

Article

In Vitro Fermentation Characteristics and Methane Mitigation Responded to Flavonoid Extract Levels from *Alternanthera sissoo* and Dietary Ratios

Sukruthai Sommai ¹, Anusorn Cherdthong ^{1,*} , Chanon Suntara ¹, Sarong So ¹, Metha Wanapat ¹  and Sineenart Polyorach ²

¹ Tropical Feed Resources Research and Development Center (TROFREC), Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen 40002, Thailand; fon_sukruthai@hotmail.com (S.S.); chanon_su@kkumail.com (C.S.); sarong.so@kkumail.com (S.S.); metha@kku.ac.th (M.W.)

² Department of Animal Production Technology and Fisheries, Faculty of Agricultural Technology, King Mongkut's Institute of Technology, Bangkok 10520, Thailand; sineenart.po@kmitl.ac.th

* Correspondence: anusorn@kku.ac.th; Tel.: +66-43-202-362; Fax: +66-43-202-362

Abstract: Two experiments were conducted under this study: Experiment 1 was to study production yield, chemical composition, and in vitro degradability of Brazilian spinach (*Alternanthera sissoo*; BS) leaf and leaf + leaf-stalk at various maturity ages of 15, 30, 45, and 60 days after plantation and regrowth and Experiment 2 was to evaluate the effect of flavonoid extract from BS leaf and leaf + leaf-stalk and dietary ratios on ruminal gas production, fermentation characteristics, and in vitro degradability. Experiment 1 showed that maturity ages after planting and regrowth increased, the yield significantly increased. Increasing maturity ages significantly ($p < 0.05$) increased neutral detergent fiber and acid detergent fiber content and decreased crude protein content, total flavonoid (TF) content, and degradability for both leaf and leaf + leaf-stalk. Maturity ages from 15 to 30 days after plantation and regrowth resulted ($p < 0.05$) the highest TF content and degradability for both leaf and leaf + leaf-stalk. Thus, BS leaf and leaf + leaf-stalk samples from 15 to 30 days of age were used for flavonoid extraction and used in the Experiment 2. Experiment 2 was conducted according to a 3×5 factorial experiment. Three roughage to concentrate (R:C) ratios at 50:50, 40:60, and 30:70 were used, and five levels of flavonoid extract (FE) at 0, 10, 20, 30, and 40 mg of substrate dry matter (DM) were supplemented. Experiment 2 showed that R:C ratio and FE had an interaction effect only on acetate to propionate ratio. Varying R:C ratios significantly increased ($p < 0.05$) in vitro DM degradability, total volatile fatty acids (VFA), and propionate (C3) concentration. FE supplementation linearly ($p < 0.05$) increased total VFA and C3 concentration and decreased methane production and protozoal population. This study could conclude that FE from BS could effectively modulate ruminal fermentation and decrease methane production. However, in vivo study needs to elucidate in order to validate the present results.

Keywords: maturity; flavonoids; Brazilian spinach; methane mitigation; protozoa



Citation: Sommai, S.; Cherdthong, A.; Suntara, C.; So, S.; Wanapat, M.; Polyorach, S. In Vitro Fermentation Characteristics and Methane Mitigation Responded to Flavonoid Extract Levels from *Alternanthera sissoo* and Dietary Ratios. *Fermentation* **2021**, *7*, 109. <https://doi.org/10.3390/fermentation7030109>

Academic Editor: Mohamed Koubaa

Received: 14 June 2021

Accepted: 6 July 2021

Published: 8 July 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Livestock farming is a significant source of anthropogenic greenhouse gas (GHG) emissions [1,2], in which methane (CH₄) produced from ruminants represents 18% of total GHG [3]. CH₄, in ruminants, is produced biologically by methanogen bacteria using hydrogen and carbon dioxide as primary substances [4,5]. Besides its negative effect on global warming, CH₄ contributes to energy loss, approximately 5 to 7% of feed intake in ruminants [6]. Thus, CH₄ mitigation does not only decreases global warming but also improves feed utilization efficiency. Regarding CH₄ mitigation, plant secondary compounds such as flavonoids presented in a wide range of plants may reduce CH₄ production by shifting the fermentation pathway of ruminal microbes [7,8]. Flavonoids derive

from benzo-L-pyrone and balance ruminal pH and antimicrobial property [9]. Flavonoids against Gram-positive microbes by inhibiting cytoplasmic membrane function and cell wall synthesis [10]. Flavonoid incorporation in diet could mitigate CH₄ production by shifting the fermentation pathway toward propionate (C3) synthesis [9,11]. Oskoueian et al. [12] revealed flavonoid naringin, and quercetin decreased in vitro CH₄ emission and ciliate protozoal number. Sinz et al. [13] showed that flavonoid luteolin-7-glucoside decreased in vitro CH₄ production. However, Stoldt et al. [14] found that rutin (glucrohamnoside of quercetin) did not differ in the CH₄ production of Holstein cows. Variation in their effect on CH₄ mitigation depends on metabolite type, characteristics, and rations [7]. Brazilian spinach (*Alternanthera sissoo*, BS) originates from Brazil and is widely grown in the tropics, it is a good source of flavonoids [15]. Manurung et al. [16] reported that maturity ages significantly affected the flavonoid content in Tabat Barito. However, studies on the production yield and chemical composition of BS at various maturity ages and flavonoid extract from BS supplementation's effect on CH₄ mitigation and in vitro degradability have not yet been evaluated. Based on the potential effect of flavonoids on CH₄ mitigation, the authors hypothesized that flavonoid extract from BS could mitigate CH₄ emission.

The objectives of this study were: Experiment (1) to study production yield, chemical composition, and in vitro degradability of BS leaf and leaf + leaf-stalk at various maturity ages after plantation and regrowth and Experiment (2) to evaluate the effect of flavonoid extract from BS leaf and leaf + leaf-stalk and dietary ratios on ruminal gas production, fermentation characteristics, and in vitro degradability.

2. Material and Methods

2.1. Experiment 1: Preliminary Study

2.1.1. Location and Plantation

The current study was conducted at the Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand. BS was taken from Surin province to plant at the Tropical Feed Resources Research and Development Center (TROFREC) at Khon Kaen University. Nine plots (1 × 1 m²) were prepared, and 2636 BS roots were planted manually at distances between and within rows of 5 × 5 cm, respectively. Plants were watered twice daily at 7 am and 5 pm.

2.1.2. Yield Collection and Chemical Analysis

BS plant was cut 5 cm above the soil surface at 15, 30, 45, and 60 days of age and re-collected at the same age of regrowth, and yield was recorded by the plot. Ten kilograms of leaf and leaf + leaf-stalk based on the fresh matter were collected from each plot and dried at 60 °C for 72 h. Then, dried BS leaf and leaf + leaf-stalk were ground through a 1 mm screen using Cyclotech Mill (Tecator, Hoganas, Sweden) to analyze the chemical composition according to AOAC [17], including dry matter (DM, ID 967.03), crude protein (CP, ID 984.13), and ash (ID 942.05), and acid detergent fiber (ADF, ID 973.18). Neutral detergent fiber (NDF) was analyzed according to Van Soest et al. [18]. Total flavonoid content (TFC) was measured in triplication according to Chang et al. [19] with some modification. Five grams of BS leaf and leaf + leaf-stalk samples were soaked in 100 mL of methanol for 24 h, and then the mixture was filtered through Whatman paper no. 42. After that, 250 µL of the filtered solution was pipetted into a 20 mL glass tube, added with 75 µL of 5% NaNO₂, and incubated at room temperature for 5 min. Then, 150 µL of 10% AlCl₃·6H₂O was added into the mixture, left for 6 min, and added 500 µL of 1 M NaOH. Finally, deionized water was added to make a total volume of 1 mL and incubated at room temperature for 30 min. The absorbance was measured at 415 nm wavelength. The TFC was expressed as mg of quercetin equivalent (mg QE/g).

2.1.3. In Vitro Degradability

Animal Ethics

The animals used for rumen fluid collection were approved (IACUC-KKU-43/63) by the Institutional Animal Care and Use Committee of Khon Kaen University (Date of approval, 21 May 2020).

Sample Processing

The BS leaf and leaf + leaf-stalk samples were collected at 15, 30, 45, and 60 days of age, oven-dried at 60 °C for three days, and milled through a 1 mm screen (Cyclotech Mill; Tecator, Hoganas, Sweden). The milled leaf and leaf + leaf-stalk samples were used as a substrate to evaluate the in vitro degradability.

