplementary Material Table S11](#). Routine mastitis diagnostics was completed following calving and cows included in this study had no mastitis.

Cows received identical diets as TMR to allow *ad libitum* feed intake throughout the study. Twenty-five days before the expected time of parturition, cows were assigned to the close-up diet ($NE_L = 1.60$ Mcal/kg; [Table S1](#); [Supplementary material](#)). The lactation diet ($NE_L = 1.71$ Mcal/kg; [Table S1](#)) was provided for all animals postpartum until day 30 of lactation. All offered diets were initially formulated to either meet or exceed the NRC (2001) requirements. The feed was offered in two equal portions at 7 and 18 h, and cows had free access to water throughout the study. All animals were randomly assigned to two experimental groups: (1) treated group, T; ($n = 17$) received oral

supplementation of organically modified clinoptilolite (Minazel Plus[®], Patent Co., Serbia; Milosevic and Tomasevic-Canovic [2003](#), [2009](#)) 150 g/animal/day in 1 L of drinking water, starting at 24 ± 4 days before the expected calving term up to 2 days after calving, and (2) control group, C; ($n = 14$) received, instead of the clinoptilolite suspension, only 1 L water. Clinoptilolite suspension was administered by a glass bottle and given orally in one portion, 2 h after the morning meal.

Blood samples collection and isolation of blood plasma and erythrocytes

Blood samples were collected on days 24 ± 4 (-24 D) and 4 ± 2 (-4 D) before calving, and on days 1 ($+1$ D), 2 ($+2$ D), and 7 ($+7$ D) postpartum (pp) by jugular venepuncture into sterile, plastic, vacutainer tubes with sodium citrate as an anticoagulant. The first blood sample (-24 D) was taken before adding the supplement. Blood components were separated by centrifugation at $2450 \times g$ for 25 min within 2 h of blood collection. Plasma was collected, aliquoted, and stored at -20°C until the use. Leucocytes were discarded by vacuum aspiration, and the remaining pelleted erythrocytes were resuspended in isotonic (0.9% w/v) saline solution, three times washed via centrifugation, aliquoted, and stored at -20°C until the use.

Collection of colostrum and isolation of colostrum whey

Colostrum was collected 2–3, 12, 24 and 36 h pp and labelled as (2 h), (12 h), (24 h), and (36 h) representing 1st, 2nd, 3rd, and 4th milking, respectively. Colostrum samples (200 mL) were immediately frozen and stored at -20°C until use. Just before the analysis the colostrum samples were thawed in a warm water bath (37°C), brought up to room temperature (20°C), centrifuged ($800 \times g$, 20 min), and the fatty layer (formed as a result the freezing induced partial deemulsification of colostrum) was removed by vacuum aspiration. Casein was precipitated by adding seven drops (approximately 175 μL) of calf rennet to 10 ml of the supernatant. After the 30 min incubation at 37°C , samples were centrifuged (15 min, $3000 \times g$), and supernatant (colostrum whey) was aliquoted and stored at -20°C until the use for biochemical analysis.

Analysis of colostrum whey, blood plasma and erythrocyte lysates

Colostrum whey and blood plasma total protein concentration was determined by the biuret method (Dumas et al. 1981). Colostrum whey proteins were separated by agarose gel electrophoresis and the γ globulin level was determined as described in Stojić et al. (2017). The cyanmethemoglobin method (Zwart et al. 1996) was used to determine haemoglobin concentration in erythrocyte lysates after thawing.

The total antioxidant capacity of colostrum whey, blood plasma, and erythrocyte was measured by ferric reducing antioxidant power (FRAP) colorimetric assay (Benzie and Strain, 1999). The assay was performed with undiluted blood plasma and colostrum whey and 48 times diluted (in 0.9% NaCl) erythrocyte lysates.

Lipid peroxidation in blood plasma, erythrocytes, and colostrum whey was estimated by thiobarbituric acid reactive species (TBARS) assay (Ohkawa et al. 1979). This assay is based on the reaction of malondialdehyde (MDA), as a lipid peroxidation marker, with thiobarbituric acid (TBA). TBA reacts with MDA to form a pink chromogen, which can be detected spectrophotometrically at 530 nm. The modification was made in sample/TCA volume ratio. For plasma: the sample in a volume of 300 μ L was mixed with 100 μ L 28% TCA. For colostrum sera: the sample in a volume of 100 μ L was mixed with 1000 μ L 28% TCA. For erythrocyte: erythrocyte lysates were 48 times diluted in 0.9% NaCl, and 100 μ L of the diluted sample was mixed with 500 μ L 28% TCA. The absorption of TBARS was measured at 530 nm (OD_{530}). A standard curve was constructed by plotting the MDA standards against OD_{530} . MDA standard was prepared by the acid hydrolysis of 1,1,3,3-tetramethoxypropane (malondialdehyde bis(dimethyl acetal)), purchased from Sigma Aldrich.

