Effects of anthocyanin-rich purple corn (*Zea mays* L.) stover silage on nutrient utilization, rumen fermentation, plasma antioxidant capacity, and mammary gland gene expression in dairy goats[1]

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ABSTRACT: Eight healthy multiparous Saanen dairy goats (41.50 ± 1.84 kg) were assigned to a double 4 × 4 Latin square design. The four treatment diets were: (i) negative control, rice straw (**NC**); (ii) first positive control, sticky corn stover silage (**PC1**); (iii) second positive control, PC1 with 1 g/d commercial purple corn pigment (**PC2**); and (iv) anthocyanin-rich purple corn stover silage (**PSS; AR**). DMI did not differ ($P > 0.05$) among the treatments. Goats receiving the NC tended ($P < 0.05$) to reduce nutrient apparent digestibility, nitrogen (**N**) absorption, N retention, and volatile fatty acid production relative to the other groups. The levels of 2,2-diphenyl-1-picrylhydrazyl (**DPPH**) scavenging activity and superoxide dismutase (**SOD**) in plasma were greater ($P < 0.05$) in goats fed PC2 and AR compared with NC and PC1. The inclusion of PC2 and AR increased ($P < 0.05$) the abundance of nuclear factor (erythroid-derived 2)-like 2 (**NFE2L2**), but decreased ($P < 0.05$) the level of tumor necrosis factor in the mammary gland. Moreover, goats receiving AR tended to increase ($P < 0.05$) the levels of SOD2, GPX1, and GPX2 mRNA expression in the mammary gland. There were significant ($P < 0.05$) positive correlations between DPPH scavenging activity, total antioxidant capacity, SOD, catalase enzymes in plasma, and the abundance of NFE2L2 in the mammary gland. In addition, stronger ($P < 0.05$) positive correlations were noted between the expression of several inflammation related and antioxidant genes. Collectively, the results from the current study indicated that the consumption of anthocyanin-rich PSS by dairy goats had the potential to enhance antioxidant potential by improving antioxidant capacity in plasma and by modulating the abundance of several inflammation related and antioxidant genes in the mammary gland.

Key words: anthocyanin, antioxidant capacity, dairy goat, gene expression, mammary gland, purple corn stover silage

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INTRODUCTION

Free radicals (**FRs**) and other reactive oxygen species (**ROS**) are constantly formed in the animal body (Aruoma, 1998). In general, animals are protected against FR or ROS by their wide range of natural antioxidant enzymes (Miller et al., 1993). However, animals from tropical and subtropical countries/areas are prone to oxidative stress (**OS**)

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due to exposure to high temperature for a long period of time (Salles et al., 2010).

Superoxide ($O_2^{-\cdot}$), an anion radical, is generated during normal metabolism by the activity of the NADPH oxidase; it is converted into hydrogen peroxide (H_2O_2) by superoxide dismutase (**SOD**) enzyme and is then converted into H2O via glutathione peroxidase (**GPX**) and catalase (**CAT**) enzymes (Simkó, 2007). Thus, these enzymes can neutralize excessive FR before they react with metal catalysts to form more reactive compounds. Roberts et al. (2006) showed that rats were under OS status, NADPH oxidase was up-regulated, pointing to increased ROS production capacity and down-regulation of the key enzymes in the antioxidant defense system.

A recent study has shown that supplementing the ruminant diet with antioxidants could alleviate OS status (Rizzo et al., 2013). Anthocyanins are water-soluble natural pigments, which have been reported to show a stronger antioxidant capacity than other antioxidants (Rice-Evans et al., 1995). Furthermore, anthocyanins have the ability to modulate the mRNA gene expression of related antioxidants (Kruger et al., 2014). For example, Han et al. (2006) indicated that the inclusion of anthocyanin-rich purple potato flake extract in rats increased FR scavenging because it had the ability to increase the levels of hepatic SOD and GPX mRNA expression.

Purple corn, a field crop widely grown in Thailand, has abundant anthocyanins compared to other nutritional sources (Phinjaturus et al., 2016). Anthocyanin-rich purple corn stover silage (**PSS**) has a high level of anthocyanin composition and a stronger 2,2-diphenyl-1-picrylhydrazyl (**DPPH**) scavenging activity relative to sticky corn stover silage (**SSS**), making it a potential new super roughage for ruminants (Tian et al., 2018). However, anthocyanins were found to have poor palatability owing to their bitter taste (Jöbstl et al., 2004), thereby leading to low digestibility and a negative effect on rumen fermentation. To our knowledge, studies on the effect of anthocyanins on the goat health status in in vivo are scarce. We hypothesized that the inclusion of anthocyanin-rich PSS could enhance antioxidant potential in dairy goats. Accordingly, the present study investigates the effects of anthocyanin-rich PSS on nutrient apparent digestibility, nitrogen (**N**) utilization, rumen fermentation, plasma antioxidant capacity, the mRNA abundance of inflammation related and antioxidant genes in the mammary gland, and the relationship between antioxidant capacity and inflammatory markers of Saanen dairy goats.

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

All goats were handled in accordance with the Rules of Animal Welfare of Suranaree University of Technology (**SUT**; SUT 4/2558). The experiment was carried out at the SUT Goat Farm, Nakhon Ratchasima, Thailand (14°53′37.9″N, 102°01′22.0″E). Eight healthy multiparous Saanen dairy goats (BW, 41.50 ± 1.84 kg; mean ± SD) were assigned to a double 4 × 4 Latin square design. The treatment period was 21 d (14-d adaptation to new diet; 7 d sampling). The four treatments were: (i) negative control, rice straw (**NC**); (ii) first positive control, sticky corn stover silage (**PC1**); (iii) second positive control, PC1 with 1 g/d commercial purple corn pigment (**PC2**); and (iv) anthocyanin-rich purple corn stover silage (**AR**). Purple corn pigment was the commercial product (Nanjing Herd Source Bio-technology Co., Ltd, Nanjing, China), and 1 g/d purple corn pigment was used in this study according to Hosoda et al. (2012c). All goats were housed in clean individual pens with free access to water and were fed diets with 50:50 roughage: concentrate ratio. Food was offered in equal amounts twice daily at 08:00 and 17:00 for *ad libitum* intake. The diets (Table 1) were formulated to meet requirements for dairy goats having 40 kg of BW according to the NRC (1981).

Sample Collection

Data were obtained for continuously monitored DMI per day. Samples (100 g) of each diet were collected once weekly and pooled the whole time period. All samples were mixed and dried at 65 °C in a vacuum oven for 72 h, and then ground and passed through a 1-mm sieve.

