Feeding tannins to dairy cows in different seasons improves the oxidative status of blood plasma and the antioxidant capacity of cheese

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ABSTRACT

The aim of the present study was to assess the dietary supplementation of tannins to grazing dairy cows in 2 seasons characterized by a good quality pasture (spring) or a poor-quality pasture (summer). The effects of dietary tannins were assessed on plasma antioxidant status and cytokines profile and on the antioxidant properties of cheese and cheese in vitro digestates. Fourteen lactating dairy cows were divided into 2 homogeneous groups (n = 7): a control group (CON), and an experimental group (TAN) receiving 150 g/head per day of tannins supplementation. The experiment was performed twice, in spring and in summer. The animals were free to graze on spontaneous pasture (spring) or on dry stubble (summer). Blood was sampled at the beginning (d 0), at the midpoint (d 11), and at the end (d 22) of the trial. Individual cheese was produced before the beginning (d −1) and at the end (d 22) of the trial from the milk collected from each cow. On blood plasma, the reactive oxygen metabolites (ROM), biological antioxidant potential (BAP), nonesterified fatty acids quantification, and cytokines profile in terms of IL-10, IL-8, IL-1β, and IFN-γ were determined. Data on ROM demonstrated that tannins supplementation lowered oxidative stress both in spring and in summer. Accordingly, TAN diet increased BAP levels compared with the CON during summer trial. Thus, feeding tannins resulted in lower ratio between ROM and BAP (oxidative stress index) in both spring and summer. Cytokines’ profile showed lower IL-1β values in TAN group at d 22 during spring season, with a concomitant higher IL-10 level, during summer season. Moreover, TAN group had a lower level of IFN-γ in plasma than CON group, both in spring and in summer. On cheese samples, the in vitro digestion was performed and on cheese and cheese digestates (gastric and intestinal digestate) the free radical scavenging antioxidant activity was evaluated. The intestinal digestate fraction registered the highest antioxidant activity compared with cheese and gastric digestate, in both spring and summer seasons. Furthermore, an improvement of the antioxidant property of cheese and cheese digestates was found. Present data demonstrated that tannins supplementation contributed to reduce the oxidative stress of lactating dairy cows and showed an increase of anti-inflammatory cytokines ratio. Key words: immunity, cheese quality, cheese digestate antioxidant capacity, pasture

INTRODUCTION

Tannins from plant feed resources used for livestock feeding have been reported to affect several important aspects of dairy cow management, such as feed intake and digestion, methane emission, ruminal fermentation (D scaff et al., 2011), as well as milk quality traits, especially in terms of milk fatty acid profile (Benchaar and Chouinard, 2009; Frutos et al., 2020). Some plant compounds, particularly phenols, are known to be resistant to the rumen microbial degradation and could reach the small intestine in a biologically active form. It has been reported that postruminal administration of plant derived bioactive compounds appeared to have an immune-stimulatory effect by activating and inducing the expansion of CD4 cells in dairy cows (Oh et al., 2017). Moreover, Asiamah et al. (2016) suggested that Sericea lespedeza water extract, a tannins rich legume, can affect gene transcription and translation involved in innate and adaptive immunity of cow, sheep, and goat. In particular, toll-like receptor (TLR)2, TLR4, and the promotion of wingless (WNT) signaling-dependent process may serve as the molecular basis for the immunomodulatory properties of Sericea lespedeza in response to different pathogen associated molecular patterns. The in vivo antioxidant activities of tannins were observed in rabbits (Liu et al., 2011), lambs (Luciano
et al., 2011; López-Andrés et al., 2013), and transition dairy cows (Liu et al., 2013).

Limited information is available on the in vivo antioxidant and immune properties of tannins supplementation in dairy cows. Moreover, it has been demonstrated that there were significant interactions between antioxidants and extreme environmental conditions, such as heat stress (Dunshea et al., 2013). Heat stress was found to impair animal performance, and it was implicated in promoting oxidative stress either enhancing reactive oxygen species production or decreasing antioxidant defenses (Chauhan et al., 2014a). Dietary supplementation of sheep with high levels of vitamin E and Se ameliorated the negative effects of heat stress on feed intake, respiratory physiology, rectal temperature, and oxidative stress (Chauhan et al., 2014a,b).