Ruminal Fluid Donors and Preparation of Inoculum

Rumen fluid was collected from two male rumen-fistulated dairy steers with body weight (BW) of 400 ± 50 kg. Dairy steers were placed in a separate pen and offered concentrate at 0.5% BW/day, and rice straw (RS) was fed ad libitum at 7:00 am in the morning and at 4:00 pm in the evening for seven days before rumen fluid was collected. The ingredients and chemical composition of concentrate and RS are provided in Table 1. For each sample of milled leaf and leaf + leaf-stalk, two replications were undertaken, and 34 serum bottles were prepared (2 bottles $\times$ 2 types of samples (leaf and leaf + leaf-stalk) $\times$ 4 harvesting times (15, 35, 45, and 60 days) $\times$ 2 incubation times (12 and 24 h) + 2 replications of blank). The 500 mg sample of milled leaf and leaf + leaf-stalk was weighed into serum bottles and closed with a rubber lid and aluminum cap. Then, the bottles were flushed with carbon dioxide to remove oxygen before being injected with artificial saliva. Approximately 1500 mL of rumen fluid was collected from each steer, filtered through four layers of cheesecloth into a pre-warmed thermos flask, and then transferred to the laboratory. Rumen fluid from each dairy steer was combined at a 50:50 ratio and mixed with the artificial saliva solution of Menke and Steingass [20] at a 2 (artificial saliva) to 1 (ruminal fluid) ratio in a 5000 mL pre-warmed volumetric flask at 39 °C. The flask was flushed with carbon dioxide to remove oxygen. A 40 mL of inoculum was injected into the serum bottles containing a sample of milled leaf and leaf + leaf-stalk of BS. After injecting, the bottles were incubated at 39 °C until sample collection. After 12 h and 24 h of incubation, the bottles were removed from the incubators and frozen until analysis. Briefly, the bottles were thawed and poured through 40 mm of porosity of pre-weighed Gooch crucibles by emptying the bottles with dH₂O. Then, the Gooch crucibles were dried in a hot-air oven at 100 °C for 24 h and weighed to calculate for residue DM. The residue DM of each sample was subtracted with the blank residue DM and used to calculate the DMD_{vt} according to Tilley and Terry [21]. Then, the Gooch crucibles containing residues were used to analyze the in vitro NDF (NDFD_{vt}) degradability by using Ankom A200i Fibre Analyser (Ankom Technology Co., New York, NY, USA.) according to Van Soest et al. [18] and ADF (ADFD_{vt}) degradability according to AOAC [22]. After fiber analysis, Gooch crucibles were ashed at 500 °C to calculate the in vitro OM degradability (OMD_{vt}) according to Tilley and Terry [21].

2.2. Experiment 2: In Vitro Gas Production Technique (Main Experiment)

Experiment 1 showed that TFC in BS leaf and leaf + leaf-stalk was highest during 15 to 30 days. Therefore, BS leaf + leaf-stalk samples during 15 to 30 days were used for flavonoid extract and used as a supplementation in the substrate.

Table 1. Ingredients and chemical composition of substrate used in this study.

Items	Concentrate	Rice Straw
Ingredients (g/kg DM)		
Cassava chip	530	
Soybean meal	165	
Rice bran	120	
Palm kernel meal	136	
Urea	10	
Premix	10	
Molasses	14	
Sulfur	5	
Salt	10	
Chemical composition		
Dry matter (DM), g/kg	870.7	926
Crude protein, g/kg DM	143.8	29
Neutral detergent fiber, g/kg DM	216.1	716
Acid detergent fiber, g/kg DM	121.4	558
Ash, g/kg DM	49.7	133

Premix composes of vitamin A 4×10^6 IU, vitamin D3 4×10^5 IU, vitamin E 4000 IU, vitamin B12 0.002 g, Mn 16 g, Fe 24 g, Zn 10 g, Cu 2 g, Se 0.05 g, Co 0.2 g, and I 0.5 g.

2.2.1. Flavonoid Extract

BS leaf + leaf-stalk samples for 15 to 30 days were used to extract flavonoids. The flavonoid extraction was measured according to Bohm and Kocipai-Abyazan [23] with minor modification. Briefly, 10 g of BS leaf + leaf-stalk samples were repeatedly extracted in 80% methanol of 100 mL at ambient temperature. Then, the solution from each extraction time was combined and filtered through Whatman No 42 (125 mm). The filtrate was transferred into crucibles and evaporated in the water bath at 65 °C until dry. Finally, crucibles were sent to a hot-air oven for 24 h at 60 °C, and flavonoid extract (FE) in powder form was obtained and used in this study.

2.2.2. Study Design and In Vitro Fermentation

The study was designed according to a 3×5 factorial experiment in a completely randomized design. Three levels of roughage (RS) to concentrate ratios were 50:50, 40:60, or 30:70 based on DM, and five levels of FE at 0, 10, 20, 30, or 40 mg based on DM basis. RS and concentrate were ground to pass a 1 mm sieve (Cyclotech Mill, Tecator, Hoganas, Sweden) before being used for the in vitro gas-production test. Gas production was conducted in triplicate for each treatment with another triplication for a blank sample, in which 48 serum bottles were prepared (3 replications $\times$ 15 treatments + 3 replications of blank). Rumen fermentation parameters including ammonia nitrogen ($\text{NH}_3\text{-N}$), volatile fatty acid (VFA), and the protozoal population at two sampling time of 4 h and 8 h of incubation were conducted in duplication, in which 62 bottles were arranged (2 replications $\times$ 15 treatments $\times$ 2 sampling times + 2 replications of blank). Another 62 bottles were prepared (2 replications $\times$ 15 treatments $\times$ 2 sampling times + 2 replications of blank) to study in vitro DM degradability (DMD_{vt}) at 12 h and 24 h of incubation. A 500 mg of RS and concentrate was weighed into 50 mL serum bottles, and FE was added at their respective amounts (0, 10, 20, 30, or 40 mg based on DM basis). After substrates were added, the bottles were flushed with carbon dioxide and injected with artificial saliva of 40 mL. The artificial saliva was prepared as mentioned above according to Menke and Steingass [20]. After injecting, serum bottles were capped with an aluminum lid and incubated at 39 °C until sampling.

2.2.3. In Vitro Gas Production Sampling and Analysis

A series of incubation times at 0, 0.5, 1, 2, 4, 6, 8, 12, 18, 24, 48, 72, and 96 h were performed for the gas production. Gas produced at each time of incubation was used to study the gas cumulation by fitting to the model of Ørskov and McDonald [24] Equation (1):

$$Y = a + b(1 - e^{-ct}) \quad (1)$$

where a is the gas production from the immediately soluble fraction (mL); b is the gas production from the insoluble fraction (mL); c is the gas production rate constant for the insoluble fraction (mL/h); t is incubation time (h); $a + b$ is the potential extent of gas production (mL), e is the exponential function in the equation, Y is the gas produced at times “ t ” (mL).

After 4 h and 8 h of incubation, serum bottles were removed from the incubator and immediately measured for pH using a HANNA pH meter (HANNA instruments, Nusfalau, Romania). Then, fermentation liquid was filtered through four-layers of cheese-cloth and separated into two portions, in which the first portion was used for $\text{NH}_3\text{-N}$ and VFA concentration analysis and the second portion was used for protozoal population analysis. The first portion of samples was centrifuged for 15 min at $16,000 \times g$, and then the supernatant was collected and kept at -20°C until analysis. The concentration of $\text{NH}_3\text{-N}$ was analyzed using the micro-Kjeldahl method [17], and VFA concentration including acetic acid (C2), propionic acid (C3), and butyric acid (C4) was measured with gas chromatography (GC; Shimadzu, Model: GC-2014, Kyoto, Japan) according to So et al. [25]. The second portion of samples was handled by keeping in formalin at a ratio of 1 (fermentation liquid 1 mL) to 9 (formalin 9 mL) and stored in the refrigerator until enumerating. The protozoal population was enumerated using a direct-count technique according to Galyean [26].

After 4 h and 8 h of incubation, serum bottles for the IVDM study were used for gas collection to study CH_4 production. Ten milliliters of gas were collected using a 10 mL syringe, injected into 25 mL serum bottles closed with a rubber lid and aluminum cap, and covered with parafilm. The CH_4 production was measured using gas chromatography (Instruments by GC-17A System, Shimadzu; TCD detector; column Shin carbon; column size $3\text{ m} \times 3\text{ mm}$, activated charcoal 60/80 mesh, Kyoto, Japan) according to Sittijunda et al. [27] with minor modifications.

After 12 h and 24 h of incubation, serum bottles were removed from the incubator and frozen until analysis. The DMD_{vt} was calculated according to [20]. Briefly, serum bottles were removed from the freezer and thawed at ambient temperature. Then, fermentation liquid samples were poured through a 40 mm porosity of pre-weighed Gooch crucibles, and Gooch crucibles were then oven-dried at 100°C for 24 h. After drying, Gooch crucibles were weighed and calculated for the residue DM. Before calculating DMD_{vt} , the residue DM values were subtracted with the residue DM of the blank.

