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Chaya (*Cnidoscolus aconitifolius*, Mill. Johnston) pellet supplementation improved rumen fermentation, milk yield and milk composition of lactating dairy cows

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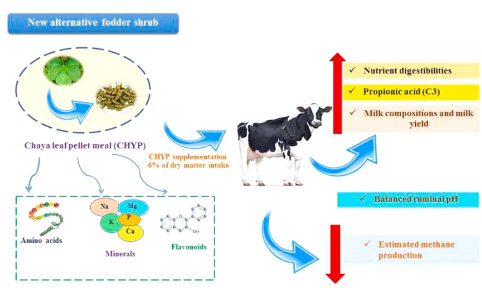
HIGHLIGHTS

- Chaya (*Cnidoscolus aconitifolius*) leaf pellet (CHYP) contains high level of crude protein and minerals.
- CHYP supplementation enhanced nutrient digestibility, propionic acid (C_3) and balanced rumen pH and reduced calculated methane emission.
- CHYP can improve milk yield and milk composition.

GRAPHICAL ABSTRACT



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ABSTRACT

Chaya (*Cnidoscolus aconitifolius*, Mill. Johnston) is a fodder shrub that contains flavonoid compounds and uses as feeds, medicine and food, respectively. This study examined the effects of Chaya leaf pellet (CHYP) supplementation on nutrient digestibilities, feed intake, rumen fermentation, milk yield and milk compositions. Four lactating crossbred (75% Holstein-Friesian × 25% Thai native cows) dairy cows with average body weight (440 ± 10), milk production (12 ± 2 kg/h/d) and days- in- milk (126 ± 20) were randomly assigned to a 2 × 2 factorial arrangement in a 4 × 4 Latin square design. Two ratio of concentrate to milk yield, (1:1; CM1 and 1:2; CM2) and two CHYP levels at 0 and 6% of total dry matter intake (TDMI) were imposed. The results revealed that concentrate to milk yield ratio and CHYP supplementation had interactive effect ($P < 0.05$) on nutrient intake except ADF, fat -corrected milk and total solids. Rumen pH was decreased ($P < 0.05$) in cows receiving high concentrate, while the pH was in normal range for animals fed with CHYP. Ruminal NH_3-N concentration was increased ($P < 0.05$) by the 6% CHYP supplementation. The level of blood urea nitrogen (BUN) ($P < 0.05$) was increased with the increase of ruminal NH_3-N ($P < 0.01$), and bacterial population, while the, protozoal counts were slightly reduced by the supplementation of CHYP. The CHYP supplementation significantly increased total VFA and propionate concentration, while decreased acetate to propionate ratio and estimated methane emission.

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In conclusion, concentrate to milk yield at 1:1 ratio and CHYP supplementation at 6% significantly modulated rumen fermentation end-products and milk composition in lactating dairy cows.

1. Introduction

Plant secondary metabolites or phytonutrients are characterized as plant-derived bioactive compounds with antimicrobial properties, commonly found in fruits, vegetables, and seeds (Middleton et al., 2000). Phytonutrients have also been reported as rumen modifiers for fermentation activities in ruminants (Wallace et al., 2002; Calsamiglia et al., 2007; Patra, 2012; Oh and Hristov, 2016). There are several plant-derived phytonutrients; however, flavonoids have been studied more intensively because of their various biological benefits (Oskoueian et al., 2013). Flavonoids are polyphenolic compounds which consist of a diphenyl propane carbon skeleton along with two benzene rings A and B linked through the linear three-carbon chain (C6–C3–C6). Closed pyran ring C is formed by this central carbon chain. (Crozier et al., 2010). Moreover, flavonoid supplementation could modulate ruminal fermentation in lactating dairy cows (Theodorou et al., 1994), protecting ruminal acidosis (Balcells et al., 2012), reducing methane production and changing microbial populations such as protozoa and methanogens (Baker, 1999). Additionally, plant extract containing 300 mg/kg DM flavonoids decreased acidosis or bloat occurrence (Balcells et al., 2012), which caused by consumption of high concentration diets (Lee et al., 2014). Indeed, approximately 70% of the costs related to subclinical mastitis are associated with temporary or permanent decreases in milk production, mainly due to inflammatory damage of the mammary tissue. Moreover, flavonoid supplementation enhanced milk production (Theodorou et al., 1994) and improved milk quality by improving milk somatic cell scores and increased antioxidant properties in dairy cattle and ewes (Aguiar et al., 2014; Simitzis et al., 2019).