Although there is sufficient evidence in literature suggesting degradation of hydrolyzable tannins by rumen microorganism (McSweeney et al., 2001; Makkar, 2003), the fate in the gastrointestinal tract of ruminants of condensed tannins is still controversial. Several reports showed that condensed tannins do not get hydrolyzed by rumen microbes (Makkar et al., 1995), others suggest that condensed tannins may be partially absorbed or degraded in sheep and goat (Terrill et al., 1994; Perez-Maldonado and Norton, 1996) and dietary supplemented phenol compounds may pass the intestinal barrier and may be transferred to milk and cheese (Hilario et al., 2010). Indeed, polyphenols could interact with milk proteins, particularly proline-rich proteins such as α-CN and β-CN, via both hydrophilic and hydrophobic interactions (Lamothe et al., 2014).

The present study is part of a previously published experiment that evaluated the effect of dietary tannins supplementation in grazing dairy cows on milk and cheese quality during spring and summer (Menci et al., 2021a). According to that study, the seasonal variation of the diet characterizing pasture-based farming systems influenced the effect of dietary tannins on milk and cheese quality. In particular, the positive effect of dietary tannins was enhanced in summer, when green pasture is not available, and diet is relatively poor in crude protein and antioxidant vitamins.

Our hypothesis is that supplementation with tannins can have beneficial effects on the health of the dairy cow and on some health-promoting characteristics of the cheese. In addition, we hypothesized that the effects of dietary tannins on cow health and cheese antioxidant capacity could be influenced by the seasonal variation of diet and weather. Therefore, the present study aimed at evaluating (1) the effect of tannins supplementation on oxidative stress and cytokines profile in lactating dairy cow in spring and summer under Mediterranean climate, and (2) the antioxidant capacity of cheese and cheese digestates for the possibility of conveying antioxidant bioactive compounds from diet to cheese produced.

MATERIALS AND METHODS

All procedures were approved by the animal welfare committee (OPBA) of the University of Catania (UNCTCLE-0015295).

Animals, Experimental Protocol, and Milk Sampling

The study was conducted on a dairy farm located in Ragusa (Sicily, Italy) and lasted 23 d in spring (March–April mean temperature 10°C) and 23 d in summer (July mean temperature 24.5°C). In both seasons, 14 dairy cows (Modicana breed) were divided into 2 groups, balanced for milk yield [spring: 11.9 ± 1.5 kg/d (± SE); summer: 13.6 ± 2.6 kg/d], DIM (spring: 190 ± 38 d; summer: 137 ± 43 d), number of lactation (spring: 4 ± 1; summer: 4.2 ± 1), and BCS (spring: 2.8 ± 0.2; summer: 3.6 ± 0.1).

The animals were enrolled in the study and assigned to 2 dietary treatments in a randomized control study. The dietary treatments were control group (CON, n = 7), with no additional supplement, and tannins group (TAN, n = 7) supplemented with 150 g/head per day of tannins extract composed by a mixture of hydrolyzable and condensed tannins from chestnut and quebracho (60:40), respectively (ByPro Silvateam). The dose of tannin supplementation was equal to 1% of estimated DMI. Total phenolic compounds concentration in tannins extract was 688 g of tannic acid equivalents per kilogram of DM, with 90.2% of tannins, according to the method of Makkar et al. (1993).

Animals were free to graze in 20 Ha of spontaneous pasture (spring) or 20 Ha of dry stubble (summer) and had free access to drinking water. In addition, all the animals received the same concentrate at a rate of 6.4 and 9.6 kg/head per day in spring and summer, respectively. The basal diet of the animals was already used on farm before the beginning of the experiment. Cows were individually milked twice a day, at 0700 and 1700 h, using a milking machine. Detailed experimental design, diets chemical composition, and composition and antioxidant activity of milk are reported and discussed in the companion paper of Menci et al. (2021a).

Blood Samplings and Oxidative Marker Assays

Blood sampling collection was performed at 3 time points (d 0, 11, and 22) for both spring and summer trials. Blood samples were obtained by the middle caudal venipuncture and collected in vacutainer tubes with
Na-heparin anticoagulant, between 1700 and 1730 h at evening milking. Plasma was collected by centrifugation at 1,500 × g for 10 min at 25°C, rapidly stored at −80°C until analyses on cytokines profile, reactive oxygen metabolites (ROM), biological antioxidant potential (BAP), and nonesterified fatty acids (NEFA) quantification.