Oxidative damage of proteins was determined by measuring advanced oxidation protein products (AOPP) in an acidic condition in the presence of potassium iodide, following the method of Selmeçli et al. (2005), with minor modifications. Briefly, 20 μ L sample was transferred (in duplicate) to wells in a 96-well microplate, followed by the addition of 180 μ L 0.2 M citric acid, and potassium iodide (10 μ L 1.19 M). After 10 min on a microplate shaker, the absorbance of AOPP was read at 340 nm. The calibration curve was constructed with chloramine - T.

The values of examined parameters in erythrocytes were normalised to the haemoglobin content (Figure S1, Supplementary Material).

Statistical analysis

Statistical analysis of the results obtained in the experiment was carried out using statistical software GraphPad Prism version 6 (GraphPad, San Diego, CA, USA). Untransformed data are presented in Figures and Supplementary material's Tables. Given that some data were not normally distributed (Shapiro–Wilk normality test $p < 0.05$), several transformations of data have been attempted until adequate transformation was found. For the data of colostrum whey following transformations were applied: $y = x + 2$ for FRAP, TBARS, and AOPP/proteins; $y = x + 50$ for AAOP and Proteins; In blood plasma: $y = x + 2$ for TBARS and AOPP/proteins, and $y = x + 50$ for AOPP; In RBC: $y = x + 2$ for TBARS/gHb and AOPP/gHb. After transformations all data were normally distributed (Shapiro–Wilk normality test $p > 0.05$). Groups were compared using two-way ANOVA with repeated measures in one factor (time of sampling) followed by Tukey's test within groups over sampling time and Sidak's test between groups through each sampling time.

Correlation between analysed redox status parameters was tested, and Pearson product–moment correlation coefficients (r) were calculated using the above-mentioned software. Differences with p -values of < 0.05 were considered significant.

Results

Effect of the oral supplementation with organically modified clinoptilolite on redox status parameters of peripheral blood plasma and erythrocytes of primiparous dairy cows

Time-course changes in the blood plasma total antioxidant capacity, lipid peroxides, and AOPP in control and the clinoptilolite supplemented dairy cows, from (–24 D) to (+7 D) is shown in Figure 1 and Tables S2 and S3 (Supplementary Material).

The concentration of total antioxidant capacity detected by FRAP method in the blood plasma of control animals fluctuated, reaching their maximum and minimum levels on (+1 D) and (+2 D), respectively. In the blood plasma of cows supplemented with clinoptilolite, total antioxidant capacity gradually increased until the (+2 D) and then remained unchanged until the end of the experiment. A significant increase in the total antioxidant capacity in blood plasma of supplemented cows, compared to healthy ones, was detected on (+2 D) and (+7 D) (41% and 19%, respectively).

