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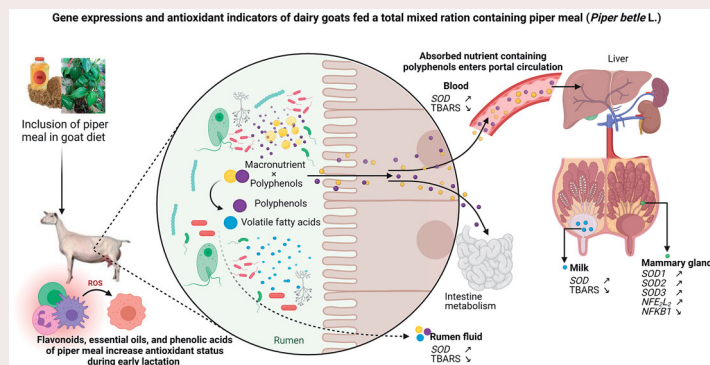
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ABSTRACT

The study evaluated the effect of piper meal which contains flavonoids, essential oils, and phenolic acids on the activity of antioxidant enzymes such as superoxide dismutase (*SOD*), glutathione peroxidase (*GPx*) and catalase (*CAT*), and as well as oxidative stress indicators such as 1,1-diphenyl-2-picrylhydrazyl-scavenging activity and thiobarbituric acid-reactive substances (TBARS) in the ruminal fluid, mammary tissue, milk, and blood of lactating goats. Fourteen early lactating Saanen goats (body weight, 44 ± 2.51 kg; 14 ± 3 days in milk) were allotted in equal number to two experimental groups, each received one of the two total mixed rations: control (CON) diet (containing 0% piper meal) and CPM diet (CON diet containing 1.3% piper meal per kg dry matter). Compared with goats fed the CON diet, those fed CPM diet had similar 1,1-diphenyl-2-picrylhydrazyl-scavenging, *GPx* and *CAT* activity in ruminal fluid, mammary tissue, milk, and blood; however, those samples had increased *SOD* activity. Dietary CPM diet had a tendency to decrease TBARS production in ruminal fluid, mammary tissue, milk, and plasma by about 2.4, 1.2, 1.1, and 1.4 folds, respectively. Furthermore, alleviated values of TBARS were associated with dwindling mRNA expression of κ light polypeptide gene enhancer in nuclear factor kappa B subunit 1 (*NFKB1*) but greater expression of *SOD1*, *SOD2*, *SOD3*, and nuclear factor (erythroid-derived 2)-like 2 (*NFE2L2*) in mammary tissue. Our findings showed that the inclusion of 13.0 g piper meal in per kg diet (CPM) can improve the oxidative status of Saanen goats in early lactation.

GRAPHICAL ABSTRACT



HIGHLIGHTS

- Flavonoids, essential oils, and phenolic acids of piper meal increased antioxidant status in early lactation of dairy goats.

ARTICLE HISTORY

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- Dietary piper meal attenuates oxidative stress indicators in physiological fluids of early lactating goats.
- Dietary piper meal modulated antioxidant and anti-inflammatory properties in physiological tissues of early lactating goats.

Introduction

Supplementation of dietary lipid such as polyunsaturated fatty acids (PUFA)-rich oils (omega-3 or omega-6) in ruminant diets can improve the nutritional quality of dairy products (Chilliard et al. 2007). Abundant PUFA sources could support isomerisation and hydrolysis in ruminal biohydrogenation, resulting in an accumulation of fatty acid (Purba et al. 2020d). However, in long term, this dietary strategy increases the level of lipid peroxidation which is prone to oxidative stress and as a consequence, the animal failed to meet the metabolic requirements for maintenance and production (Gobert et al. 2009). This indicates that lipoperoxidation mechanisms are referred (mainly) to PUFA intake. Activity of this peroxidation might lead to oxidative damage, whereby lipid substrate containing carbon-carbon double bond is attacked by a free radical and other reactive oxygen (Vasilaki and McMillan 2011). It is reported that cells attacked by peroxidation expresses the natural immune response by promoting cell survival or inducing cell death (Ayala et al. 2014). A survival cell in animal body exerts the extent of natural antioxidant system in correspondence with intracellular enzymes such as superoxide dismutase (SOD: 1.15.1.1), glutathione peroxidase (GPx: 1.11.1.9), and catalase (CAT: 1.11.1.6), which have a fundamental role to prevent superoxides and peroxides for further deteriorated reaction (Saleh et al. 2017; Jafari et al. 2021).

A plunged oxidative stress associated with decrease pro-oxidant properties on lactation performance of dairy cows could be rectified by supplementing antioxidant agent in diets, which could help to mitigate an initiation of lipid peroxidation (Celi 2010). In general, supplementation of potential antioxidant agents, such as vitamin C and/or vitamin E in diets can protect lipids and lipid structures against peroxidation (Saleh et al. 2018; Azimi et al. 2020). Nevertheless, sole supplementation of vitamin E as antioxidant replacer had no effect on lipoperoxidation, whereas vitamin E together with polyphenol-containing diets resulted in lowered lipoperoxidation by boosting the resistance time against peroxidation (+47%) and by alleviating the oxidation rate (−48%; Gobert et al. 2009). More recently, Côrtes et al. (2012) and Schogor et al. (2013)

reported that dietary polyphenols from plant (lignans) without combination of vitamin C or E increased antioxidant properties, and sheltered mammary and other tissues of dairy cows from oxidative stress damage.

Polyphenol-rich plants are natural strong antioxidants and anti-inflammatory agents, and *Piper betle* L. leaf has been reported to be rich in polyphenols containing flavonoids, essential oils, and phenolic acids as major active compound (Purba, et al. 2021a). This *Piperaceae* leaf which also contained some amount of food nutrient (Guha 2006) is edible by human and animal. More recently, it was reported that supplementing *P. betle* L. in *in vitro* studies using rumen fluid from goats increased organic matter degradability (Purba, et al. 2020b; Purba, et al. 2020c). Flavonoids could be degraded to intermediate metabolites (McSweeney et al. 2001) and transferred to milk (Purba, et al. 2020d). Tian et al. (2019) evaluated the effect of typical flavonoids such as anthocyanin in rumen fluid, mammary tissue, milk, and plasma in Saanen goats. The levels of 2,2-diphenyl-1-picrylhydrazyl scavenging activity and SOD activity in plasma, the relative expressions of nuclear factors (erythroid-derived 2)-like 2 (*NFE₂L₂*), *SOD2*, *GPx1*, and *GPx2* mRNA expression in the mammary gland were increased with a higher content of anthocyanin in milk. This indicates that, in ruminating system, the animal antioxidant performance largely depends on the status of rumen metabolic pathways, when the diet is supplemented with abundance of polyphenol-rich plants. To our knowledge, the effects of piper meal on the activity and expression of antioxidant enzymes in dairy goats during lactation are scanty. We hypothesised that dietary piper meal attenuates oxidative stress indicators in physiological fluids (e.g. blood, ruminal fluid, and milk) and physiological tissues (e.g. in mammary gland). The present study thus investigated the effects of piper meal on the activity of antioxidant enzymes as SOD, GPx, and CAT and oxidative stress indicators as 1,1-diphenyl-2-picrylhydrazyl-scavenging activity, and TBARS in the above-mentioned physiological fluids and mammary tissue, and the expression of studied antioxidant enzymes and oxidative stress-related genes in early lactating goats over different sampling times.

