

Chestnut tannin supplementation can improve immune response and kidney function in prepartum dairy cows

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ABSTRACT. Due to their antiketogenic and antioxidant effects, chestnut tannins may offer a viable approach to manage the impaired immune and renal functions in transition cows. This study aimed to investigate the effects of dietary supplementation with chestnut tannins on haematological, biochemical and antioxidant indices, as well as cortisol levels in prepartum dairy cows. Forty multiparous Holstein cows were divided into two homogeneous groups ($n = 20$): a control (CON), and an experimental group (CNT) receiving 20 g/day of chestnut tannins for the last 25 ± 2 days of pregnancy. Haematological and biochemical indices, cortisol concentration and total antioxidant capacity (T-AOC) were measured in blood samples collected 25 (day -25) and 5 days (day -5) before the expected parturition. The addition of chestnut tannins exerted no significant effect on red blood cells indices; however, white blood cell ($P = 0.02$), lymphocyte ($P = 0.05$) and platelet ($P < 0.01$) counts were higher, while the neutrophil to lymphocyte ratio ($P = 0.03$) was lower on day -5 in the CNT group compared to the CON group. Significantly higher values of T-AOC ($P = 0.03$) and significantly lower levels of triglycerides ($P = 0.03$) and gamma-glutamyl transferase ($P = 0.02$) were also found in CNT compared to CON on day -5. The improved haematological profile in CNT cows was accompanied by lower serum creatinine concentration ($P = 0.04$), while total protein, calcium, phosphorus and cortisol did not differ significantly between CNT and CON cows. These data demonstrate that dietary chestnut tannin supplementation in a close-up diet has antioxidant and anti-inflammatory effects, and could potentially mitigate immune suppression and kidney dysfunction near parturition. Further research should be conducted concerning the mechanisms underlying these responses.

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Introduction

Dairy cows face a range of physiological challenges during the transition period from 3 weeks

before calving to 3 weeks post-calving. During this time, cows are exposed to pronounced endocrine and metabolic changes, including decreases in thyroid hormones, insulin and insulin-like growth factor-1

(IGF-1) concentrations, and increases in growth hormone, cortisol, and catecholamine levels. These hormonal changes result in elevated non-esterified fatty acids (NEFAs) and beta-hydroxybutyrate (BHB) concentrations as a response to negative energy balance (NEB) (Kneysel et al., 2014). Moreover, periparturient cows experience a suppression of the immune response, which can increase susceptibility to periparturient diseases and disorders. Changes in the immune system around the time of parturition have been the subject of extensive investigations, particularly in the context of immunosuppression, which may raise the incidence of immune-related disorders in transition dairy cows, such as retained placenta, mastitis and metritis (Drackley et al., 2005; Wankhade et al., 2017; Guan et al., 2020). This has been linked to various factors, including changes in the concentrations of circulating sex hormones during this time (Jonsson et al., 2013), elevated cortisol levels during the prepartum period, and a concomitant reduction in redox imbalance in these animals (Bionaz et al., 2007). In addition, alterations in metabolic status caused by a negative energy balance is an important determinant of immunosuppression in transition cows, because metabolites derived from lipomobilisation are associated with immune dysfunction (Lacetera et al., 2005). In particular, energy deficiency accompanied by a loss of hormonal responsiveness, as well as an increase in circulating NEFA and BHB levels during the periparturient period, can lead to a decrease in lymphocyte counts (Qi et al., 2022).

Over the past fifteen years, concepts related to nutrition and ration formulation have been based on the discovery of alternatives such as plant extracts and their secondary metabolites, especially since the European Union banned the use of antibiotics in livestock feeds in 2006. Among these alternatives, the concept of tannin application has attracted great interest in dairy cow research due to their ability to affect several aspects of nutrition, such as feed intake, digestion, ruminal fermentation and methane emission (Orzuna-Orzuna et al., 2021), as well as to modulate oxidative stress response (Liu et al., 2013). The diet of dairy cows can be supplemented either with condensed or hydrolysable tannins, with the dietary effects differing depending on the source and/or their biological activity of individual tannins (Waqas et al., 2023). Dietary supplementation with tannins can enhance plasma antioxidant capacity, thereby reducing oxidative stress and proinflammatory cytokine profile, as evidenced by studies involving dairy cows (Liu et al., 2013;

