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Effects of chestnut tannins supplementation of prepartum moderate yielding dairy cows on metabolic health, antioxidant and colostrum indices

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**Effects of chestnut tannins supplementation of prepartum moderate yielding dairy cows
on metabolic health, antioxidant and colostrum indices**

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Short title: Chestnut tannins supplementation of prepartum cows

Abstract

This study investigated the effects of dietary supplementation with chestnut tannins (CNT) on metabolic and antioxidant status of prepartum cows along with their colostrum quality.

Pregnant multiparous Holstein cows were paired according to parity and body condition score, and assigned either to a diet supplemented with 20 g/d of commercially available product containing chestnut tannins (CNT, n=20) or to an unsupplemented control diet (CON, n=20) for the last 25±2 d of pregnancy. Serum metabolite, insulin and antioxidant capacity indices were measured in blood samples taken at d 25 and d 5 before expected parturition. Chemical composition and IgG concentration were determined in colostrum samples collected from the first milking postpartum. The addition of CNT led to lower BUN (P=0.02) and consequently higher serum glucose (P=0.02) and insulin (P<0.01) concentrations which were associated with lower circulating NEFA (P<0.01) and BHBA (P<0.01) in CNT group than those of CON. The serum paraoxonase 1 (PON 1) activity and total antioxidant capacity (T-AOC) were higher at -5 d in CNT than in CON (P<0.01, P=0.03; respectively). Close-up CNT improved lactose percentage and IgG concentration (P=0.03, P=0.04; respectively) and tended to improve percentage of protein and SNF (Solid Not Fat) in primary colostrum (P=0.06, respectively), without affecting colostrum fat and total solid (P=0.98, P=0.43; respectively). Supplementation of CNT in the diet during close-up period did not have adverse effects on metabolic profiles prepartum. Instead, this feeding regimen was more beneficial to antioxidant capacity and colostrum quality than feeding the control diet.

Key words: antioxidant status, chestnut tannin, colostrum quality, dairy cows, metabolic status.

The inclusion of plant extracts in livestock feed supplements has been widely researched as a strategy to replace synthetic feed additives and improve animal health and

production traits. Among several plant metabolites, tannins have attracted significant attention in regards to dairy cows. Tannins are water soluble plant polyphenol metabolites known for a binding affinity for proteins, amino acids, metal ions and polysaccharides (Makkar, 2003; Mueller-Harvey, 2006). They have the ability to affect several aspects of ruminant nutrition and to decrease environmental pollution (Huang et al., 2018).

Tannins from sweet chestnuts, i.e., *Castanea sativa* Mill., are hydrolysable tannins (HTs) with molecular weights of 500-3,000 Da and multiple hydroxyl groups (Cieslak et al., 2013). Due to hydrophobic and ionic interactions, these groups give the HTs an affinity and capacity for forming pH-dependent reversible tannin protein complexes (Makkar, 2003; Huang et al., 2018). These complexes are stable in rumen pH (pH 5.0 to 7.0), and dissociations occur in low (abomasum) and high (intestine) pH environments (Makkar, 2003; Huang et al., 2018). Moreover, chestnut tannins (CNT) have a tendency to decrease ammonia production in rumen by decreasing rates of rumen protein degradation (Sliwinski et al., 2002; Hassanat and Benchaar, 2013); this is primarily done by inhibiting the growth and activities of proteolytic bacterial populations (McSweeney et al., 2001). This can lead to a significant non-ammonia nitrogen flow to the duodenum and to improved protein utilisation (Jayanegara et al., 2015).

In addition to these beneficial effects in regards feed protein utilisation, feeding CNT to a dairy cow can improve the cow's antioxidant status, wellbeing and performance (Liu et al., 2013; Huang et al., 2018). The abundance of hydroxyl groups and the groups' conformations can cause CNT to be effective at scavenging ROS (O_2^-) and at protecting liposomes from lipid peroxidation (Živković et al., 2010). Indeed, Liu et al. (2013) found that CNT fed to transition cows had the capacity to significantly decrease lipid peroxidation and to increase superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and T-AOC activities in plasma. Further, the CNT could increase SOD and GSH-Px activities in the cows'

liver during a transition period (Liu et al., 2013). However, no information is available on dietary factors in regards to modulate paraoxonase 1 (PON 1) activities or expressions in dairy cows, despite the relevance of PON 1 to overall reductions in antioxidant capacities and several pathological conditions (Farid et al., 2013; Cao et al., 2017; Nedić et al., 2019).