Chaya (*Cnidoscolus aconitifolius,* Mill. Johnston) is a plant of the genus *Cnidoscolus*, which belongs to the family Euphorbiaceae. Chaya leaf contains many phytonutrients including compounds, tannins, crude protein, and essential amino acid profile (Donkoh et al., 1990; Adaramoye et al., 2011; Adanlawo and Elekofehinti, 2012; García-Rodríguez et al., 2014). Furthermore, Chaya contains various kinds of vitamins (carotene, vitamin A, vitamin C, niacin, riboflavin, and thiamine) and minerals (P, K, Na, Ca, Mg, Zn, and Fe) (Victor et al., 2016). Recently, Totakul et al. (2021a) and Totakul et al. (2021b) revealed that Chaya leaf pellet supplementation could improve feed utilization, propionate concentration and methane mitigation. However, the effect of Chaya leaf pellet supplementation on milk yield and milk composition in dairy cows has not been studied.

Therefore, the purpose of this study was to investigate effects of Chaya leaf pellet (CHYP) supplementation on feed intake, digestibility, rumen fermentation, milk yield and milk compositions in lactating dairy cows.

2. Materials and methods

The experimental protocols were approved by the Institute of Animals for Scientific Purpose Development (IAD), Thailand (record no. U1−06565−2526).

2.1. Preparing of Chaya leaf pellet

Chaya leaves and young stems of about 6 months after regrowth were harvested from the Khon Kaen province, Thailand. The harvested Chaya was then chopped to 2−3 mm in length and sun-dried for 3 days. Dried Chaya was ground to pass 1−mm screen using Cyclotech Mill (Cyclotech Mill, Tecator, Hoganas, Sweden). Pellets containing 90% ground Chaya, 1% molasses, 9% cassava starch, and water (Table 1) were produced by Ryuzo-kun pelleting machine (Kakiuchi Co., Ltd, Nankoku, Kochi, Japan) then sundried for 2−3 days. Chaya leaf (CHY) and Chaya leaf pellet (CHYP) were analyzed for condensed tannins contents by using the vanillin−HCl method. Minerals in CHYP were analyzed using Atomic absorption spectrometer (Model analytic jena nova 350). The total flavonoid concentrations were measured by Folin–Ciocalteu reagent method (Hafizah et al., 2010). Absorbance was evaluated at 425 nm spectrophotometrically (Shimadzu, Kyoto, Japan).

2.2. Animals and design

Four, lactating crossbreds (75% Holstein-Friesian × 25% Thai native cows) dairy cows with average live weight (440 ± 20 kg), milk production (12 ± 2 kg/h/d), and days-in-milk (126 ± 20 d) were randomly assigned to a 2 × 2 factorial arrangement in a 4 × 4 Latin square. Each cow was assigned to different diets combining two ratios of concentrate to milk yield (1:1; CM1 and 1:2; CM2), two Chaya leaf pellet levels (0 and 6%) of total dry matter intake (TDMI) /h/d. Treatments were: T1= CM1 + 0 %CHYP, T2 = CM1 + 6 %CHYP, T3 = CM2 + 0 %CHYP, T4 = CM2 + 6 %CHYP). All animals were kept individually in a pen (6 × 6 m), fed ad libitum rice straw, mineral block and water. Concentrate, rice straw, and CHYP were offered two times per day at 06.00 and 16.00 after the milking process. The animals were injected with vitamin AD_3E before imposing the respective dietary treatment. The experiment was conducted for four periods consisting of 21 days per period; the first 14 days were used for feed intake adaptation and measurement. During the last 7 days samples of feed and feces were taken for chemical analysis. The average milk yield of individual cow was recorded during each period.

2.3. Sampling procedure, data collection, and chemical analysis

Feed offered and refusals were sampled daily throughout the experimental period for DM intake measurement. Samples of concentrate, rice straw, CHYP and feces were collected daily during the last 7 days of each period. About 200g of feces were collected by rectal grab sampling at the morning, the feces sample were taken 3 h intervals, the successive sample were then combined and used as one sample. The combined samples were dried at 60 °C, ground to pass 1-mm screen and analyzed for DM, CP and ash (AOAC, 2012). Acid-insoluble ash (AIA) was used to evaluate digestibility of nutrients, following the protocol of Van Keulen and Young (1977). ADF was determined and was expressed inclusive of residual ash, NDF in samples fiber was estimated according to Van Soest et al. (1991).

Milk yields were sampled every day for each animal. Samples of milk were collected and composited from the morning and afternoon milkings on the last 7 day of each period (using the ratio of 60 ml in the morning: 40 ml in the afternoon). Milk samples were immediately preserved with 2−bromo−2 nitropropane−1, 3−dial and stored at 4°C. The milk samples were analyzed for fat, protein, lactose, total solids and solids-not-fat contents according to the AOAC (2012), using infrared methods (Milko−Scan 33; Foss Electric, Hillerod, Demark). Somatic cell count (SCC) was analyzed using the Fossomatic 5000 Basic, Foss Electric, Hillerod, Demark). Milk urea nitrogen (MUN) was analyzed using a commercial kit (Sigma kits#640, Sigma Diagnostics, St. Louis, MO).

