grasses

MDPI

Article

Evaluation of Associative Effects on Degradability, Fermentation Parameters, and In Vitro Methane Production as a Result of Variation in the Ruminants Diets Constituents

Danielle Ferreira Baffa †, Tadeu S. Oliveira *,†, Alberto Magno Fernandes, Michele Gabriel Camilo, Ismael N. Silva, Elon Souza Aniceto, José Ribeiro Meirelles Júnior and Elvanio José Lopes Mozelli Filho

Laboratory of Animal Science, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes 28013-602, RJ, Brazil; danibaffa@gmail.com (D.F.B.); alberto@uenf.br (A.M.F.); michelegabrielc@hotmail.com (M.G.C.); ismaelnacarati@gmail.com (I.N.S.); elon1995@hotmail.com (E.S.A.); junioribeiromeirelles@gmail.com (J.R.M.J.); elvaniomozelli@hotmail.com (E.J.L.M.F.)

* Correspondence: tsoliveira@uenf.br; Tel.: +55-22-27397083

† These authors contributed equally to this work.

Abstract: This study aimed to investigate the effect of different forage sources and forage-to-concentrate ratios on digestibility, energy concentration, fermentation parameters, and in vitro estimate of methane. The experiment was carried out in a completely randomized design using a 3 × 6 factorial arrangement with three forages varying the chemical composition (pineapple crop waste silage [PS], corn silage [CS], and Tifton hay [TH]) associated with concentrate feed (C) in six combinations, using triplicates for each ratio. We evaluated in vitro digestibility, metabolizable and net energy, pH, redox potential, volatile fatty acids (VFA), and methane production. The in vitro neutral detergent fiber digestibility (IVNDFD) decreased ($p = 0.0011$) with the inclusion of concentrate. It was also affected by the forage source, but this fact was only observed in CS up to the 50:50 ratio. In TH, this fact occurred from the 80:20 ratio, and this behavior was not observed in the PS. Data on methane production, VFA, and fermentation parameters varied according to forage source and concentrate inclusion. In conclusion, the inclusion of concentrate reduces methane production, increasing the system's energy contribution. Overall, the different forage sources and the inclusion of concentrate change digestion and fermentation parameters.

Keywords: chemical composition; digestion; grains; roughage

Citation: Baffa, D.F.; Oliveira, T.S.; Fernandes, A.M.; Camilo, M.G.; Silva, I.N.; Aniceto, E.S.; Meirelles Júnior, J.R.; Lopes Mozelli Filho, E.J. Evaluation of Associative Effects on Degradability, Fermentation Parameters, and In Vitro Methane Production as a Result of Variation in the Ruminants Diets Constituents. *Grasses* **2024**, *3*, 274–286. https://doi.org/10.3390/grasses3040020

Academic Editor: Fabio Gresta

Received: 9 August 2024
Revised: 18 September 2024
Accepted: 14 October 2024
Published: 23 October 2024

1. Introduction

The ruminal environment is a complex ecosystem where nutrients consumed by microorganisms such as bacteria, protozoa, and fungi are digested anaerobically [1]. Ruminal microorganisms transform plant cell walls into products (e.g., volatile fatty acids [VFA]) through microbial fermentation. However, ruminal fermentation is only partially efficient due to the production of methane gas (CH_4) [2] and ammonia (NH_3) in excess [3]. A negative implication of methane production by ruminants can compromise 2–12% of the gross energy from the feed [4]. Reducing CH_4 emissions from ruminants is crucial for mitigating the rise in global temperature over the next 30 years. Researchers and breeders are exploring various strategies to influence methanogenesis in the rumen [5]. Several methods have been developed to lower CH_4 emissions related to ruminant digestion, including improved farm management, optimized feeding strategies, the use of feed additives and chemical compounds to alter rumen biochemical processes, direct manipulation of rumen microflora, animal immunization, and genetic breeding approaches [6]. Common feed additives that help reduce methanogenesis include saponins, tannins, flavonoids, probiotics, organic acids, fats, and fiber [6]. The marked reductions in CH_4 emissions can be expected beyond the 35% to 40% inclusion of grain in the diet [7].