Plasma ROM were determined by a colorimetric assay (d-ROMs Test, Diacron International), following the manufacturer’s instructions. Briefly, the ROM test was considered a specific marker of oxidative damage measuring the concentration of hydroperoxides that in the presence of free iron, can generate free radicals. Results are expressed in Caratelli units (UCarr), where 1 UCarr is equivalent to the oxidizing power of 0.08 mg of H2O2/dL. Plasma antioxidant activity was assessed by a colorimetric assay using the BAP test (Diacron International), as recommended by manufacturer’s instructions. The principle of this test is based on the plasma BAP capacity to reduce ferric (Fe3+) to ferrous (Fe2+) iron. The results are expressed in micromoles per liter of reduced iron. Both ROM and BAP were measured using a spectrophotometer (PowerWave XS, Biotek). In addition, a better indication of redox balance was obtained with the combination of the ROM/BAP in the oxidative stress index (OSi), as previously proposed by Celi (2011) for the measurement of the oxidative stress degree.

Plasma NEFA were analyzed using a commercial kit according to manufacturer’s instructions (Free Fatty Acid Quantitation Kit, Sigma-Aldrich).

**Determination of Cytokines in Plasma Supernatants by ELISA**

The concentration of IL-10, IL-8, IFN-γ, and IL-1β in plasma were determined by ELISA, according to Ciliberti et al. (2020, 2017). Briefly, the 96-well microtiter plates (Sterilin) were coated overnight at 4°C with 100 μL of mouse anti-ovine IL-8 antibody (Kingfisher Biotech), antibovine IL-10 (AbD Serotec), antibovine IFN-γ (AbD Serotec), and rabbit polyclonal anti-ovine IL-1β (Kingfisher Biotech). After each step, the plates were washed for 4 times with PBS, 0.05% Tween 20 (PBST). The blocking solution constituted by PBST and bovine serum albumin at 3% was added into all wells for 1 h to block nonspecific binding. Then, standards and samples were added into wells and incubated for 1 h. Biotinylated antibovine IFN-γ antibody (AbD Serotec), rabbit antisheep IL-8 (AbD Serotec), biotinylated antibovine IL-10 (Serotec), and rabbit polyclonal anti-ovine IL-1β (AbD Serotec) were added to plates. Finally, 100 μL of streptavidin-horseradish peroxidase (AbD Serotec) was added for IL-10 and IFN-γ assay, respectively, whereas, for IL-1β and IL-8 the sheep antirabbit IgG horseradish peroxidase (HRP) conjugated antibody (Sigma-Aldrich) was added into wells. The 3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma-Aldrich) substrate solution was added to each well and the colorimetric reaction was stopped adding 50 μL of H2SO4 (2 M).

All plates were read at 450 nm by a spectrophotometer (Power Wave XS, Biotek). Secretion of cytokines in plasma was calculated against a standard curve generated using a specific recombinant protein for each interleukin, data were expressed as nanograms per milliliter for IL-10, IL-8, and IL-1β, whereas IFN-γ levels were expressed as picograms per milliliter. The intra-assay coefficient of variation was around 10%.

**Cheese Production**

Cheeses were produced the day before the beginning (d −1) and at the end (d 22) of each seasonal trial, using individual raw whole milk according to the protocol described in the companion paper by Menci et al. (2021b). Briefly, a batch of 7 kg of milk from each cow enrolled in the experiments was heated at 38°C and commercial liquid veal rennet was added at a final concentration of 37 international milk clotting units/L. After 1 h, the curd was cut into small cubes of 5 to 10 mm, drained, and cooked at 75°C in hot water. Curds were brine salted (300 g/L NaCl, for 2 h at 10°C) and then ripened under controlled parameters (10°C, 80% relative humidity) for 25 d. Ripened cheese was portioned, vacuum packaged, and stored refrigerated until analyses.

**Enzymatic Activities in Cheese**

Plasmin (PL) and plasminogen (PG) activities in cheese were determined according to the method of Richardson and Pearce (1981) with some modification. Briefly, 5 g of grated cheese was added to 20 mL of 0.4 M sodium citrate (pH 8.5); after equilibration at 38°C for 15 min, the mixture was homogenized in a Stomacher Lab-Blender 400 (PBI International) for 5 min. Then, the PL and PG activities were measured according to the method of Baldi et al. (1996); in which the dissociation of PL and PG from cheese casein micelles was obtained by incubation with 50 mM of ε-aminocaproic acid for 2 h at room temperature (Ko-rycka-Dahl et al., 1983). The reaction mixture was of 250 μL of 0.1 M Tris-HCl buffer (pH 7.4), 0.6 mM Val-Leu-Lys-p-nitroanilide (V7127; Sigma Chemical Co.), 30 plough units (2.5 μL) of urokinase (U0633; Sigma Chemical Co.), and 30 μL of cheese serum. The PL activity was determined in the same reaction mixture.
without added urokinase. The PG-derived activity was the difference (PL+PG) − PG. A similar reaction mixture without sample was used as a control. The reaction mixture was incubated at 37°C for 3 h, and A405 was measured at 30-min intervals with a microtitre plate reader (Anthos 2020, version 1.0; Diessechem). One unit of PL or PG activity was defined as the amount of the enzyme that produces a change in absorbance at 405 nm of 0.1 in 60 min.