3. Statistics

Yield, chemical composition, TF content, and DMD_{vt} data were analyzed according to a completely randomized design (CRD) using Proc ANOVA of SAS [28]. The model was $Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$ where Y_{ij} is the observations, μ is overall means, τ_i is effect of maturity ages at 15, 30, 45, and 60 days, and ε_{ij} is error. Different means were compared using Tukey's multiple comparison test [29] and considered statistically different at $p < 0.05$.

Gas kinetics, gas cumulation, $\text{NH}_3\text{-N}$, VFA, CH_4 , DMD_{vt} , OMD_{vt} , NDFD_{vt} , and ADFD_{vt} data were analyzed according to a 3×5 factorial experiment in a CRD using PROC GLM of SAS [27]. The model was $Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$ where Y_{ijk} is observations, μ is overall means, α_i is the effect of roughage to concentrate (R:C) ratios, β_j is the effect of FE supplementation, $\alpha\beta_{ij}$ is the interaction effect of R:C ratio and FE, and ε_{ijk} is error. Treatment means were compared using Tukey's multiple comparison test [29] and considered statistically different at $p < 0.05$. The orthogonal polynomial was tested for FE levels.

4. Results

4.1. Experiment 1: Preliminary Study

4.1.1. Production of Brazilian Spinach

BS yield was significantly ($p < 0.05$) affected by maturity ages after plantation and regrowth (Figure 1). The maturity ages after planting and regrowth increased, the yield significantly increased. The BS yield after planting at 15, 30, 45, and 60 days were at 0.68, 1.36, 1.60, and 1.81 kg/m², respectively. Additionally, the yield after regrowth at 15, 30, 45, and 60 days were at 2.16, 2.31, 2.39, and 2.51 kg/m², respectively.

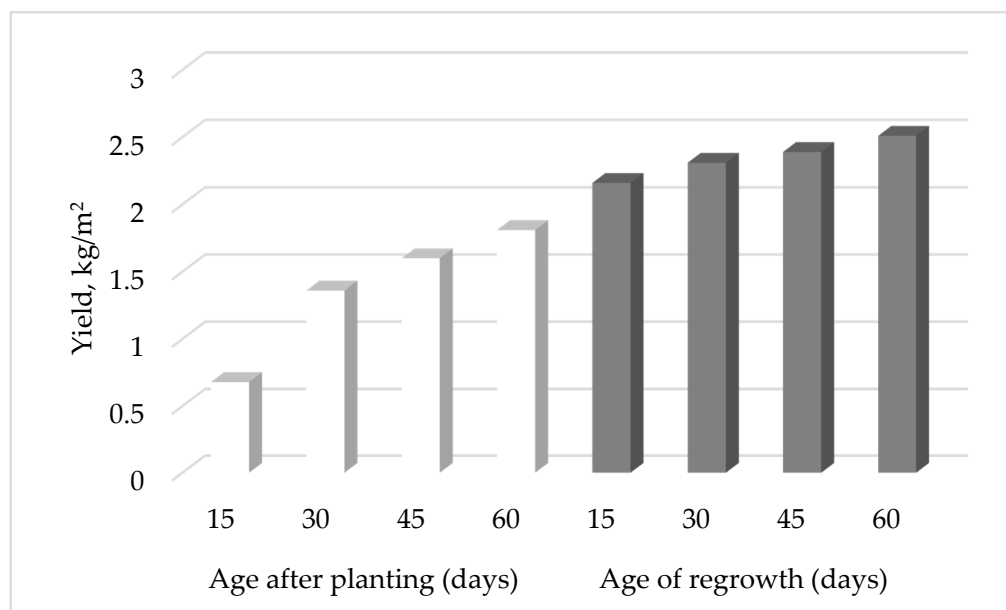


Figure 1. Brazilian spinach yield at 15, 30, 45, 60 days of age after planting and regrowth.

4.1.2. Chemical Composition and Digestibility of Brazilian Spinach

Table 2 shows the chemical compositions and TFC of BS leaf and leaf + leaf-stalk at 15, 30, 45, and 60 days after planting and regrowth. The DM, CP, NDF, ADF, and TF content in leaf was significantly ($p < 0.05$) different among harvesting ages. The TF content in the leaf (82.16 g/kg DM) was highest at 15 days of age after planting. The DM, NDF, and CP content in leaf + leaf-stalk were significantly ($p < 0.05$) different among harvesting ages. The TF content (78.14 g/kg) in leaf + leaf-stalk was highest at 15 days. DM and NDF content in both leaf and leaf + leaf-stalk significantly increased when the harvesting ages increased; however, CP and TF content significantly decreased.

In vitro nutrient degradability in leaf and leaf + leaf-stalk at 15, 30, 45 and 60 days of harvesting after 12 h and 24 h of incubation was presented in Table 3. Harvesting ages significantly ($p < 0.05$) affected nutrient degradability in both leaf and leaf + leaf-stalk. After 12 h of incubation, DM, OM, NDF, and ADF degradability significantly ($p < 0.05$) decreased as harvesting ages increased for leaf + leaf-stalk. For leaf, harvesting ages significantly affected the in vitro degradability of OM, NDF, and ADF except for DM. After 24 h of incubation, increasing harvesting ages significantly ($p < 0.05$) decreased the degradability of DM, OM, NDF, and ADF for both leaf and leaf + leaf-stalk.

Table 2. Chemical composition in leaf and leaf + leaf-stalk of Brazilian spinach at 15, 30, 45 and 60 days of harvesting.

Items	Leaf				SEM	<i>p</i> -Value	Leaf + Leaf-Stalk				SEM	<i>p</i> -Value
	15	30	45	60			15	30	45	60		
Chemical Composition (g/kg)												
DM	118.2 ^b	126.1 ^a	128.2 ^a	129.9 ^a	1.14	0.004	148.5 ^d	153.2 ^c	159.9 ^b	168.9 ^a	0.14	0.001
OM	973.0 ^b	973.6 ^b	976.7 ^a	976.9 ^a	0.03	0.001	958.8	958.6	957.4	955.3	0.10	0.092
CP	256.4 ^a	233.8 ^b	214.7 ^c	212.0 ^c	0.17	0.000	196.2 ^a	170.1 ^b	163.0 ^b	149.6 ^c	0.33	0.001
NDF	343.2 ^b	303.4 ^a	406.1 ^a	417.0 ^a	0.71	0.002	477.5 ^b	481.9 ^b	485.7 ^b	497.3 ^a	0.35	0.019
ADF	173.8 ^c	192.7 ^b	200.6 ^b	210.9 ^a	0.33	0.002	207.1	209.9	212.1	212.9	0.68	0.202
TF (g/kg)	82.16 ^a	72.21 ^b	50.73 ^c	49.57 ^c	0.48	0.000	78.14 ^a	71.34 ^b	50.04 ^c	45.52 ^d	1.06	0.000

DM = Dry matter, OM = Organic matter, CP = Crude protein, NDF = Neutral detergent fiber, ADF = Acid detergent fiber, TF = Total flavonoids content, SEM = standard error of mean. ^{a, b, c, d} means within row with different superscript letter showed significantly different at various harvesting times ($p < 0.05$).

Table 3. In vitro nutrient degradability in leaf and leaf + leaf-stalk of Brazilian spinach at 15, 30, 45 and 60 days of harvesting.

Items (g/kg)	Leaf				SEM	p-Value	Leaf and Leaf + Leaf-Stalk				SEM	p-Value
	15	30	45	60			15	30	45	60		
12 h												
DM	559.4	556.0	551.4	542.1	0.70	0.220	468.5 ^a	406.4 ^b	366.0 ^c	346.6 ^d	0.37	0.000
OM	871.2 ^a	863.9 ^b	857.0 ^c	839.2 ^a	0.76	0.000	862.2 ^a	844.1 ^b	841.9 ^b	839.9 ^b	0.15	0.002
NDF	398.1 ^a	389.3 ^a	364.9 ^b	358.7 ^b	0.43	0.002	341.6 ^a	312.6 ^b	262.6 ^c	234.0 ^d	0.07	0.000
ADF	229.0 ^a	218.1 ^b	207.6 ^c	186.0 ^d	0.32	0.001	177.1 ^a	134.7 ^b	130.1 ^c	129.2 ^c	0.04	0.000
24 h												
DM	629.4 ^a	615.8 ^b	611.0 ^c	602.8 ^d	0.08	0.000	606.0 ^a	600.0 ^b	574.6 ^c	542.7 ^d	0.07	0.000
OM	934.6 ^a	927.6 ^b	912.7 ^c	900.6 ^d	0.07	0.000	906.0 ^a	899.4 ^b	881.7 ^c	879.2 ^d	0.07	0.000
NDF	458.8 ^a	457.2 ^a	449.3 ^b	438.9 ^c	0.07	0.000	423.1 ^a	396.9 ^b	385.5 ^{bc}	374.7 ^c	0.71	0.009
ADF	259.3 ^a	257.4 ^a	246.6 ^b	238.8 ^c	0.07	0.000	247.0 ^a	224.8 ^b	212.2 ^c	207.0 ^d	0.07	0.000

DM = Dry matter, OM = Organic matter, NDF = Neutral detergent fiber, ADF = Acid detergent fiber, SEM = standard error of mean. ^{a, b, c, d} means within row with different superscript letter showed significantly different at various harvesting time ($p < 0.05$).