Santillo et al., 2022). The mechanisms responsible for these effects appear to be linked to the antioxidant and antiradical properties of hydrolysable tannins, which can disrupt the activities of inflammatory transcription factors, enzymes and ion channels. This disruption is believed to underlie the anti-inflammatory and immunomodulatory effects associated with tannin supplementation (Piazza et al., 2022). Hydrolysable tannins derived from sweet chestnut (*Castanea sativa* Mill.) have been proven to effectively scavenge reactive oxygen species (O₂⁻) and protect tissues against oxidants, as well as liposomes from lipid peroxidation (Liu et al., 2013). Therefore, the modulatory role of tannin supplementation on oxidative stress and inflammatory response in dairy cows may contribute to maintaining immunocompetence in the periparturient period. However, limited information is available on the *in vivo* immune properties of chestnut tannin supplementation in dairy cows during late gestation.

The fluctuations in the metabolic and/or antioxidant status near parturition were shown to influence the effect of dietary tannins on colostrum quality, indicating an additional positive effect of chestnut tannins on the immune system of the mammary gland (Prodanović et al., 2021). The positive effect of dietary tannins has also been attributed to the reciprocal regulation between thyroid response and oxidative stress (Prodanović et al., 2023).

Although research into the beneficial effects of hydrolysable tannins on dairy cattle nutrition and health have been extensive and provided many practical applications, little attention has been devoted to haematological and biochemical indices, which may indicate the relationship of tannin supplementation to the immune response and function of certain organs such as the kidneys. Therefore, the objective of this study was to investigate the impact of dietary supplementation of chestnut tannins during the close-up dry period on selected haematological and biochemical indices in dairy cows. Additionally, the study sought to evaluate the concentration of cortisol, a hormone regulating the immune response and total antioxidant capacity, as an indicator of antioxidant defence.

Material and methods

The experimental aspects of the study related to animals were approved by the Ethics Committee of

the Faculty of Veterinary Medicine (No. 05/2015), University of Belgrade, in accordance with the National Regulation on Animal Welfare. The data reported here were obtained as part of a larger experiment, and details regarding the experimental design, chemical composition of the diets and selected biochemical and antioxidant indices have been previously documented (Prodanović et al., 2021). Briefly, 40 clinically healthy and late-pregnant Holstein cows without a history of metabolic disorders in previous lactation were selected and divided into two dietary treatments: a control group (CON, $n = 20$), with no additional supplementation, and a group supplemented with chestnut tannins (CNT, $n = 20$). CNT cows received 20 g/day of a commercially available product containing chestnut tannins (Tanimil SCC, Tanin Sevnica, Slovenia) during the final 25 ± 2 days of pregnancy. Tanimil SCC contains 48% hydrolysable and 2.1% condensed tannins derived from chestnut extract; 10 g of the product were mixed twice daily with 50 g of concentrate as part of the total mixed ration (TMR), and fed to each CNT cow shortly before the morning and evening TMR delivery. The diets, formulated to meet or exceed NRC (2001) requirements, were administered to cows in two equal portions at 6:30 and 17:30. At the beginning of the study, the groups were balanced for the number of lactations (CON: 3.0 ± 0.19 ; CNT: 2.95 ± 0.18) and body condition score (BCS; CON: 3.45 ± 0.04 ; CNT: 3.44 ± 0.04). The cows were kept in a tie-stall housing system with individual feeding control and had continuous access to water via automatic water bowls. Animal health was monitored daily using general clinical examination methods.

Blood sampling

Blood samples were collected from each cow on the first day of chestnut tannin supplementation (i.e. on day 25 before the expected calving date; day -25) and daily thereafter until calving; blood taken on day 5 before calving (day -5) was used for analysis. Blood was sampled before morning feeding via jugular vein puncture into Vacutainer tubes (Becton Dickinson, Plymouth, UK) for haematological analysis, with a clot activator for serum separation. The tubes were immediately placed in an icebox and transferred to the laboratory within an hour.