Fecal and urine samples were collected on days 15 through 21 (2 d of the adaptation period and 5 d of the metabolic trial period). At the conclusion of each period, fecal samples were oven-dried at 65 °C for 72 h, ground and passed through a 1-mm sieve, and kept at 4 °C until further analysis. The urine collected daily from each animal was acidified with 20% H_2SO_4 to keep the pH < 3, and was then stored at −20 °C. Fecal and urine samples were also composited by each period.

On day 20 of each period, blood (6 mL) and ruminal fluid (~30 mL) samples were collected at 0, 2, and 4 h from the jugular vein by VACUETTE tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) with K_3-EDTA and by a vacuum pump

Table 1. Ingredients and nutrient composition of experimental diets

Item	Treatment[1]			
	NC	PC1	PC2	AR
Ingredient, % DM				
Rice straw	50.0			
Sticky corn stover silage		50.0	50.0	
Purple corn stover silage				50.0
Soybean meal	9.5	7.7	7.7	7.2
Rice bran	10.0	10.0	10.0	10.0
Cassava chip	10.9	24.2	24.2	24.7
Corn	15.0	6.5	6.5	6.5
Salt	0.4	0.4	0.4	0.4
Limestone	0.2	0.2	0.2	0.2
Vitamin premix[2]	0.5	0.5	0.5	0.5
Mineral permix[3]	0.5	0.5	0.5	0.5
Palm olein	3.0			
Total	100.0	100.0	100.0	100.0
Purple corn pigment, g/d[4]			1.0	
Chemical composition				
DM, %	93.84	58.79	58.74	57.93
OM, % DM	90.08	92.74	92.84	93.20
CP, % DM	10.06	10.07	10.10	10.06
GE, kJ/g DM	16803.05	16739.33	16795.85	16879.16
NDF, % DM	47.44	43.23	43.37	43.37
ADF, % DM	27.64	25.26	25.16	24.51
Hemicellulose, % DM	19.79	17.97	18.21	18.86
Ash, % DM	9.92	7.26	7.16	6.80

[1]NC = negative control of goats fed rice straw; PC1 = positive control 1 of goats fed sticky corn stover silage; PC2 = positive control 2 of goats fed sticky corn stover silage with 1 g/d purple corn pigment; AR = goats fed anthocyanin-rich purple corn stover silage.

[2]Vitamin premix contained (per kg of premix): vitamin A 12,000,000 IU, vitamin D_3 2,400,000, vitamin E 750 mg, vitamin B_1 980 mg, vitamin B_2 960 mg, vitamin B_6 654 mg, vitamin B_{12} 1,658 µg, vitamin B_9 133 mg, calcium pantothenate 2,940 mg, nicotinamide 8,910 mg, K 637 mg, vitamin B_4 446 g, Na 289.4 g, and citrate 850.5 mg.

[3]Mineral premix contained (per kilogram of premix): Fe 12 g, Cu 2 g, Mn 7 g, Mg 5 g, Zn 15 g, Co 2 g, I 2 g, Ca 195.27 g, and P 144.33 g.

[4]Purchased from Nanjing Herd Source Bio-technology Co., Ltd.

through the mouth, respectively. For the blood samples, after centrifugation at 1,788.6 × *g* for 15 min at 4 °C (Sorvall Legend XT/XF Centrifuge Series, Thermo Fisher Scientific, Waltham, MA), the plasma was transferred to 1.5-mL tubes and was stored at −80 °C until further analysis. For the ruminal fluid samples, pH was determined immediately by a portable pH meter; and then 20 mL ruminal fluid sample and 5 mL HCl (6 mol/L) were mixed together, placed in a container, and stored at −20 °C.

Mammary gland tissue biopsies were conducted on day 21 of each period by the methods of Farr et al. (1996) and Zhang et al. (2015). Each goat received a subcutaneous injection of a Penomycin (contains Procaine and sodium penicillins with Streptomycin; Manufacturing Co., Ltd, Samutprakam, Thailand), and the sample was taken using a semi-automatic biopsy needle (16 G × 90 mm, SAG-16090, TSK Corporation, Tochigi, Japan). The sample was immediately transferred to a 1.5-mL tube, snap frozen in liquid nitrogen, and stored at −80 °C until the assay was performed.

Chemical Analysis

DM, ash, and CP/N were determined according to the AOAC Official Methods 930.15, 942.05, 988.05, respectively (AOAC, 1990). NDF and ADF were detected by the method of Van Soest et al. (1991). OM and hemicellulose were calculated using the respective equations: OM = 100 − ash; hemicellulose = NDF − ADF. Gross energy was analyzed using a Parr 6200 calorimeter (Moline, IL).

The anthocyanin composition in the silage extract was analyzed by HPLC(1260 Infinity II LC, Agilent Technologies, Santa Clara, CA) according to Hosoda et al. (2009). The sample was filtered through a 13-mm 0.45-µm nylon syringe filter (Xiboshi, TNL1345PP, Tianjin Fuji Science & Technology Co., Ltd, Tianjin, China), and the composition was determined with an HPLC and diode array detector (**DAD**). Separation of anthocyanin composition was accomplished on a Symmetry C_{18} column (mobile phase: A, acetonitrile; B, 10% acetic acid, 5% CH3CN, 1% phosphoric acid in deionized water). The run time was 30 min with a 5-min delay before the next injection. The other conditions were as follows: sample temperature 4 °C; injection volume 20 µL; flow rate 0.8 ml/min; column temperature 25 °C; DAD detector wavelength 520 nm. The anthocyanin composition of the two types of corn stover silage is shown in Table 2.

Ammonia nitrogen (**NH_3-N**) was assayed according to the method of Bremner and Keeney (1965). The ruminal fluid sample was filtered through a 13-mm 0.45-µm nylon syringe filter and was analyzed for individual VFAs: acetic acid (**HAc**), propionic acid (**PA**), and butyric acid (**BA**) by HPLC. Total volatile fatty acid (**TVFA**) = HAc + PA + BA.

Antioxidant Capacity Analysis

The DPPH scavenging activity in plasma was assayed spectrophotometrically according to Wei and Chiang (2009), using a stable FR DPPH (Sigma-Aldrich, St. Louis, MO; Pcode: 101845869) with a slight modification. Briefly, 50 µL of each sample

Table 2. Nutrient composition and anthocyanin composition of two types of corn stover silage

Item	Type of corn stover silage[1]	
	SSS	PSS
Nutrient composition		
DM, %	23.16	21.58
OM, % DM	92.94	93.81
CP, % DM	6.56	7.11
GE, kJ/g DM	16,949.06	17,044.35
NDF, % DM	62.42	61.27
ADF, % DM	39.36	38.12
Hemicellulose, % DM	23.06	23.16
Ash, % DM	7.06	6.19
Anthocyanin composition, mg/100 g DM[2]		
C3G	3.06	2.92
Del	8.36	8.82
P3G	—	—
Peo	3.27	4.62
M3G	3.85	7.14
Cya	11.25	14.01
Pel	9.01	10.17
Mal	-	39.77
Total	38.80	87.46

[1]SSS = sticky corn stover silage; PSS = anthocyanin-rich purple corn stover silage.