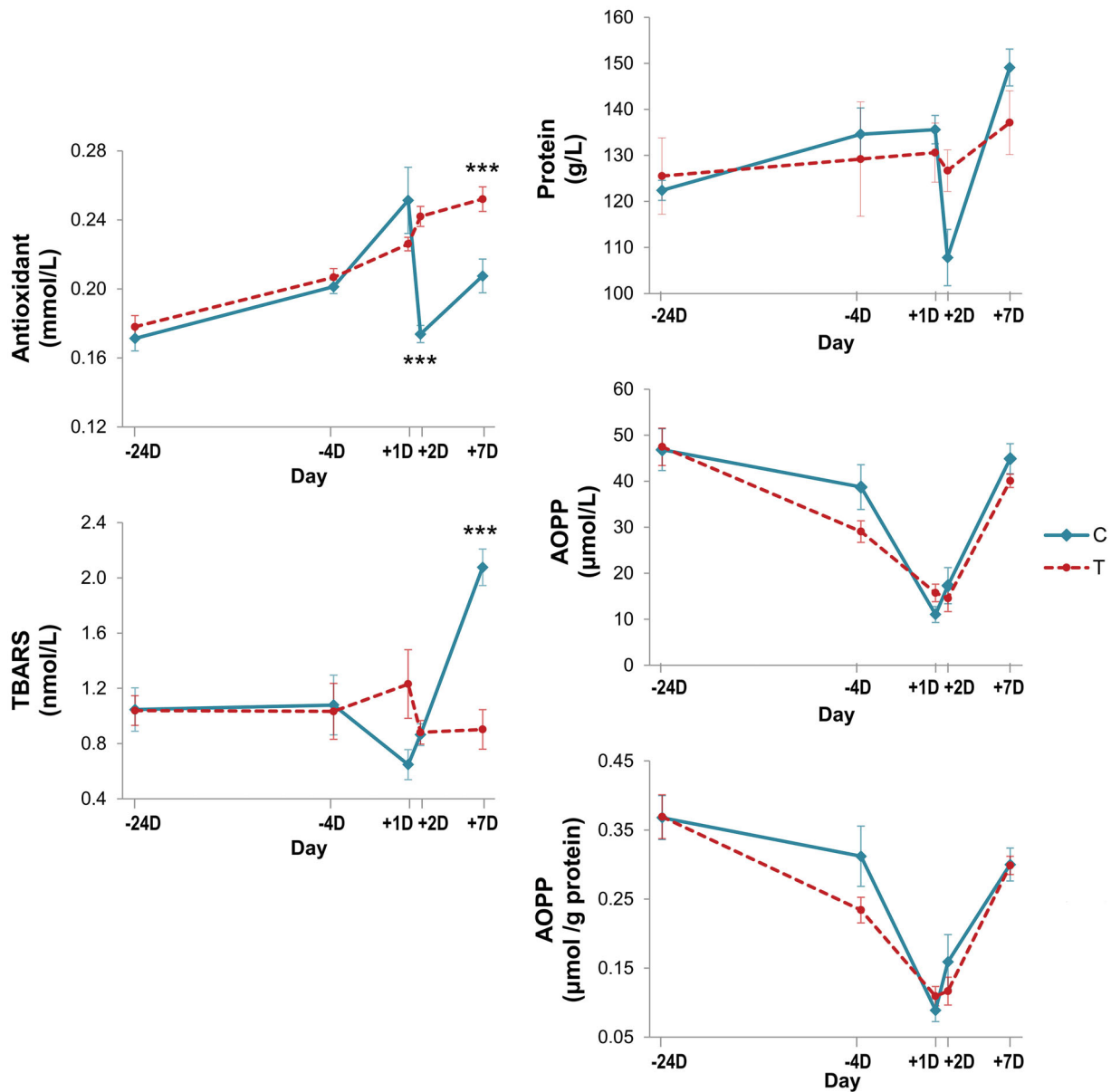


Figure 1. Effect of the oral supplementation with organically modified clinoptilolite during prepartum period on the level of redox status parameters of peripheral blood plasma of primiparous dairy cows. C – Control cows (solid line); T – Clinoptilolite supplemented cows (dashed line); (-24 D) and (-4 D) – days 24 ± 4 and 4 ± 2 prior to parturition; (+1 D), (+2 D), (+7 D) – days 1, 2, and 7 postpartum. The concentration of antioxidants expressed as mM equivalent to $\text{FeSO}_4 \times 7\text{H}_2\text{O}$; TBARS: thiobarbituric acid reactive species; the concentration of lipid peroxides expressed as nM equivalent to malondialdehyde (MDA); AOPP: advanced oxidation protein products; (***) – $p < 0.001$.

In the blood plasma of control group of cows, the TBARS concentration remained unchanged from (-24D) to (+2D), but increased on (+7D). In the supplemented group, the TBARS level was unchanged during the experimental period. The significant difference in blood plasma TBARS level between groups was detected on (+7D), when the TBARS level in the treated group was 57% lower than in the control group.

The concentration of proteins in the blood plasma of control cows varied over time. It stayed unchanged from (-24D) to (+2D), after which it grew until (+7D). The concentration of proteins in the blood plasma of the clinoptilolite supplemented cows remained unchanged throughout the experimental period. The statistical analysis did not reveal a significant effect of this supplementation on the concentration of blood plasma protein.