Materials and methods

Animals and diets

The National Research Council of Thailand (U1-02632-2559) and the Animal Ethics Committee of Suranaree University of Technology (SUT 4/2558) approved all the experimental procedures. The experiment began on October, 2018, and ended on December, 2018. Detailed descriptions of the animal management, sample collection and analyses, and production responses (e.g. dry matter intake, milk yield, and milk composition) have been reported previously (Purba, et al. 2020d). Briefly, a total of 14 healthy third-parity Saanen goats (BW, 44 ± 2.51 kg; mean \pm SD) at their early lactation period (14 ± 3 DIM; mean \pm SD) was used in a randomised, complete, balanced block design experiment with seven goats per treatment. Goats were paired on the basis of milk yield. The trial lasted for six weeks (0–42 days) after 14 days of adaptation to feeding regimen. The two experimental diets (iso-caloric, iso-nitrogenous, and iso-lipidic) were diet (containing 0% piper meal) and CPM diet (CON diet containing 1.3% piper meal per kg dry matter) prepared in the form of total mixed ration (TMR), formulated following to NRC requirement for goats (NRC 2007; Table 1).

P. betle L. leaves were harvested from several locations in the Suranaree University of Technology organic farm, Nakhon Ratchasima, Thailand (elevation of 243 m above sea level). The leaves from different plants were pooled and dried in hot air convection oven (Memmert 217 GmbH + Co.KG., ULM600, Germany) at 40 °C for three days, ground using a mesh size of 1 mm (Retsch SM 100 mill; Retsch GmbH, Haan, Germany), and kept in sealed plastic bags pending for the experiment (and hereafter referred to piper meal). The dosage level of piper meal used for this study was based on earlier *in vitro* (Purba, et al. 2020b; Purba, et al. 2020c) and *in vivo* (Purba, et al. 2020a; Purba, et al. 2020d) studies which reported that 1–6% DM of *P. betle* L. per kg of feed improved the ruminal fermentation and considered safe for the animals. In the present study, a moderate amount (<2%) of piper meal was chosen as a cost-effective way to augment the antioxidant status of animal response. The TMR were prepared every alternate day, with the portion for the following day kept in sealed plastic bags and store in room temperature. All the feed ingredients were purchased from the university (SUT) commercial feed mill. Samples of the TMR were regularly collected and analysed following to regular protocols (Van Soest et al. 1991; AOAC 2005), except the gross energy was

Table 1. Ingredients and chemical compositions of the experimental diets^a.

Item	Experimental diets		
	CON ^b	CPM ^c	Piper meal
Ingredient, g/kg DM			
Pangola hay	239	232	
Soybean meal	120	120	
Cassava chip	109	107	
Urea	14	14	
Sulphur ^d	0.4	0.4	
Sugarcane molasses	15	15	
Palm meal	134	131	
Cassava pulp	184	183	
Rice bran	137	137	
Mineral mix ^e	10	10	
Premix ^f	20	20	
Sunflower oil	17.6	17.6	
Piper meal	0	13	
Chemical composition			
Dry matter, g/kg as fresh weight	911.24	904.43	910.70
Organic matter, g/kg DM	903.17	901.44	908.63
Crude protein, g/kg DM	172.42	171.61	25.10
Ether extract, g/kg DM	40.14	39.78	3.20
Neutral detergent fibre, g/kg DM	520.64	519.92	751.24
Acid detergent fibre, g/kg DM	352.31	351.05	623.13
Gross energy (MJ/kg DM)	21.03	20.97	24.91
Net energy lactation (Mcal/kg DM) ^g	1.31	1.31	1.80

^aTMR diet containing the two different diets fed to goats for a six-week feeding trial after two weeks of adaptation period. ^bCON = control, TMR diet with no piper meal supplementation. ^cCPM = control with piper meal, TMR diet with containing piper meal 1.3% DM. ^dSulphur cube was derived from commercial purchase (Sand Sea Sun Shop: TG-6731, Bangkok, Thailand) and ground (sieve size of 1 mm). ^eContained (g/kg): NaCl (600), P (160), Ca (240). ^fVitamin A (4,200,000 IU/kg), vitamin A3 (840,000 IU/kg), vitamin E (10,000 IU/kg), vitamin K3 (2 g/kg), vitamin B1 (2.4 g/kg), vitamin B2 (3.5 g/kg), vitamin B6 (1.8 g/kg), vitamin B12 (0.01 g/kg), vitamin B5 (4.6 g/kg), vitamin C (12 g/kg), folic acid (0.28 g/kg), vitamin 7 (0.4 g/kg), copper (12 g/kg), manganese (40 g/kg), zinc (3.2 g/kg), iron (42 g/kg), iodine (0.8 g/kg), cobalt (0.8 g/kg), selenium (0.35 g/kg). ^gEstimated according to NRC (2007).

determined using a bomb calorimeter with O₂ carrier according to the manufacturer's instructions. Concentration of polyphenol compounds in the TMR was assayed in water, methanol, ethanol, chloroform, and hexane extracts and quantified based on mean signal of peak rate in the High-performance liquid chromatography with a diode-array detector (HPLC-DAD) principle (Purba, et al. 2021a). The present study, no significant different was found in their chemical compositions (Table 1), except for composition and polyphenol (phenolic acids, flavonoids, and essential oils) content of the TMR (Table 2).

Sampling

On weeks 1, 3, and 6, consecutive morning and afternoon milk samples from each individual goat were collected. Milk samples were directly stored at –80 °C fixed with 0.02% (w/w) of sodium azide (Labchem, Ajax Finechem Pty, Australia) for analysis of milk antioxidant (DPPH, TBARS, SOD, CAT, and GPx).

Table 2. Composition of polyphenol contents^a of piper meal and the experimental diets.