High-grain diets can cause pH drops, high concentrations of acids (i.e., lactic acid), and an increase in osmolality, exacerbating the accumulation of acid within the rumen by inhibiting VFA absorption [7]. In addition, the high inclusion of grains (starch) can reduce fibrolytic microorganisms' activity, resulting in decreased fiber digestibility and increased risks of subacute ruminal acidosis [8]. Amylolytic bacteria have rapid growth rates in the rumen as they ferment starch and soluble sugars [9]. The high rate of starch fermentation may contribute to the accumulation of lactic acid and volatile fatty acids (VFA). However, these microorganisms have low sensitivity to low pH (lower 5.0) [9,10]. Conversely, ruminants fed high proportions of forage (e.g., 90:10 forage-to-concentrate ratio), forage low quality, and fiber high content can limit feed intake and decrease microbial protein synthesis (due to an increase in passage rate and consequently increase in the maintenance required by the ruminal microbes) and energy efficiency by favoring CH_4 production [11]. Thus, great inclusions of concentrate feed in the diet can improve the efficiency of microbial utilization and NH_3 utilization in the rumen [12]. The inclusion of concentrate can also increase energy efficiency by reducing methanogenesis, as the metabolism of the amylolytic bacteria favors the conversion of pyruvate to propionic acid, which would improve efficiency due to the fact that there is no loss of CO_2, while in the fibrolytic bacteria, the metabolism favors the conversion of pyruvate to acetic acid would result in a CO_2 loss, which is a loss of energy [13,14].

Therefore, we hypothesize that fermentation parameters and digestion can be changed because of the associative effect among forages with different chemical compositions and their interaction with concentrate. This study aimed to investigate the effect of forage sources and forage-to-concentrate ratios on digestibility, energy concentration, fermentation parameters, and in vitro estimate of methane.

2. Materials and Methods

2.1. Location

The experiment was conducted between May and September 2023 in the municipality of Campos dos Goytacazes, RJ, Brazil (21°45′45″ S, 41°17′06″ W, and 8 m a.s.l.). The climate of northern Rio de Janeiro is Aw, a humid tropical climate with rainy summers and dry winters according to the classification of Köppen-Geiger, with an annual rainfall of 1020 mm.

The experiment was conducted following the approval of the Ethics Committee on Animal Use of the Universidade Estadual do Norte Fluminense (UENF)—Darcy Ribeiro (no. 419/2017 protocol).

2.2. Experimental Design and Substrates

Pineapple crop waste silage, corn silage, and Tifton 85 hay were used as forage sources. Tifton 85 (*Cynodon* sp. cv. Tifton 85) was harvested at 60 days, while the corn (*Zea mays*) used for silage production was the UENF 506-11 variety. Both Tifton 85 and corn were sourced from the UENF forage sector. Pineapple crop waste (*Ananas comosus*) was purchased from local producers, and the variety used was 'Pérola'. These materials were chosen based on their chemical variation, firstly, dry matter (DM). Corn silage contains approximately 30% DM, so we consider it as a baseline feed. Silage from pineapple crop residue is close to 20% DM, and Tifton 85 hay is around 80% DM. The experiment was carried out in a completely randomized design using a 3 × 6 factorial arrangement with three forage sources (pineapple crop waste silage, corn silage, and Tifton hay) and concentrate feed (binary mixture) in 6 combinations, using triplicates for each ratio. The adjusted combinations for each forage (as fed) were as follows: 100% forage + 0% concentrate; 90% forage + 10% concentrate; 80% forage + 20% concentrate; 50% forage + 50% concentrate; 20% forage + 80% concentrate; and 10% forage + 90% concentrate. In brief, 100-CS (100% corn silage), 90CS-10C (90% corn silage + 10% concentrate), 80CS-20C (80% corn silage + 20% concentrate), and so forth.

The concentrate feed was 61.25% ground corn and 38.75% soybean meal for all treatments. Table 1 shows the chemical composition of the feedstuffs for the in vitro measurements.

Table 1. Chemical composition of the forages and their combinations used in the study.