[2]C3G = cyanidin-3-glucoside; Del = delphinidin; P3G = pelargonidin-3-glucoside; Peo = peonidin; M3G = malvidin-3-*O*-glucoside; Cya = cyanidin; Pel = pelargonidin; Mal = malvidin; — = not detected.

was added to 1 mL of methanolic solution of DPPH reagent (25 μmol/L) in a 1.5-mL tube. The mixture was shaken vigorously and incubated in the dark at room temperature for 30 min, and then centrifuged at 1,788.6 × *g* for 10 min at 4 °C. The supernatant (200 μL) was immediately transferred to a 96-well plate, and the absorbance was detected at 517 nm via a microplate reader (Epoch, BioTek, Luzern, Switzerland). DPPH scavenging activity = (Ac − As) × 100/Ac, where Ac is the absorbance of the control, and As is the absorbance of the sample.

The total antioxidant capacity (**TAC**), SOD, GPX, and CAT activities were determined using the respective commercially available kits: MAK187-1KT, 19160-1KT-F, CGP1-1KT, and CAT100-1KT (Sigma-Aldrich).

Mammary Gland mRNA Gene Expression Analysis

Total RNA was extracted from the mammary gland using the TRIzol reagent method described by Labrecque et al. (2009), with a minor modification. Briefly, the sample was transferred into a 2-mL tube, and 1 mL of TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) was added per 0.1 g of 0.5-mm dia. Zirconia/silica (Cat. No. 11079105z, BioSpec Products) and was then run in a homogenizer. The total RNA was dissolved with DEPC water (Ambion by Life Technologies, NY), and template DNA was then digested by RNase-free DNase I. Next, the purity of the extracted RNA was analyzed by assaying the 260/280 absorbance ratio using a NanoVue spectrophotometer (GE Healthcare Bio-Sciences, Pittsburgh, PA). The integrity of the extracted RNA was assessed by verifying the presence of 18S and 28S RNA bands using an ImageQuant LAS 500 imager (GE Healthcare Bio-Sciences) by electrophoresis on a 1% agarose gel. cDNA synthesis was performed as described by the ImProm-II Reverse Transcription System (Promega Corporation, Madison, WI).

All samples were assayed using quantitative real-time PCR amplification by Roche LightCycler 480 real-time PCR System (Roche Diagnostics GmbH, Penzberg, Germany). The nine target genes were: nuclear factor (erythroid-derived 2)-like 2 (**NFE2L2**), heat shock transcription factor 1 (**HSF1**), heat shock 70 kDa protein 1A (**hspA1A**), tumor necrosis factor (**TNF**), SOD2, SOD3, GPX1, GPX2, and CAT; the housekeeper gene was glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**). Nucleotide sequences were obtained from the National Center for Biotechnology Information, and the primers set for all genes were designed using the GenScript real-time PCR (TaqMan) Primer Design tool and were synthesized by the Bio Basic Inc. Company (Table 3). The real-time PCR amplifications were performed in a 10-μL reaction volume, which consisted of 5 μL of 2× Roche 04707516001 LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany), 2 μL of 10× diluted cDNA, and 1 μL of forward and reverse primers. Next, the plates (LightCycler 480 multiwell plate 96, white; Roche Diagnostics GmbH) were centrifuged at 4 °C, 1,500 rpm for 3 min (Universal 320, Hettich Zentrifugen, Germany). The cycling conditions were 10 min at 95 °C for pre-incubation, 40 cycles of 30 s at 95 °C for amplification, and 55 to 57.5 °C for 1 min (annealing temperature optimized depending on primers; Table 3), and cooling at 40 °C for 30 s. Amplifications were performed in triplicate for each gene.

Statistical Analysis

All calculations were analyzed as a 4 × 4 double Latin square design using the GLM procedure of the Statistical Analysis System 9.1.3 (SAS Institute, Cary, NC) according to the general model: $Y_{ij(k)} = \mu + Ri + Cj + \tau_{(k)} + \varepsilon_{ij(k)}$, where $_{Yij(k)}$

Table 3. Primer sequences used for real-time PCR amplifications in this study

Gene[1]	Primer sequences (5′ to 3′)[2]	Accession number	Product size, nt	Annealing temperature, °C	Amplification efficiency, %
NFE2L2	(F) GGCCCATTGACCTCTCTGAT	NM_001314327.1	131	57	108.64
	(R) TGATGCCATGCTTGGACTTG				
HSF1	(F) GCCATGAAGCATGAGAACGA	NM_001314344.2	111	55.5	100.50
	(R) CAGCGAGATGAGGAACTGGA				
hspA1A	(F) GGACATCAGCCAGAACAAGC	JN656104.1	137	57	101.16
	(R) TGGACGTGTTGAAGTCGATG				
TNF	(F) CGTATGCCAATGCCCTCAAG	NM_001286442.1	82	57	101.67
	(R) ATGAGGTAAAGCCCGTCAGT				
SOD2	(F) CTGTTGGTGTCCAAGGTTCC	XM_018053428.1	153	56	93.73
	(R) AATAAGCATGCTCCCACACG				
SOD3	(F) ATCCACGTGCACCAGTTTG	NM_001285675.1	74	56	99.03
	(R) ACGGACATCGGGTTGTAGTG				
GPX1	(F) CCTGGTGGTACTCGGCTT	XM_005695962.3	91	57.5	94.47
	(R) TCGGACGTACTTCAGGCAAT				
GPX2	(F) CCGCACCTTCCAAACCATTA	XM_005685982.3	103	55.5	102.23
	(R) AAAGACACTCAGGGCAGGAG				
CAT	(F) CCTGTTCAGTGATCGAGGGA	XM_005690077.3	97	57	107.46
	(R) AACTGCCTCTCCATTGGCAT				
GAPDH	(F) CACAGTCAAGGCAGAGAACG	XM_005680968.3	109	56	91.84
	(R) GTACTCAGCACCAGCATCAC				

[1]NFE2L2 = nuclear factor (erythroid-derived 2)-like 2; HSF1 = heat shock transcription factor 1; hspA1A = heat shock 70 kDa protein 1A; TNF = tumor necrosis factor; SOD = superoxide dismutase; GPX = glutathione peroxidase; CAT = catalase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

[2]F = forward; R = reverse.