Colostrum immunoglobulin G (IgG) concentration is one of the most important factors in the adequate transfer of Ig in newborn calves. Several risk factors associated with low colostrum quality have been elucidated (Gulliksen et al., 2008; Kehoe et al., 2011; Conneely et al., 2013), but dietary approaches to improving colostrum quality and IgG content require further investigation. Indeed, there is no universal strategy for formulating pre-fresh diets that promote colostrum quality in cows. Only a few studies have shown that colostrum IgG levels can be affected by energy intake (Panigrahi et al., 2004; Gulliksen et al., 2008; Nowak et al., 2012) and dietary protein levels (Stockdale and Smith, 2004; Toghyani and Moharrery, 2015). Moreover, Rezai et al. (2012) reported that increasing dietary rumen undegradable protein (RUP) can promote colostrum quality, and this corresponds with the effects of CNT, i.e., a decreased protein degradation rate in rumen and an increased protein flow to intestines. However, no studies have investigated the possibility that HTs are involved in colostrum formation in dairy cows

Supplementation of CNT extract in dry cow diets has not been extensively investigated; the literature lacks information on how the CNT feeding regimen can affect metabolic profiles and health prepartum. Therefore, the objective of this study was to investigate the effects of dietary supplementation of CNT during close-up dry period on metabolic and antioxidant status of dairy cows (particular on PON 1) and their colostrum composition.

Material and Methods

Animals, diets and housing

Forty late pregnant Holstein cows were selected on a commercial farm (PKB) in the central part of Serbia, City of Belgrade, at latitude 44°49 '14" North, longitude 20°27'44" East (total number of cows 1250, average milk production per year 8200L). Chosen animals were subjected to the Program of Animal Health Protection Monitoring. All animals were clinically healthy, between second and fourth lactation and with body condition score ranged from 3.0 to 3.25. The cows were kept in a tie-stall housing systems with individual control of feeding and had free access to water at all times via automated water bowls.

During whole dry period (60 days before expected parturition until parturition) cows were fed ad libitum with diets in the form of total mix ration (TMR) formulated to meet or exceed NRC (2001) requirements. Initially, cows were assigned to the far off diet (NEL=1.40 Mcal/kg DM) and then, 25 days before expected parturition, switched to the close-up diet (NEL=1.57 Mcal/kg DM) (Table 1). Diets were fed in two equal portions at 6.30 AM and 5.30 PM. Animals were ranked by parity and body condition score in a decreasing order and alternately distributed into two groups: control (CON, n=20), which was not supplemented, and group supplemented with chestnut tannins (CNT, n=20). CNT cows received 20 g/d of commercially available product containing chestnut tannins (Tanimil SCC, Tanin Sevnica, Slovenia) for the last 25±2 d of pregnancy. Ten grams of product was mixed twice a day with 50 g of concentrate used in TMR, and was given to each CNT cow soon before the morning and evening TMR delivery. The animal-related component of the study was approved by the Ethical Committee of the Faculty of Veterinary Medicine (05/2015), University of Belgrade in accordance with the National Regulation on Animal Welfare.

Table 1. The ingredients and chemical compositions of the far-off and close-up diets.

Item	Diet	
	Far off	Close up
Ingredient, g/kg of DM		
Alfalfa hay	173.3	170.8
Corn silage	374.0	369.0
Alfalfa haylage	115.3	91.26
Molasses		38.27
Corn grain	36.48	112.9
Barley	59.70	29.44
Soybean cake (42% CP)		15.70
Soybean meal (44% CP)		1.96
Sunflower meal (34% CP)	17.41	85.38
Wheat bran	153.4	64.77
Calcium carbonate	60.53	4.90
Monocalcium phosphate	2.49	1.96
Sodium chloride	1.66	4.90
Sodium bicarbonate		0.98
Vitamin Mineral Mix	5.80	7.85
DMI (kg/day)	12.06	10.19
Chemical composition		
NE _L (Mcal/kg of DM)	1.4	1.57
CP (g/kg of DM)	122	140
RDP (g/kg of DM)	92	110
RUP (g/kg of DM)	30	30
MP (g/kg of DM)	76.36	81.76
NDF (g/kg of DM)	477	382
ADF (g/kg of DM)	305	236
NFC (g/kg of DM)	335	407
Ether Extract (g/kg of DM)	21	25
Ca (g/kg of DM)	7	7
P (g/kg of DM)	4	4
DCAD* (mEQ/kg of DM)	196	249
Ash (g/kg of DM)	112.3	82.5