The pH 4.6-insoluble nitrogen fractions of cheeses were analyzed by urea-PAGE using a Protean II xi vertical slab gel unit (BioRad) in a continuous buffer system. The gels were stained according to the method of Blakesley and Boezi (1977) with Coomassie Brilliant Blue G250. The destained gels were acquired by the Gel Doc EQ system (BioRad) using a white light conversion screen and analyzed with the Quantity One software (BioRad) to determine the signal intensity (optical density) of the defined bands. Identification of bands was performed by comparison with the NaCN standard, with the sum of the intensity of the defined bands in a lane set at 100%, the relative quantity of each band was determined as the percentage of the signal intensity of the defined band in a lane. Data on β-CN and γ-CN were used to determine the γ/β CN ration.

Cheese In Vitro Digestion

The simulated in vitro digestion model on cheese samples was performed according to Minekus et al. (2014) with some modification. The sample was minced to simulate the chewing mouth with a mincer. During the simulation of oral phase, 5 g of cheese sample, oral phase stock solution, CaCl₂, and 975 μL of water were mixed. During gastric phase simulation, 10 mL of liquid cheese samples after oral phase (oral bolus) were mixed with 7.5 mL of electrolyte stock gastric fluid, 1 mL of porcine pepsine (25,000 U/mL stock solution) in simulated gastric fluid (SGF) electrolyte stock solution (pepsin from porcine gastric mucosa 3,200–4,500 U/mg, Sigma), 5 μL of CaCl₂ (0.3 M), and x-mL of HCl (1 M) to reach pH 3 and water up to 20 mL. Time of gastric digestion was 2 h at 37°C. During the last phase, the intestinal phase, 20 mL of samples after gastric phase (gastric chime) was mixed with simulated intestinal fluid (SIF) electrolyte solution, 4.0 mL of a pancreatin solution 800 U/mL made up in SIF electrolyte stock solution based on trypsin activity, 2.5 mL of fresh bile (160 mM), 40 μL of CaCl₂ (0.3 M), x-mL of NaOH (1 M) to reach pH 7.0 and water up to 40 mL. Time of intestinal digestion was 2 h at 37°C. After the final step the sample was snap-frozen immediately and stored at −80°C until performing the subsequent analysis.

Statistical Analysis

Statistical analysis was conducted using SAS, University Edition (SAS Institute Inc.). Data from spring and summer trials were statistically analyzed separately. Data on blood parameters were analyzed using ANOVA for mixed models using the MIXED procedure of SAS, having the treatment (CON or TAN), time of sampling (d 11 or 22), and their interaction as fixed effects, and the animal as random factor. Data on cheese parameters was analyzed using one-way ANOVA with the treatment (CON or TAN) as fixed factor. The measures on blood and cheese sampled at the beginning of the experiments were used as covariates. Linear simple correlations between cytokines, BAP, ROM, NEFA according to the treatment, time of sampling and season were investigated using PROC CORR of SAS. The interaction between treatment and time of sampling did
not result in any significant results, therefore it was removed from the statistical model. Significant differences among treatments were declared at $P<0.05$.

**RESULTS**

**Animal Blood Parameters: Oxidative Markers and Cytokine Profile**

Results on blood oxidative markers of the grazing cows subjected to CON and TAN diet evaluated by ROM, BAP, OSi, and NEFA are presented in Table 1. In both spring and summer trials, ROM were significantly lower in TAN than in CON, with significant differences emerging from d 11. Results on BAP evidenced higher levels in TAN group compared with the CON in the summer trial on d 11 and d 22 of the experimental diet supplementation. The ratio values of ROM on BAP were significantly different between the treatments, with lower values in TAN than CON in both spring ($P<0.05$) and summer ($P<0.01$) trials.