denotes the observation, μ represents the overall mean; R_i represents the effect of period (i = 1 to 4), C_j expresses the effect of dairy goat (j = 1 to 8), $\tau_{(k)}$ is the fixed effect of treatment (k = NC, PC1, PC2, and AR), and $\varepsilon_{ij(k)}$ describes random error with mean 0 and variance σ^2. Goat and period are random effects, and dietary treatment is the fixed effect. The treatment means were calculated by LSMEANS, with differences between treatment means using a Tukey test (Kaps and Lamberson, 2004). Amplification efficiency (**AE**) of each gene was measured using the standard curve method according to Fink et al. (1998): AE = ($10^{-1/s}$ -1), where S is the slope of the generated standard curve. For all of the calculated values, AE varied from 91.84% to 108.64% (Table 3). The relative mRNA abundance was calculated using the $2^{-\triangle\triangle Ct}$ method, in which $\triangle\triangle Ct = (Ct_{\text{target gene unknown sample}} - Ct_{\text{GAPDH unknown sample}}) - (Ct_{\text{target gene calibrator sample}} - Ct_{\text{GAPDH calibrator sample}})$. The averaged abundance of the genes in the NC data was considered as the calibrator. Pearson correlation coefficients (r) used to analyze the relationship between antioxidants in plasma and the inflammation-related and antioxidant gene expressions in the mammary gland. Significant differences were considered at $P < 0.05$.

RESULTS

DMI and Digestibility

There were no significant differences ($P > 0.05$) in the DMI, BW (%), and g/kg $BW^{0.75}$ (%) for the four treatments (Table 4). However, the apparent digestibility of DM, OM, CP, GE, NDF, and ADF in the goats receiving PC1, PC2, and AR were greater ($P < 0.05$) than those in the NC.

No significant differences ($P > 0.05$) were observed in the N intake and N excretion in urine among all treatments (Table 5). N excretion in feces and total N excretion of the NC treatment were greater ($P < 0.05$) than those of the PC1 and AR treatments. In contrast, N absorption and N retention were significantly decreased ($P < 0.05$) in NC relative to the other treatments.

Rumen Fermentation Parameters

The treatments had no effect ($P > 0.05$) on the pH, NH_3-N, and HAc values (Table 6). PA, BA, and TVFA were less ($P < 0.05$) in goats receiving NC compared to the other treatments. For VFA (% Molar), the NC group decreased ($P < 0.05$) PA and BA, whereas it increased ($P < 0.05$) the

Table 4. Effect of anthocyanin-rich PSS on apparent digestibility of dairy goats

	Treatment[2]					
Item[1]	NC	PC1	PC2	AR	SEM	*P*-value
DMI, g/d	908	933	931	941	12.9796	0.4231
BW, %	2.19	2.25	2.24	2.27	0.0313	0.4428
g/kg $BW^{0.75}$, %	55.54	57.07	56.92	57.51	0.7946	0.4428
Apparent digestibility, %						
DM	56.80[b]	63.92[a]	64.78[a]	66.90[a]	1.7657	0.0065
OM	61.19[b]	67.14[a]	68.16[a]	69.85[a]	1.5208	0.0066
CP	47.71[b]	59.65[a]	56.32[a]	57.84[a]	1.9374	0.0363
GE	59.20[b]	64.43[a]	65.53[a]	67.43[a]	1.5391	0.0201
NDF	32.45[b]	44.28[a]	45.95[a]	43.35[a]	3.2529	0.0418
ADF	31.95[b]	43.69[a]	45.14[a]	42.58[a]	2.4924	0.0207

[a, b]Different letters within a row are significantly different ($P < 0.05$).

[1]Values represent the means of eight replicates ($n = 8$).

[2]NC = negative control of goats fed rice straw; PC1 = positive control 1 of goats fed sticky corn stover silage; PC2 = positive control 2 of goats fed sticky corn stover silage with 1 g/d purple corn pigment; AR = goats fed anthocyanin-rich purple corn stover silage.

AA and ratio of AA to PA compared to the other groups.

Antioxidant Activity in Plasma

As shown in Table 7, the dietary treatments had no effect ($P > 0.05$) on the activity of plasma TAC, GPX, and CAT, whereas a significant increase ($P < 0.05$) was observed in goats receiving PC2 and AR in terms of the DPPH scavenging activity and SOD in plasma.

Relative mRNA Abundance of Inflammation-Related Genes

As shown in Figure 1, the abundance of NFE2L2 mRNA was downregulated ($P < 0.05$) in the mammary gland of goats receiving NC relative to the other treatments. Goats fed the PC1 diet showed increased ($P < 0.05$) levels of HSF1, hspA1A, and TNF mRNA abundance in the mammary gland compared with those fed the NC and PC2 treatments. There was no difference ($P > 0.05$) in the HSF1, hspA1A, and TNF mRNA abundance of goats receiving AR relative to the NC. Furthermore, the addition of PC2 in goats decreased ($P < 0.05$) mammary gland TNF mRNA expression when compared with the NC treatment.

Relative mRNA Abundance of Antioxidant Genes

As shown in Figure 2, goats receiving PC1 tended to increase ($P < 0.05$) the level of GPX1 mRNA abundance in the mammary gland compared with NC. AR treatment led to significant elevations ($P < 0.05$) in the levels of the mRNA abundance of SOD2, GPX1, and GPX2 compared

Table 5. Effect of anthocyanin-rich PSS on nitrogen utilization of dairy goats

	Treatment[2]					
Item[1]	NC	PC1	PC2	AR	SEM	*P*-value
N intake, g/d[3]	14.62	15.04	15.04	15.15	0.2092	0.4034
N excretion in feces, g/d	7.65[a]	6.07[b]	6.57[ab]	6.39[b]	0.2922	0.0464
N excretion in urine, g/d	5.22	4.35	4.31	4.09	0.3263	0.2466
Total N excretion, g/d	12.87[a]	10.42[b]	10.88[b]	10.48[b]	0.3125	0.0036
N absorption, g/d	6.97[b]	8.97[a]	8.47[a]	8.76[a]	0.2922	0.0213
N absorption, %	47.71[b]	59.65[a]	56.32[a]	57.84[a]	1.9374	0.0363
N retention, g/d	1.75[b]	4.62[a]	4.16[a]	4.67[a]	0.3087	0.0009
N retention, %	11.95[b]	30.72[a]	27.67[a]	30.85[a]	2.1708	0.0011

[a, b]Different letters within a row are significantly different ($P < 0.05$).

[1]Values represent the means of eight replicates ($n = 8$).

[2]NC = negative control of goats fed rice straw; PC1 = positive control 1 of goats fed sticky corn stover silage; PC2 = positive control 2 of goats fed sticky corn stover silage with 1 g/d purple corn pigment; AR = goats fed anthocyanin-rich purple corn stover silage.

[3]N = nitrogen.