On day-21 of each period, approximately 200 ml of fluid sample from the rumen were collected using vacuum pump attached with stomach tube at 0 h before feeding and 4 h after feeding. After that, fluid samples were passed through four-layers cheesecloth and divided into 3 portions. The first portion was immediately measured for temperature and pH using portable pH meter (HANNA instrument HI8424 microcomputer, Singapore). The second portion of 50 ml of ruminal fluid was added 5 ml

Table 1
Feed ingredients and chemical composition of the feeds.

Items	Concentrate	Rice straw	CHY	CHYP
Ingredients, % as fed				
Cassava chip	47			9
Rice bran	5			-
Palm kernel meal	9.5			-
Corn	7			-
Soybean meal	20			-
Soybean hull	4			-
Di-calcium phosphate	1			-
Urea	2.5			-
Molasses	3			1
Mineral mixture [1]	0.5			-
Salt	0.5			-
Chaya leaf meal				90
Chemical compositions				
Dry matter, %	87.3	89.7	90.1	91.5
——— % of dry matter ———				
Organic matter	90.8	85.1	87.3	87
Ash	9.2	14.9	12.7	13.5
Crude protein	18.7	2.9	25.1	24.2
Neutral - detergent fiber	18.8	76.1	22.5	19.8
Acid - detergent fiber	10.6	56.4	17.2	16.8
Condensed tannins	-	-	2.2	2
Total flavonoids	-	-	7.8	7.6
Macro mineral, %				
Potassium			0.61	0.58
Calcium			5.12	4.96
Magnesium			1.9	1.84
Phosphorus			0.79	0.7
Sodium			0.16	0.12
Amino acid profile, g/100g CP				
Aspartic acid				1.56
Glutamic acid				3.72
Serine				2.19
Asparagine				6.79
Threonine				2.85
Glycine				0.67
Proline				7.66
Alanine				12.01
Methionine				0.09
Valine				10.88
Phenylalanine				28.6
Isoleucine				17.41
Leucine				20.34
Tryptophan				6.97
Cysteine				0.05
Histidine				0.74
Lysine				1.65
Arginine				6.65

CHY, Chaya leaf meal; CHYP, Chaya leaf meal pellet.
[1] Contains per kg: 4,000,000 IU vitamin A; 400,000 IU Vitamin D3; 4,000 IU vitamin E; 0.002 g vitamin B12; 16 g Mn; 24 g Fe; 10 g Zn; 2 g Cu; 0.05 g Se; 0.2 g Co, 0.5 g I.

of 1 M H_2SO_4 and used for analysis of NH_3-N (AOAC, 2012) and volatile fatty acid (VFA) concentrations, using HPLC (Instruments by controller water model 600E; water model 484 UV detector; column Novapak C18; column size 3.9 mm × 300 mm; mobile phase 10 mM H_2PO_4 pH 2.5) (Samuel et al., 1997). A third portion was used for total direct count of bacteria and protozoa by the methods of Galyean (1989) based on the use of a haemacytometer (Boeco).The estimation of methane production was calculated according to Moss et al. (2000) [estimation of methane = 0.45 (acetic acid; C_2) – 0.275 (propionic acid; C_3) + 0.4 (butyric acid; C_4)].

Blood samples (10 ml) were drawn from the jugular vein into tubes containing EDTA at the same time of rumen fluid sampling. Blood samples were immediately placed in an ice box and transported to laboratory to separate plasma from the whole blood. Samples were kept in refrigeration for 1 h at 4°C and then centrifuged at 500 × g for 10 minutes (Table Top Centrifuge PLC−02, U.S.A.). Plasma was collected and stored at – 20°C for later blood urea nitrogen (BUN) analysis according to Crocker (1967).

2.4. Statistical analysis

All data were statistically analyzed in a 2 × 2 factorial arrangement in a 4 × 4 Latin square design by analysis of variance using GLM procedure (SAS, 2013). The results are presented as mean values and standard error of the means. The statistical model included ratio of concentrate to milk yield (CM), supplementation of CHYP and interactions between CM and CHYP supplementation. Treatment effects were tested using Duncan's new multiple range test and evaluated using the method of least significant difference at the 5% significance level ($P < 0.05$).

3. Results

3.1. Feed ingredients and chemical compositions

The feed ingredients and chemical compositions composed of concentrate, rice straw, Chaya leaf meal (CHY), and Chaya leaf pellet (CHYP) are shown in the Table 1. The concentrate mixture, rice straw, CHY and CHYP contained 18.7%, 2.9%, 25.1% and 24.2% CP on DM basis, respectively. Additionally, CHYP consisted of 91.5 %DM, 87.0 % OM, 13.5 %ash, 19.8 %NDF and 16.8 %ADF. Moreover, condensed tannins and total flavonoids content in CHYP was 2.0 % and 7.6 %DM, respectively. Macro minerals were 0.58 %K, 4.96 %Ca, 1.84 %Mg, 0.70 %P and 0.12 %Na.