In the present study, values of blood NEFA did not differ significantly between the CON and TAN treatment during both spring and summer season.

Results on immunological status determined by plasma IL-10, IL-8, IL-1β, and IFN-γ cytokines are presented in Table 2. During spring trial, lower IL-1β values ($P<0.01$) were found in TAN on d 22 of the trial than CON group; whereas, at the same time point in the summer trial, the levels of IL-10 were higher in TAN than CON group ($P<0.001$). Tannins supplementation led to lower levels of IFN-γ in TAN than in CON group, both in spring and in summer trials. In particular, both in summer and in spring seasons, the IL-1β/IL-10 and IFN-γ/IL-10 ratios were significantly ($P<0.01$) reduced by tannins supplementation.

**Antioxidant Activity of Cheese and Cheese Digestate**

The changes in antioxidant activity of cheese and gastric and intestinal digestates expressed as DPPH free radical scavenging activity are shown in Figure 1.
Compared with vitamin E, the cheese made from milk of cows fed with tannins (Figure 1A, cheese antioxidant activity) showed higher ($P < 0.05$) antioxidant activity in both spring and summer season, with 50 and 70% of DPPH inhibition, respectively, compared with the control cheese showing 32 and 54% in spring and summer, respectively. The gastric (Figure 1B) and intestinal (Figure 1C) digestates showed higher ($P < 0.05$) percentage of DPPH inhibition in TAN treatment, only for cheese produced in the summer trial, with a free radical reduction of 43% for gastric and 80% for intestinal digestate, compared with the CON group showing 33 and 46%, respectively. Whereas, in spring season the scavenging activity levels were comparable between control and tannins treatment ($P > 0.05$).

**Cheese Plasmin System**

Plasmin activity, plasmin plasminogen-derived activity, and the ratio of PG/PL in cheese made from individual milk of cow supplemented dietary tannins after 25 d of ripening are presented in Table 3. Cheese made from the milk of cows receiving CON diet showed significantly higher plasmin, plasmin plasminogen-derived activity and PG/PL than cheese made from milk of the TAN group in both spring and summer trials. Figure 2 showed the $\gamma/\beta$ ratio in cheese manufactured in spring and summer season, using milk from cows receiving a control diet and a diet supplemented with tannins. Although no significant differences were found in the optical density of the principal casein fractions of the individual cheeses ripened 25 d, values of $\gamma/\beta$ ratio turned out to be almost doubled in summer cheese compared with spring cheese.

**DISCUSSION**

**Animal Blood Parameters: Oxidative Markers**

The plasma level of ROM, that includes radical and nonradical oxygen species, is considered an indicator of total oxidative stress status (Celi, 2011). Levels of ROM registered in the present research were in accordance with previous reports in mid-lactating cows (Abuelo et al., 2013), where the authors highlighted that ROM levels progressively increase during lactation period. The study of serum oxidative stress biomarkers in dairy and beef cows in a daytime grazing system showed lower serum ROM together with increased serum $\alpha$-tocopherol and $\beta$-carotene concentrations in the time of the year characterized by rich pasture and fresh forage availability (Mirzad et al., 2017).

The fate of condensed tannins upon digestion is controversial. The polymeric nature and high molecular weight of these tannins should limit their absorption (Manach et al., 2005); however, the direct antioxidant activity of a dietary compound would imply its absorption along the gastrointestinal tract and deposition in the tissues (Vasta and Luciano, 2011). Local activity in the gastrointestinal tract has been proposed as an indirect antioxidant mechanism for the phenolic
compounds, which are poorly absorbed in the digestive tract, potentially resulting in improvements of the animal’s antioxidant status (Halliwell et al., 2005) increasing the expression and activity of endogenous antioxidant enzymes (Sgorlon et al., 2006). Thus, it could be hypothesized that the lack of differences in BAP during the spring season may be likely attributed to the antioxidant compounds supplied by available pasture that sustained the antioxidant potential in the serum of the grazing cows during the spring season. Likewise, when investigating the quality of milk from the very same experiment of the present study, Menci et al. (2021a) found dietary tannins to improve the antioxidant capacity of milk from grazing cows only in summer, whereas they observed no effect in spring. The effect on milk could be related to the effects on blood parameters found in the present study. Anyway, no matter the grazing season, tannins supplementation affected the antioxidant status of dairy cows, resulting in a significant change in plasma antioxidant concentrations, and played a role in mitigating the oxidative stress, especially during summer season when pasture provides a lower intake of antioxidant compounds.