Polyphenol compounds (g/kg DM)	Experimental diets		Piper meal
	CON ^b	CPM ^c	
Phenolic acids			
Gallic acid	ND	0.02	1.31
Caffeic acid	ND	0.01	1.17
Syringic acid	ND	0.02	1.46
<i>p</i> -coumaric acid	ND	0.01	0.81
Sinapic acid	ND	0.01	0.83
Ferulic acid	ND	0.00	0.39
Flavonoids			
Catechin	ND	0.08	6.55
Rutin	ND	0.03	2.74
Quercetin	ND	1.00	84.75
Apigenin	ND	0.12	10.10
Myricetin	ND	0.01	1.03
Kaempferol	ND	0.08	6.67
Essential oils			
Eugenol	ND	0.22	18.52
Caryophyllene	ND	0.07	6.12

^aQuantification of polyphenol contents evaluated using the methods of Purba, et al. (2021a). ^bCON: control, TMR diet with no piper meal supplementation; ND: non-detectable. ^cCPM: control with piper meal, TMR diet with containing piper meal 1.3% DM.

After morning milking, rumen fluids at 0 h (before feeding) and those of three and six-hour post-feeding were collected from each goat using a stomach tube connected to a manual pump as given by Purba, et al. (2021b) with a slightly modification (Paengkoum, et al. 2021a; Vorlaphim et al. 2021). Rumen fluid and substrate were collected from rumen routes by attaching approximately 2.5 m of poly tubing (0.4 cm i.d.), which has approximately 50 holes in one end of poly tubing. The other end of the poly tube was attached to a manual pump, which was fitted into the top of a 500 mL polypropylene filter flask (Fisher Scientific, Pittsburgh, PA). One end of a 0.7 m piece of vinyl tubing was attached to an 85-mL rubber bulb, whereas the other end was attached to side port of the filter flask. Oral lavage samples were collected from goats with this apparatus by passing the modifying poly tube through a Frick speculum (stainless steel 50.8 cm length, 3.2 cm diameter; Navel, Punjab, Pakistan) placed in the mouth, and the manual pump was controlled to provide suction once the modifying poly tube was in the rumen. Immediately after collection, each sample of rumen fluids was strained through four layers of cheesecloth, and the filtered ruminal fluids of each sampling time were stored at -80°C for DPPH, TBARS, SOD, CAT, and GPx analyses.

Blood samples (10 mL) were collected immediately before the morning feeding (0 h), 3 h, and 6 h post-feeding from the jugular vein into using evacuated tubes containing heparin. Blood samples were centrifuged at $3000 \times g$ for 15 min at 4°C (Sorvall Legend

XT/XF Centrifuge Series, Thermo Fisher Scientific, Waltham, MA, USA). Plasma was stored at -80°C to determine plasma antioxidant (DPPH, TBARS, SOD, CAT, and GPx) and erythrocyte was stored at -80°C for subsequent analysis of enzyme activity.

A day after collection of rumen fluid and blood, biopsies of the mammary gland were performed following the method by Farr et al. (1996) with minor modification (Tian et al. 2019). Before the biopsy procedure, goats received a subcutaneous injection of antibiotic (Penomycin containing procaine, sodium penicillin, and Streptomycin, Manufacturing Co., Ltd, Samutprakam, Thailand). Mammary biopsies were collected at a midpoint (about 10 cm^2 area of skin) on the left or right rear quarter gland from seven goats per treatment, alternating mammary rare quarters between time points. The area was strictly checked about 2–3 cm from a recent scar that indicated earlier biopsy area. The selected area was cleaned and shaved. The samples of biopsy (approximately 1 mg) were taken using a semi-automatic biopsy needle (16 G \times 90 mm, SAG-16090, TSK Corporation, Tochigi, Japan), after receiving local anaesthesia. The skin or punctured region was closed with a simple interrupted nondegradable suture. This activity caused a minor inflammation, but the wound area was completely disappeared within six days. During this period, extreme care during manual milking was performed to minimise possible blood clots lodged in the gland. Mammary gland was alternated among repeated measurement to collect mammary tissue biopsies. Biopsy samples of mammary tissue were subsequently rinsed in sterile saline solution to remove all traces of blood and then divided into two portions: the first part kept into 1.5-mL microcentrifuge tube was instantly frozen in liquid N_2 and stored consecutively at -80°C for gene expression analyses. The second part was ground promptly in 1 mL of sterile saline solution for 20 seconds with a rotor–stator homogeniser (IKA-T50 ultra-turrax, USA) and kept at -80°C for further analysis of antioxidant enzyme activity.

Determination of antioxidant indicators

Determination of DPPH in plasma, milk, ruminal fluid, and mammary tissue was performed spectrophotometrically (Varioskan-LUX multimode microplate reader, Thermo Scientific, USA) using a stable free-radical DPPH in quadruplicate as described by Schogor et al. (2013). Radical scavenging capacity was expressed as percentage DPPH (DPPH%) and calculated using the following equation: $\text{DPPH}\% = [(\text{Abs}$

control -Abs sample)/(Abs control)] \times 100. A calibrated curve of antioxidant capacity was calculated as described by Li et al. (2009), and a linear relationship of antioxidant and volume dilution including the 50% effective concentration (EC50) was performed according to previous reports (Chen et al. 2013).

Lipid peroxidation was assessed in plasma, milk, ruminal fluid, and mammary tissue from the original samples using a commercially available TBARS assay kit (CN: E BC K184 OXI-TEK; Elabscience, USA) under the fluorescence procedure ($\lambda_{ex}/\lambda_{em}$ = 560 nm/585 nm) and was performed according to the manufacturer's instructions. The assay was performed in quadruplicate.

The activity of *GPx*, *CAT*, and *SOD* in plasma, erythrocytes, ruminal fluid, milk, and mammary tissue was determined enzymatically in Microplate (96 wells, UV plate), in quadruplicate under monitoring absorption by microreader (Varioskan-LUX multimode microplate reader, Thermo Scientific, USA), according to the manufacturer's instructions. The sensitivity, intra assay CV, inter assay CV, and recovery were 0.2 U/mL, 2.9, 3.7 and 96.6%, respectively. Total protein concentration in the blood, mammary tissue, and milk was determined with a bicinchoninic acid protein assay (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions with a minor modification (Paengkoum, Petlum et al. 2021).

Quantitative real-time PCR amplifications of the studied genes

Total RNA was isolated from 150 mg of mammary biopsy using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocols. Samples of mammary biopsy was prepared in 2 mL of Eppendorf tubes and dehydrated by evaporating tissue using nitrogen. The RNA samples were digested with RQ1 RNase-Free DNase (M6101, Promega, Madison, WI, USA) and quantification was accessed using a NanoDrop NanoVue spectrophotometer (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Abundant purity of extracted RNA obtained in average 260/280 ratio throughout the experiment was 1.99 (1.98–2.04). Integrity of the RNA was performed by verifying the presence of 18S and 28S RNA bands using an ImageQuant LAS 500 imager (GE Healthcare BioSciences) after electrophoresis on a 1% agarose gel. The RNA integrity number (RIN) was calculated in triplicate by using the Agilent 2100 Expert software (Schroeder et al. 2006). RIN-values were consistently within the minimum required for cDNA

synthesis (RIN, 3.9–7; values $>$ 5 obtained from at least duplicate). A GoScriptTM Reverse Transcriptase with random primers (Promega Corporation, Madison, WI, USA) was used, according to the manufacturer's instructions, to synthesise cDNA. A sample of 100 ng total RNA was used for cDNA synthesis. The cDNA therefore obtained was stored at -20°C until ready to use.