Table 6. Effect of anthocyanin-rich PSS on rumen fermentation parameters of dairy goats

	Treatment[2]					
Item[1]	NC	PC1	PC2	AR	SEM	*P*-value
pH	6.82	6.88	6.78	6.80	0.0584	0.7056
NH_3-N, mg/dL[3]	11.94	12.33	12.20	11.65	0.7302	0.9277
HAc, mmol/L	40.13	40.87	42.35	41.40	1.5851	0.7912
PA, mmol/L	14.44^{b}	16.99^{a}	19.34^{a}	17.74^{a}	0.8640	0.0039
BA, mmol/L	5.11^{b}	8.64^{a}	9.18^{a}	8.52^{a}	0.4030	<0.0001
TVFA, mmol/L	59.68^{b}	66.50^{ab}	70.87^{a}	67.66^{a}	2.5167	0.0283
VFA, % molar						
AA	67.21^{a}	61.60^{b}	59.71^{b}	61.18^{b}	0.8132	<0.0001
PA	24.22^{b}	25.50^{ab}	27.27^{a}	26.23^{ab}	0.7536	0.0527
BA	8.57^{b}	12.89^{a}	13.02^{a}	12.59^{a}	0.3613	<0.0001
Ratio of AA to PA	2.90^{a}	2.50^{b}	2.28^{b}	2.50^{b}	0.1241	0.0126

$^{a, b}$Different letters within a row are significantly different ($P < 0.05$).

[1]Values represent the means of eight replicates ($n = 8$).

[2]NC = negative control of goats fed rice straw; PC1 = positive control 1 of goats fed sticky corn stover silage; PC2 = positive control 2 of goats fed sticky corn stover silage with 1 g/d purple corn pigment; AR = goats fed anthocyanin-rich purple corn stover silage.

[3]NH_3-N = ammonia nitrogen; HAc = acetic acid; PA = propionic acid; BA = butyric acid; VFA = volatile fatty acid; TVFA = total volatile fatty acid.

to NC. GPX1 mRNA abundance was more upregulated ($P < 0.05$) in the mammary gland of goats fed PC2 than of goats fed NC. To note, no differences ($P > 0.05$) were observed in SOD3 and CAT mRNA abundance among all treatments.

Pearson Correlation Coefficients

Significant ($P < 0.05$) positive correlations were observed between DPPH scavenging activity, TAC, SOD, and CAT enzymes in plasma and NFE2L2 (Table 8). There were significant ($P < 0.05$) negative correlations between TAC, SOD, and GPX enzymes and the abundances of hspA1A and TNF. Moreover, significant ($P < 0.05$) positive correlations were detected between several antioxidant enzymes (DPPH scavenging activity, TAC, and CAT) and the mRNA expression of some antioxidant genes (SOD2, GPX1).

As shown in Table 9, there were significant ($P < 0.05$) positive correlations between the expression of some inflammation-related and antioxidant genes (NFE2L2 and SOD2, GPX1; HSF1 and SOD3, GPX1; hspA1A and GPX1, CAT; and TNF and CAT).

DISCUSSION

DMI and Digestibility

Anthocyanins are phenolic compounds that contribute a characteristic bitter flavor to plants.

Table 7. Antioxidant activity in plasma of dairy goats in different treatments

	Treatment[2]					
Item[1]	NC	PC1	PC2	AR	SEM	*P*-value
DPPH scavenging activity, %[3]	19.92^{b}	22.90^{ab}	24.81^{a}	24.88^{a}	1.0340	0.0069
TAC, nmol/μL	37.32	37.62	38.95	40.11	0.8399	0.1303
SOD, inhibition rate %	87.12^{b}	87.08^{b}	90.61^{a}	90.48^{a}	0.9254	0.0094
GPX, mmol·min^{-1}.mL^{-1}	0.142	0.141	0.146	0.144	0.0019	0.3689
CAT, μmol·min^{-1}.mL^{-1}	63.00	66.30	66.84	67.27	2.6556	0.6863

$^{a, b}$Different letters within a row are significantly different ($P < 0.05$).

[1]Values represent the means of eight replicates ($n = 8$).

[2]NC = negative control of goats fed rice straw; PC1 = positive control 1 of goats fed sticky corn stover silage; PC2 = positive control 2 of goats fed sticky corn stover silage with 1 g/d purple corn pigment; AR = goats fed anthocyanin-rich purple corn stover silage.

[3]DPPH = 2,2-diphenyl-1-picrylhydrazyl; TAC = total antioxidant capacity; SOD = superoxide dismutase; GPX = glutathione peroxidase; CAT = catalase.