Analyses of haematological indices

Venous blood samples for complete blood count and white cell differential count (WCDC) were

collected into K₂EDTA anticoagulant tubes (Becton Dickinson, Plymouth, UK). Haematological indices, including total white blood cell (WBC), red blood cell (RBC) and platelet (Plt) counts, haemoglobin (Hb) concentration, haematocrit (Hct) value, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were determined using a Phoenix NCC-30 Vet automated haematology analyser (Neomedica, Serbia). WCDC included neutrophil and lymphocyte counts and was determined manually using standard smear preparations stained with May Grunwald Giemsa, as described by Jones and Allison (2007). The neutrophil to lymphocyte ratio (NLR) was calculated by dividing the percentage of neutrophils by the percentage of lymphocytes.

Analyses of biochemical indices and cortisol levels

Samples for the determination of total protein, calcium, phosphorus, triglycerides (TG), gamma-glutamyl transferase (γ -GT), creatinine and cortisol were collected to gel-coated blood tubes (Becton Dickinson, Plymouth, UK), centrifuged at 1800 g for 10 min, and aliquoted into 2-ml microfuge tubes. Serum aliquots were stored at -20 °C until analysis. Biochemical metabolites were analysed at the Department for Ruminants and Swine Diseases (Belgrade, Serbia) using appropriate BioSystems S.A. (Barcelona, Spain) kits: total protein (Biuret method), calcium (Arsenazo method), phosphorus (phosphomolybdate/UV method), TG (Glycerol phosphate oxidase/reductase method), γ -GT (IFCC method), creatinine (compensated Jaffe method). These analyses were performed automatically using spectrophotometry (A15; BioSystems S.A., Barcelona, Spain). Serum cortisol levels were detected photometrically on an AIA 360 automated immunoassay analyser (Tosoh, Japan) using a commercially available competitive enzyme immunoassay kits from the same manufacturer.

Estimation of total antioxidant capacity in blood serum

The total antioxidant capacity (T-AOC) was evaluated using the 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolourisation assay with Trolox as a standard. The ABTS test was performed spectrophotometrically, following a modified method of Re et al. (1999). This method is based on the ability of antioxidant bioactive compounds to capture the cation

radical ABTS (ABTS+) and reduce the blue-green radical form to a colourless neutral compound. The reduction in absorption, i.e. the intensity of the colour change of the solution, is proportional to the concentration of total antioxidants present in the sample. ABTS+ solution was obtained by adding potassium persulphate ABTS solution and stored in the dark. The change in absorbance was read at 734 nm, and the results were expressed as micrograms of Trolox equivalents (TEq) per ml of sample.

Statistical analyses

Data were analysed using the commercial software Statistica v.8 (Stat Soft, Inc., Tulsa, OK, USA). The normality of data distribution was tested using the Shapiro-Wilk test and all data had a normal distribution ($P > 0.05$) and were presented as mean \pm SE (standard error) for all parameters examined. The data in this study were analysed according to the model published by Prodanović et al. (2021). To compare the significance of differences in mean parameter values between the CON and CNT groups of cows, an independent samples t-test was applied. This test was conducted once at the beginning of the trial (day -25) and once at the end of the trial (day -5). Results were considered statistically significant at $P \leq 0.05$ and tendencies were noted at $0.05 < P < 0.1$.

Results

The animals did not show any clinical health problems and no significant difference in average dry matter intake (DMI) was observed between the CON and CNT groups during the trial period ($P > 0.05$). No differences were observed for any of the monitored parameters between the CON and CNT group of cows on day -25, i.e. before the start of the study ($P > 0.05$).

As shown in Table 1, there was no effect of the treatment, i.e. CNT supplementation (comparison of values on day -5 relative to calving) on RBC count, Hb concentration, and Hct, MCV, MCH and MCHC values. At the same time, the addition of CNT had a significant effect on WBC ($P = 0.02$), lymphocyte ($P = 0.05$) and platelet ($P < 0.01$) counts, leading to higher values in the CNT group compared to the CON group on day -5 relative to calving. The NLR value prepartum was lower in the CNT group than in the CON group ($P = 0.03$).