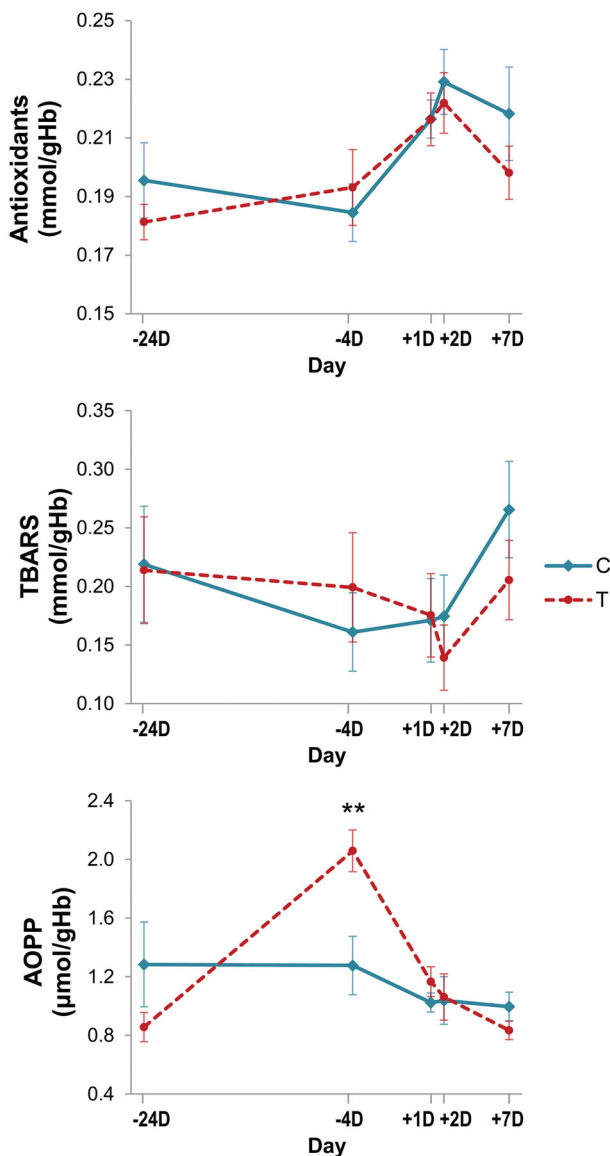


Figure 2. Effect of the oral supplementation with organically modified clinoptilolite during prepartum period on the level of redox status parameters of erythrocytes of primiparous dairy cows. C – Control cows (solid line); T – clinoptilolite supplemented cows (dashed line); (–24 D) and (–4 D) – days 24 ± 4 and 4 ± 2 prior to parturition; (+1 D), (+2 D), (+7 D) – days 1, 2, and 7 postpartum. The concentration of antioxidants expressed as mM equivalent to $\text{FeSO}_4 \times 7\text{H}_2\text{O}$; TBARS: thio-barbituric acid reactive species; the concentration of lipid peroxides expressed as nM equivalent to malondialdehyde (MDA); AOPP: advanced oxidation protein products; (***) – $p < 0.01$.

The AOPP level (expressed either as $\mu\text{mol/L}$ or $\mu\text{mol/g}$ protein) in blood plasma of both, control and supplemented group of cows was constantly decreasing from (–24D) to (+1D), stayed unchanged from (+1D) to (+2D), and increased on (+7D). A significant difference in the AOPP level between control and supplemented groups was not found.

The total antioxidant capacity, lipid peroxides and AOPP in erythrocyte of clinoptilolite treated and control cows in the period from (–24D) to (+7D) was shown in Figure 2 and Tables S4 and S5 (Supplementary Material).

The total antioxidant capacity in the erythrocyte lysates of the control group did not change from (–24D) to (–4D), increased on (+1D), stayed unchanged till (+2D), and then decreased on (+7D). In the erythrocytes of clinoptilolite supplemented cows, the increase in the antioxidant level was already detected on (–4D) and the upward trend continued until (+2D). As in the control group, in the treated group a decrease in the total antioxidant capacity was registered on (+7D). A significant difference in the erythrocytes' antioxidants status of the control and the supplemented group was not found.

The TBARS level stayed unchanged during the experimental period in both clinoptilolite supplemented and control group. Also, TBARS assay did not reveal any difference in the erythrocytes' lipid peroxides level between the groups.

In the control group, the erythrocyte AOPP level stayed unchanged during the examination period. In the clinoptilolite supplemented group the erythrocyte AOPP level increased on (–4D), and at this point it was 61% higher than in the control group.

A significant correlation between total antioxidant capacity and lipid peroxides or AOPP level in peripheral blood plasma or erythrocytes was not found in any group of cows (Tables S6, Supplementary Material).

Effect of the supplementation with organically modified clinoptilolite on redox status parameters of colostrum whey of primiparous dairy cows

First milking colostrum (2 h) of control groups of cows contained 129 ± 30 g/L IgG and the colostrum of supplemented group contained 152 ± 33 g/L IgG (Figure S2, Supplementary Material). Both groups of cows produced high quality colostrum (>50 g/L IgG; McGuirk and Collins 2004) and the effect of difference in the colostrum quality on results of our study was not expected. Redox status indices were analysed in the colostrum whey (Figure S2, Supplementary Material) whose concentration of γ globulin (predominately IgG) almost perfectly correlated with the concentration IgG in whole colostrum IgG (Table S7, Supplementary Material).