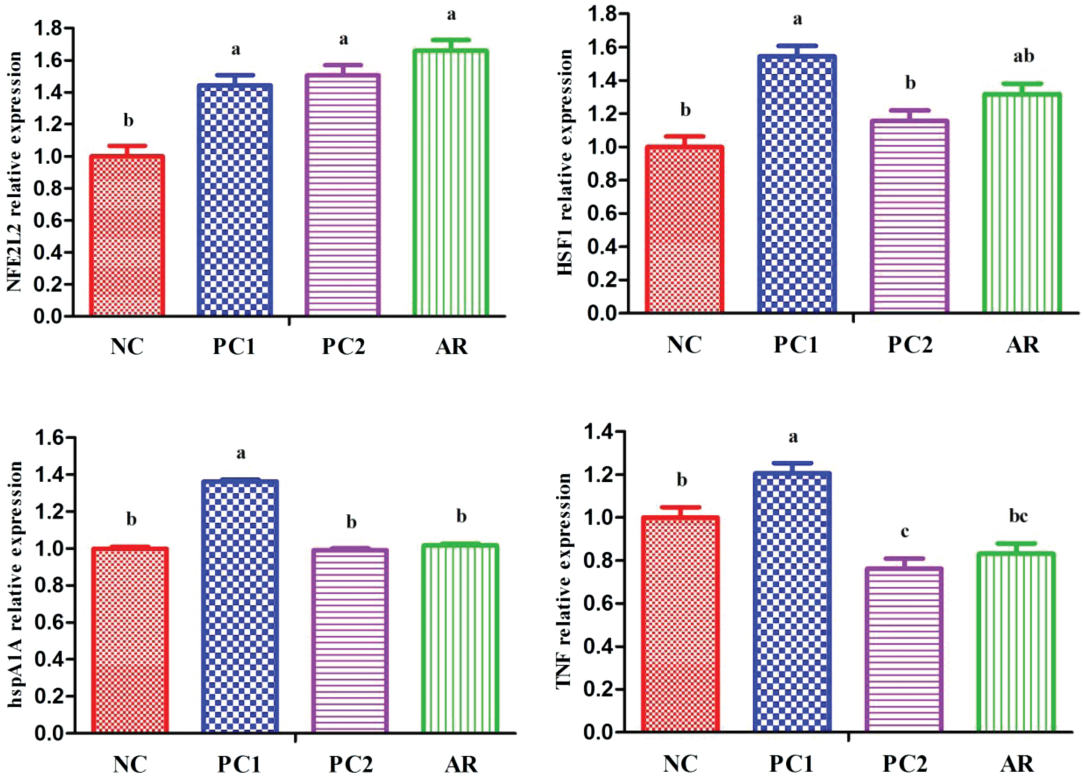

Figure 1. Relative mRNA abundance of inflammation-related genes in the mammary gland of dairy goats in different treatments. NC = negative control of goats fed rice straw; PC1 = positive control 1 of goats fed sticky corn stover silage; PC2 = positive control 2 of goats fed sticky corn stover silage with 1 g/d purple corn pigment; AR = goats fed anthocyanin-rich purple corn stover silage. Data reported as least-squares means ± SEM ($n = 8$). Relative quantification of mRNA abundance for each gene was analyzed by the $2^{-\triangle\triangle Ct}$ method with the NC treatment as the reference expression point. NFE2L2 = nuclear factor (erythroid-derived 2)-like 2; HSF1 = heat shock transcription factor 1; hspA1A = heat shock 70 kDa protein 1A; TNF = tumor necrosis factor. $^{a-c}$ Different letters are significantly different ($P < 0.05$).

Our current study revealed that goats consumed all the feed, indicating that anthocyanins did not lead to poor palatability for goats. It is well known that the nutritional value of the feed usually depends on its digestive characteristics and nutrient composition. Hosoda et al. (2012b) demonstrated that apparent nutrient digestibility increased in a purple rice silage treatment than in control group due to purple rice silage having a greater level of fiber fractions in comparison to the control rice silage. Additionally, anthocyanins might be bound to the nutrients to be digested, inhibiting the digestive enzymes and exerting an anti-microbial effect (Acosta-Estrada et al., 2014). In the present experiment, goats receiving PC1, PC2, and AR diets increased their levels of apparent digestibility and N retention relative to the NC. Alternatively, the rice straw had a high level of fiber, resulting in dropping of the level of nutrient utilization in the NC group. As a result, it appears that anthocyanins do not affect digestibility when the nutrient balance is unchanged. However, the exact reasons remain unclear and further observation is required.

Rumen Fermentation Parameters

Ruminal pH values at both extremes, either <5.6 or >7.3, should impact ruminal bacterial growth (Keunen et al., 2002), suggesting that the inclusion of anthocyanin-rich PSS will not affect the rumen microflora. True protein and nonprotein nitrogen can be degraded into NH_3-N by microbial extracellular enzymes and deamination, respectively. Consequently, NH_3-N is generally affected by the CP content in the diet (Hristov et al., 2004). In the current study, no significant difference was observed in the NH_3-N concentration among the treatments, perhaps because all diets were calculated to be isonitrogenous. Anthocyanins might be able to affect carbohydrate metabolism to provide more energy for ruminants by inhibiting AA production, thus increasing the proportion of PA (Tian et al., 2018). The results of the current study showed that the addition of PC2 or AR to the dairy goats increased the levels of PA, BA, and TVFA, whereas they decreased the ratio of AA to PA compared to NC, perhaps because the NC treatment diet had a high level of fiber, making it

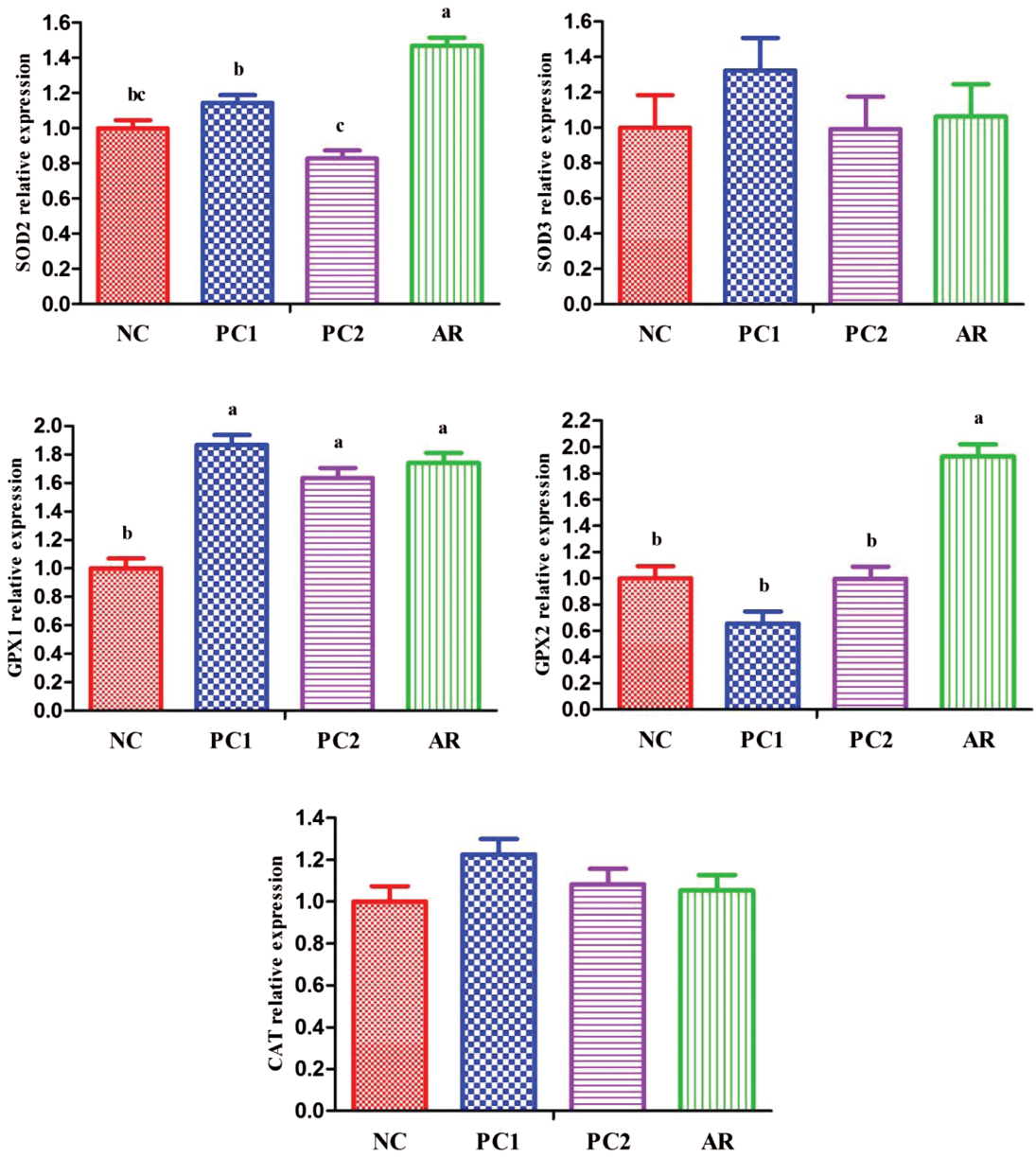

Figure 2. Relative mRNA abundance of antioxidant genes in the mammary gland of dairy goats in different treatments. NC = negative control of goats fed rice straw; PC1 = positive control 1 of goats fed sticky corn stover silage; PC2 = positive control 2 of goats fed sticky corn stover silage with 1 g/d purple corn pigment; AR = goats fed anthocyanin-rich purple corn stover silage. Data reported as least-squares means ± SEM (n = 8). Relative quantification of mRNA abundance for each gene was analyzed by the $2^{-\triangle\triangle Ct}$ method with the NC treatment as the reference expression point. SOD = superoxide dismutase; GPX = glutathione peroxidase; CAT = catalase. $^{a-c}$ Different letters are significantly different ($P < 0.05$).