The data from Figure 1 shows that on day -5 relative to calving, T-AOC activity was higher ($P = 0.03$),

Table 1. Mean (\pm SE) values for selected haematological indices in the CON and CNT groups of cows 25 and 5 days before expected parturition

Indices	CON	CNT	P-value
RBC, $\times 10^{12}/l$			
day -25	5.35 \pm 0.09	5.28 \pm 0.07	0.48
day -5	5.54 \pm 0.09	5.42 \pm 0.10	0.37
Hb, g/dl			
day -25	9.52 \pm 0.10	9.46 \pm 0.13	0.73
day -5	11.28 \pm 0.20	10.74 \pm 0.18	0.06
Hct, %			
day -25	25.14 \pm 0.21	24.93 \pm 0.08	0.35
day -5	25.26 \pm 0.45	24.33 \pm 0.43	0.15
MCV, fl			
day -25	47.11 \pm 0.60	47.38 \pm 0.50	0.73
day -5	45.68 \pm 0.74	45.03 \pm 0.76	0.55
MCH, pgHb			
day -25	17.83 \pm 0.25	17.94 \pm 0.12	0.69
day -5	20.38 \pm 0.27	19.87 \pm 0.31	0.22
MCHC, g/dl			
day -25	37.88 \pm 0.36	37.94 \pm 0.42	0.91
day -5	44.66 \pm 0.23	44.16 \pm 0.38	0.27
WBC, $\times 10^9/l$			
day -25	6.89 \pm 0.12	7.02 \pm 0.09	0.37
day -5	7.70 \pm 0.25	8.59 \pm 0.26	0.02
Ly, %			
day -25	60.36 \pm 1.73	62.61 \pm 1.69	0.36
day -5	49.48 \pm 2.16	59.97 \pm 1.73	0.05
Ne, %			
day -25	32.50 \pm 1.68	31.28 \pm 1.87	0.63
day -5	42.93 \pm 2.16	37.79 \pm 1.75	0.07
NLR			
day -25	0.56 \pm 0.04	0.52 \pm 0.05	0.58
day -5	0.93 \pm 0.08	0.71 \pm 0.05	0.03
Plt, $\times 10^9/l$			
day -25	131.4 \pm 2.60	135.1 \pm 3.16	0.36
day -5	154.4 \pm 5.78	218.2 \pm 10.36	<0.01

CON – control group of cows that did not receive chestnut tannin supplementation, CNT – experimental group of cows that received chestnut tannin supplementation; RBC – red blood cells, Hb – haemoglobin, Hct – haematocrit, MCV – mean corpuscular volume, MCH – mean corpuscular haemoglobin, MCHC – mean corpuscular haemoglobin concentration, WBC – white blood cells, Ly – lymphocytes, Ne – neutrophils, NLR – neutrophil-to-lymphocyte ratio, Plt – platelets. Data are presented as mean value \pm standard error (SE). $P < 0.05$ indicates significant difference between the CON and CNT groups of cows on days 5 and 25 before expected parturition.

while TG ($P = 0.03$), creatinine ($P = 0.04$) and γ -GT ($P = 0.02$) levels were significantly lower in the CNT group compared to the CON group. On the other hand, serum concentrations of total protein, calcium, phosphorus and cortisol were not affected ($P > 0.05$) by CNT supplementation.