The results of the analysis of the effect of the clinoptilolite oral supplementation on redox status

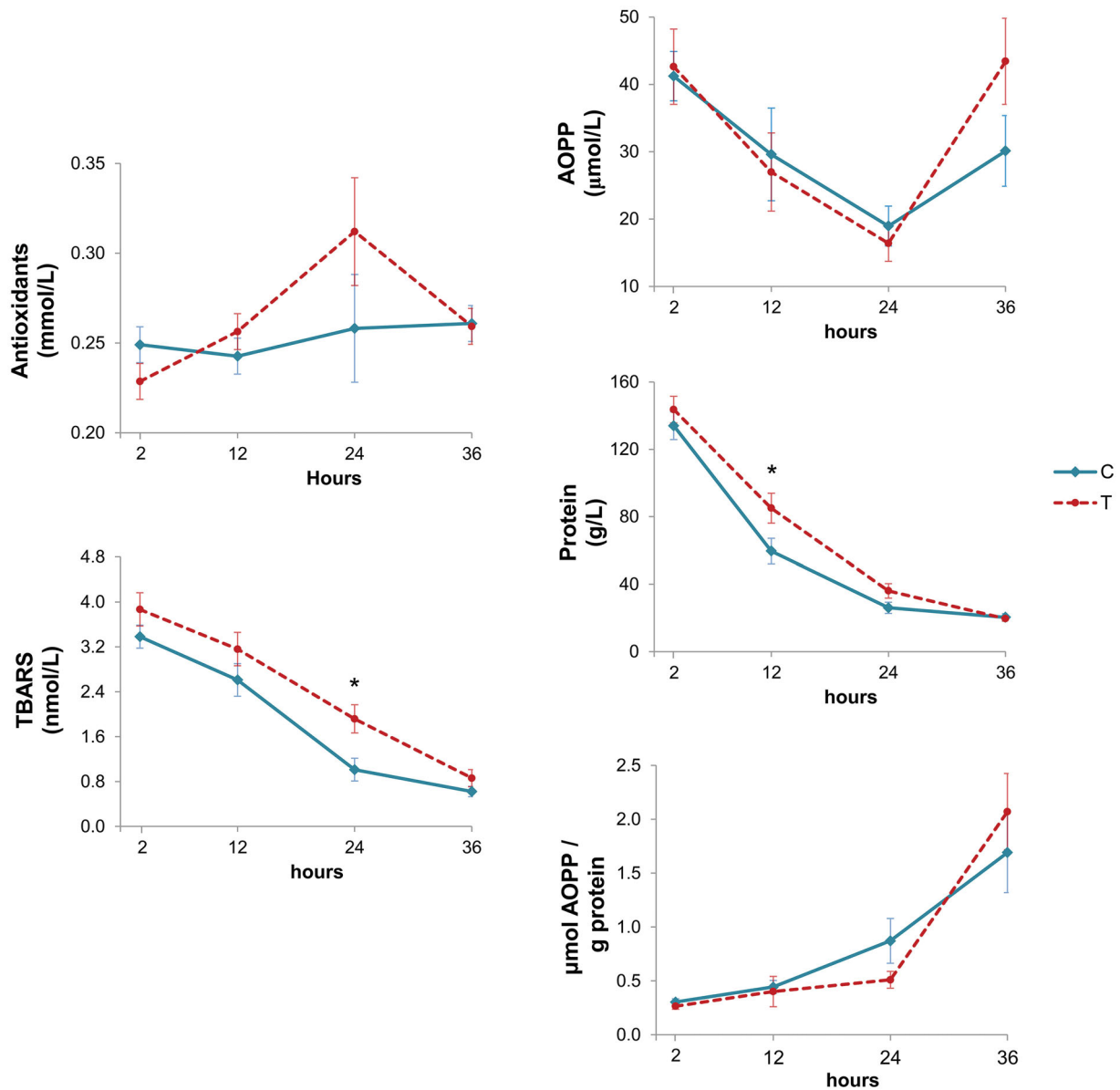


Figure 3. Effect of the oral supplementation with organically modified clinoptilolite during prepartum period on the level of redox status parameters of colostrum whey of primiparous dairy cows. C – Control cows (solid line); T – clinoptilolite supplemented cows (dashed line); Colostrum was collected 2–3, 12, 24 and 36 h pp and labelled as (2 h), (12 h), (24 h), and (36 h). The concentration of antioxidants expressed as mM equivalent to $\text{FeSO}_4 \times 7\text{H}_2\text{O}$; TBARS: thiobarbituric acid reactive species; The concentration of lipid peroxides expressed as nM equivalent to malondialdehyde (MDA); AOPP: advanced oxidation protein products; (*) – $p < 0.05$.

parameters of colostrum whey of dairy cows are given in Figure 3, and Tables S8 and S9 (Supplementary Material).