The relative mRNA abundance of the selected genes was conducted using quantitative real-time PCR. Quantitative PCR amplification, detection, and data analyses were equipped using a Roche Lightcycler 480-II (Roche Applied Science, Switzerland). Reference of targeted genes and housekeeper genes in present study used for real-time qPCR is listed in Table 3. In the present study, we used all listed references and four housekeeper genes (*PPIA*: peptidyl-prolyl isomerase A, *ACTB*: actin β , *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase, and *UBB*: ubiquitin B), therefore, the geometric mean of four housekeeper genes was used for normalisation of datasets. To note, publicly available goat sequences were used to design the primer pair for all genes. Online NCBI (<http://www.ncbi.nlm.nih.gov/>) was used to perform database research, alignments, and sequence analyses. The referred organism utilised was *Capra hircus* (taxid:9925) with no preference at exon junction span. Primer pair specificity was Refseq mRNA. The primers set of selected genes were purchased from the commercial company (Vivantis Technologies Sdn Bhd, Selangor Darul Ehsan, Malaysia). The RT-qPCR amplifications were assayed in a 10 μL reaction volume, which consisted of 5 μL of 2 \times Roche 04707516001 LightCycler 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany), 2 μL of 10 \times diluted cDNA and 1 μL of forward and reverse primers (final concentration ranging from 200 to 1000 nM). The mixed assay was prepared in the PCR plates (LightCycler 480 multiwell plate 96, white; Roche Diagnostics GmbH) with plastic cover. The plates were centrifuged at 4 $^{\circ}\text{C}$, 24900 $\times g$ for 3 min (Universal 320, Hettich Zentrifugen, Germany). The cycling conditions were 10 min at 95 $^{\circ}\text{C}$ for pre-incubation, 40 cycles of 30 s at 95 $^{\circ}\text{C}$ for amplification, and 55 to 59 $^{\circ}\text{C}$ for 1 min and cooling at 40 $^{\circ}\text{C}$ for 30 s. Amplifications were performed in quadruplicate for each gene. Amplification efficiency (AE) of each gene was calculated 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. The calculated values of AE were 94.67–104.11%. The relative mRNA abundance was calculated using the $2^{-\Delta\Delta\text{Ct}}$

Table 3. Primer sequences used for real time qPCR amplification of targeted and housekeeper genes selected genes in the mammary of lactating goats.

Gene name ^a	Primer sequences (5'→3')	Size (bp)	Reference Sequence ^b
<i>SOD1</i>	(F) ACACAAGGCTGTACCAAGTGC (R) TTCACATTGCCAGGCTCC	104	NM_001285550.1
<i>SOD2</i>	(F) CTGTTGGTGTCCAAGGTTCC (R) AATAAGCATGCTCCACACG	153	XM_018053428.1
<i>SOD3</i>	(F) ATCCACGTGCACCAAGTTTG (R) ACGGACATCGGGTTGTAGTG	74	NM_001285675.1
<i>GPX1</i>	(F) CCTGGTGGTACTCGGCTT (R) TCGGACGTAICTCAGGCAAT	91	XM_005695962.3
<i>GPX2</i>	(F) CCGCACCTTCCAACCATTA (R) AAAGACACTCAGGGCAGGAG	103	XM_005685982.3
<i>GPX3</i>	(F) TTCGGTCTGGTCACTTCTGGG (R) CGAACATACTTGAGGGTGGC	95	XM_005683183.3
<i>CAT</i>	(F) CCTGTTCACTGATCGAGGGA (R) AACTGCCTCTCCATTGGCAT	97	XM_005690077.3
<i>NFE₂L₂</i>	(F) GGCCCATGACCTCTCTCTG (R) TGATGCCATGCTTGGACTTG	131	NM_001314327.1
<i>NFKB1</i>	(F) CTGGAAGCACGAATGACAGA (R) GCTGTAACATGAGCCGTACC	197	XM_018049265.1
<i>PPIA</i>	(F) GGATTTATGTGCCAGGGTGGTGA (R) CAAGATGCCAGGACCTGTATG	120	XM_018047036.1
<i>ACTB</i>	(F) CGTGATGGTGGGCATGGG (R) GTGTGGTGCCAGATCTTCT	141	NM_001314342
<i>UBB</i>	(F) AGATCCAGGATAAGGAAGGCAT (R) GCTCCACCTCCAGGGTGAT	198	XM_005693600.3
<i>GAPDH</i>	(F) TGGTGAAGGTCGGAGTGAAC (R) TGTAGACCATGTAGTGAAGGTCA	129	XM_005680968.3

^a*SOD1*: superoxide dismutase 1; *SOD2*: superoxide dismutase 2; *SOD3*: superoxide dismutase 3; *GPx1*: glutathione peroxidase superoxide dismutase 1; *GPx2*: glutathione peroxidase superoxide dismutase 2; *GPx3*: glutathione peroxidase superoxide dismutase 3; *CAT*: catalase; *NFE₂L₂*: nuclear factors (erythroid-derived 2)-like 2; *NFKB1*: nuclear factor kappa B subunit 1; *PPIA*: peptidylprolyl isomerase A; *ACTB*: actin β; *UBB*: ubiquitin B; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase. ^bThe reference sequence numbers are given for primers whose source is the National Centre for Biotechnology Information (NCBI) GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/> . . .).

as given by Livak and Schmittgen (2001). Threshold (Ct) values of target genes were normalised to the geometric mean of four reference genes to determine $\Delta\Delta C_t$ values ($C_{t \text{ target gene unknown sample}} - C_{t \text{ housekeeper unknown sample}} - (C_{t \text{ target gene calibrator sample}} - C_{t \text{ housekeeper calibrator sample}})$). The calibrator was the mean of abundant genes in the CON data. Ct values were averaged Ct value from each tested gene-condition-sample combination. Data relative mRNA abundance of genes was normalised by \log_2 transformation before statistical computation.

Statistical analysis

The data were analysed by repeated measures ANOVA for a randomised block design using the MIXED procedure of SAS 9.4; the normality of residuals was tested by the Shapiro–Wilk test. The statistical model included the fixed effects of diet (CON vs. CPM), week (1, 3, and 6), and their interaction (diet × week) and the random effects of goat within block, assuming an autoregressive order one covariance structure fitted on the basis of Akaike information and Schwarz Bayesian model fit criteria. Least-squares means are reported, and significance was declared at $p < .05$.