Substrate	DM	Ash	CP	CF	NDF	ADF	LIG	NFC	OM
Corn silage (CS)									
100-CS	301.7	44.81	79.18	14.76	411.59	250.21	28.93	449.66	256.89
90CS-10C	365.39	44.77	95.22	33.41	378.64	230.09	26.46	447.96	320.62
80CS-20C	429.08	42.33	107.15	21.72	360.57	223.33	25.35	468.24	386.75
50CS-50C	620.14	35.22	160.36	23.41	255.36	156.8	18.82	525.65	584.92
20CS-80C	811.2	32.42	226.08	31.76	154.77	96.74	16.61	554.97	778.78
10CS-90C	874.89	33.99	222.68	28.32	106.86	66.77	6.44	608.14	840.9
SEM	57.847	1.338	15.767	2.096	30.446	18.2	1.964	15.728	84.394
Tifton hay (TH)									
100-TH	867.4	66.59	48.01	15.09	777.85	417.55	52.05	92.45	800.81
90TH-10C	874.52	62.66	55.44	13.41	730.69	379.6	50.72	137.79	811.86
80TH-20C	881.63	57.68	85.63	15.43	634.96	334.15	41.38	206.3	823.95
50TH-50C	902.99	50.42	166.13	15.79	431.9	235.7	29.1	335.76	852.57
20TH-80C	924.34	37.16	202.15	28.88	231.16	130.54	14.33	500.65	887.18
10TH-90C	931.46	36.59	224.27	25.59	155.92	86.16	8.35	557.63	894.87
SEM	6.465	3.319	19	1.708	64.022	32.275	4.403	47.333	13.472
Pineapple silage (PS) [1]									
100-PS	191.45	62.63	89.01	45.65	595.13	381.01	56.56	207.57	128.82
90PS-10C	266.17	56.07	105.2	33.05	502.25	334.11	50.02	303.43	210.10
80PS-20C	340.88	50.42	112	47	471.17	310.64	43.68	319.4	290.46
50PS-50C	565.01	43.11	163.56	32.29	355.23	213.06	26.72	405.81	521.9
20PS-80C	789.15	38.42	214.33	33.4	190.34	109.79	14.2	523.51	750.73
10PS-90C	863.86	37.75	227.35	29	143.64	81.92	9.37	562.27	826.11
SEM	67.861	2.374	14.492	2.178	43.436	30.244	4.777	32.848	99.977
100-Concentrate (C)	938.58	31.71	242.34	30.82	95.01	50.37	5.3	600.11	906.87
Soybean meal	869.04	66.38	485.64	18.86	122.51	47.42	5.86	306.62	802.66
Ground corn	856.3	11.01	84.93	31.54	77.3	24.12	8.16	795.23	845.29
SEM	20.877	13.188	95.167	3.356	10.739	6.777	0.716	115.941	21.271

[1] Pineapple crop waste silage; SEM = standard error of mean; DM = dry matter; CP = crude protein; CF = crude fat; NDF = neutral detergent fiber; ADF = acid detergent fiber; LIG = lignin; NFC = non-fibrous carbohydrates; and OM = organic matter, all expressed as g/kg DM, except for DM, which is expressed as g/kg as-fed.

2.3. Chemical Composition

The samples were analyzed for DM (AOAC 967.03; [15]), crude fat (CF; AOAC 2003.06; [15]), and ash (AOAC 942.05; [15]) contents. The crude protein (CP) was determined according to AOAC 984.13 and AOAC 2001.11 [15]. The neutral detergent fiber was determined using sodium sulfite and two additions of a standardized heat-stable amylase solution, excluding ash, according to the AOAC 2002.04 (aNDFom) [16]. Non-fibrous carbohydrate (NFC) content was estimated as NFC $(g/kg) = 1000 - CP - CF - Ash - NDF$. Acid detergent fiber (ADF), with residual ash, and lignin (sa) were analyzed as described by [17].

2.4. In Vitro Fermentation

We used three sheep with permanent rumen cannulas and 45 kg body weight (standard deviation = 3.2 kg). The animals were housed in collective stalls with free access to feeders and drinkers. Before the ruminal fluid collection, the sheep were adapted to a diet with forage and concentrate to meet the maintenance requirements for 14 days. After this period, ruminal fluid collections began, always moments before the morning feeding, as recommended by [18].