The results show that colostrum sera of the control cows revealed stable total antioxidant capacity during the experimental period. In colostrum sera of clinoptilolite supplemented cows, total antioxidant capacity increased significantly from second milking (12 h) to third milking (24 h) and then decreased reaching at fourth milking (36 h) the same concentration as in the

second milking. A difference in the colostrum whey total antioxidant capacity, between control and treated cows was not significant.

In both groups of cows, the TBARS level (nmol/L) was the highest at first milking and continuously decreased up to fourth milking. In addition, it was found that the (24 h) colostrum of supplemented cows contained significantly more (90%) lipid peroxides than the control colostrum. The decrease in the colostrum whey TBARS level followed the trend of

decreasing in the colostrum fat content (Figure S3, Supplementary Material), and to avoid a misinterpretation of TBARS level measurement, TBARS to colostrum lipid ratio was calculated. The data also showed that colostrum of supplemented cows contained significantly more lipid peroxides than the control colostrum (65%, as expressed as $\mu\text{mol TBARS}/\% \text{ fat}$).

The concentration of proteins in colostrum of both groups of cows decreased from first to fourth milking, but the protein concentration in the second colostrum of supplemented cows was 43% higher than in control cows. In the colostrum of control cows, the concentration of AOPP ($\mu\text{mol/L}$) decreased from first (2 h) to second (24 h) milking and stayed unchanged till fourth milking (36 h). On the other hand, in the control group AOPP level appeared to increase at (36 h). When expressed as AOPP/protein ratio, it was shown that AOPP level was significantly higher in the fourth colostrum of both examined groups of cows. A significant disparity in AOPP level between these two groups of cows was not found.

A significant correlation between analysed colostrum redox indices was not found in either the control or the supplemented group Table S10 (Supplementary Material).

Discussion

It has been shown that dietary mineral supplementation has a vital role in the oxidative/antioxidant balance of dairy cows and their productive and reproductive performances (Vázquez-Añón et al. 2008). In the last few decades, natural zeolite (clinoptilolite), as an additive to feed, has been applied successfully in animal breeding for different purposes. The study conducted by Karatzia et al. (2013) has shown that the dietary administration of 200 g per day of a natural zeolite, clinoptilolite, during the last 2 months of pregnancy and the subsequent lactation improves the energy status and the reproductive performance of dairy cows in their first lactation (Karatzia et al. 2013). We have recently shown that organically modified oral clinoptilolite supplementation at 150 g/day significantly increases the IgG concentration in colostrum and has no adverse effects on the energy status, protein, lipid, and mineral metabolism in primiparous dairy cattle during the prepartum period (Stojić et al. 2020). Although there are several reports on natural zeolites' antioxidant properties *in vitro* and *in vivo* (Zarkovic et al. 2003; Dogliotti et al. 2012; Montinaro et al. 2013), data on such effects on peripheral blood

and colostrum of dairy cows during prepartum period are still scarce.

In this work, we have studied the effect of orally administered organically modified clinoptilolite on the total antioxidative capacity of blood plasma, erythrocytes and colostrum of primiparous dairy cows by FRAP method, starting from 24 ± 4 days before the expected calving term up to 2 days pp. Since correctly evaluating oxidative stress needed determination of both pro-oxidants and antioxidants (Kerwin et al. 2019), we have also examined the level of lipid peroxides by the TBARS method and level of AOPP during the same investigation period. Calving itself also causes temporary but significant changes in the antioxidant system of cow blood (Albera and Kankofer, 2011). Thus, it was meaningful to investigate whether clinoptilolite possesses antioxidant effects on peripheral blood and colostrum before and after calving since antioxidant supplementation only had an outcome when the nutritional/oxidant status is deficient (Ipek et al. 2012). Otherwise, healthy cows' biological antioxidant system is effective enough to combat these transient changes of redox homeostasis, as demonstrated in the study of Ipek et al. (2012). Ipek et al. (2012) examined 60 days' influence of 2.5% zeolite supplementation on oxidant/antioxidant status in 3–4 years old healthy non-pregnant Holstein dairy cows in the first period of lactation (2 months after calving), and showed no changes of oxidative and antioxidant indicators in blood plasma, except for lipid hydroperoxides that were lowered. Recently, Kerwin et al. (2019) reported that feeding synthetic zeolite during the prepartum period of multiparous Holstein cows effectively improves serum calcium status during the postpartum period.