All data related to DPPH, TBARS, and enzyme activities in ruminal fluid and blood observed after 0, 3, and 6 h of a post-feeding effect were processed as a completely randomised design with repeated measures using the MIXED procedure of SAS 9.4. The normality of the distribution of variables was tested by the Shapiro–Wilk test. Akaike's information criterion of the mixed model of SAS was performed to obtain the compound symmetry of the covariance structure. Least-square means are reported, and significance was calculated by Tukey's HSD at $p < .05$.

Results

Oxidative indicators and antioxidant enzyme activity

Radical-scavenging activities in plasma, milk, ruminal fluid and mammary tissue were not affected by dietary experimental diets which ranged from 29.63–68.43% of DPPH inhibition (SE 0.99). Also, dietary supplement of piper meal resulted in similar value of the EC₅₀ among diets and sampling times, with average values of 1.86, 0.45, 0.60, and 1.21 μL/mL, for plasma, milk, ruminal fluid and mammary tissue, respectively.

Table 4. Thiobarbituric acid-reactive substances (TBARS) in physiological fluid, mammary tissue, and milk of Saanen goats fed the experimental diets^P.

TBARS (nmol/mL)	Diet ^Q			p-Value		
	CON	CPM	SEM	Diet	Week	Diet × Week
Rumen fluid	26.91	11.1	0.322	<.001	.474	.503
Mammary tissue	6.43	5.49	0.022	.014	.015	.020
Milk	24.21	22.09	0.136	.015	.018	.001
Plasma	18.79	13.75	0.109	<.001	.663	1.163

^PData are presented as a least square means ± standard error of the mean (SEM). ^QCON: control, TMR diet with no piper meal supplementation; CPM: control with piper meal, TMR diet with containing piper meal 1.3% DM.

In addition, inclusion of piper meal (CPM) reduced TBARS production in plasma, milk, ruminal fluid and mammary tissue by approximately 1.4-, 1.1-, 2.4- and 1.2-fold, respectively as compared to the control (CON, Table 4). Interaction between diet and time of sampling (weeks 1, 3, and 6) for TBARS production of mammary tissue and milk were noted, with lower values ($p < .05$) at week six as compare to the earlier two sampling times (Table 4). Furthermore, there was a post-feeding effect for TBARS in rumen fluids and plasma observed in Saanen goats fed the CPM diet (Figure 1(a)). The concentration of TBARS in ruminal fluids and plasma declined after 3 h of feeding time (both at $p < .001$), but no significant difference between 3 and 6 h feeding times ($p > .05$).

The activity of glutathione peroxidase (GPx) and catalase (CAT) profiles in rumen fluid, mammary tissue, milk, plasma and erythrocyte were not affected by dietary supplementation of piper meal (Table 5). In contrast, the CPM diet increased ($p < .05$) superoxide dismutase (SOD) in rumen fluid, mammary tissue, milk, plasma and erythrocyte. There were interactions between diet and sampling time (weeks 1, 3, and 6) for SOD observed in mammary tissue and milk, with those in the CPM diet increased, but it was remarkably lower ($p < .05$) at week 6 as compares to the earlier two sampling times (weeks 1 and 3; Table 5). Regardless of diet influences, there was post-feeding effect on SOD in rumen fluid and blood observed in Saanen goats fed the CPM diet (Figure 1(b)). The SOD concentrations of rumen fluids and erythrocytes were higher ($p < .01$) after a 3-h post-feeding, and remained unchanged thereafter. However, SOD concentration in plasma did not affected by feeding time ($p > .05$).

Relative mRNA abundance of antioxidant genes and inflammation-related genes

There was interaction ($p < .001$) between diet and sampling time (weeks 1, 3, and 6) for *SOD1*, *SOD2*,

SOD3, and *NFE2L2* (except *NFKB1* decreased) mRNA abundance in the mammary tissue, with a significant increase for goats in the CPM diet group (Figure 2). The mRNA abundance of the *CAT*, *GPx1*, *GPx2*, and *GPx3* genes was not affected by supplementation of piper meal ($p > 0.05$).

Discussion

In the present study, we evaluated the effect of supplementation of piper meal on the oxidative stress on lactating dairy goats using the level of malondialdehyde (MDA) production as indicator. MDA production has been broadly assessed as mutagenic product of lipid peroxidation by omega-3 and omega-6 fatty acids due to its facile reaction with thiobarbituric acid (Ayala et al. 2014). The data reported here, together with those from the previous reports (Purba, et al. 2020d; Jafari et al. 2021), suggested that thiobarbituric acid assay (TBARS) could be used to monitor the MDA performance and inclusion of sunflower oils as a predominantly omega-6 fatty acid source in the total mixed ration (TMR) was successful in making all the experimental goats under the status of abundant peroxidation. As expected, the bioactive polyphenols in the CPM diet decreased the TBARS concentration in the ruminal fluid, plasma, mammary tissue, and milk in goats. Dietary plant polyphenol was expected to prevent oxidative stress by decreasing TBARS concentration in rumen fluids (Schogor et al. 2013) due to the improvement of ruminal epithelial cells and defence systems (Ma et al. 2018). Similar to our results, Li et al. (2020) observed reduced early lactation TBARS coinciding with increasing metabolic status of dairy buffaloes receiving mulberry leaf. Our result suggested that 3-h post-feeding of the CPM diet, antioxidant activity in ruminal fluid (Figure 1) gradually increased as the result of activated defence by the bioactive polyphenols to protect available lipid towards the propagation of lipid peroxidation in rumen (Oteiza et al. 2005). Although the supplementation of dietary polyphenols presents in *P. betle* leaves affecting rumen fermentation and biohydrogenation (Purba, Yuangklang, Paengkoum, Paengkoum 2020), its effect of flavonoids as a major regulator on subsequent metabolisms is still not totally clear. Flavonoids could be degraded in the rumen and metabolised via hydrolysing glycosides and cleavages of heterocyclic compounds among polyphenol structures, absorbed from the lumen of the gut into the portal vein, and synthesised with other milk constituents through either the Golgi or the milk fate routes of secretion across the mammary