4.2. Experiment 2: In Vitro Gas Technique (Main Experiment)

4.2.1. Gas Production Kinetics and In Vitro True Dry Matter Digestibility (DMD_{vt})

The kinetics of gas, gas cumulation, and DMD_{vt} are presented in Table 4. The R:C ratio and FE had no interaction effect on gas kinetics, cumulative gas, and DMD_{vt}. The R:C ratios did not affect gas kinetics and gas cumulation but significantly affected DMD_{vt} at 12 h and 24 h of incubation. The R:C ratio at 40:60 was not different compared with 50:50 and 30:70 ratio for DMD_{vt} at 12 h of incubation but DMD_{vt} at 12 h and 24 h of incubation was significantly different between 50:50 and 30:70 ratios. Increasing concentrate in the ratio from 50% to 70% significantly increased the DMD_{vt} degradability. FE supplementation significantly affected the gas production rate constant for an insoluble fraction (c). Increasing FE level quadratically affected the (c) value. FE supplementation did not affect gas production from a soluble fraction (a), potential extent of gas production (a + b), gas cumulation, and DMD_{vt}.

4.2.2. Rumen Fermentation, Volatile Fatty Acids (VFAs) and Protozoal Population

Ruminal pH, NH₃-N, and protozoal population are presented in Table 5. The interaction effect between the R:C ratio and FE was not observed for ruminal pH, NH₃-N, and the protozoal population. The R:C ratio significantly ($p < 0.05$) affects ruminal pH, NH₃-N, and the protozoal population. Varying R:C ratios from 50:50 to 30:70 increased NH₃-N concentration and protozoal population. FE supplementation significantly affected the protozoal population but did not affect ruminal pH and NH₃-N concentration. Increasing FE the level linearly decreased the protozoal population. The lowest protozoal population was 1.79×10^5 cells/mL when FE was supplemented at 40 mg of the substrate.

Table 4. Effect of R:C ratios and flavonoid extract levels on in vitro gas production and degradability.

Treatments	R:C	FE (mg)	a (mL)	B (mL)	c (mL)	a + b (mL)	Gas Production at 96 h (mL/0.5 g Substrate)	DMD _{vt} (%)	
								12 h	24 h
T1	50:50	0	−6.17	109.17	0.039	103.03	93.1	51.72	63.65
T2		10	−3.1	127.8	0.02	124.7	99.5	51.58	65.2
T3		20	−1.6	116.9	0.027	115.43	102.7	51.13	67.9
T4		30	−2.3	110.13	0.024	107.93	88.9	46.93	68.52
T5		40	−1.33	107.43	0.078	106.03	101.7	57.23	68.9
T6	40:60	0	−1.3	117.53	0.019	116.4	105.4	60	62.98
T7		10	−4.23	121.07	0.018	116.83	94.4	62.33	72.25
T8		20	−5.53	111.93	0.032	108.4	100.9	61.22	70.48
T9		30	−4.2	130.6	0.019	126.4	101.5	65.05	72.02
T10		40	−2	107.7	0.018	105.7	82.4	64.77	71.6
T11	30:70	0	−1.25	126	0.038	124.75	113.1	68.8	78.3
T12		10	−2.07	119.53	0.022	117.7	98.7	67.28	79.37
T13		20	−3.4	112.75	0.021	119.3	95.6	70.2	86.17
T14		30	−3.57	100.5	0.03	97	88.7	74.57	84.88
T15		40	−3.1	113.73	0.028	110.63	97.3	70.6	74.8
SEM			3.098	17.87	0.01	15.9	14.18	12.46	18
R:C	50:50		−1.29	114.31	0.029	111.41	97.2	51.72 ^b	66.84 ^b
	40:60		−2.88	112.17	0.027	114.55	94.51	62.68 ^{ab}	69.86 ^b
	30:70		−2.89	111.71	0.028	110.41	98.71	68.83 ^a	80.73 ^a
FE (mg)	0		−1	113.12	0.034 ^a	112.13	103.87	60.18	68.31
	10		−3.04	122.80	0.020 ^b	119.73	97.54	60.40	72.28
	20		−2.43	113.64	0.028 ^{ab}	111.2	99.73	60.85	74.84
	30		−3.36	113.78	0.024 ^{ab}	110.43	93.07	59.75	75.14
	40		−1.93	100.29	0.032 ^a	107.13	89.81	64.20	71.80
Orthogonal	Lin		0.510	0.075	0.985	0.259	0.621	0.651	0.678
polynomial	Quad		0.172	0.107	0.030	0.485	0.620	0.722	0.548
	Cub		0.925	0.784	0.359	0.424	0.264	0.746	0.924
R:C × FE			0.061	0.234	0.832	0.533	0.153	0.999	0.999

R:C = Rice straw to concentrate ratio, FE = flavonoid extract, a = gas production from immediately soluble fraction, b = gas production from insoluble fraction, c = gas production rate constant for insoluble fraction, a + b = potential extent of gas, DMD_{vt} = in vitro true dry matter digestibility, SEM = standard error of mean. ^a, ^b means within column with different superscript letter showed significantly different ($p < 0.05$).

Table 5. Effect of R:C ratios and flavonoid extract levels on ruminal pH, ammonia nitrogen (NH₃-N), and protozoal population.

Treatments	R:C	FE (mg)	pH	NH ₃ -N (mg/dL)	Protozoa (×10 ⁵ Cells/mL)
T1	50:50	0	6.85	10.82	3.50
T2		10	6.87	17.03	3.25
T3		20	6.91	10.63	3.00
T4		30	6.94	17.13	1.25
T5		40	6.91	17.92	1.50
T6	40:60	0	6.61	20.02	3.50
T7		10	6.66	18.43	2.75
T8		20	6.60	20.63	3.25
T9		30	6.64	16.52	2.75
T10		40	6.65	21.42	2.00
T11	30:70	0	6.49	22.92	5.50
T12		10	6.40	22.22	4.52
T13		20	6.50	24.32	3.50
T14		30	6.41	22.22	4.00
T15		40	6.60	21.82	1.87
SEM			0.013	0.255	0.052
R:C	50:50		6.90 ^a	14.71 ^b	2.50 ^b
	40:60		6.63 ^b	19.41 ^b	2.85 ^b
	30:70		6.48 ^b	22.70 ^a	3.88 ^a
FE (mg)	0		6.65	17.92	4.17 ^a
	10		6.65	19.23	3.51 ^{ab}
	20		6.67	18.53	3.25 ^{ab}
	30		6.66	18.62	2.67 ^{bc}
	40		6.72	20.39	1.79 ^b
Orthogonal	Lin		0.556	0.394	0.000
polynomial	Quad		0.773	0.773	0.534
	Cub		0.895	0.467	0.502
R:C × FE			0.995	0.437	0.299

R:C = rice straw to concentrate ratio; FE = flavonoid extract; SEM = standard error of mean. ^a, ^b, ^c means within column with different superscript letter showed significantly different ($p < 0.05$).

Total VFA, molar portions of VFA, C2 to C3 ratio, and CH₄ production were presented in Table 6. The R:C ratio and FE had an interaction effect on the C2 to C3 ratio but total

VFA, molar portions of VFA, and CH₄ concentration were not observed. The C2 to C3 ratio, equal 2.43, was lowest at a 30:70 ratio and 20 mg FE supplementation. The R:C ratio significantly ($p < 0.05$) influenced total VFA, molar portions of VFA (C2, C3, and C4), and C2 to C3 ratio. Varying R:C ratios significantly decreased the C2 to C3 ratio and C2 concentration but significantly increased C3, C4, and total VFA concentration. The R:C ratio significantly affects the CH₄ production, decreasing from 21.55 mmol/L to 12.97 mmol/L. FE supplementation significantly ($p < 0.05$) influenced total VFA, C2, C3, and CH₄ concentration. FE supplementation linearly ($p < 0.05$) increased C3 and total VFA concentration and decreased C2 concentration. In addition, FE supplementation linearly ($p < 0.05$) decreased CH₄ production, ranging from 20.22 mmol/L to 13.48 mmol/L.

Table 6. Effect of R:C ratios and Brazilian spinach levels on ruminal organic acids and methane (CH₄) production.