Our results obtained in the control group of animals indicated changes of antioxidant status in blood plasma by calving itself. Our findings demonstrated significantly higher FRAP activities in plasma samples 7 days after calving than in samples obtained 20 days before calving. These results followed study of Mudroň et al. (1999) but were opposed to the results of Gaál et al. (2006), who found no changes in FRAP activities before and several days after calving. Furthermore, our results were in contrast to Albera and Kankofer (2011) findings, showing that during the first 24 h after parturition, the antioxidant capacity of colostrum measured by FRAP (correspond to 3rd milking in our study) was significantly lower in comparison to the antioxidant capacity value in blood plasma. Our study showed that supplementation with organically modified clinoptilolite induced changes in antioxidant

capacity of blood plasma of primiparous dairy cows. The differences in supplemented animals were revealed on days 2 and 7 after parturition, with an increase in FRAP activities of the blood plasma of 41% and 14%, respectively, compared to control animals. The concentration of specific antioxidants, total antioxidant capacity, the levels of oxidative damages of lipid and proteins in peripheral blood, colostrum, and milk, are significantly dependent on the age of cows (Albera and Kankofer, 2010; Puppel et al. 2012). We believe that the discrepancy between the results obtained in our study and some other reports could be at least partially explained by variations in the experimental setup: use of different zeolites, distinct duration time of supplementation, and research on cows in different stage of lactation or age.

AOPP are well-known biomarkers of the oxidative modification of proteins, where the plasma proteins are the key targets (Witko-Sarsat et al. 1996). Nevertheless, this study revealed no differences between AOPP levels in the blood plasma of the control and treated animals, while the AOPP level in the erythrocytes of supplemented animals was increased 5 days before calving (on the twentieth day after the start of the supplementation with clinoptilolite). Such results could be explained by the relatively short half-life of proteins in the peripheral blood (half-life of serum albumin: 2–3 weeks, transferrin: 9 days; prealbumin 2: days; C reactive protein: 4–6 h, etc. (Tariq and Morley, 2004); half-life of bovine IgG is up to 4 weeks; Murphy et al. 2014) as opposed to the 130 days erythrocyte lifespan (Wood and Quiroz-Rocha, 2010) and their cell properties. Namely, in the circulation there are several populations of erythrocytes of different ages, and as the cells without a nucleus, erythrocytes are not able to respond to some environmental changes by protein synthesis *de novo*.

An increase of thiobarbituric acid reactive substances (TBARS) plasma levels may be considered as a sign of cellular lipid oxidation and used as a marker of oxidative status (Da Silva et al. 2013). The result of TBARS analysis of blood plasma of primiparous dairy cows supplemented with organically modified clinoptilolite in our study indicated its positive effect on lipid peroxidation level 7 days after calving, since the level of measured MDA was 40% lower in the treated group in comparison to the control group. Our results are consistent with a study of Yarovan (2008), who measured the level of MDA and some others oxidative markers and reported that zeolite has a positive effect on the oxidant status in the blood plasma of dairy cows.