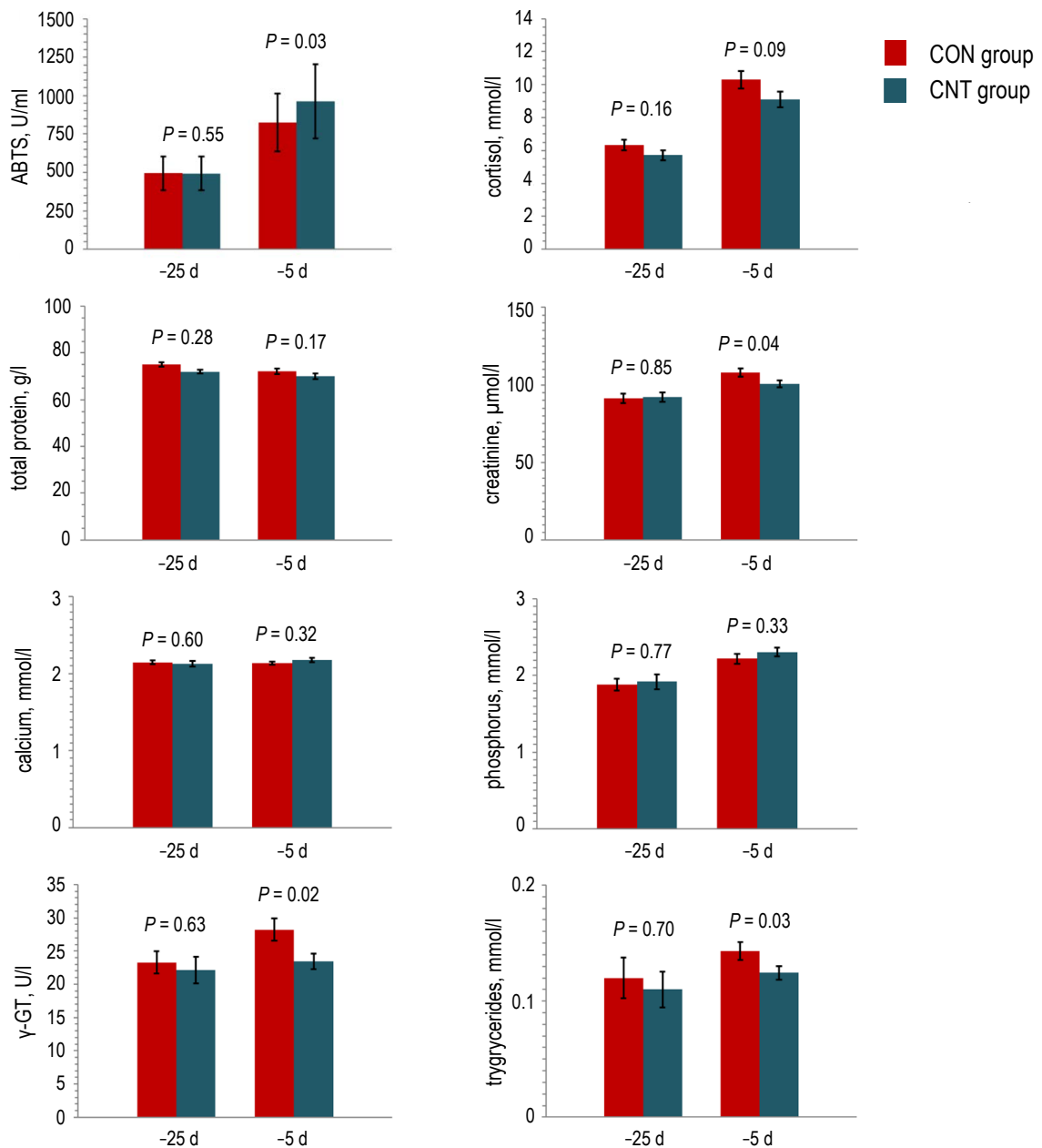


Figure 1. Effect of dietary supplementation of chestnut tannins during the close-up dry period on selected biochemical and antioxidant indices and cortisol concentration in dairy cows 25 and 5 days (-25 d and -5 d, respectively) before expected parturition

CON – control group of cows that did not receive chestnut tannin supplementation; CNT – experimental group of cows that received chestnut tannin supplementation; ABTS – 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), γ -GT – gamma-glutamyl transferase, $P < 0.05$ indicates significant difference between the CON and CNT groups of cows on days 5 and 25 before expected parturition