The BAP may be decreased as a consequence of increasing ROM concentration. Accordingly, changes in the antioxidant complex are often the result of the oxidative stress causing a depletion of antioxidant availability (Celi, 2011). To obtain more accurate information about the oxidative status of dairy cows, ROM and BAP values were combined to obtain the OSI parameter. The result on the ratio values of ROM on BAP highlights that the combined evaluation of oxidants and antioxidants is more accurate to interpret the complex effects of tannins supplementation and pasture availability. Therefore, it can be argued that tannins supplementation exerted a boosting activity in balancing the oxidative status of grazing dairy cows. Blood NEFA concentrations have been found to be an accurate measure of negative energy balance and a strong indicator of disease, reproductive performance, and milk production used especially in transition period (McArt et al., 2013). In the present study, the NEFA concentrations were always found lower than 0.6 mEq/L, which is set as a threshold for prediction of clinical diseases in transition dairy cattle (Ospina et al., 2010). Furthermore, this result was in accordance with concentrations found in mid-lactating dairy cows supplemented or not supplemented with Yerba Mate tea as source of dietary antioxidants (Celi and Raadsma, 2010).

**Animal Blood Parameters: Cytokine Profile**

There are many factors able to promote cytokines expression in vivo, including immune complexes, local complement activation, heat stress, lactation stages, microbial species and their soluble products, reactive oxygen species, trauma, and cytokines in autocrine loop (Mohamed Ibrahim et al., 2016).

Dietary antioxidants can have a modulatory role on inflammatory response via indirect and direct mechanisms; the indirect one involves the enhancement of antioxidant defense or silencing some oxidative stress signaling. The direct action is characterized by the suppressive action on the pro-inflammatory signaling

![Figure 2](image_url). The γ/β casein ratio in cheese made from the milk from cows subjected to different diets in spring and summer. Error bars represent SEM. Control = cows fed with no additional supplement; tannin = cows fed with control diet plus 150 g/cow per day of tannins.

| Table 3. Mean values of cheese enzymatic activity made by cows subjected to different diets in spring and summer1 |
|---------------------|-----|-----|-----|---------------------|-----|-----|-----|
| Item                |     |     |     | Item                |     |     |     |
|                     | CON | TAN | SEM | P-value2            | CON | TAN | SEM |
| PL, U/g             | 4.83| 1.73| 0.63| **                  | 10.32| 5.02| 0.61| *    |
| PG, U/g             | 19.43| 4.75| 1.67| *                   | 3.47 | 0.20| 0.19| *    |
| PG/PL, U/g          | 4.02| 2.75| 0.34| *                   | 0.34 | 0.04| 0.02| *    |

1PL = plasmin; PG = plasminogen; PG/PL = plasminogen/plasmin; CON = cows fed with no additional supplement; TAN = cows fed with control diet plus 150 g/cow per day of tannins.

2Significance declared at \( P \leq 0.05 \). *\( P < 0.05 \); **\( P < 0.01 \).
transduction (Zhang and Tsao, 2016). Cytokines can be divided into Th1 type immunity, including IFN-γ, IL-2, IL-12, and tumor necrosis factor (TNF-α) and Th2 characterized by IL-4, IL-5, IL-10, and IL-13 (Viallard et al., 1999; Janeway et al., 2001). It has been demonstrated that polyphenols, such as tannins, were able to suppress the Th1-type immune response (Schroechsnelad et al., 2007). Indeed, IL-10 is defined as an anti-inflammatory cytokine and is considered the key antagonist of Th1 response by regulating cytokine production and acting on posttranscriptional mechanisms (Moore et al., 2001), thus suppressing IFN-γ and other pro-inflammatory cytokines (O’Shea et al., 2002). In the present paper, the key regulatory role of IL-10 was confirmed by the IL-1β/IL-10 and IFN-γ /IL-10 ratios as a measure of activation of immune cells reflecting the balance or imbalance of the immune profile (Santillo et al., 2012). In particular, both in summer and in spring seasons, the IL-1β/IL-10 and IFN-γ / IL-10 ratios in TAN group confirmed the suppressing action of tannins on Th1 cytokines.