The ruminal fluid (liquid and solid) was collected at several points of the liquid-solid interface of the ruminal environment for each incubation battery. The ruminal fluid collected from all 3 animals was pooled together. Then, the ruminal fluid was mixed in a blender for 30 s to homogenize the liquid and solid phases. The homogenized material was filtered through four layers of gauze in 2 L Erlenmeyer flasks connected to a hose with CO_2 and kept in a water bath at 39 °C. We used the buffer solution described by [19], composed

of $NaHCO_3$ (9.80 g/L), anhydrous Na_2HPO_4 (3.71 g/L), KCl (0.57 g/L), NaCl (0.47 g/L), $MgSO_4$ heptahydrate (0.12 g/L) and $CaCl_2$ dihydrate (0.05 g/L). Feed samples (200 mg, standard deviation = 10 mg) were air-dried and added to amber flasks (100 mL) with 50 mL of the previously prepared inoculum (1:4 ratio, ruminal fluid and buffer solution), according to [20]. The pH adjustment was performed by bubbling CO_2 until the buffer pH reached 6.8. The free space in the flasks was immediately saturated with CO_2, and the flasks were sealed and taken to a water bath at 39 °C. Incubations were conducted in two consecutive batches, each with triplicates of samples.

2.5. In Vitro Digestibility, Metabolizable Energy (ME), and Net Energy (NE)

The determination of in vitro digestibility focused on one stage of digestion in ruminal fluid, omitting the stage with pepsin, as recommended by [20]. The flasks were withdrawn from the water bath after 48 h of incubation. Then, the incubated samples were immediately filtered on quantitative filter paper (55 L/s m^2 air permeability) and washed with hot distilled water (above 90 °C). Later, the samples were dried at 55 °C for 24 h, followed by 105 °C for 16 h. The resulting material was weighed, obtaining the apparent undigested residue of dry matter (DM). We used the methodology described by [16] to evaluate the in vitro digestibility of NDF.

The digestibility (D) of DM and NDF was calculated according to Equation (1):

$$D = (M - [R - B] / M) \times 1000 \quad (1)$$

where M = mass (g) of incubated DM or NDF; R = DM or NDF residue from incubation (g); B = DM or NDF residue from the 'blanks' (g).

Concomitant with in vitro digestibility, we measure gas production up to 48 h to use gas production at 24 h time to estimate metabolizable (ME) and net energy (NE). In brief, we measured pressure and volume at 0, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 30, 36, and 48 h after adding the ruminal inoculum. The cumulative gas production time profiles were obtained by a non-automated method. The estimate of metabolizable (ME) and net energy (NE) were calculated using the equations proposed by [21]:

$$ME, MJ/kgDM = 0.157 \times GP + 0.0084 \times CP + 0.022 \times CF - 0.0081 \times Ash + 1.06 \quad (2)$$

$$NE, MJ/kgDM = 0.115 \times GP + 0.0054 \times CP + 0.014 \times CF - 0.0054 \times Ash + 0.36 \quad (3)$$

where GP is the net gas production in 24 h (mL/200 mg DM).

2.6. Fermentation Parameters

The pH, redox potential, and concentration of ammoniacal nitrogen (NH_3-N) were measured in five incubation times (3, 6, 12, 24, and 48 h). Three sample flasks were withdrawn from the water bath at each incubation time, totaling 15 flasks. The content of each flask was filtered in a triple layer of gauze in Falcon tubes, so pH and redox potential were measured with a digital potentiometer (MPA-210, Tecnopon, Piracicaba, SP, Brazil). This device uses a specific ORP electrode to measure redox potential, which works by recording the difference in electrical potential between the reference electrode and the measuring electrode (E0). The C is the potential of the reference electrode used relative to the standard hydrogen electrode, i.e., +199 mV at 39 °C. Thus, the equation used was Eh = E0 + C. This potential is expressed in millivolts (mV). After measuring pH and redox potential, an aliquot was taken from each tube. The aliquot (10 mL) was used to determine NH_3-N concentration, with 1.0 mL of H_2SO_4 solution (500 mL/L) added to each tube, and they were refrigerated (4 °C) for further analysis. The concentration of NH_3-N in the ruminal medium was determined by a distillation system with potassium hydroxide (2.0 N) without acid digestion, according to [22].