Treatments	R:C	FE (mg)	C ₂ :C ₃ Ratio	Molar Proportions of VFA (mmol/L)			Total VFA (mmol/L)	CH ₄ (mmol/L)
				C ₂	C ₃	C ₄		
T1	50:50	0	4.14 ^a	74.75	18.06	10.43	94.85	24.96
T2		10	3.92 ^a	72.44	18.46	10.62	94.53	21.99
T3		20	3.08 ^{bc}	72.59	24.24	10.71	102.12	20.93
T4		30	3.17 ^{bcd}	72.65	24.48	12.12	107.31	20.30
T5		40	3.86 ^a	72.28	20.16	10.56	99.09	19.59
T6	40:60	0	3.21 ^b	69.97	21.79	12.51	104.28	19.42
T7		10	2.87 ^{bcd}	68.36	23.86	11.41	103.62	16.30
T8		20	3.25 ^{bc}	68.18	21.57	11.01	102.76	11.55
T9		30	2.84 ^{bcd}	66.24	24.52	10.3	104.55	10.74
T10		40	2.83 ^{bcd}	63.48	25.62	12.02	110.13	10.04
T11	30:70	0	2.8 ^{bcd}	66.35	23.73	11.52	109.99	16.29
T12		10	2.65 ^{de}	65.45	24.73	11.71	108.88	13.25
T13		20	2.43 ^e	65.34	27.66	11.84	114.08	12.55
T14		30	2.66 ^{cde}	65.22	26.58	12.59	116.81	11.91
T15		40	2.49 ^e	64.87	27.5	12.61	117.89	10.82
SEM			0.25	0.100	1.93	1.076	4.25	0.156
R:C	50:50		3.64 ^a	72.94 ^a	21.22 ^c	10.89 ^b	99.61 ^c	21.55 ^a
	40:60		3.02 ^b	67.25 ^b	23.47 ^b	11.45 ^{ab}	104.87 ^b	13.61 ^b
	30:70		2.61 ^c	65.45 ^c	26.24 ^a	12.06 ^a	113.53 ^a	12.97 ^b
FE (mg)	0		3.41 ^a	70.36 ^{ab}	20.59 ^b	11.49	103.04 ^b	20.22 ^a
	10		3.15 ^{ab}	68.75 ^{ab}	22.87 ^{ab}	11.25	101.44 ^b	17.18 ^b
	20		2.94 ^b	68.70 ^{ab}	24.49 ^a	11.19	106.38 ^{ab}	15.01 ^{bc}
	30		2.89 ^b	68.04 ^b	25.19 ^a	11.67	109.56 ^a	14.32 ^{bc}
	40		3.06 ^b	66.88 ^b	24.43 ^a	11.74	109.04 ^a	13.48 ^c
Orthogonal	Lin		0.010	0.001	0.002	0.523	0.003	0.000
polynomial	Quad		0.019	0.904	0.120	0.493	0.932	0.121
	Cub		0.595	0.308	0.265	0.670	0.091	0.743
R:C × FE			0.042	0.356	0.122	0.398	0.483	0.785

R:C = rice straw to concentrate ratio; FE = total flavonoid content; SEM = standard error of mean. ^{a, b, c, d, e} means within column with different superscript letter showed significantly different ($p < 0.05$).

5. Discussion

5.1. Brazilian Spinach Yield

In this study, BS yield was 1.81 kg/m² at 60 days of age after plantation and 2.51 kg/m² after regrowth. Increasing maturity significantly increased the BS yield. There were no studies reported about the maturity effect on the yield of BS. Martin and Ruberté [30] reported that yield increased for second harvesting compared to first harvesting suggesting that frequent cutting could stimulate regrowth resulting increase yield. It is clearly seen in Figure 1 that BS yield after regrowth was higher than first harvesting.

5.2. Chemical Composition and Digestibility of Brazilian Spinach

Various factors including genotypes, pre, and post-harvesting, cropping practices, maturity, handling, and/or interaction these factors could affect the chemical quality of vegetables [31,32]. Generally, increasing maturity resulted in a decrease in CP content and increasing structural fiber content in plant leaves [33]. This study showed that CP content in leaf and leaf + leaf-stalk significantly decreased as the maturity ages increased. In contrast, the structural carbohydrate including NDF and ADF content significantly increased as the maturity stage increased. The nutrient content of vegetables at various harvesting ages may be genotype dependent [34]. Spinach is rich in phytonutrient sources [35]. Bergquist et al. [36] reported that spinach contains relatively high amounts of carotenoids, flavonoids, and phenolic acids [37]. This study showed that TF content ranged from 49.57 to 82.16 g/kg DM in leaf and 45.52 to 78.14 g/kg DM in leaf + leaf-stalk. The TF content in leaf significantly decreased during 15 to 30 days but from 45 to 60 days the TF content was not different. In leaf + leaf-stalk, TF content significantly decreased from 15 to 60 days of regrowth. The highest content of TF in both leaf and leaf + leaf-stalk was 82.16 g/kg DM and 78.14 g/kg DM. Flavonoid concentration and composition may change during plant growth [38]. Furthermore, environmental conditions may affect the rate of plant growth and development, thereby influencing flavonoid content directly [39]. Bergquist et al. [40] reported that TF content in *Spinacia oleracea* L. was higher at a younger age stage than older age. This study assumed that leaf and leaf + leaf-stalk of BS should be harvested at younger age after plantation and regrowth to obtain a high TF content.

As mentioned above, increasing maturity resulted in a decrease of CP and an increase of structural carbohydrates [33]. Increasing structural carbohydrate content may affect nutrient degradability. This study found that in vitro DM, OM, NDF, and ADF degradability at 12 h and 24 h for both leaf and leaf + leaf-stalk significantly decreased as the maturity increased. This could be due to the increase of NDF and ADF content in both leaf and leaf + leaf-stalk as the harvesting ages increased (Table 1). The effect of maturity on nutrient degradability in leaf and leaf + leaf-stalk of BS has never been reported until now. In fodders, Khan et al. [41] reported that in situ DM, OM, and NDF digestibility were affected by the maturity stages, by which digestibility of DM, OM, and NDF significantly decreased as the maturity stages increased with the increase of the total DM and NDF portions in stem tissue.

5.3. Gas Production Kinetics and In Vitro Dry Matter Degradability (DMD_{vt})

The R:C ratio and FE had no interaction effect on gas kinetics, gas cumulation, and DMD_{vt} . Thus, attention was paid to the main factors. R:C ratios did not affect gas kinetics and gas cumulation. The highest gas cumulation was found in the R:C ratio at 30:70, which was 98.71 mL/0.5 g substrate DM. This could be due to the high concentrate portion in the substrate ratio compared to other ratios. Insoongnorn and Wachirapakorn [42] similarly revealed that R:C ratios (40:60 vs. 30:70) did not affect gas kinetics and gas cumulation at 48 h of incubation. In addition, R:C ratios significantly affected DMD_{vt} at 12 h and 24 h of incubation. The highest DMD_{vt} at 12 h and 24 h was 68.83% and 80.73%, which was found in the R:C (30:70) ratio. Decreasing roughage in the substrate ration significantly increased DMD_{vt} . This could be due to the decrease of fiber content and increase of concentrate in the substrate ratio. Phesatcha et al. [43] similarly reported that R:C ratios (80:20 to 20:80) significantly affected the DMD_{vt} at 12 h and 24 h of incubation, in which R:C ratio at 20:80 resulted from the highest DMD_{vt} of 72.2% at 12 h and 85.5% at 24 h of incubation. Insoongnorn and Wachirapakorn [42] similarly showed that increased concentrate portion in the substrate ration from 60 to 70% significantly increased DMD_{vt} at 12 h and 24 h of incubation. Nagadi [44] similarly showed that increasing the concentrate portion from 0% to 100% in the substrate ration significantly increased in vitro true DM digestibility, which ranged from 75.03% to 88.85%. FE supplementation quadratically affected gas production rate constant (c) of insoluble fraction (b). A significant in gas production rate constant (c) of insoluble fraction (b) was observed between 0 mg and 10 mg of FE supplementation,

which decreased from 0.034 mL to 0.020 mL. This could be due to the increase in the potential extent of gas (a + b) from 112.13 mL to 119.73 mL. The potential extent of gas (a + b) had a negative relationship with the gas production rate constant (c) of insoluble fraction (b), by which increasing potential extent of gas (a + b) leads to a decrease of the gas production rate constant (c) of insoluble fraction (b) as stated by Ørskov and McDonald [24]. Oskoueian et al. [12] similarly reported that 4.5% (*w/w*) of naringin, rutin, and quercetin in the substrate significantly decreased the gas production rate constant (c) of insoluble fraction (b).