DM – dry matter; CP – crude protein; DMI – dry matter intake; NEL – net energy for lactation; RDP – rumen degradable protein; RUP – rumen undegradable protein; MP – metabolisable protein; NDF – neutral detergent fibre; ADF – acid detergent fibre; NFC – non-fibre carbohydrate and DCAD – dietary cation-anion difference.

* in order to prevent milk fever, each cow was supplemented with 1 oral Ca bolus immediately after calving

Blood samples and analyses of metabolite, insulin and antioxidant capacity indices

Blood samples were collected from each cow on days 25 and 5 before expected parturition. Blood were sampled before morning feeding by jugular vein puncture into BD Vacutainer (Becton Dickinson, Plymouth, UK) tubes with clot activator for serum separation. Tubes were placed in an icebox immediately, and transferred to the laboratory within an hour. After clotting for 2 h on ice, samples were centrifuged at $1800 \times g$ for 10 min, and aliquoted into 2-mL microfuge tubes. Aliquots of serum were stored at -20°C until analysis.

Each blood sample was analyzed for glucose, non-esterified fatty acids (NEFA), beta-hydroxybutyrate (BHBA), albumin, blood urea nitrogen (BUN), total bilirubin, total cholesterol, HDL-cholesterol (HDL-C) and insulin. Biochemical metabolites were analyzed by the Departments for Ruminants and Swine Diseases (Belgrade, Serbia) using the respective kits: NEFA (Colorimetric method) and BHBA (Enzymatic method) both from Randox Laboratories Ltd. (Crumlin, UK), albumin (Bromcresol green method), BUN (Urease/Glutamate dehydrogenase method), total bilirubin (Diazotized sulphanilic acid method), total cholesterol (Cholesterol oxidase/peroxidase method) and HDL-C (Direct detergent method) from BioSystems S.A. (Barcelona, Spain). Analyses were performed automatically by spectrophotometry (A15; BioSystems S.A., Barcelona, Spain). Glucose was measured in whole blood enzymatically (Glucose dehydrogenase, GDH-NAD method) using commercial test strips (Abbott Diabetes Care Ltd., Oxon, UK). Insulin concentration was determined by radioimmunoassay technique using a commercially available RIA kits (INEP, Zemun, Serbia) according to the manufacturer's guidelines. The Revised Quantitative Insulin Sensitivity Check Index (RQUICKI), commonly used for estimation of insulin sensitivity in dairy cows, was calculated as described by Pantelic et al. (2018).

Estimation of total antioxidant capacity in blood serum

The total antioxidant capacity (T-AOC) was evaluated by method of α , α -diphenyl- β -picrylhydrazyl (DPPH) radical scavenging ability. The estimation of T-AOC was done by measuring the reduction of DPPH radical spectrophotometrically according to Thaipong et al. (2006). 50 μ L of sample were mixed with 2.95 mL of DPPH solution and incubated in dark for 1 h. The percentage of reduction was monitored by measuring the DPPH absorbance at 515 nm (the extent of the solution discolouration is proportional to the concentration of total antioxidants in the samples). A standard curve was prepared using different concentrations of Trolox, and the results were expressed as micrograms of Trolox equivalents (TEq) per mL of sample.

Estimation of paraoxonase 1 activity in blood serum

The serum paraoxonase 1 (PON 1) activity was determined by measuring the initial rate of the synthetic paraoxon (O,O-diethyl-O-p-nitrophenyl phosphate) hydrolysis to p-nitrophenol. The absorbance was monitored at 412 nm in the assay mixture containing 2.0 mM paraoxon, 2.0 mM CaCl₂, 5 M NaCl, and 40 μ L of the serum sample. PON 1 activity was calculated using the molar extinction coefficient of phenol (18,500 M⁻¹cm⁻¹), and the results are presented as U/mL (Hashemi et al., 2012).