A second aliquot was taken only at 24 h to determine the concentrations of volatile fatty acids (acidic, propionic, and butyric acid). A solution of metaphosphoric acid 25%

(w/v) was added to the aliquot and frozen at −18 °C for further analysis. The VFA was determined using High-Performance Liquid Chromatography (HPLC; YL9100 HPLC system [Young Lin]), equipped with a Rezex RCM—Monosaccharide Ca^{+2} (8%) column and dimension of 300 × 7.8 mm. Ultra-pure water was used as a mobile phase with a 0.7 mL/min flow, the column temperature was 60 °C, and a refractive index detector was used. Previously, a calibration curve was performed with a linearity interval of the analyzed compounds between 0.5 to 1 g/L for butyric and acetic acid and 1 to 2 g/L for propionic acid.

2.7. Stoichiometric Calculations

The theoretical gas production (CO_2 and CH_4) was estimated based on the stoichiometric balance using the VFA measured after 24 h of fermentation, according to [13]. It was assumed that glucose equivalents were fermented for the production of acetic (HAc), propionic (HPr), and butyric (HBu) acid, as well as CO_2 and CH_4 gases. From this, we used the following stoichiometric Equations:

$$CO_2 = HAc/2 + Hpr/4 + 3HBu/2 \quad (4)$$

$$CH_4 = HAc + 2HBu - CO_2 \quad (5)$$

2.8. Statistical Analysis

Data regarding in vitro digestibility of DM and NDF, gas production in 24 h, metabolizable and net energy, organic acids, CO_2, and CH_4 were compared by the Tukey test with a significance level of 0.05 using the MIXED package of the SAS software (SAS OnDemand for Academics).

The following statistical model was used:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + e_{ijk} \quad (6)$$

In which Y_{ijk} is the observed value for the variable under study referring to the k-th replicate of the combination of the i-th level of the α factor with the j-th level of the β factor; μ is the mean of all experimental units for the variable under study; α_i is the effect of forage source with i = 1, 2, 3; β_j is the effect of the forage-to-concentrate ratio with j = 1, 2, 3, 4, 5, 6; $\alpha\beta_{ij}$ is the interaction between forage sources and forage-to-concentrate ratio; e_{ijk} is the error associated with the observation Y_{ijk}.

Data on redox potential, pH, and $N\text{-}NH_3$ were analyzed as repeated measures over time through regression analysis with a significance level of 0.05 using the MIXED package from SAS software (SAS OnDemand for Academics).

The following statistical model was used:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \tau_k + \beta\tau_{jk} + e_{ijkl} \quad (7)$$

where Y_{ijkl} is the observed value for the variable under study referring to the l-th replicate of the combination of the i-th level of the α factor with the j-th level of the β factor in the k-th hour; μ is the mean of all experimental units for the variable under study; α_i is the forage source effect with i = 1, 2, 3; β_j is the effect of the forage-to-concentrate ratio with j = 1, 2, 3, 4, 5, 6; τ_k is the random effect of the evaluation hours with k = 3, 6, 12, 24, 48; $\beta\tau_{jk}$ is the interaction between the forage-to-concentrate ratio and the hours of evaluation; e_{ijkl} is the error associated with the observation Y_{ijkl}.

3. Results

We did not observe any interaction effect ($p > 0.05$) for in vitro digestibility, ME, and NE estimates between the forage sources and forage-to-concentrate ratios (Table 2). The in vitro dry matter digestibility (IVDMD) did not differ between forages ($p = 0.107$); however, it was affected ($p < 0.001$) by the inclusion of concentrate. The IVDMD increase was already