Discussion

In the present study, the values of haematological indices were consistent with the reference range previously reported for RBC count ($4.9\text{--}7.5 \times 10^{12}/l$), Hb concentration (8.4–12 g/dl), and Hct value (21–30%) (Wood and Quiroz-Rocha, 2010). Furthermore, the values of these parameters did not differ between the study groups before and after the feeding trial. These results indicate the lack of adverse

effects of chestnut tannins on erythropoiesis, although a tendency towards lower Hb concentration in CNT cows could be ascribed to Fe-binding properties of tannins (Huang et al., 2018). However, the addition of chestnut tannins to the close-up diet led to higher total WBC, lymphocyte and platelet counts, and a lower neutrophil-to-lymphocyte ratio near parturition when compared to non-supplemented cows, suggesting an immunostimulatory effect of chestnut tannins. This pattern observed in the mean WBC and lymphocyte

counts in CNT cows contrasted with the results obtained by Olafadehan et al. (2014), who determined that WBC and lymphocyte counts were negatively associated with condensed tannin content in goat feed. The explanation for this discrepancy may be associated with differences in methodologies and/or tannin origin, as well as supplementation levels (Jayanegara et al., 2015). On the other hand, the results of our study were consistent with the findings of Xiong et al. (2014), who observed protective effects of tannins on myelosuppression in mice. This phenomenon has not been reported in cows; however, if it does exist, it could help alleviate the adverse effects in the periparturient period on immune response caused by stress. Furthermore, the neutrophil-to-lymphocyte ratio, which was reduced in the CNT group, is used as valuable indicator of systemic inflammatory response and shows prognostic potential in certain diseases (Guan et al., 2020). Although this ratio is also utilised as a stress indicator in dairy cows (Hong et al., 2019), no significant differences were found in terms of cortisol levels between the two groups. Therefore, several other mechanisms may be responsible for the observed changes in the haematological profile in CNT cows. First, the change in the immune status of dairy cows around the calving period depends on alterations in the endocrine status, which may also affect white blood cell production and functioning towards lactation. According to Jonsson et al. (2013), increases in serum oestradiol levels in response to stressors could cause haematological changes, including an increase in the number of neutrophils and a decrease in lymphocytes. However, as reported previously and demonstrated in the present experiment, tannin supplementation did not affect the concentration of this hormone compared to non-supplemented cows (Bešlo et al., 2022; Prodanović et al., 2023). Therefore, sex steroid probably did not contribute to the differences observed in the immune status between the groups after tannin supplementation. Second, thyroid hormones are thought to be involved in maintaining immune function in response to environmental stimuli and stress-mediated immunosuppression (Dorshkind and Horseman, 2000). Therefore, increased serum T3 and T4 concentrations demonstrated in cows supplemented with chestnut tannins in a previous study (Prodanović et al., 2023) could indicate that tannins modulated the immune system response in dry dairy cows. Third, the improved metabolism of CNT cows during the periparturient period (Prodanović et al., 2021) could be a contributing factor to the higher lymphocyte counts in CNT cows. Accordingly, chestnut tannins

could contribute to the increased lymphocyte counts in the blood of CNT cows by mitigating the adverse effects of NEFA and BHB on lymphocyte development and function (Lacetera et al., 2005; Qi et al., 2022). Lastly, the direct beneficial effects of tannins on multiple immune and inflammatory cell functions should also be considered (Santillo et al., 2022). The higher lymphocyte counts in the blood of CNT cows, compared to those of CON cows, could be attributed to an enhancement of the effect of tannins on lymphopoiesis caused by a reduction in IL-6-induced signalling (Piazza et al., 2022).

It is generally known that WBC, Ne, and Ly counts, as well as NLR can be used as indicators of immune status. Therefore, our data show that alleviating oxidative stress through CNT supplementation can prevent possible immune suppression induced by late gestation, thereby mitigating disease susceptibility (Guan et al., 2020).