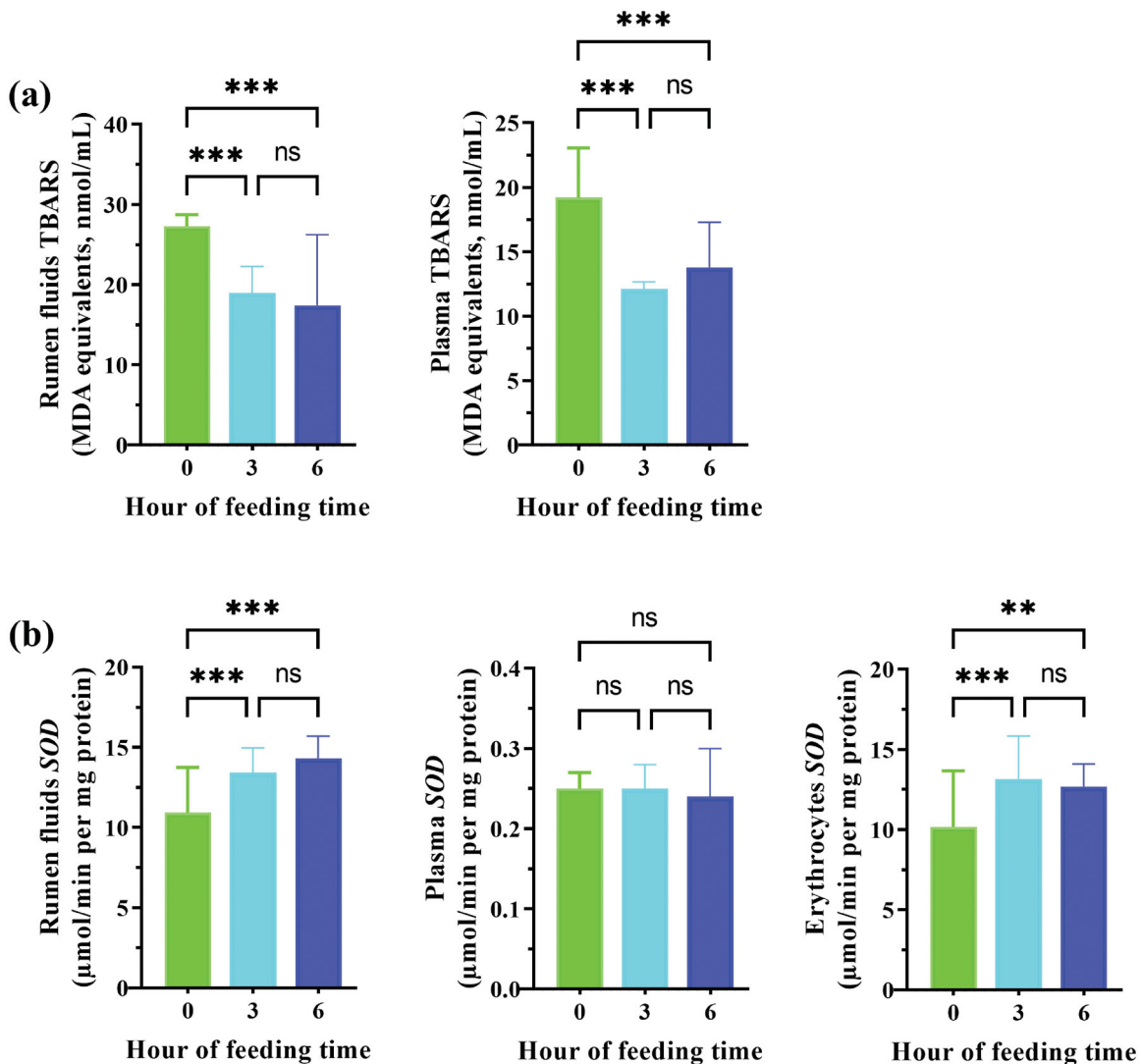


Figure 1. TBARS in rumen fluids and plasma observed in Saanen goats fed the CPM diet after feeding time (a) and SOD in rumen fluids and blood observed in Saanen goats fed the CPM diet after feeding time (b). Values are means, with their standard errors represented by vertical bars. Values was different (***, $p < .001$; **, $p < .01$; ns, $p > .05$).

epithelium (Shennan and Peaker 2000; McSweeney et al. 2001; Purba, et al. 2020d). Therefore, a lower TBARS in plasma, mammary gland, and milk of goats fed with the CPM diet might be originating from a lower TBARS content in rumen, which likely indicated optimum interaction of polyphenols with bilayers by surging membrane fluidity.

Superoxide dismutase (SOD) is the endogenous enzyme in first line of the defence system against reactive oxygen species. In term of rendering the potentially harmful superoxide anion, SOD has an important role to catalyse the dismutation of two molecules of the superoxide anion radical to hydrogen peroxide and molecular oxygen, whereas further transformation of hydrogen peroxide to water is handled by the GPx and CAT (Ayala et al. 2014). Over the last decades, only two studies reported on the effect of

secondary metabolites of *P. betle* L. on activity of anti-oxidant enzymes, especially the effect on SOD, GPx, and CAT in rats (Aliahmat et al. 2012; Venkadeswaran et al. 2014). To the best of the author's knowledge, the current data show for the first time an investigation that bioactive polyphenols in the CPM diet increased SOD concentrations in the mammary tissue, milk, rumen fluid, plasma, and erythrocyte and those were reaffirming results of previous studies (Tian et al. 2019). The CPM diet increased SOD activity could be attributed to the sufficient removal of endogenously superoxide, tending to a direct reduction of the reactive oxygen species. According to McSweeney et al. (2001) and Purba, et al. (2020d), bioactive compounds of polyphenols, especially flavonoids, could be degraded in rumen and absorbed across the rumen wall or subsequently transferred to intestine

metabolism. Those absorbed then were carried by the blood vessels to the liver before secreted to the milk via mammary gland. It is worthy to note that those absorbed might provoke an increase of blood response and messenger RNA expression (Yang et al. 2010). In the present study, a post-feeding effect for *SOD* was occurred in rumen fluids and erythrocytes, although, remaining unchanged in plasma. These results suggested that antioxidant activity to inhibit the propagation of lipid peroxidation needs sufficient time. The CPM diet was expected to increase catalysation for dismutation from reactive oxygen species at least 3-h post-feeding. A lack of alteration in *SOD* activity observed in plasma could be due to different transport mechanism compared with erythrocytes. Erythrocytes has been shown to increase the defence response when this site obtained an impairment such a deterioration of erythrocyte membrane integrity (Köse et al. 2002). MDA or TBARS affected oxidative status in the present study may involve later destruction in membrane structure and function, resulting in alleviation in membrane integrity. Concurrently, polyphenols from piper meal may be assessed by the erythrocytes as foreign molecules and these compounds seemed to have capacity to induce erythrocytic membrane as reminder signal (Revin et al. 2019). Taken altogether, our results suggested that digestion of antioxidant substrates from piper meal may interact directly on the *SOD* enzymatically system which render the protection to lipid peroxidation.