Colostrum samples and analyses

Colostrum samples were collected from each cow during the first milking, which was between 2 and 4 hours of calving. The samples were collected in two plastic bottles with a total volume of 300 mL for each cow, frozen and stored at -20°C until they were used to determine chemical compositions and colostrum IgG concentrations. The samples were also analysed for fat, protein, lactose, SNF and total solids. Prior to the analyses, they were thawed at room temperature and then diluted with a phosphate buffer solution of 1:3 and a pH of 7.4; this was done to reduce the samples' viscosities and to prevent technical difficulties

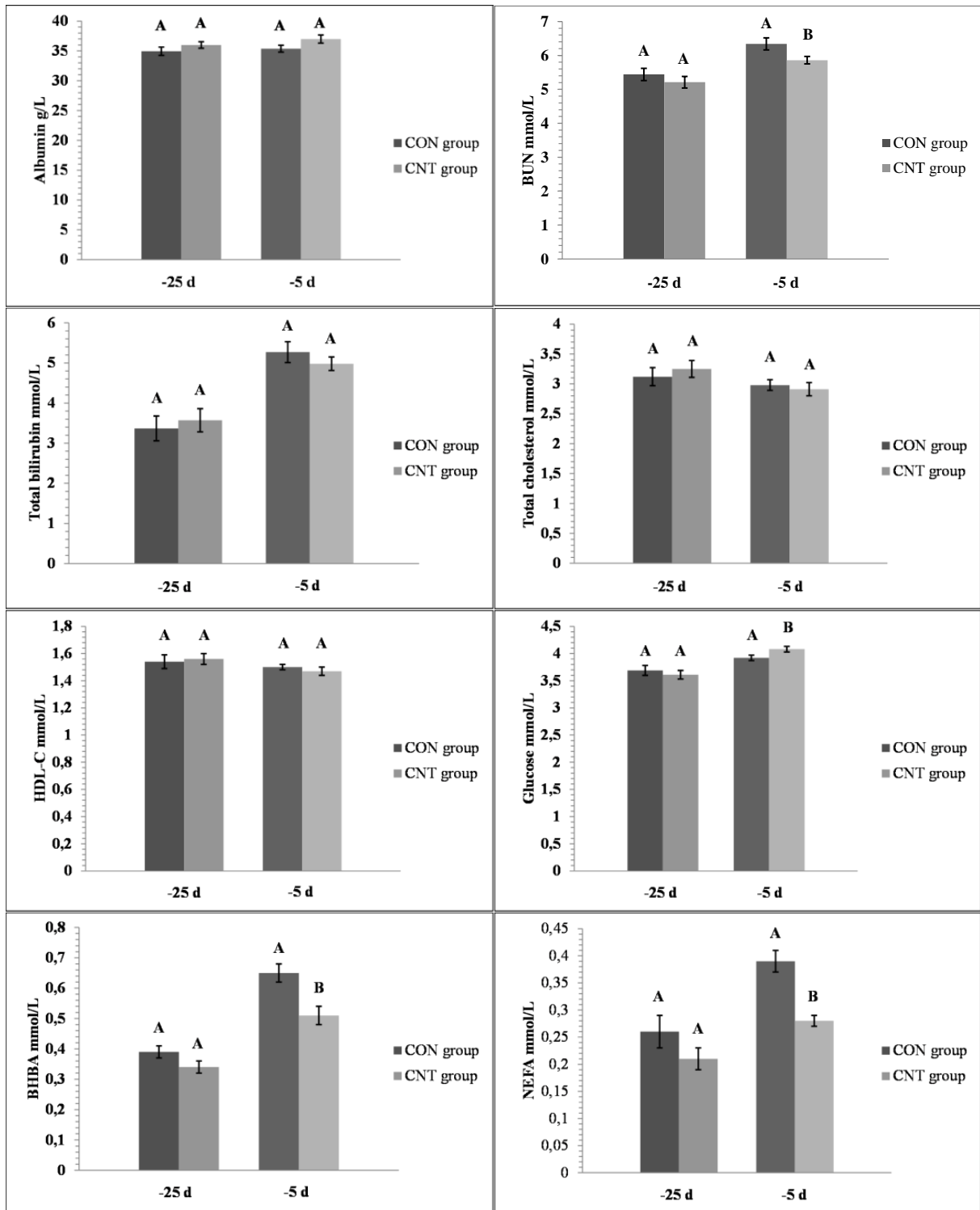
commonly encountered when using highly viscous samples. Content of fat, protein, lactose and total solids were determined using infrared technology, i.e., a LactoScope C4 (Delta Instruments, Drachten, the Netherlands). Colostrum IgG concentrations were determined using radial immunodiffusion (RID) assays conducted with commercially available bovine IgG RID test plates (Binding Site group Limited in Birmingham, the United Kingdom); this was done by following a method used by Stojić et al. (2017).