difficult to degrade in the rumen, thereby reducing the level of VFA production. To note, no significant difference was observed in the PC1, PC2, and AR treatments regarding VFA production in this study, which might be because although anthocyanin-rich PSS showed a greater level of anthocyanins, they did not appear to be not broken down in the rumen (Hosoda et al., 2009). Additionally, the type of VFA formed in the rumen depends on the type of substrate fermented, the rumen environment, and the microbial population (Messana et al., 2013). The formation of AA is always accompanied by H_2 and CO_2 production, whereas PA production requires H_2 to be formed as PA (Luthfi et al., 2018). Thus, the fermentation of cell wall fiber increased the AA:PA ratio in the NC treatment. Our results are consistent with those of Hosoda et al. (2012a), who revealed that in lactating dairy cows receiving anthocyanin-rich corn silage, there was no change in the ruminal fluid VFA production compared with those fed the control silage.

Table 8. Pearson correlation coefficients between antioxidant enzymes in plasma and gene expressions in the mammary gland

Item[1]		NFE2L2[2]	HSF1	hspA1A	TNF	SOD2	SOD3	GPX1	GPX2	CAT
DPPH scavenging activity	*r*	0.917	0.303	–0.093	–0.510	0.208	0.011	0.692	0.433	0.157
	P	<0.0001	0.292	0.764	0.090	0.422	0.972	0.003	0.159	0.608
TAC	*r*	0.785	0.073	–0.436	–0.694	0.503	–0.160	0.338	0.829	–0.168
	P	0.003	0.803	0.137	0.012	0.040	0.619	0.201	0.001	0.583
SOD	*r*	0.690	–0.139	–0.592	–0.853	0.096	–0.262	0.227	0.636	–0.221
	P	0.013	0.635	0.033	0.0004	0.714	0.411	0.397	0.026	0.468
GPX	*r*	0.509	–0.300	–0.668	–0.891	–0.223	–0.329	0.069	0.430	–0.265
	P	0.091	0.297	0.013	<0.0001	0.389	0.297	0.800	0.163	0.318
CAT	*r*	0.940	0.489	0.134	–0.314	0.310	0.128	0.826	0.354	0.285
	P	<0.0001	0.076	0.664	0.320	0.227	0.691	<0.0001	0.259	0.345

[1]Values represent the means of eight replicates (n = 8).

[2]NFE2L2 = nuclear factor (erythroid-derived 2)-like 2; HSF1 = heat shock transcription factor 1; hspA1A = heat shock 70kDa protein 1A; TNF = tumor necrosis factor; SOD = superoxide dismutase; GPX = glutathione peroxidase; CAT = catalase; DPPH = 2,2-diphenyl-1-picrylhydrazyl; TAC = total antioxidant capacity.

Antioxidant Activity in Plasma

Studies have shown that anthocyanins are absorbed intact into blood (McGhie et al., 2003; Passamonti et al., 2003). Moreover, anthocyanins have strong antioxidant activity (Dixon et al., 2005). DPPH is a type of FR, which is reduced in aqueous solution containing antioxidant molecule (Mensor et al., 2001). Thus, anthocyanins in plasma have the ability to provide electrons to the DPPH, thereby increasing the level of DPPH scavenging activity. As discussed elegantly by Toaldo et al. (2016), anthocyanin-rich grape juices can increase blood antioxidant capacity in humans.

PC2 and AR significantly increased plasma SOD activity, suggesting that anthocyanins were absorbed into the blood, providing electrons to $O_2^{-\cdot}$, thereby alleviating the OS condition. Moreover, anthocyanin-rich plants might cause elevations in messenger RNA expression, resulting in an effect on the activity of SOD in blood (Yang et al., 2010). The present findings are in line with those of Hosoda et al. (2012a), in which a significant increase in SOD in the plasma of lactating dairy cows fed anthocyanin-rich purple corn silage was reported. To note, the activity of GPX and CAT in plasma did not change in goats receiving PC2 and AR, perhaps because they can improve SOD activity in plasma that alleviated the burden of the antioxidant defense system, and therefore, other enzymes did not differ (Mittler, 2002). These observations are in agreement with Tian et al. (2019), who indicated that anthocyanin-rich PSS can increase plasma antioxidant capacity in lactating dairy goats.

Table 9. Pearson correlation coefficients between inflammation-related and antioxidant gene expressions

Item[1]		SOD2[2]	SOD3	GPX1	GPX2	CAT
NFE2L2	*R*	0.450	–0.089	0.760	0.484	0.142
	P	0.040	0.783	0.001	0.111	0.643
HSF1	*r*	0.362	0.588	0.698	–0.032	0.253
	P	0.154	0.044	0.003	0.922	0.404
hspA1A	*r*	0.119	0.491	0.524	–0.541	0.619
	P	0.649	0.105	0.037	0.069	0.024
TNF	*r*	0.084	0.486	0.202	–0.586	0.607
	P	0.749	0.109	0.454	0.055	0.028

[1]Values represent the means of eight replicates (n = 8).

[2]SOD = superoxide dismutase; GPX = glutathione peroxidase; CAT = catalase; NFE2L2 = nuclear factor (erythroid-derived 2)-like 2; HSF1 = heat shock transcription factor 1; hspA1A = heat shock 70 kDa protein 1A; TNF = tumor necrosis factor.