Despite increased *SOD* activity by ingestion of the CPM diet, activity of *GPx* and *CAT* observed in mammary tissue, milk, rumen fluid, and blood of the present study was stagnant. These occurrences could be assessed either from unvaried DPPH results which polyphenols gave a consistent effect on mitigating oxidative stress through performing massively donation into the first line of the defence system (Schogor et al. 2013; Tian et al. 2019). Notably, the first line defence system is *SOD* enzymatically site. As a consequence, there were no remaining superoxide anion radical in the dismutation tending to lack of *GPx*, and *CAT* activities (Mittler 2002). Observation of dietary flavonoids on blood metabolism revealed no difference in activities of antioxidant enzymes involved *SOD*, *GPx*, and *CAT*; nevertheless, increase in antioxidant status was detected in that study by increasing heat shock proteins and serum metabolic hormones (Li et al. 2020). The above findings were for early lactation buffaloes fed with different quantities of mulberry leaf supplementations (15–45 g/day). Using piper meal as supplement, our results seemed to have a higher efficacy

and consistency over the six-week study. The present study revealed that inclusion of piper meal at 13.0 g/d in the diet is promising in enhancing antioxidant enzymes activity in goats. Here, the dose-dependent effect of dietary flavonoids observed in the present study is also occurred and might corroborate Li and co-workers' findings, which the number of *SOD* in the current findings were similar to prior studies observed in dairy goats (Tian et al. 2019) and cows (Hosoda et al. 2012).

During the periparturient stage, a change in antioxidant enzyme activity may associate with antioxidant gene expression including their corresponding linking with proinflammatory gene expression in mammary tissue (Aitken et al. 2009; Côrtes et al. 2012; Schogor et al. 2013). In the present study, the bioactive polyphenols in the CPM diet influenced mammary *NFKB1*, *NFE₂L₂*, *SOD1*, *SOD2*, and *SOD3* mRNA abundances and did not affect *CAT*, *GPx1*, *GPx2*, and *GPx3* genes. *NFE₂L₂*, also known as nuclear factor E2-related factor-2 is a transcription factor that owns a well-documented detoxification benefit to alleviate oxidative stress (Nguyen et al. 2009). Previous studies that used dairy animals had highlighted enhancement of *NFE₂L₂* mRNA abundance as affected by inclusion of polyphenols in the diets (Schogor et al. 2013; Ma et al. 2018; Tian et al. 2019). *NFE₂L₂* generated an elaborated expression of the antioxidant response element in the promoter region of a number of genes (Zhu et al. 2005). For this reason, *SOD1*, *SOD2*, *SOD3*, and *NFKB1* to be signalled on modulating antioxidant response, thereby caused lack of CPM effect on *CAT*, *GPx1*, *GPx2*, and *GPx3* genes. Furthermore, the present study showed an upregulated gene expression of *SOD1*, *SOD2*, and *SOD3* by dietary CPM diet, and that was consistent with prior study (Tian et al. 2019). In elaboration, *SOD3* acknowledged as indispensable enzyme had been reported to have a favourably communication with proinflammatory agent, thereof acted antioxidant defence against inflammatory conditions (Ighodaro and Akinloye 2018). In other reports, *SOD3* could be influenced by antioxidant vitamin C, butylated hydroxyanisole and other antioxidant agents such as plant polyphenols (Singh and Bhat 2012). Accordingly, *NFKB1* gene was down-regulated as affected by the dietary CPM, which was in agreement with prior study (Subbaramaiah et al. 2013). The present results indicated that there was a link between *NFKB1* and *NFE₂L₂* which down-regulated *NFKB1* gene resulted in up-regulation of *NFE₂L₂* gene. Therefore, the present study implied that ingestion of piper meal as antioxidant source could improve defence

Table 5. Antioxidant enzymes in physiological fluid, mammary tissue, and milk of Saanen goats fed the experimental diets^P.

Item ^Q	Diet ^r			p-Value		
	CON	CPM	SEM	Diet	Week	Diet × Week
Rumen fluid						
<i>SOD</i> , μmol/min per mg protein	11.96	13.63	0.107	.004	.562	.059
<i>GPx</i> , nmol/min per mg protein	11.74	11.37	0.104	.067	.863	.928
<i>CAT</i> , μmol/min per mg protein	10.78	10.40	0.088	.059	.378	.665
Mammary tissue						
<i>SOD</i> , μmol/min per mg protein	20.26	25.86	0.129	.002	.017	.014
<i>GPx</i> , nmol/min per mg protein	21.94	21.74	0.140	.209	.176	.342
<i>CAT</i> , μmol/min per mg protein	26.33	26.33	0.136	.390	.774	.276
Milk						
<i>SOD</i> , μmol/min per mg protein	138.36	144.40	0.372	.026	.020	.031
<i>GPx</i> , nmol/min per mg protein	121.18	118.25	0.319	.353	.776	.163
<i>CAT</i> , μmol/min per mg protein	101.99	102.25	0.290	.454	.806	.195
Plasma						
<i>SOD</i> , μmol/min per mg protein	0.13	0.16	0.134	.010	.275	.216
<i>GPx</i> , nmol/min per mg protein	0.29	0.29	0.156	.089	.41	.092
<i>CAT</i> , μmol/min per mg protein	0.14	0.14	0.130	.077	.299	.373
Erythrocyte						
<i>SOD</i> , μmol/min per mg protein	10.55	13.27	0.105	.004	.225	.174
<i>GPx</i> , nmol/min per mg protein	11.76	11.76	0.086	.061	.419	.098
<i>CAT</i> , μmol/min per mg protein	11.52	11.52	0.084	.059	.527	.335

^PData are presented as a least square means ± standard error of the mean (SEM). ^Q*SOD* = superoxide dismutase; *GPx* = glutathione peroxidase; *CAT* = catalase. ^rCON: control, TMR diet with no piper meal supplementation; CPM: control with piper meal, TMR diet with containing piper meal 1.3% DM.