3.2. Feed intake and nutrient digestibility

Intake of rice straw in terms of g/kg $BW^{0.75}$ was unaffected by the 6% CHYP supplementation ($P > 0.05$). Concentrate and CHYP were fed individually, and no refusals were found. Moreover, there was interaction of CM and CHYP supplementation for all nutrient intakes ($P < 0.01$), except for ADF. Nutrient digestibility was improved by the concentrate ratio and CHYP supplementation ($P < 0.05$) (Table 2).

3.3. Rumen fermentation characteristics and blood metabolite

Rumen pH was decreased ($P < 0.05$) in cows receiving CM1 when compared with CM2. However, ruminal pH was increased when animals consumed CHYP, especially fed with CM1. The NH_3−N concentration in the rumen was found increasing by supplementing the 6 %CHYP. The value of BUN (16.8−20.9 mg/l) were increased with the increasing ruminal NH_3−N concentration ($P < 0.01$) when CHYP were supplemented with CM1. In addition, protozoal population were slightly reduced with supplementation of CHYP with both of CM1 and CM2, whereas, bacterial population was increased (Table 3).

3.4. Volatile fatty acids and methane production

After feeding, the total VFA concentration found in cows fed with CM1 was significantly increased to 115.6 mmol/l (CHYP supplementation) and 110.8 mmol/l (non−CHYP supplementation). In addition, in cows fed with CM2, the values were found at 106.4 and 104.6 mmol/l, with CHYP and non−CHYP supplementation, respectively. However, the overall treatment C_2 concentration tended to be decreased with CHYP supplementation. Nevertheless, the C_3 concentration was significantly higher than in the group with non−supplementation ($P < 0.01$) for both of CM1 and CM2. The C_4 concentration was slightly increase by CHYP supplementation. Furthermore, feeding CM1 and CHYP supplementation resulted in reduction of C_2:C_3 ratio ($P < 0.01$), and CH_4 production ($P < 0.01$).

Table 2
Effect of Chaya leaf meal pellet (CHYP) supplementation on feed dry matter intake and nutrient digestibility in dairy cows.

Items	CM1		CM2		SEM	$P-$value		
	0 %CHYP	6 %CHYP	0 %CHYP	6 %CHYP		CM	CHYP	CM × CHYP
DM intake								
Rice straw								
kg/d	5.3	5.4	5.5	5.4	0.02	0.37	0.76	0.06
g/kg BW $^{0.75}$	55.9	56.7	57.2	56.2	0.21	0.36	0.74	0.05
Concentrate								
kg/d	10.8	10.7	4.9	5	-	-	-	-
g/kg BW $^{0.75}$	111.3	110.6	49.6	51.4	-	-	-	-
CHYP								
kg/d	-	0.9	-	0.5	-	-	-	-
g/kg BW $^{0.75}$	-	9.1	-	5.4	-	-	-	-
Nutrients intake, kg/d								
Dry matter	15.3	16.5	8.6	9.3	0.12	<0.01	<0.01	0.02
Organic matter	13.6	14.7	7.6	8.2	0.11	<0.01	<0.01	0.03
Crude protein	2.7	3.2	1.3	2.1	0.18	<0.01	<0.01	0.04
Neutral detergent fiber	5.6	6.2	3.8	4.1	0.18	<0.01	0.04	0.01
Acid detergent fiber	3.8	4.1	2.7	3	0.03	<0.01	0.03	0.06
Nutrients digestibility, %								
Dry matter	68.1	70.7	66.5	68.2	0.84	<0.01	0.03	0.69
Organic matter	71.1	73.2	68.9	70	0.62	<0.01	0.05	0.42
Crude protein	61.1	63.1	56.9	58.5	0.42	<0.01	0.03	0.58
Neutral detergent fiber	51.6	55	50.9	52.4	1.07	<0.01	0.03	0.38
Acid detergent fiber	37	38.1	34.7	36.3	0.59	0.04	0.06	0.39

CM1, Ratio of concentrate to milk yield (1:1); CM2, ratio of concentrate to milk yield (1:2); CHYP, Chaya leaf meal pellet supplementation; SEM, standard error of mean; Means in the same row with different ($P < 0.05$).

Table 3
Effect of Chaya leaf meal pellet (CHYP) supplementation on rumen fermentation and blood metabolite in dairy cows.