As expected, CNT cows had higher activities of the total antioxidants (ABTS), confirming the strong antioxidant properties of chestnut tannins (Santillo et al., 2022). These results are consistent with the findings of Liu et al. (2013) and Prodanović et al. (2023), who observed the stimulatory effect of chestnut tannins on endogenous and general antioxidant defence against free radicals in the liver and plasma of transition dairy cows. The consistency of the results of two different methods (DPPH and ABTS scavenging) in the assessment of antioxidant capacity confirmed their reliability in determining the antioxidant activity of chestnut tannins, and demonstrated the beneficial role of CNT in attenuating oxidative damage during the periparturient period. In addition, the obtained decreased TG concentrations in CNT cows was consistent with the lower NEFA and BHBA levels previously observed in tannin-supplemented cows (Prodanović et al., 2021), further indicating the antiketogenic potential of chestnut tannins. Moreover, the decreased TG levels in the presence of supplemented tannins, were to some extent consistent with reduced γ -GT levels. In addition to the role of γ -GT in secretory and absorptive processes in the hepatobiliary system, it is now considered one of the most potent indicators of whole-body oxidative stress (Koenig and Seneff, 2015). These statements are further supported by the results of the present study, which showed that transition cows supplemented with tannins had significantly lower γ -GT activity on day -5 to calving. This could also suggest that the liver in CNT cows was in better condition compared to CON cows; however, it is well known that the cow's prepartum liver accumulates lower amounts of lipids since it is

able to metabolise NEFA supplied in late gestation (Prodanović et al., 2016). Overall, prepartum TG and γ -GT concentrations were within the normal range expected for dry cows without clinical disease (0.1–0.56 mmol/l for TG and 9–42 U/l for γ -GT) (Brscic et al., 2015). Therefore, these results further support a lower degree of oxidative stress in CNT cows rather than less damage to liver cells.

Another fact that emerges from the results of our study is the lower serum creatinine levels in CNT cows compared to matched controls, suggesting at least two potential effects or outcomes of tannin supplementation. Creatinine is one of the indicators of kidney function (Issi et al., 2016), thus its lower concentrations along with concomitant lower γ -GT activity and higher T-AOC may indicate a more preserved functional integrity of this organ in tannin-supplemented cows. Specifically, the level of creatinine in the blood, which indicates the kidneys' capacity to remove it, is a reliable predictor of glomerular filtration efficiency (Issi et al., 2016). Reduced glomerular filtration rate of the kidney in late gestation has been documented in many species, including ruminants (Van Drongelen et al., 2014), and the high levels of circulating NEFA and systemic inflammation that occur in dairy cows during the transition period may also contribute to renal dysfunction in late gestation (Ma et al., 2016). In line with the previously reported reduced blood urea nitrogen levels (Prodanović et al. 2021), the lower concentration of serum creatinine found in CNT cows could indicate an improved renal glomerular filtration rate, supporting the beneficial influence of chestnut tannins on kidney function. Similar results were reported by Ahmad and Sultana (2012), who investigated the renal effects of tannic acid, which is usually obtained from the aqueous extract of sweet chestnut. The latter authors reported that tannic acid reduced kidney damage and decreased creatinine levels in mice treated with cisplatin. Furthermore, since several tannin plant extracts have already been demonstrated to inhibit angiotensin-converting enzyme (Liu et al., 2003), chestnut tannins may exert an additional effect. This mechanism would limit the kidney's ability to absorb nutrients, causing an increase in urine volume and creatinine excretion. However, our data do not exclude the possibility that CNT cows had increased creatinine accumulation in muscles, which could be attributed to the role of insulin in creatinine metabolism. It has been reported that insulin plays an important role in creatinine muscle transport, leading to lowering blood creatinine levels (Steenge et al., 1998).

Conclusions

Our results indicate that dietary supplementation with chestnut tannins is effective not only in improving the antioxidant status, but also in enhancing the anti-inflammatory and/or immune responses in prepartum cows. Although the present study showed for the first time the immunomodulatory effects of chestnut tannin supplementation in close-up dairy cows, in-depth studies are required to elucidate the molecular pathways responsible for the immunomodulatory properties of chestnut tannins, focusing on changes in lymphocyte subsets and parallel cytokine levels. Furthermore, lower creatinine concentration in cows supplemented with chestnut tannins may indicate improved kidney function in these cows near parturition.

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Conflicts of interest

The Authors declare that there is no conflict of interest.

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