Statistical analyses

Data were analyzed using the Statistica v.8 commercial software (Stat Soft, Inc., Tulsa, OK, USA). The normality of data distribution was tested using the Shapiro-Wilk test. All data were normally distributed ($P > 0.05$) and presented as mean \pm SE (standard error) for all examined parameters. The significance of differences between the two groups, i.e. effect of treatment, was estimated with Student's t-test. Results were considered significant if $P \leq 0.05$ and trends were noted if $0.05 < P < 0.1$.

Results

The animals exhibited no clinical health problems or signs of tannin toxicity during the close-up period. During the trial period, no significant difference ($P = 0.32$) in average DMI was observed between CON (9.81 ± 0.05 kg of DM) and CNT group (9.72 ± 0.06 kg of DM). As shown in Figure 1, there was no effect of treatment i.e. CNT supplementation (comparison of values determined at d -5 relative to calving in both groups) on albumin, total bilirubin, total cholesterol, HDL-C concentrations as well as RQUICKI.



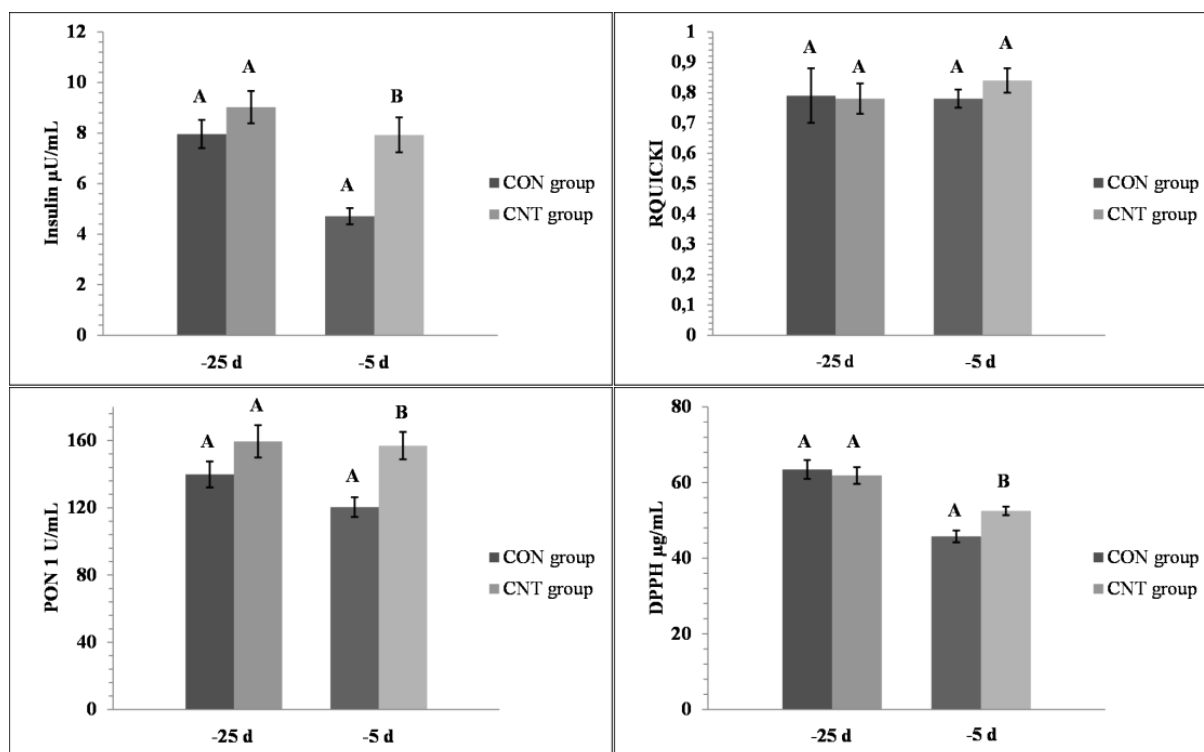


Figure 1. Indices associated with metabolic and antioxidant status in CON and CNT groups of cows at 25 and 5 days before expected parturition. Values are expressed as mean±SE.