expected with the concentrate inclusion, but the increments in IVDMD varied between the forages. For example, we compared the ratios of 100:00 and 90:10 and observed an increase of 21.78% for corn silage, 14.87% for Tifton hay, and 19.56% for the silage of pineapple crop waste. Nevertheless, the increase was lower in the ratios of 90:10 and 80:20, 15.65% for corn silage, 6.75% for Tifton hay, and 3.5% for the silage of pineapple crop waste (Table 2). The IVNDFD of the corn silage was higher (p = 0.002) than the other forages (Table 2). The IVNDFD decreased (p = 0.001) with the inclusion of concentrate. It was also affected by the forage source, but this fact was only observed in corn silage up to the 50:50 ratio. In Tifton hay, this fact occurred from the 80:20 ratio, and this behavior was not observed in the silage of pineapple crop waste (Table 2). The GP was not affected by the forage sources (p = 0.080), but there was a difference (p < 0.001) for the concentrate. The inclusion of concentrate decreased the GP in corn silage by 29.42% compared to 100:00 and 10:90 ratios. This behavior was contrary to Tifton hay and silage of pineapple crop waste. The GP increased with the inclusion of concentrate by 34.08 and 33.86%, respectively, for the same comparison previously mentioned (Table 2). ME and NE were affected by forage sources (p < 0.001) and concentrate ratios (p < 0.001) (Table 2). Corn silage showed higher concentrations of ME and NE than Tifton hay. However, they did not differ from the silage of pineapple crop waste. The inclusion of concentrate increased the concentrations of ME and NE regardless of the forage source (Table 2).

Table 2. In vitro digestibility and estimating of energy of corn silage, Tifton hay, and pineapple crop waste silage incubated as single forages and in combinations with concentrate.

Substrates	IVDMD (g/kg)	IVNDFD (g/kg)	GP (mL/g DM)	ME (MJ/kg DM)	NE (MJ/kg DM)
Corn Silage (CS)					
100-CS	445.29 ^{e}	626.24 ^{Aa}	20.65 ^{c}	5.36 ^{Ac}	3.45 ^{Ac}
90CS-10C	569.29 ^{d}	546.50 ^{Ab}	23.63 ^{b}	5.47 ^{Ac}	3.48 ^{Ac}
80CS-20C	674.98 ^{c}	469.87 ^{Ac}	23.43 ^{b}	5.81 ^{Abc}	3.73 ^{Abc}
50CS-50C	747.84 ^{b}	368.47 ^{Ad}	26.53 ^{ab}	5.87 ^{Ab}	3.69 ^{Ab}
20CS-80C	854.50 ^{a}	362.02 ^{Ad}	27.32 ^{a}	6.93 ^{Ab}	4.51 ^{Ab}
10CS-90C	892.23 ^{a}	341.55 ^{Ad}	29.26 ^{a}	7.99 ^{Aa}	5.21 ^{Aa}
Tifton hay (TH)					
100-TH	514.72 ^{d}	443.45 ^{Ba}	17.04 ^{Ac}	2.95 ^{Bd}	1.61 ^{Bc}
90TH-10C	604.67 ^{cd}	435.01 ^{Ba}	18.18 ^{Ac}	3.93 ^{Bd}	2.82 ^{Bb}
80TH-20C	648.45 ^{c}	314.15 ^{Bb}	21.33 ^{Ab}	4.51 ^{Bc}	2.43 ^{Bb}
50TH-50C	725.99 ^{bc}	280.93 ^{Bb}	22.45 ^{Ab}	4.84 ^{Bc}	3.09 ^{Bb}
20TH-80C	853.35 ^{ab}	274.82 ^{Bb}	23.53 ^{Aab}	6.56 ^{Bb}	4.19 ^{Ba}
10TH-90C	878.42 ^{a}	267.68 ^{Bb}	25.85 ^{Aa}	7.15 ^{Ba}	4.63 ^{Ba}
Pineapple crop waste silage (PS)					
100-PS	565.77 ^{d}	411.87 ^{Ba}	14.61 ^{Ac}	4.560 ^{ABb}	2.82 ^{ABc}
90PS-10C	703.40 ^{c}	387.79 ^{Ba}	17.18 ^{Abc}	4.91 ^{ABb}	4.33 ^{ABa}
80PS-20C	728.99 ^{c}	357.49 ^{Bab}	17.86 ^{Abc}	5.50 ^{ABb}	3.83 ^{ABab}
50PS-50C	772.48 ^{bc}	341.47 ^{Bb}	19.16 ^{Ab}	5.80 ^{ABab}	3.46 ^{ABb}
20PS-80C	790.43 ^{bc}	296.63 ^{Bc}	18.33 ^{Ab}	6.09 ^{ABa}	3.67 ^{ABb}
10PS-90C	868.04 ^{a}	365.10 ^{Bb}	22.09 ^{Aa}	6.77 ^{ABa}	3.06 ^{ABbc}
100-Concentrate (C)	931.10 ^{a}	633.73 ^{a}	27.90 ^{a}	7.89 ^{b}	5.14 ^{b}
Soybean meal	930.03 ^{a}	651.75 ^{a}	28.59 ^{a}	9.51 ^{a}	6.18 ^{a}
Ground Corn	823.19 ^{b}	612.64 ^{a}	26.95 ^{a}	6.61 ^{c}	4.30 ^{c}
SEM	14.959	13.422	0.616	0.137	0.095
p-value Forage	0.107	0.002	0.080	<0.001	<0.001
p-value Forage-to-concentrate ratio	<0.001	0.001	<0.001	<0.001	<0.001
p-value Interaction	0.668	0.267	0.866	0.103	0.116