Items	CM1		CM2		SEM	$P-$value		
	0 %CHYP	6 %CHYP	0 %CHYP	6 %CHYP		CM	CHYP	CM × CHYP
Rumen ecology								
Ruminal temperature,°C								
0 h post feeding	38.9	38.9	38.6	38.7	0.23	0.06	0.71	0.37
4	38.6	38.3	38.5	38.4	0.25	0.88	0.42	0.66
Mean	38.7	38.4	38.6	38.6	0.13	0.92	0.25	0.22
Ruminal pH								
0 h post feeding	6.714	6.923	6.946	7.021	0.08	0.16	0.13	0.66
4	6.305	6.681	6.571	6.693	0.11	0.1	0.06	0.22
Mean	6.51	6.802	6.758	6.857	0.06	0.04	0.04	0.17
NH_3-N concentration, mg/dl								
0 h post feeding	17.2	17.6	16.4	16.8	0.41	0.06	0.33	0.97
4	19.9	22.1	18.2	19.6	0.28	<0.01	<0.01	0.22
Mean	18.5	19.9	17.3	18.2	0.26	<0.01	0.02	0.49
Bacteria, × 10^9 cells/ml								
0 h post feeding	4.3	4.7	3.9	4.1	0.13	0.03	0.04	0.45
4	6	6.7	5.1	6.2	0.22	0.02	0.04	0.4
Mean	6	6.7	5.1	6.1	0.23	0.03	0.04	0.39
Protozoa, × 10^6 cells/ml								
0 h post feeding	3.4	3.1	3	2.7	0.16	0.04	0.1	0.94
4	4.1	3.8	3.6	3.1	0.15	<0.01	0.04	0.52
Mean	3.7	3.5	3.3	2.9	0.09	<0.01	0.04	0.64
Blood urea$-$N concentration, mg/dl								
0 h post feeding	15.8	19.5	15.3	17.5	0.34	0.04	<0.01	0.04
4	18.5	22.3	18.3	22.8	0.33	0.71	<0.01	0.28
Mean	16.8	20.9	18.8	18	0.23	0.11	<0.01	<0.01

CM1, Ratio of concentrate to milk yield (1:1); CM2, ratio of concentrate to milk yield (1:2); CHYP, Chaya leaf meal pellet supplementation; NH_3-N, ammonia nitrogen; SEM, standard error of mean; Means in the same row with different ($P < 0.05$).

3.5. Milk yield and milk compositions in dairy cow

The CM × CHYP interaction was significant ($P < 0.05$) for milk yield and milk composition (Table 5). However, milk yield (kg/d), protein, fat and lactose showed increasing when supplementation with CHYP. The total solids were found interactively different ($P > 0.05$) by the CM and CHYP supplementation. Moreover, the SCC of milk exhibited a significant decrease ($P < 0.05$) in the animal receiving 6% CHYP than in the non-supplement group. The MUN concentration in milk was higher by CM ($P < 0.01$) and CHYP supplementation ($P < 0.05$).

4. Discussions

4.1. Feed ingredients and chemical composition

The crude protein content of CHYP (26%) was close to that reported by Donkoh et al. (1999) and lower than that reported by Sarmiento--Franco et al. (2003) (29 and 27%, respectively). Moreover, CHYP had high content of macro minerals, especially calcium (Kuri-GarcÃa and GuzmÃ, 2017). The amino acid profile in CHYP was lower than the value reported by Victor et al. (2016). The higher amino acid profile could be explained by the different harvesting periods.

In addition, CHYP contained flavonoids, which are phenolic compounds that are reported to act as an endogenous antioxidants. In the present study, the total flavonoid content in CHYP was 7.6% DM, which was higher than the value of 4.3% DM in Chaya leaf meal reported by Loarca-Piña et al. (2010). Additionally, Aye (2012) reported that CHYP contains a higher amount of condensed tannins (3.7% DM). Since CHYP was readily consumed by the animals, it could be a good source of protein, macro minerals, and phytonutrients for dairy cows. However, factors like soil, climate, and growth stage may alter its chemical compositions and nutritive properties (Waterman, 1994).

4.2. Feed intake and nutrient digestibility

The experiment showed that supplementation of 6% CHYP improved the DM intake. CHYP contained condensed tannins (CT) (2.2% DM) in this experiment. Min et al. (2006) reported that a level of CT below 2% did not show a negative effect on DM intake and palatability. Feeding ruminants more than 5% CT could decrease palatability and reduce DM feed intake (Mueller-Harvey 2006; Waghorn 2008). In the present experiment, the nutrient digestibility of DM, OM, CP, and NDF was improved by 6% CHYP supplementation for both CM1 and CM2.This could be due to the lower CT content in CHYP at 2.0% DM. Additionally, Kaplan et al. (2014) stated that using *Sanguisoba minor* hay containing CT at 1.6% DM did not show a negative effect on nutrient digestibility.