5.4. Rumen Fermentation, VFAs and Protozoal Population

Ruminal pH critically affects the ruminal functions, too high or low pH negatively affects fermentation, the activity of microbes, microbial population, and digestion as well as absorption in the rumen [4,5,25,45,46]. The optimal pH ranges from 6.5 to 7.0 [47]. Increasing concentrate portion in the ration normally decreases ruminal pH due to the accumulation of VFAs and propionic acid [48]. This study showed that varying R:C ratios from 50:50 to 30:70 significantly decreased ruminal pH from 6.90 to 6.48. This could be explained by the increase of concentrate portion from 50% to 70% in the substrate ration. Phesatcha et al. [43] similarly found that increasing concentrate portions from 20% to 80% in the substrate ration significantly decreased ruminal pH from 6.83 to 6.46. Cherdthong et al. [48] similarly reported that increasing concentrate portions from 0% to 75% in the diet of buffaloes significantly decreased from 7.0 to 6.2. Increasing concentrated portions in the ration resulted in an increase in energy supply, proteins, minerals, and vitamins [49]. Increasing protein in the ration increases protein degradation resulting in an increase of $\text{NH}_3\text{-N}$ production in the rumen [4,5]. This study showed that varying concentrate portions from 50% to 70% in the substrate ration significantly increased $\text{NH}_3\text{-N}$ concentration, ranging from 14.71 to 22.70 mg/dL. Varying R:C ratios significantly affected the protozoal population, in which the increase of concentrate portions in the substrate ration increased the protozoal population from $2.50\text{--}3.88 \times 10^5$ cells/mL. Dehority and Orpin [50] reported that concentrate portions from 40% to 60% could maximize ruminal protozoal population because protozoa could degrade protein [51] resulting in an increase in $\text{NH}_3\text{-N}$ concentration. Aziz et al. [52] similarly showed that 70% concentrate in the ration was the best condition in maximizing ruminal protozoal population. Phesatcha et al. [43] showed that 80% concentrate in the substrate ration resulted in the highest protozoal population. Natural bioactive compounds have recently been paid more attention due to their various functional physiological advantages including CH_4 mitigation and ruminal fermentation modification [6]. Flavonoids are benzo-L-pyrone derivatives [53] and have antimicrobial properties acting like monensin and other types of antibiotic [54]. This study showed that FE supplementation significantly influenced the protozoal population, by which increasing FE supplementation from 0 mg to 40 mg linearly decreased the protozoal population from 4.17×10^5 cells/mL to 1.79×10^5 cells/mL. Oskoueian et al. [12] similarly showed that flavone, myricetin, naringin, catechin, quercetin, and kaempferol at 4.5% (*w/w*) of substrate significantly decreased the total protozoal population from $3.8\text{--}1.5 \times 10^6$ copies/mL. FE supplementation did not affect ruminal pH and $\text{NH}_3\text{-N}$ concentration. Similarly, Oskoueian et al. [12] showed that various flavonoid compounds addition at 4.5% (*w/w*) did not change ruminal pH and $\text{NH}_3\text{-N}$ concentration. This suggested that any flavonoid compounds and their levels of supplementation had no effect on ruminal pH and $\text{NH}_3\text{-N}$ concentration.

Organic acids produced from anaerobic fermentation of OM by microbes in the rumen are called volatile fatty acids (VFAs) [55]. The main molar portions of VFAs are C2, C3, and C4 and are used as a nutrient source for the host [56,57]. Many factors could affect the production of VFA including substrate composition, availability of substrate, and microbial population. This study showed that varying R:C ratios significantly total VFA, C2, C3, and C4 concentration. Total VFA, C3, and C4 increased when concentrate portions in the substrate increased from 50% to 70%. In contrast, C2 decreased as the concentrate

portion increased. Increasing total VFA, C3, and C4 and decreasing C2 concentration could be explained by the increase of concentrate in the substrate. Ruminal pH sensitively determines the production of VFA and their molar portions. Normally, lowering pH (7.0–5.5) resulted in a decrease of C2 and increase of C3 and C4 concentration, which supported the current finding of this study as shown in Table 5 that ruminal pH significantly dropped as the concentrate increased. In addition, an interaction effect between R:C ratios and FE was observed for the C2 to C3 ratio. This mechanism in promoting C2 to C3 ratio by R:C ratios and FE was not clear; they might have a synergistic mechanism in lowering C2 to C3 ratio which indicated the efficiency of energy utilization. These findings were in agreement with Phesatcha et al. [43] who found an increase of total VFA, C3, and C4 and a decrease of C2 when R:C ratios (80:20 to 20:80) increased. Varying R:C ratios significantly decreased CH₄ production. This could link to the increase of C3 concentration because hydrogen as the main substrate for the CH₄ production pathway was used to synthesize C3 instead [25,45,58,59]. Saini et al. [60] similarly found that increasing the concentrate portion in the ration from 30% to 70% significantly decreased CH₄ production, ranging from 54.75 mL/g DOM to 49.67 mL/g DOM. Similarly, Phesatcha et al. [43] showed that 20% to 80% of concentrate significantly decreased CH₄ emission, ranging from 27.72 mM to 23.32 mM. It has been widely claimed that flavonoid compounds can reduce CH₄ emission and modulate ruminal fermentation [6]. This study showed that C3 and total VFA linearly increased, and C2 linearly decreased when FE supplementation increased. This showed a positive effect of FE on shifting ruminal fermentation patterns and FE may act as a fermentable substrate for microbes in the rumen. Some flavonoid derivatives such as rutin, naringin, and quercitrin functioned as the substrate for microbes after being degraded in the rumen [61,62]. In addition, FE may affect methanogen bacteria and enhance hydrogen availability for C3 synthesis. Ramos-Morales et al. [63] showed that 1 g/L of isoflavonoid-rich liquorice extract significantly increased C3 and total VFA and decreased C2 concentration; in contrast, 2 g/L of isoflavonoid-rich liquorice extract decreased C3 and total VFA, and this could be due to the strong effect of isoflavonoid-rich liquorice extract given its purity. Similarly, Sinz et al. [13] showed that epicatechin supplementation at 50 mg/g substrate significantly increased C3 concentration. FE supplementation linearly decreased CH₄ production. This could link to the increase of C3 concentration because FE may affect the methanogenesis pathway resulting in more available hydrogen as substrate in synthesizing C3. Kim et al. [64] indicated that flavonoid-rich plant extracts appear to have a potential possibility as a bio-active regulator for ruminants with decreasing ruminal methane emission. Oskoueian et al. [12] similarly showed that 4.5% (*w/w*) of flavonoids significantly decreased CH₄ production. Similarly, Sinz et al. [13] showed that tannic acid, quercetin, and luteolin-7-glucoside addition at 50 mg/g substrate significantly decreased CH₄ volume by 22.82%, 9.57%, and 10.55%.

6. Conclusions

This study showed that BS should be harvested during 15 to 30 days of age after plantation and regrowth to obtain a high total flavonoid content and *in vitro* degradability. Varying R:C ratios from 50:50 to 30:70 significantly increased *in vitro* DM degradability, total VFA, and C3 concentration. Flavonoid extract supplementations significantly increased total VFA and C3 concentration and decreased CH₄ production and protozoal population. However, an *in vivo* study is needed to confirm the present findings.

Author Contributions: Planning and design of the study, S.S. (Sukruthai Sommai) and A.C.; Conducting and sampling, S.S. (Sukruthai Sommai), A.C., C.S. and S.S. (Sarong So); Samples analysis, S.S. (Sukruthai Sommai), C.S. and S.S. (Sarong So); Statistical analysis, S.S. (Sukruthai Sommai) and S.S. (Sarong So); Manuscript drafting, S.S. (Sukruthai Sommai), A.C., C.S. and S.S. (Sarong So); Manuscript editing and finalizing, S.S. (Sukruthai Sommai), A.C., C.S., S.S. (Sarong So), M.W. and S.P. All authors have read and agreed to the published version of the manuscript.

Funding: The authors express their most sincere gratitude to the Research Program on Research and Development of Winged Bean Root Utilization as Ruminant Feed, Increase Production Efficiency and Meat Quality of Native Beef and Buffalo Research Group, and Research and Graduate Studies, Khon Kaen University (KKU). Thailand and Thailand Research Fund (TRF) through the Thailand Science Research and Innovation (TSRI) (TRF-IRN5702PHDW06) is gratefully acknowledged.