Notably, the hypothesis of a bimodal regulation between inflammatory response and oxidative stress was corroborated by the significant negative correlation between ROMs and IL-10 (−0.67, P < 0.001), found in both spring and summer trials. The reduction of oxidative stress can lead to a decrease in inflammatory cytokines. Indeed, when bovine mammary epithelial cells were treated in vitro with (-)-Epigallocatechin-3-gallate, the major phenolic compound of green tea, a decrease in gene expression of the main pro-inflammatory cytokines and an increase in IL-10 was observed (Basiricò et al., 2019). Moreover, beef steers supplemented with Pinus taeda hydrolyzed lignin, a source of phenolic compounds, resulted in a control of inflammatory responses due to a decrease in TNF-α and reactive oxygen species production with a concomitant increase in IL-8 secretion in peripheral blood mononuclear cells supernatants (Ciliberti et al., 2020). On the contrary, our data did not support a different IL-8 secretion after TAN supplementation. In addition, Aitken et al. (2009) showed that changes in the expression of several antioxidant factors were correlated with changes in the expression of adhesion molecules and some pro-inflammatory cytokines, during the transition period in bovine mammary tissue. In the present research, the stress induced by the high temperature exposure during summer, may have sustained the protective effect of tannins supplementation on the immune status of mid-lactating dairy cows.

Collectively, all these findings support that tannins supplementation can enhance plasma antioxidant capacity, reducing the oxidative stress and the pro-inflammatory cytokines profile; this relationship may play a role in sustaining mid-lactating dairy cows in extensive production systems, particularly during summer season and when, in Mediterranean areas, animals might be exposed to chronic mild heat stress and low-quality pasture represent the main component of the diet. In such conditions dietary tannins may contribute to prevent a possible immune depression.

**Antioxidant Activity of Cheese and Cheese Digestate**

The comparable antioxidant activity found in the spring season, for both gastric and intestinal digestates, could be related to the high quality of the basal diet, composed by green pasture which likely provided high levels of antioxidants. As reported in the companion paper, Menci et al. (2021a) found that dietary tannins did not affect milk antioxidant capacity in the spring experiment, whereas tannins supplementation increased the antioxidant capacity of the hydrophilic fraction of summer milk assessed as reducing power (ferric reducing antioxidant power, FRAP) and radical scavenging capacity (Trolox-equivalent antioxidant capacity, TEAC). However, the high content of TEAC and FRAP found in milk from the spring experiment was explained by the contribution of grazing pasture to the antioxidant content of milk without any additional synergic effect mediated by tannins supplementation. The green grass is typically able to supply an adequate level of antioxidants for dairy cows; according to this result, previous study showed that pasture-based systems improve the animal oxidative status due to the antioxidant substances content of green grass, such as vitamin A and E (Gatellier et al., 2004). In a study of Descalzo et al. (2005), animals fed with pasture showed the same level of antioxidants in plasma compared with animals fed with pasture plus vitamin E, thus, the pasture only was able to supply the same quantity of antioxidant than antioxidant-supplemented diet.

Branciari et al. (2015) verified that polyphenols in rosemary leaves underwent slight modifications due to animal metabolism, contributing to increase the total phenolic content and the antioxidant properties in cheese. The different antioxidant capacity between gastric and intestinal phase may be related to different levels of tannins-derived bioactive compounds released. As suggested in a study of Bhatt and Patel (2013), a gradual release of polyphenols occurs during the digestive process, passing from the gastric to the intestinal tract. Moreover, the molar antioxidant capacity of condensed tannins is directly proportional to the pH values (Riedl and Hagerman, 2001); in the present research, the greater free radical reduction detected in the intestinal digestate than the gastric digestated in the TAN group, may be likely ascribed to the higher
pH that characterize the SIF than the SGF. The digestion process generally affects the composition of cheese digestate, depending on the physiological conditions and the sequence of events within the gastrointestinal tract (Pavan et al., 2014). Our study demonstrated that after digestion, the antioxidant activity of cheese, in terms of free radical scavenging activity, was increased after digestion, the antioxidant activity of cheese, in tract (Pavan et al., 2014). Our study demonstrated that and the sequence of events within the gastrointestinal digestion process generally affects the composition of cheese pH that characterize the SIF than the SGF. The diges-

**Cheese Plasmin System**

Plasmin is the predominant and most completely studied endogenous protease in milk; it is part of a complex protease–protease inhibitor system that consists of active and inactive forms of the enzyme, activators, and inhibitors (Ismail and Nielsen, 2010). Furthermore, in cheese, plasmin activity can be involved in flavor and texture development being one of the proteolytic agents in the ripening process.