■ - CON = not supplemented; □ - CNT = 1.96 g of chestnut tannins /kg of DM

A,B- Different uppercase determine significant difference (P<0.05) between CON and CNT groups at the same time

BUN, glucose, BHBA, NEFA and insulin concentrations were affected by the treatment. Namely, at d -5 relative to calving BUN concentration was lower (P=0.02), glucose concentration was higher (P=0.02), BHBA and NEFA concentrations were lower (P<0.01, respectively) and insulin concentration was higher (P<0.01) in CNT group compared to CON. At the same time, the addition of CNT had significant effect on PON 1 and DPPH leading to higher values in CNT group than those of CON at d -5 relative to calving (P<0.01 and P=0.03, respectively).

Table 2. The colostrum compositions of the CON and CNT cows (mean \pm SE).

Index	CON	CNT	<i>P</i>
Colostrum yield (L)	7.80 \pm 0.45	8.50 \pm 0.53	0.29
Fat (%)	6.31 \pm 1.30	6.28 \pm 0.79	0.98
Protein (%)	14.70 \pm 0.46	15.74 \pm 0.31	0.06
Lactose (%)	2.47 \pm 0.08	2.70 \pm 0.06	0.03
Total solids (%)	24.85 \pm 1.52	26.19 \pm 0.86	0.43
SNF (%)	18.54 \pm 0.59	19.92 \pm 0.40	0.06
IgG (mg/mL)	92.52 \pm 3.80	104.82 \pm 4.25	0.04

As shown in table 2, the addition of CNT had no significant effect on colostrum yield ($P=0.29$). Colostrum lactose percentage and IgG concentration were higher in CNT than in CON group ($P=0.03$ and $P=0.04$, respectively). Colostrum percentage of protein and SNF were not significantly affected by CNT supplementation ($P=0.06$, respectively) although their levels were higher in CNT group than those of CON.

Discussion

In the present study, the selected indices were within the normal range of variations that has been reported for healthy dry cows (Quiroz-Rocha et al., 2009; Brscic et al., 2015). The decreasing effect of the addition of tannins on the serum BUN was also found by Aguerre et al. (2016), Dschaak et al. (2011) and Sliwinski et al. (2002) who explain it by tannins suppressive effect on rumen protein degradation. The higher prepartal concentration of insulin, in response to plant extracts supplementation in the present and previous studies (Devant et al., 2007) might reflect metabolic changes toward gluconeogenesis and/or peripheral insulin resistance. However, the absence of differences in calculated RQUICKI, the

method used to measure insulin resistance in ruminants, do not support a compromised insulin resistant states in the CNT cows. Therefore, it is reasonable to suggest that the higher intestinal availability of glucogenic amino acids, and/or the reduced need for urea synthesis in the liver, i.e., the lower BUN, accounts, at least in part, for higher concentrations of glucose (Avila et al., 2014; Jayanegara et al., 2015; Noro and Wittwer, 2011) which subsequently stimulated pancreatic insulin secretion in CNT cows. Moreover, this adaptation helps explain the lower adipose lipolytic rate, i.e., the lower NEFA levels, and the lower concentrations of BHBA in the CNT cows on d -5 relative to calving. These results are in agreement with the observation of Senturk et al. (2015), who reported antiketogenic effect of tannins. The decreased circulating NEFA and BHBA levels could indirectly mitigate the adverse effects of these fatty acids on the antioxidant system and liver functions (Li et al., 2016). Further, PON 1 activity, a valuable part of the antioxidant system, is a reliable indicator for evaluations of oxidative stress and liver functions in periparturient dairy cows (Cao et al., 2017; Farid et al., 2013). There are several possible explanations for the differences in the PON 1 patterns of the CON and CNT groups. Less possible option is that the higher PON 1 activity observed in the CNT cows was a consequence of differences in liver fat deposition and/or liver dysfunction near parturition (Bionaz et al., 2007); the assembly and secretion of VLDL are enough to keep up with NEFA supply in late gestation (Prodanović et al., 2016). Only blood liver indices were used for this study, so little can be said about liver fat content; however, no differences were noted between the groups in regards to liver function indicators, i.e., albumin, total bilirubin and HDL-C. It could be argued that this would implicate more changes in the prooxidant/antioxidant status rather than liver function in these cows. In other words, a reduction in PON 1 activity in CON group near parturition might have been related to oxidative stress development. This is conceivable because elevated NEFA and BHBA concentrations are common factors in regards to provoking oxidative stress in late pregnancy