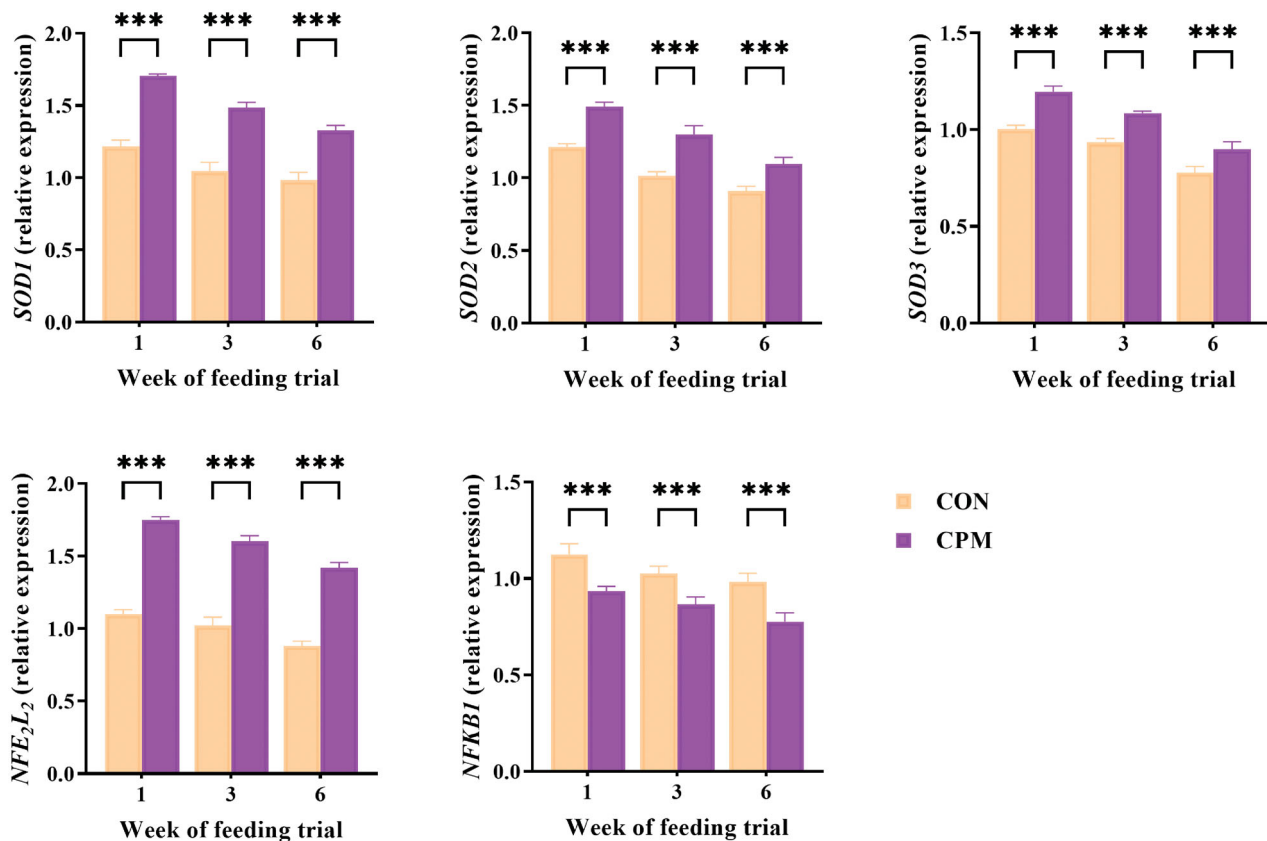


Figure 2. Relative mRNA abundance of *SOD 1, 2, 3*, *NFE₂L₂*, and *NFKB1* genes in the mammary tissue shown the interaction of diet and sampling time (weeks 1, 3, and 6). Values are means, with their standard errors represented by vertical bars. Gene expression of *SOD1*, *SOD2*, *SOD3*, and *NFE₂L₂* was up-regulated (***, $p < .001$), and gene expression of *NFKB1* was down-regulated (***, $p < .001$); in the mammary gland of goats receiving polyphenols in diet (CPM) relative to the no polyphenols in diet (CON).

mechanisms through eliminating reaction of inflammatory mediators such as *NFKB1* and alleviating oxidative stress by increasing response of *NFE2L2*, *SOD1*, *SOD2*, and *SOD3* mRNA abundances.

As aforementioned, goats receiving the CPM diet experienced less oxidation. Abundance of sample size ($n = 7$ /experimental diet) in the present study, the possibly reason of these enhanced performances in comparable DM intake (CON vs CPM) seemed to indicate the capability of piper meal to reduce metabolic stress, lipid peroxidation, and inflammation leading to a more efficient utilisation of energy and crude protein for milk production (Winkler et al. 2015; Purba, et al. 2020d). Furthermore, polyphenol and essential oil intake may also influence other aspects such as improved ruminal fermentation, nutrient absorption, and gut function which may be also responsible for improved milk production (Purba, et al. 2020d). The present findings suggested the benefits of supplementing piper meal in diet of lactating goats, although, almost all of the above parameters were lower at week 6 as compared to weeks 1 and 3 samplings. Notably, the quantity and quality of milk involving antioxidant deposition therein, could be influenced by breed, age, parity, management and lactation stage, where goats had been reported to produce higher milk yield, protein and fat components during the first 60 days of lactation but decrease thereafter (Clark and García 2017; Purba, et al. 2020d). In addition, in studies on performances in enzymatically antioxidant activities and oxidative stress indicators in cows and goats reported gradual reduction from peak lactation to the next lactation (Kapusta et al. 2018; Giorgio et al. 2020). Also, dietary polyphenols regulation on antioxidant activity and deposited levels in milk could plunge in the next day after peak lactation (Lakram et al. 2019; Tian et al. 2019). Ultimately, our results demonstrated that enhanced antioxidant status in lactating goats could be obtained by dietary plant polyphenols to reduce lipid peroxidation and directly effecting producing milk, but the above effect was dependent on lactation period.

Conclusions

Inclusion of piper meal rich in secondary metabolites including phenolic acids, flavonoids and essential oils in the diet of dairy goats significantly influenced the activity of antioxidant enzymes and oxidative stress indicators in the ruminal fluid, mammary tissue, milk and plasma. These outcomes reflect a positive benefit by using *Piper betle* L. and the inclusion of piper meal

at 13.0 g/kg diet can improve the oxidative status of early lactation Saanen goats. Further studies are, however, warranted to substantiate the current findings with a larger sample size and to clarify the role of piper meal on the oxidative status of goat during the transition or late dry, mid and late lactations.

Ethical approval

The research was carried out in accordance with regulations on animal experimentation and the Guidelines for the Use of Animals in Research as recommended by the National Research Council of Thailand (U1-02632-2559). The Animal Ethics Committee of Suranaree University of Technology issued a statement approving the experimental protocol (SUT 4/2558).

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Author Contributions

Conceptualization, R.A.P.P., S.P., C.Y., P.P. and J.B.L.; data curation, R.A.P.P., S.P., C.Y., P.P. and J.B.L.; methodology, R.A.P.P., S.P., C.Y., P.P., A.Z.M.S. and J.B.L.; project administration, R.A.P.P. and P.P.; formal analysis, R.A.P.P., C.Y. and P.P.; investigation, R.A.P.P.; resources, R.A.P.P., S.P. and P.P.; visualization, R.A.P.P. and J.B.L.; writing—original draft preparation, R.A.P.P., S.P., P.P., A.Z.M.S. and J.B.L.; writing—review and editing, R.A.P.P., P.P., A.Z.M.S. and J.B.L.; supervision, S.P., C.Y., P.P. and J.B.L.; funding acquisition, S.P. and P.P.; All authors have read and agreed to the published version of the manuscript.


Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

All data relevant to the study are included in the article.

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