Institutional Review Board Statement: The study was conducted under approval procedure no. IACUC-KKU-43/63 of Animal Ethics and Care issued by Khon Kaen University (Date of approval, 21 May 2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Thanks to the Tropical Feed Resources Research and Development Center (TROFREC), Department of Animal Science, Faculty of Agriculture, KKU for the use of the research facilities.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Beauchemin, K.A.; Ungerfeld, E.; Eckard, R.J.; Wang, M. Review: Fifty years of research on rumen methanogenesis: Lessons learned and future challenges for mitigation. *Animal* **2020**, *14*, s2–s16. [\[CrossRef\]](#)
2. Halmemies-Beauchet-Filleau, A.; Rinne, M.; Lamminen, M.; Mapato, C.; Ampapon, T.; Wanapat, M.; Vanhatalo, A. Review: Alternative and novel feeds for ruminants: Nutritive value, product quality and environmental aspects. *Animal* **2018**, *12*, s295–s309. [\[CrossRef\]](#)
3. Steinfeld, H.; Gerber, P.; Wassenaar, T.D.; Castel, V.; Rosales, M.; Rosales, M.; de Haan, C. *Livestock's Long Shadow: Environmental Issues and Options*; Food and Agriculture Organization: Rome, Italy, 2006; p. 377.
4. Unnawong, N.; Cherdthong, A.; So, S. Influence of supplementing *Sesbania grandiflora* pod meal at two dietary crude protein levels on feed intake, fermentation characteristics, and methane mitigation in Thai purebred beef cattle. *Vet. Sci.* **2021**, *8*, 35. [\[CrossRef\]](#)
5. Unnawong, N.; Cherdthong, A.; So, S. Crude saponin extract from *Sesbania grandiflora* (L.) Pers pod meal could modulate ruminal fermentation, and protein utilization, as well as mitigate methane production. *Trop. Anim. Health Prod.* **2021**, *53*, 1–9. [\[CrossRef\]](#)
6. Hristov, A.N.; Ott, T.; Tricarico, J.; Rotz, A.; Waghorn, G.; Adesogan, A.; Dijkstra, J.; Montes, F.; Oh, J.; Kebreab, E.; et al. Mitigation of methane and nitrous oxide emissions from animal operations: III. A review of animal management mitigation options1. *J. Anim. Sci.* **2013**, *91*, 5095–5113. [\[CrossRef\]](#)
7. Ku-Vera, J.C.; Jiménez-Ocampo, R.; Valencia-Salazar, S.S.; Montoya-Flores, M.D.; Molina-Botero, I.C.; Arango, J.; Gómez-Bravo, C.A.; Aguilar-Pérez, C.F.; Solorio-Sánchez, F.J. Role of secondary plant metabolites on enteric methane mitigation in ruminants. *Front. Vet. Sci.* **2020**, *7*, 584. [\[CrossRef\]](#) [\[PubMed\]](#)
8. Manh, N.S.; Wanapat, M.; Uriyapongson, S.; Khejornsart, P.; Chanthakhoun, V. Effect of eucalyptus (*Camaldulensis*) leaf meal powder on rumen fermentation characteristics in cattle fed on rice straw. *Afr. J. Agric. Res.* **2012**, *7*, 2142–2148.
9. Olagaray, K.E.; Bradford, B.J. Plant flavonoids to improve productivity of ruminants—A review. *Anim. Feed Sci. Technol.* **2019**, *251*, 21–36. [\[CrossRef\]](#)
10. Xie, Y.; Yang, W.; Tang, F.; Chen, X.; Ren, L. Antibacterial activities of flavonoids: Structure-activity relationship and mechanism. *Curr. Med. Chem.* **2015**, *22*, 132–149. [\[CrossRef\]](#) [\[PubMed\]](#)
11. Kang, S.; Wanapat, M.; Pakdee, P.; Pilajun, R.; Cherdthong, A. Effects of energy level and *Leucaena leucocephala* leaf meal as a protein source on rumen fermentation efficiency and digestibility in swamp buffalo. *Anim. Feed Sci. Technol.* **2012**, *174*, 131–139. [\[CrossRef\]](#)
12. Oskoueian, E.; Abdullah, N.; Oskoueian, A. Effects of flavonoids on rumen fermentation activity, methane production, and microbial population. *BioMed Res. Int.* **2013**, 1–9. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Sinz, S.; Kunz, C.; Liesegang, A.; Braun, U.; Marquardt, S.; Soliva, C.R.; Kreuzer, M. In vitro bioactivity of various pure flavonoids in ruminal fermentation, with special reference to methane formation. *Czech J. Anim. Sci.* **2018**, *63*, 293–304.
14. Stoldt, A.-K.; Derno, M.; Daş, G.; Weitzel, J.M.; Wolfram, S.; Metges, C.C. Effects of rutin and buckwheat seeds on energy metabolism and methane production in dairy cows. *J. Dairy Sci.* **2016**, *99*, 2161–2168. [\[CrossRef\]](#) [\[PubMed\]](#)
15. Tiveron, A.P.; Melo, P.S.; Bergamaschi, K.B.; Vieira, T.M.; Regitano-d'Arce, M.A.; Alencar, S.M. Antioxidant activity of Brazilian vegetables and its relation with phenolic composition. *Int. J. Mol. Sci.* **2012**, *13*, 8943–8957. [\[CrossRef\]](#) [\[PubMed\]](#)
16. Manurung, H.; Kustiawan, W.; Kusuma, I.W.; Marjenah, M. Total flavonoid content and antioxidant activity of tabat Barito (*Ficus deltoidea* Jack) on different plant organs and ages. *J. Med. Plants* **2017**, *5*, 120–125.
17. AOAC. *Official Methods of Analysis*, 16th ed.; AOAC: International—Association of Official Analytical Chemists: Gaithersburg, MD, USA, 1995.