(Li et al., 2016), implying an increase in the production of free radicals and lipoperoxidative products which in turn might inactivate PON 1. This is supported by research conducted by Cao et al. (2017) and Farid et al. (2013), who found high correlations between NEFA, BHBA and PON 1 activity, and research by Liu et al. (2013), who found that CNT had inhibitory effects on lipid peroxidation. Addressing previously mentioned, the lower NEFA and BHBA in the CNT group on d -5 relative to calving were likely factors in the lower rate of PON 1 inactivity. Finally, the tannins could directly contribute to higher PON 1 activity in blood of the CNT fed cows. Change in serum PON 1 activity supported by higher T-AOC in the CNT group might reflect a change in regards to antioxidant capacity, which is in accordance with the results of Liu et al. (2013). Accordingly, improvement to the antioxidant status of the CNT cows appear to have been, at least in part, mediated by positive modulations of PON 1, which may have been caused by polyphenol mixtures of CNT extracts (Barreira et al., 2008; Costa et al., 2011). Nonetheless, despite the fact that prepartal DMI and insulin sensitivity did not differ between the groups, the significantly lower prepartum NEFA and BHBA levels, combined with the higher PON 1 activity and T-AOC in the CNT cows, provide an opportunity for designing dietary strategies for improving transition success.

Dietary approaches to minimize the periparturient metabolic disorders have the potential to promote colostrum quality and the acquisition of immunity in newborn calves (Stockdale and Smith, 2004; Mann et al., 2014; Toghiani and Moharrery, 2015). The improved metabolism of the CNT cows near parturition, indicated by the reduced NEFA, BHBA and BUN levels, may have involved decreased mobilisation of body fat. Thus, decreased risk of developing clinical or subclinical ketosis in these cows might have, at least in part, improved colostrum quality (Klimes et al., 1989; Mann et al., 2014). There is a lack of information in available literature concerning the effect of dietary tannins on colostrum composition in dairy cattle. Improvements to colostrum constituents and IgG concentrations

were observed in this study in response to the CNT supplementation during the close-up period. These results were in agreement with the findings of Rezai et al. (2012), who reported that increasing dietary RUP increased colostrum protein, lactose and IgG concentrations. However, the scope of the study did not allow for firm conclusions to be drawn regarding the higher lactose content of colostrum in the CNT cows than in the CON cows; despite this, increases in glucose production could not be excluded (Lin et al., 2016). The increased lactose content that was noted in the colostrum of CNT cows was not supported by findings reported by Jafari et al. (2018), who evaluated the effects of oak acorn on the colostrum compositions of goats. This disagreement could be related to differences in methodologies and/or tannin origins and supplementation levels (Jayanegara et al., 2015). The tendency towards higher colostrum protein and SNF percentages in the cows supplemented with CNT could be related to increased IgG concentrations (Aragona et al., 2016). Additionally, the improved IgG concentrations could have been reflections of the improved antioxidant status in the prepartal period (Lee et al., 2013; Moeini et al., 2011). Chestnut tannins extract has been reported to increase antioxidant capacities in plasma and liver (Liu et al., 2013), and this was corroborated by the higher PON 1 activity and T-AOC in CNT fed cows, which in turn might have promoted colostrum quality.

Finally, the direct positive effects of tannins on the diverse immune and inflammatory cell functions should be considered (Malisan et al., 1996; Wu et al., 2019). The higher IgG levels in the colostrum of CNT cows, compared to that of CON cows, could be attributed to an enhancement of the effects of tannins on IgG production caused by stimulating IL-10 induced signalling (Malisan et al., 1996; Zhong et al., 2014).

Conclusions

The present study showed that chestnut tannins in close-up diets have the potential to improve the colostrum quality and the metabolic and antioxidant status of cows near parturition. Further research into the implications of this dietary approach is needed in regards to metabolic adaptation as carryover effects can persist into early lactation.

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