CHYP seems to have a potential beneficial effect when included in ruminant diets without decreasing nutrient digestibility. Increasing CHYP supplementation improves the NH_3−N concentration, which supports microbial growth, resulting in greater nutrient digestibility in the present work. According to Hannah et al. (1991) and Giang et al. (2016), nutrient digestibility and microbial activity are enhanced when degradable protein is added to the rumen. Phesatcha et al. (2016) used fodder tree (*Flemingia macrophylla*) for dairy steers and observed an improvement in CP and NDF digestibility. Additionally, Wanapat et al. (2009) reported that supplementation with fodder tree leaves resulted in improved microbial fermentation in the rumen. Consequently, the increased microbial population of the rumen enhanced the nutrient degradability and intake of the diets.

4.3. Rumen fermentation characteristics and blood metabolite

The rumen pH of animals supplemented with CM1 was significantly reduced from 6.7 (0 h) to 6.3 (4 h). Normally, high concentrate increases the degradability of soluble carbohydrates, which are present in large amounts in the cereal grains in concentrate (Jayanegara et al., 2020). Thus, animals receiving a high concentrate diet have greater VFA production, which induces a critically low ruminal pH, leading to ruminal acidosis (Costa-Roura et al., 2020). However, this study, the supplementation of CHYP at 6% with CM1 (high concentrate) increased the ruminal pH to 6.7, which is the optimal level for microbial activity and rumen fermentation. This result may be due to the CHYP supplement containing flavonoids that balance rumen pH. According to Seradj et al. (2014), an increase in the population of lactate-consuming *Megasphaera elsdenii* in rumen fluid from a high-concentrate diet incubated with Bioflavex (flavonoids) suggests potential efficacy for the prevention of lactic acidosis. Additionally, Olagaray and Bradford (2019) stated that incorporation of flavonoids in high-concentrate diet can improve rumen fermentation and efficiently reduces the risk of acidosis.

The concentration of NH_3−N was increased when dairy cows received CM1 and was even higher when supplemented with 6% CHYP, indicating high CP content (24%). Similarly, Karamnejad et al. (2019) indicated that degradable nitrogen leads to high concentrations of NH_3−N in the rumen. In addition, Kazemi-Bonchenari et al. (2010) and Jayanegara et al. (2020) demonstrated that NH_3−N concentration increases with CP supplementation. Nevertheless, the ruminal NH_3−N was in the range of 17.3−19.9 mg/dl. This result is similar to that reported by Wanapat and Pimpa (1999), who noted that NH_3−N concentrations of 15−30 mg/dl were the optimal levels for microbial activity and rumen fermentation.

The high rate of ruminal NH_3−N greatly depends on the availability of protein in the rumen, where it is a key metabolite, and a large proportion of the N requirement for microbial protein synthesis is met by ruminal NH_3−N (Pisulewski et al., 1981). The BUN concentrations in dairy cows fed with CHYP supplementation were 19.6 to 21.1 mg/dl, which are normal values. Similarly, Hammond et al. (1994) and Thronton (1970) reported that the greater BUN might be related to the greater NH_3−N concentration in the rumen. Furthermore, Hammond et al. (1994) and Wanapat et al. (2009) noted that the optimal ranges for the BUN concentration were 7−20 mg/dl and 13.0−21.3 mg/dl, respectively.

CM2 significantly decreased bacterial and protozoal populations compared with CM1. In addition, CHYP supplementation at 6% increased bacteria and decreased protozoa. The presence of flavonoids in CHYP may have directly inhibited the protozoal population by interrupting cell-wall synthesis or nucleic-acid synthesis. This is in agreement with Hernandez et al. (2012), who found that flavonoids can be added to an animal diet in order to decrease protozoal populations. This result is also similar to that of Paula et al. (2016), who reported that feeding flavonoid compounds to water buffaloes can be suppress the protozoal population of *Entodinium*. Therefore, the results could probably be explained by the bacteria population being improved because of the engulfment of protozoa by bacteria. Similarly, Oskoueian et al. (2013) stated that flavonoids had no negative effect on bacterial number but decreased rumen protozoa.

4.4. Volatile fatty acid and estimated methane production

Total VFA and C_3 were increased with CM1 in comparison with CM2. It has been suggested that concentrate containing high levels of nonstructural carbohydrate is readily degradable in the rumen (Wanapat and Khampa, 2007). It is well known that increasing the concentrate proportion in feed will increase total VFA and C_3 (Lovett et al., 2003; Olijhoek et al., 2018). Moreover, in the present study, the total VFA production was greater when animals were fed concentrate supplemented with 6% CHYP.

McSweeney et al. (2001) reported that flavonoids were readily degraded in the rumen and that their derivatives are utilized by rumen microbes. Flavonoids have been suggested to act as an alternative carbon source for the metabolism of ruminal microbes, which results in greater total VFA (Smith et al., 2005). Theses findings are in agreement with those of Paula et al. (2016), who also observed that using flavonoid-containing propolis led to greater total VFA. An increase in total VFA concentration is very desirable because VFAs are an important energy substrate in ruminants (Brown et al., 2017).