18. Van Soest, P.J.; Robertson, J.B.; Lewis, B.A. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597. [\[CrossRef\]](#)
19. Chang, C.C.; Yang, M.H.; Wen, H.M.; Chern, J.C. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.* **2002**, *10*, 178–182.
20. Menke, K.H.; Steingass, H. Estimation of the energetic feed value obtained from chemical analysis and gas production using rumen fluid. *Anim. Res. Devel.* **1988**, *28*, 7–55.
21. Tilley, J.M.A.; Terry, D.R. A two-stage technique for the in vitro digestion of forage crops. *Grass Forage Sci.* **1963**, *18*, 104–111. [\[CrossRef\]](#)
22. AOAC. *Official Methods of Analysis*, 19th ed.; AOAC: International—Association of Official Analytical Chemists: Gaithersburg, MD, USA, 2012.
23. Bohm, B.A.; Koupai-Abyazani, M.R. Flavonoids and condensed tannins from leaves of Hawaiian *Vaccinium reticulatum* and *V. calycinum* (Ericaceae). *Pac. Sci.* **1994**, *48*, 458–463.
24. Ørskov, E.R.; McDonald, I. The estimation of protein degradability in the rumen from incubation measurements weighted according to rate of passage. *J. Agric. Sci.* **1979**, *92*, 499–503. [\[CrossRef\]](#)
25. So, S.; Cherdthong, A.; Wanapat, M. Growth performances, nutrient digestibility, ruminal fermentation and energy partition of Thai native steers fed exclusive rice straw and fermented sugarcane bagasse with *Lactobacillus*, cellulase and molasses. *J. Anim. Physiol. Anim. Nutr.* **2021**, in press. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Galyean, M. *Laboratory Procedures in Animal Nutrition Research*; New Mexico State University: Las Cruces, NM, USA, 1989.
27. Sittijunda, S.; Reungsang, A.; Sompong, O.T. Biohydrogen production from dual digestion pretreatment of poultry slaughterhouse sludge by anaerobic self-fermentation. *Int. J. Hydrog. Energy* **2010**, *35*, 13427–13434. [\[CrossRef\]](#)
28. SAS (Statistical Analysis System). *User's Guide: Statistic, Version 9*, 4th ed.; SAS Inst. Inc.: Cary, NC, USA, 2013.
29. Crichton, N. Information point: Tukey multiple comparison test. *J. Clin. Nurs.* **1999**, *8*, 299–304.
30. Martin, F.W.; Ruberté, R.M. *Edible Leaves of the Tropics*, 3rd ed.; Agency for International Development, Department of State: Washington, DC, USA; Agricultural Research Service, U.S. Department of Agriculture: Washington, DC, USA, 1975; p. 235.
31. Lee, S.K.; Kader, A.A. Preharvest and postharvest factors influencing vitamin C content of horticultural crops. *Postharvest Biol. Technol.* **2000**, *20*, 207–220. [\[CrossRef\]](#)
32. Chiesa, A. Factors determining postharvest quality of leafy vegetables. In *Proceedings of the International Conference on Quality in Chains: An Integrated View on Fruit and Vegetable Quality*, Wageningen, The Netherlands, 6–9 July 2003; Tijskens, L.M.M., Vollebregt, H.M., Eds.; ISHS Acta Horticulturae: Leuven, Belgium, 2003.
33. Baranga, D. Changes in chemical composition of food parts in the diet of Colobus monkeys. *Ecology* **1983**, *64*, 668–673. [\[CrossRef\]](#)
34. Oloyede, F.M.; Oloyede, F.A.; Obuotor, E.M. Effect of plant maturity on the antioxidant profile of *Amaranthus cruentus* L. and *Celosia argentea* L. *Bull. Env. Pharmacol. Life Sci.* **2013**, *2*, 18–21.
35. Mehta, D.; Belemkar, S. Pharmacological activity of *Spinacia oleracea* linn. *Asian J. Pharm. Res. Devel.* **2014**, *2*, 32–35.
36. Bergquist, S.Å.; Gertsson, U.E.; Olsson, M.E. Influence of growth stage and postharvest storage on ascorbic acid and carotenoid content and visual quality of baby spinach (*Spinacia oleracea* L.). *J. Sci. Food Agric.* **2005**, *86*, 346–355. [\[CrossRef\]](#)
37. Fallovo, C.; Roupael, Y.; Rea, E.; Battistelli, A.; Colla, G. Nutrient solution concentration and growing season affect yield and quality of *Lactuca sativa* L. var. *acephala* in floating raft culture. *J. Sci. Food Agric.* **2009**, *89*, 1682–1689. [\[CrossRef\]](#)
38. Fico, G.; Bilia, A.R.; Morelli, I.; Tomè, F. Flavonoid distribution in *Pyracantha coccinea* plants at different growth phases. *Biochem. Syst. Ecol.* **2000**, *28*, 673–678. [\[CrossRef\]](#)
39. Lucier, G.; Allshouse, J.E.; Lin, B.H. *Factors Affecting Spinach Consumption in the United States*; US Department of Agriculture, Economic Research Service: Washington, DC, USA, 2004; p. 15.
40. Bergquist, S.Å.; Gertsson, U.E.; Knuthsen, P.; Olsson, M.E. Flavonoids in Baby Spinach (*Spinacia oleracea* L.): Changes during plant growth and storage. *J. Agric. Food Chem.* **2005**, *53*, 9459–9464. [\[CrossRef\]](#)
41. Khan, S.H.; Khan, A.G.; Sarwar, M.; Azim, A. Effect of maturity on production efficiency, nutritive value and in situ nutrients digestibility of three cereal fodders. *Int. J. Agric. Res.* **2007**, *2*, 900–906.
42. Insoongnarn, H.; Wachirapakorn, C. Effect of roughage to concentrate ratio and mineral salt block supplementation to optimize rumen fermentation in vitro gas technique. *Khon Kaen Agri. J.* **2017**, *45*, 48–52.
43. Phesatcha, K.; Phesatcha, B.; Wanapat, M.; Cherdthong, A. Roughage to concentrate ratio and *saccharomyces cerevisiae* inclusion could modulate feed digestion and in vitro ruminal fermentation. *Vet. Sci.* **2020**, *7*, 151. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Nagadi, S.A. In vitro gas production, methane emission and rumen fermentation characteristics with increasing roughage to concentrate ratios. *J. King Abdulaziz Univ. Meteorol. Environ. Arid. Land Agric. Sci.* **2019**, *28*, 27–36. [\[CrossRef\]](#)
45. So, S.; Wanapat, M.; Cherdthong, A. Effect of sugarcane bagasse as industrial by-products treated with *Lactobacillus casei* TH14, cellulase and molasses on feed utilization, ruminal ecology and milk production of mid-lactating Holstein Friesian cows. *J. Sci. Food Agric.* **2021**. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Ogata, T.; Makino, H.; Ishizuka, N.; Iwamoto, E.; Masaki, T.; Kizaki, K.; Kim, Y.-H.; Sato, S. Long-term high-grain diet alters ruminal pH, fermentation, and epithelial transcriptomes, leading to restored mitochondrial oxidative phosphorylation in Japanese Black cattle. *Sci. Rep.* **2020**, *10*, 1–11. [\[CrossRef\]](#)
47. Wanapat, M. Manipulation of cassava cultivation and utilization to improve protein to energy biomass for livestock feeding in the tropics. *Asian Australas. J. Anim. Sci.* **2003**, *16*, 463–472. [\[CrossRef\]](#)

48. Cherdthong, A.; Wanapat, M.; Kongmun, P.; Pilajun, R.; Khejornsar, P. Rumen fermentation, microbial protein synthesis and cellulolytic bacterial population of swamp buffaloes as affected by roughage to concentrate ratio. *J. Anim. Vet. Adv.* **2010**, *9*, 1667–1675. [[CrossRef](#)]
49. Aziz, H. Effect of roughage to concentrate ratio on digestibility in small ruminants. *J. Anim. Poult. Prod.* **2014**, *5*, 673–696. [[CrossRef](#)]
50. Dehority, B.A.; Orpin, C.G. Development of, and natural fluctuation in, rumen microbial population. In *The Rumen Microbial Ecosystem*; Hobson, P.N., Ed.; Elsevier Sci. Publ.: New York, NY, USA, 1988; pp. 151–183.
51. Euge'ne, M.; Archimed, H.; Sauvant, D. Quantitative meta-analysis on effects of defaunation of the rumen on growth, intake and digestion in ruminants. *Livest. Prod. Sci.* **2004**, *85*, 81–97. [[CrossRef](#)]
52. Aziz, H.A.; Nassar, M.S.; Abd Badway, H.S.; Elrahaman, M.A. Rumen fermentations and rumen ciliate protozoa of goat kids fed diets with different concentrate: Roughage ratio. *Egypt. J. Nutr. Feed.* **2018**, *21*, 667–683. [[CrossRef](#)]
53. Nijveldt, R.J.; van Nood, E.; van Hoorn, D.E.; Boelens, P.G.; van Norren, K.; van Leeuwen, P.A. Flavonoids: A review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* **2001**, *74*, 418–425. [[CrossRef](#)] [[PubMed](#)]
54. Kalantar, M. The importance of flavonoids in ruminant nutrition. *Arch. Anim. Husb. Dairy Sci.* **2018**, *1*, 1–4.
55. Bergman, E.N.; Bergman, E.N. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. *Physiol. Rev.* **1990**, *70*, 567–590. [[CrossRef](#)] [[PubMed](#)]
56. Aluwong, T.; Kobo, P.I.; Abdullahi, A. Volatile fatty acids production in ruminants and the role of monocarboxylate trans-porters: A review. *Afr. J. Biotechnol.* **2010**, *9*, 6229–6232.
57. Dieho, K.; Dijkstra, J.; Schonewille, J.T.; Bannink, A. Changes in ruminal volatile fatty acid production and absorption rate during the dry period and early lactation as affected by rate of increase of concentrate allowance. *J. Dairy Sci.* **2016**, *99*, 5370–5384. [[CrossRef](#)]
58. So, S.; Cherdthong, A.; Wanapat, M. Improving sugarcane bagasse quality as ruminant feed with *Lactobacillus*, cellulase, and molasses. *J. Anim. Sci. Technol.* **2020**, *62*, 648–658. [[CrossRef](#)]
59. So, S.; Cherdthong, A.; Wanapat, M.; Uriyapongson, S. Fermented sugarcane bagasse with *Lactobacillus* combined with cellulase and molasses promotes in vitro gas kinetics, degradability, and ruminal fermentation patterns compared to rice straw. *Anim. Biotechnol.* **2020**, 1–12. [[CrossRef](#)]
60. Saini, J.K.; Hundal, J.S.; Wadhwa, M.; Bakshi, M.P.S. Effect of roughage to concentrate ratio in the diet on the rumen environment and nutrient utilization in goat and sheep. *Indian J. Anim. Nutr.* **2012**, *4*, 333–338.
61. McSweeney, C.S.; Palmer, B.; McNeill, D.M.; Krause, D.O. Microbial interactions with tannins: Nutritional consequences for ruminants. *Anim. Feed Sci. Technol.* **2001**, *91*, 83–93. [[CrossRef](#)]
62. Tassone, S.; Fortina, R.; Peiretti, P.G. In vitro techniques using the daisyii incubator for the assessment of digestibility: A review. *Animals*. **2020**, *10*, 775. [[CrossRef](#)] [[PubMed](#)]
63. Ramos-Morales, E.; Rossi, G.; Cattin, M.; Jones, E.; Braganca, R.; Newbold, C.J. The effect of an isoflavonoid-rich liquorice extract on fermentation, methanogenesis and the microbiome in the rumen simulation technique. *FEMS Microbiol. Ecol.* **2018**, *94*, fty009. [[CrossRef](#)]
64. Kim, E.T.; Guan, L.L.; Lee, S.J.; Lee, S.M.; Lee, S.S.; Lee, I.D.; Lee, S.K.; Lee, S.S. Effects of flavonoid-rich plant extracts on in vitro ruminal methanogenesis, microbial populations and fermentation characteristics. *Asian-Australas. J. Anim. Sci.* **2015**, *28*, 530–537. [[CrossRef](#)] [[PubMed](#)]