Inflammation-Related Gene Expression

Anthocyanins have been reported to modulate the inflammatory pathway by inhibiting ROS and the nuclear factor-kappa B (**NF-κB**) transcription factor (Speciale et al., 2014). The inducers can lead to the release of NFE2L2 (also known as Nrf2) and allow its nuclear translocation, activating the expression of a battery of cytoprotective genes (Espinosa-Diez et al., 2015). In this study, mammary gland NFE2L2 mRNA expression increased with supplementation of PC2 and AR. The possible reason is that anthocyanins have inherent radical scavenging activity, preventing OS damage by inducing the signaling pathways of Nrf2 (Martín et al., 2010). Indeed, anthocyanins are a source of Nrf2 activating agent, which can directly modify sensor

cysteines present in the Kelch-like ECH protein, leading to Nrf2 proteins translocating into the nucleus and binding to the antioxidant response element, thereby modulating the expression of Nrf2 target genes that have anti-inflammatory and antioxidant functions (Aboonabi and Singh, 2015). Our observations concur with those of Kruger et al. (2014), who suggested that anthocyanins can activate the expression of Nrf2, increasing the expression of inflammation-related and antioxidant genes.

HSF1 regulates the heat shock response (**HSR**) pathway in animals by acting as the major transcription factor for heat shock proteins (**HSPs**; Calderwood et al., 2009). As a result, animals elicit counterattack mechanisms, which affect the transcriptional pathways that activate chaperones for refolding misfolded proteins of the HSPs of the HSF1 cascade (Akhtar et al., 2012). The present results revealed that SSS supplementation upregulated mammary gland HSF1 mRNA abundance, perhaps due to the level of anthocyanins in SSS, which was not sufficient to alleviate the OS status of dairy goats. It was reported that feeding dietary oil could reduce mitochondrial ROS production and alleviate oxidative damage in chickens (Mujahid et al., 2009). Hence, the high level of oil in the NC treatment might have alleviated the OS status of dairy goats, resulting in decreasing the level of HSF1 gene abundance in the mammary gland relative to PC1.

Chaperones are released from HSF1 to perform their protein-folding roles, and the exportation of HSF1 to the cytoplasm is inhibited, allowing HSF1 to trimerize and accumulate in the nucleus to stimulate transcription of target genes (Shamovsky and Nudler, 2008). Accordingly, the abundance of mammary gland hspA1A mRNA increased in the SSS treatment, perhaps because a high level of hspA1A mRNA expression can protect against OS injury by activation of the HSF1 signaling pathway (Chauhan et al., 2014).

Lipopolysaccharide molecules can be embedded in the outer membrane of Gram-negative bacteria, which can activate TNF (Cohen, 2002). Moreover, anthocyanins were found to be effective inhibitors of Gram-negative bacteria (He and Giusti, 2010). As expected, the PC2 and AR treatments tended to inhibit the expression of TNF in the mammary gland, suggesting that they inhibited damage. Consistent with our results, anthocyanins have been reported to significantly inhibit the TNF transcription via an in vivo model of experimental angiogenesis (Atalay et al., 2003).

Antioxidant Gene Expression

Various articles have shown that anthocyanins can increase antioxidant and detoxifying enzymes by activation of the NFE2L2 transcription factor (Rahman et al., 2006; Speciale et al., 2014). Hence, AR treatment increased the SOD2 mRNA abundance in the mammary gland of dairy goats, which may be associated with a greater level of NFE2L2 relative expression. However, PC2 and AR had no effect on the SOD3 mRNA abundance, which might be because differences in isoforms were discriminated at the transcript levels, and there were differences in antioxidant enzyme concentrations in the mammary gland of ruminants (Côrtes et al., 2012). Specifically, SOD2 (in the mitochondria) and SOD3 (on cell surfaces and in the extracellular matrix) are located in different areas (Jung, 2014), which might also be driving factors.

AR increased the levels of mRNA GPX1 and GPX2 expression in the mammary gland, perhaps because anthocyanins in anthocyanin-rich plants can be absorbed into mammary tissue, leading to improving antioxidant capacity. GPX2 was unaffected by the inclusion of PC2 compared to NC, suggesting that these mRNA discrepancies might be the differences in bioavailability and absorption of the anthocyanin metabolites because anthocyanins in plants seem to not be broken down in the rumen (Song et al., 2012), whereas plant extract or anthocyanin powder is more susceptible to rumen fermentation (Leatherwood, 2013). However, this assumption needs to be further confirmed.

Dieterich et al. (2000) showed that CAT expression seemed to be much lesser than that of other antioxidants. Similarly, no difference was detected in the mRNA abundance of CAT in the mammary gland. This is because CAT is absent in the mitochondria, so the reduction of H_2O_2 to H_2O is carried out by GPX (Ighodaro and Akinloye, 2018). In addition, anthocyanins increased the abundance of some SOD and GPX genes that alleviate OS status in dairy goats and consequently, did not affect the CAT mRNA expression.

Pearson Correlation Coefficients

Anthocyanins can directly scavenge oxygen FR, improving antioxidant-related enzyme activity in the body (Sakano et al., 2005). Importantly, anthocyanins are able to modulate antioxidant and detoxifying enzymes by activation of Nrf2 (Speciale et al., 2014). Thus, significant positive correlations were found between enzymes in the plasma and NFE2L2.

In the current study, stronger correlations were noted between some antioxidant enzymes and the mRNA expression of inflammation-related and antioxidant genes, suggesting that the body triggered a natural protective response by modulating these antioxidants and their gene expressions. Indeed, antioxidant enzymes form the first line of defense against FR in organisms (Rodriguez et al., 2004). The levels of antioxidant-related enzyme activity can response to the abundance of mRNA expression involved in inflammatory and antioxidant activities in tissues (Polavarapu et al., 1998). One study did reveal that a significant correlation between mRNA and protein levels as well as between protein levels and enzyme activities of the antioxidant enzymes was detected in rat tissues (Tiedge et al., 1997). In addition, Aitken et al. (2009) showed that significant positive correlations were observed between some antioxidant enzymes and pro-inflammatory molecules in bovine mammary tissue during the periparturient period. However, several parameters (TAC, SOD, GPX enzymes, and the abundances of hspA1A, TNF) showed negative correlations, perhaps because of differences in the mitochondria content in the mammary gland tissue. It should be noted that oxidative damage would be reduced if anthocyanins improve the antioxidant potential in dairy goats. Furthermore, anthocyanins can inhibit NF-κB, helping to fight OS status, thus inhibiting HSR (hspA1A) and relieving the OS correlation factor (TNF). This point needs to be determined in further additional studies. Our data provided evidence that antioxidant enzymes and related gene expressions may be cooperatively or differentially involved in the defense mechanisms in plasma and the mammary gland of dairy goats exposed to OS.

CONCLUSIONS

The current study demonstrated that the inclusion of anthocyanin-rich PSS in dairy goats had no effect on the DMI, apparent digestibility, nitrogen utilization, and rumen fermentation, whereas it did improve the DPPH scavenging activity and SOD activity in the plasma. In addition, anthocyanin-rich PSS could lead to up-regulation of NFE2L2 gene expression and could improve the expression of SOD2, GPX1, and GPX2 genes in the mammary gland of dairy goats.

Conflict of interest statement. None declared.

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