In this study, using CM1 resulted in higher C_3 in comparison to CM2. However, the C_3 proportion increased with supplementation of CHYP, while CH_4 production decreased. The CHYP has a flavonoid content of 7.6% DM. In this work, the dairy cows received flavonoids at 0.61 g/kg $BW^{0.75}$ from CHYP supplementation at 6% of TDMI. This was probably due to the uptake of flavonoids in dairy cows. Flavonoids may also decrease CH_4 production by decreasing the methanogen population.

Cushnie and Lamb (2005) reported that flavonoids generally inhibited cytoplasmic membrane function or nucleic synthesis of protozoa. Wang et al. (2013) and Seradj et al. (2014) revealed that the total protozoa population was reduced by flavonoids when fed to cattle. Ma et al. (2017) stated that mulberry leaf meal containing flavonoids reduced the population of protozoa in response to methanogens, and CH_4 was decreased. The promising effects of plant phytonutrients on methane mitigation and the methanogen population both in *in vitro* and *in vivo* trials have been reported by numerous investigators (Patra et al., 2006; Bodas et al., 2008; Patra and Saxena, 2010; Oskoueian et al., 2013).

Normally, the theory of CH_4 production indicates that it is mainly

Table 4
Effect of Chaya leaf meal pellet (CHYP) supplementation on volatile fatty acids and estimated CH_4 production by Chaya leaf meal pellet (CHYP) supplementation in dairy cows.

Items	CM1		CM2		SEM	*P*−value		
	0% CHYP	6% CHYP	0% CHYP	6% CHYP		CM	CHYP	CM × CHYP
Total VFA, mmol/l								
0 h post feeding	102.6	103.1	100.7	101	1.05	0.09	0.76	0.94
4	110.8	115.6	104.6	106.4	1.1	<0.01	0.02	0.2
Mean	106.6	109.3	102.7	103.7	0.56	<0.01	0.03	0.17
Acetate, mol/100ml								
0 h post feeding	66.5	66.8	66	65.6	1.29	0.49	0.98	0.84
4	69.5	68.6	71	69.3	0.56	0.08	0.06	0.57
Mean	68	67.7	68.4	67.5	0.69	0.92	0.41	0.67
Propionate, mol/100ml								
0 h post feeding	19	18.4	17.6	17.9	0.36	0.08	0.94	0.42
4	23.8	25.1	19	20.9	0.47	<0.01	<0.01	0.78
Mean	21.4	22	18.1	19.4	0.15	<0.01	<0.01	0.29
Butyrate, mol/100ml								
0 h post feeding	12.8	12.5	14.1	13	0.59	0.36	0.31	0.51
4	8.2	9.2	8.1	8.6	0.46	0.65	0.3	0.59
Mean	10.6	11.3	10.6	10.5	0.38	0.32	0.45	0.41
Acetate:Propionate	3	2.7	3.7	3.3	0.07	<0.01	<0.01	0.16
CH_4 [1], mmol/100mol	28.5	27.1	30.5	28.7	0.31	<0.01	<0.01	0.37

CM1, Ratio of concentrate to milk yield (1:1); CM2, ratio of concentrate to milk yield (1:2); CHYP, Chaya leaf meal pellet supplementation; VFA, volatile fatty acid; CH_4, methane; SEM, standard error of mean; Means in the same row with different ($P < 0.05$).
[1] CH_4 production calculated according to (Moss et al., 2000): $CH_4 = 0.45$ (Acetate) – 0.275 (Propionate) + 0.4 (Butyrate).

produced from CO_2 and H_2 by rumen methanogens. Therefore, decreasing methanogens led to suppression of CH_4 production, and the available H_2 would be available for C_3 synthesis. The decrease in estimated CH_4 production may be due to the supplementation of CHYP, which was associated with a lower protozoa population in comparison with no supplementation.

4.5. Milk yield and milk compositions in dairy cows

Milk yield, milk protein, and lactose were significantly increased when using higher concentrate. However, in this study, CHYP supplementation improved milk yield, milk protein, and lactose. This could be due to the CHYP supplementation enhancing protein contents in the animal diet, which is important for dairy cows, as reported in Table 1. This might have been due to milk protein produced from microbes and protein that was absorbed in the blood (Morison, 2005).

Moreover, the results showed that CHYP supplementation decreased the protozoa population. The reduction of protozoa could have improved C_3 production. C_3 is a precursor for gluconeogenesis and lactose synthesis (Lemosquet et al., 2009). Increasing the content of glucogenic precursors has a favorable effect on the milk's lactose content. In addition, the milk lactose composition may be related to the increased C_3 production (Kholif et al., 2018). Therefore, the greater quantity of glucose arriving at the udder enhanced the lactose composition and led to a higher milk yield (Morison, 2005). Table 5 shows the enhancement of lactose and improved milk yield.