Lower plasmin activity found in TAN cheese may be the result of different mechanisms. Tannins partly undergo to enzymatic hydrolysis and depolymerization in the gastrointestinal tract (Huang et al., 2018), thus low molecular weight tannins-derived molecules may be available for absorption. A direct activity of dietary tannins on cheese plasmin system cannot be ruled out, probably due to formation of complex between phenolic compounds and endogenous protease system. Furthermore, phenolic compounds ingested upon graze feeding during spring trial may have enhanced the inhibitory effect on plasmin system. Recently, Yildirim-Elikoglu and Vural (2019) assessed the ability of polyphenols to inhibit plasmin in milk using a molecular and kinetic approach. In particular, epicatechin gallate, epigallocatechin gallate, quercetin, and myricetin were found to significant decrease plasmin activity by 60, 86, 65, and 90%, respectively, and it was observed that the phenolic-plasmin interactions were dominated by H-bonds and electrostatic attractions leading to signific-

an indirect effect on plasmin activity was related to the reduction in IMI.

It was reported that plasmin and its zymogen in blood are inactivated by extracellular release of oxidants, such as Cu (II) and ascorbate (Lind et al., 1993), and the author indicated the active site His residue as the possible target of oxidative inactivation. The PG/PL ratio permits to evaluate the activation degree of the enzyme system by the balance between the zymogen and the active enzyme in the cheese matrix. Plasmin system seemed to undergo lower activation in cheese made during spring as highlighted by higher level of plasmin plasminogen-derived activity, which, in turn, affected the PG/PL ratio. Under the present experimental conditions of cheese manufacturing, proteolysis in cheese could be ascribed to different agents as the residual rennet activity, proteolytic enzymes both of endogenous and exogenous origin: the γ/β ratio in the cheese after 25 d of cheese ripening may be considered an outcome of PL activity in the cheese matrix, being plasmin, the main proteolytic enzyme involved in β-CN hydrolysis yielding γ-CN and proteose-peptone in the cheese matrix.

However, the effect of dietary tannins supplementation on cheese plasmin system did not result in a differ-

c Chees e Plasmin System

Plasmin is the predominant and most completely studied endogenous protease in milk; it is part of a complex protease–protease inhibitor system that consists of active and inactive forms of the enzyme, activators, and inhibitors (Ismail and Nielsen, 2010). Furthermore, in cheese, plasmin activity can be involved in flavor and texture development being one of the proteolytic agents in the ripening process.

Lower plasmin activity found in TAN cheese may be the result of different mechanisms. Tannins partly undergo to enzymatic hydrolysis and depolymerization in the gastrointestinal tract (Huang et al., 2018), thus low molecular weight tannins-derived molecules may be available for absorption. A direct activity of dietary tannins on cheese plasmin system cannot be ruled out, probably due to formation of complex between phenolic compounds and endogenous protease system. Furthermore, phenolic compounds ingested upon graze feeding during spring trial may have enhanced the inhibitory effect on plasmin system. Recently, Yildirim-Elikoglu and Vural (2019) assessed the ability of polyphenols to inhibit plasmin in milk using a molecular and kinetic approach. In particular, epicatechin gallate, epigallocatechin gallate, quercetin, and myricetin were found to significant decrease plasmin activity by 60, 86, 65, and 90%, respectively, and it was observed that the phenolic-plasmin interactions were dominated by H-bonds and electrostatic attractions leading to significant changes in the secondary structure of plasmin. A further explanation for the results on plasmin system may be attributed to an indirect effect of dietary tannins, able to preserve other antioxidants during digestion (Menci et al., 2021a). In lactating cow, vitamin E supplementation has been found to decrease plasmin activity as a consequence of increased oxidative stability of milk (Politis, 2012). Notably, the author clarified that vitamin E increases gene expression and amount of the enzyme urokinase-plasminogen activator present on the cell membrane of neutrophils. This enzyme is critical for the ability of neutrophils to extravasate and reach the mammary gland during inflammation. Thus,
lighting that antioxidant molecules from the diet may modulate those in milk and dairy products, conferring to dairy products a boosting antioxidant property. Overall, the manipulation of key antioxidant ingredients in animal diet may be regarded as management strategy, sustaining health in mid-lactating dairy cow during extensive rearing under summer season, when animals can be exposed to mild heat stress, and improving the nutritional quality of dairy products.

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TANNINS IMPROVE DAIRY PRODUCT HEALTH FEATURES

Santillo et al.: TANNINS IMPROVE DAIRY PRODUCT HEALTH FEATURES

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