The physiological settings of periparturient period also reflect on the colostrum's redox balance, the neonates' first source of nutrients and immunity. Apart from nutrient and immunological components, colostrum possesses antioxidative systems necessary for the neonate's protection and ROS originating from easily oxidised macromolecules, lipids, or proteins. This study demonstrated that only at the third milking (24 h pp) difference in colostrum whey antioxidant capacity between control and zeolite treated animals (33% higher) was shown to be close to statistical significance ($p=0.06$) Although the literature provides data that suggest an increase of antioxidants in bovine colostrum over time, with the highest increase at 36 h pp (4rd milking in our study) (Kankofer and Lipko-Przybylska, 2008; Albera and Kankofer, 2011), our results did not show such trend in colostrum samples of control animals. This discrepancy could be due to the different approaches in results expression. Proteins, which concentration indisputably decreases in colostrum over time (Stojić et al. 2020), contribute to the total antioxidant capacity. Although other authors expressed total antioxidant capacity as mM per gram of proteins, we did not. We think that such expression of the results does not show the proper physiological state but falsely displays colostrum's timely unchanged antioxidant capacity. The discrepancy detected in studies on the use of natural clinoptilolite and other zeolites as antioxidant agents in diverse animals (dogs, cats, horses, pigs, poultry, small and large ruminants) might be attributed to the various factors such the type of zeolite tested, particle size, pre-treatment and the amounts that were used in these studies (Valpotić et al. 2017). MDA levels in colostrum sera of both groups of cows were continuously decreasing from first to fourth milking. The decreasing MDA level followed the decreasing trend of the fat level in colostrum, and we assumed that the time-dependent change of MDA levels in colostrum sera was a consequence of the changes in the content of fat in colostrum. Interestingly, lipid peroxide analysis of colostrum whey revealed MDA level in zeolite treated group 90% higher than MDA level in control animals on third milking. Although these results indicate a higher level of lipid peroxides in the third milking colostrum of treated animals, results of increased total antioxidant capacity measured by FRAP in the same colostrum samples indicate a positive effect of zeolite on the redox status of colostrum. The lipid peroxides act as immunomodulators capable to activate neutrophils, dendritic cells and macrophages (Girotti, 1998; Mushenkova et al. 2021; Zhivaki and Kagan, 2021) and in this way to activate both innate and adaptive immune response. Their

presence in colostrum might be beneficial because activated macrophages and neutrophils use ROS generating systems to kill bacteria (Albera and Kankofer, 2011). In this way, colostrum lipid peroxides may help overcome poorly developed antioxidative defence mechanisms of neonate's calves (Przybylska et al. 2007). If the observed redox status of colostrum in zeolite-treated animals could be of importance for IgG absorption in new-born calves (as proposed by Abuelo et al. 2014) remained to be elucidated.

The exact mechanisms of zeolite's antioxidant effects are not well known. Some evidence suggests that zeolite act via (1) removal of toxins from the gut, (2) improvement of the immune system through the mucosal-related intestinal lymphoid tissue, (3) increase in the bioavailability of minerals that are essential cofactors for some enzymes, and (4) arrest of generation of peroxides and free radicals (Hosseini Nia et al. 2018). In addition, it has been proposed that antioxidant properties of zeolites are attributed to their effect on macrophages' phagocytic function triggered after phagocytosis of zeolite particles, subsequently leading to the production of cytokines such as tumour necrosis factor- α , which stimulates immunologic responses and also increases the expression of superoxide dismutase (Zarkovic et al. 2003).

While speculation on the action mechanism of used organically modified clinoptilolite merits further investigation, it is indicative that the impacts on total antioxidative capacity and lipid peroxidation of blood plasma and colostrum by this agent exist, even after short-term supplementation. In this study, we do not observe significant effects on erythrocyte redox status after short-term supplementation with zeolite. To examine the clinoptilolite's effect on the erythrocyte redox status, in future studies it will be necessary to include longer (several months) supplementation. Overall, this study showed that organically modified clinoptilolite positively modulates the redox status of colostrum and blood plasma of primiparous dairy cows during adaptation for parturition. These data allow the recommendation for use of organically modified clinoptilolite as an agent for preventing the development of oxidative stress during some physiological or unfavourable housing and feeding conditions.

Conclusions

In this study, we showed that short-term oral supplementation of primiparous dairy cows with 150 g per day of organically modified clinoptilolite during the prepartum period modulate their redox homeostasis

by increasing total antioxidants and decreasing lipid peroxides level in the peripheral blood plasma. This result, together with result of our previous study showing that this supplementation had no adverse effects on the cows' energy status, protein, lipid, and mineral metabolism, indicates that this clinoptilolite has positive effect on adaptation of primiparous cows to redox imbalance in the peripartum period.

This study also showed that organically modified clinoptilolite supplementation resulted in the secretion of colostrum with increased level of lipid peroxides. This result, together with result of our previous study showing that organically modified clinoptilolite increase concentration of colostrum IgG, indicates that this mode of supplementation of cows might lead to more efficient passive immune protection of new-born calf.

Author contributions

The study conception and design: N.F., V.I. and I.D. Material preparation, data collection and analysis: M.S., I.D., M.K., and J.G.M. Statistical analysis: BV. The first draft of the manuscript was written by V.I. and I.D. Critical analysis of the entire study and manuscript: D.M., J.G.M. and D.G. All authors have read and agreed to the published version of the manuscript.

Disclosure statement

None of the authors has a financial or personal relationship with other people or organisations that could inappropriately influence this publication.

Ethical approval statement

This study has been approved by Ethical Committee of the Faculty of Veterinary Medicine, University of Belgrade [01-19/10].

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author [V.I.].

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