The production of milk fat was enhanced when feed was supplemented with CHYP. Normally, fatty acids are synthesized in the mammary glands via the malonyl−CoA pathway. Beta-hydroxybutyrate in the blood is primarily used for the first four carbons to initiate fatty acid synthesis. The milk fat composition is positively related to the proportion of C_4 in the rumen (Linn, 1988). Therefore, in this study, the digestibility of NDF was improved by the treatment with CHYP supplementation, which slightly increased the C_4 proportion while increasing the milk fat composition. Isobe et al. (2011) stated that NDF digestibility is related to improved lipogenic production rates of glucogenic VFA. The altered proportion of VFA results in an enhanced concentration of milk fat. Similarly, Viennasay et al. (2020) reported that fodder tree supplementation positively affected milk yield and milk composition.

MUN increased when supplementing CHYP at 6% of total DMI. This might have resulted from the high crude protein contained in CHYP (24%). Several studies have shown that MUN is highly correlated with BUN (Roseler et al., 1993; Hong et al., 2003). In the treatment with CHYP, the BUN concentration was associated with increased concentrations of MUN, as reported in the Table 5. Increased concentrations of MUN occurred because the amount of NH_3 absorbed from the rumen and changed to urea reflects both protein degradation to NH_3 in the rumen and absorption into the blood system. In all treatment diets, the MUN concentrations were in the range of 15.8 to 17.8 mg/dl. In treatments

Table 5
Effect of Chaya leaf meal pellet (CHYP) supplementation on milk yield and milk composition by Chaya leaf meal pellet (CHYP) supplementation in dairy cows.

Items	CM1		CM2		SEM	*P*−value		
	0 %CHYP	6 %CHYP	0 %CHYP	6 %CHYP		CM	CHYP	CM × CHYP
Milk yield, kg/d	12.8	13.3	10.6	11.4	0.19	<0.01	0.03	0.09
3.5% FCM, kg/d	13.4	14.1	11.6	12.8	0.21	<0.01	<0.01	0.04
Milk composition, %								
Protein	3.32	3.44	3.20	3.31	0.01	<0.01	<0.01	0.65
Fat	3.61	3.83	3.51	3.74	0.01	<0.01	<0.01	0.36
Lactose	4.64	4.82	4.44	4.51	0.04	<0.01	<0.01	0.16
Solids-not-fat	9.1	9.3	8.8	9.0	0.14	0.07	0.33	0.52
Total solids	12.7	12.9	12.5	12.6	0.17	<0.01	<0.01	0.04
SCC, $\times 10^5$ cells/ml	2.65	2.44	2.67	2.48	0.02	0.15	<0.01	0.59
MUN, mg/dl	17.1	17.8	15.8	16.4	0.22	<0.01	0.03	0.86

CM1, Ratio of concentrate to milk yield (1:1); CM2, ratio of concentrate to milk yield (1:2); CHYP, Chaya leaf meal pellet supplementation; FCM, fat collected milk; SCC, somatic cell counts; MUN, milk urea nitrogen; SEM, standard error of mean; Means in the same row with different ($P < 0.05$).

with CHYP supplementation and no supplementation, MUN was decreased by 0.7 and 0.6 mg/dl, respectively.

These changes occurred with the increase in dietary protein intake. Barros et al. (2017) illustrated that lactating dairy cows fed with a high protein level showed increased nitrogen intake, as well as BUN. Many researchers report that ruminal NH_3-N, BUN, and MUN have a positive correlation (Broderick et al., 2010; Wanapat et al., 2014). However, Hwang (2000) summarized that cattle produce milk that contains a level of MUN within the ranges of the standard values of 11−17 mg%, and the results in this study were similar.

5. Conclusion

In conclusion, supplementation of CHYP at 6% of total dry matter intake improved nutrient digestibilities, C_3 concentration, while the protozoal count and estimated CH_4 production were significantly reduced. Ruminal pH was enhanced by CHYP supplementation with high concentrate feeding. Supplementation CHYP both of ratio concentrate generally remarkably increased milk yield, 3.5 % fat−corrected milk especially, fat and protein compositions. Further research undertakings concerning supplemental level of CHYP in lactating dairy cows are of importance.

Credit authorship contribution statement

Pajaree Totakul: Acquisition of data**,** Analysis and/or interpretation of data, Drafting the manuscript. **Bounnaxay Viennasay:** Analysis and/or interpretation of data. **Sukruthai Sommai:** Analysis and/or interpretation of data. **Maharach Matra:** Analysis and/or interpretation of data. **Metha Wanapat:** Conception and design of study, Acquisition of data, Critical review/revision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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