IVDMD = in vitro dry matter digestibility; IVNDFD = in vitro neutral detergent fiber digestibility; GP = volume of gas produced in 24 h; ME = metabolizable energy; NE = net energy; DM = dry matter. SEM = standard error of the mean. Means followed by the different letters capital letters (forage) and letters lowercase (forage to concentrate ratio) differ significantly by the Tukey test (p < 0.05).

Regarding VFA and gases (CO_2 and CH_4), no interaction effect was observed between forage sources and concentrate ratios ($p > 0.05$) (Table 3). As for VFA, we observed effects for forage sources ($p < 0.05$) and concentrate ratios ($p < 0.05$). The HAc showed a higher concentration in Tifton hay ($p < 0.001$) than in the silages of corn and pineapple crop waste. In contrast, HPr ($p = 0.011$) and HBu ($p = 0.007$) had higher concentrations in corn silage than in Tifton hay and pineapple crop waste silage (Table 3). The inclusion of concentrate decreased the HAc in the forage sources. When we compared the proportions of 100:00 and 10:90, this decrease was 57.65, 54.15, and 72.96% for corn silage, Tifton hay, and pineapple crop waste silage, respectively (Table 3). The availability of potentially degradable carbohydrates can explain this result. However, this behavior was contrary for HPr and HBu, with an increase in their concentrations with the inclusion of concentrate, being 63.87, 94.87, and 68.18% for corn silage, Tifton hay, and pineapple crop waste silage, respectively (Table 3). The gases (CO_2 and CH_4) showed the same behavior as HAc, both for the forage sources ($p < 0.05$) and the concentrate inclusion ($p < 0.05$) (Table 3).

Table 3. Volatile fatty acids and estimates of methane of corn silage, Tifton hay, and pineapple crop waste silage were incubated as single forage and in combinations with concentrate.

Substrates	HAc (µmol/mL)	HPr (µmol/mL)	Hbu (µmol/mL)	CO_2 (mmol)	CH_4 (mmol)
Corn Silage (CS)					
100-CS	9.80 ^{Ba}	1.08 ^{ABb}	0.09 ^{Ab}	5.30 ^{Ba}	4.67 ^{Ba}
90CS-10C	7.76 ^{Bab}	1.64 ^{ABab}	0.21 ^{Aab}	4.60 ^{Bab}	3.57 ^{Bab}
80CS-20C	6.77 ^{Bab}	1.98 ^{ABab}	0.41 ^{Aab}	4.50 ^{Bab}	3.10 ^{Bab}
50CS-50C	5.62 ^{Bab}	2.34 ^{ABab}	0.50 ^{Aab}	4.14 ^{Bab}	2.47 ^{Bb}
20CS-80C	4.21 ^{Bb}	2.78 ^{ABab}	0.58 ^{Aab}	3.67 ^{Bb}	1.70 ^{Bb}
10CS-90C	4.15 ^{Bb}	2.99 ^{ABa}	0.76 ^{Aa}	3.96 ^{Bb}	1.70 ^{Bb}
Tifton hay (TH)					
100-TH	16.01 ^{Aa}	0.38 ^{Ac}	0.13 ^{Bb}	8.30 ^{Aa}	7.98 ^{Aa}
90TH-10C	14.56 ^{Aab}	1.49 ^{Ac}	0.12 ^{Bb}	7.84 ^{Aab}	6.97 ^{Aab}
80TH-20C	13.29 ^{Aab}	2.09 ^{Abc}	0.16 ^{Bb}	7.41 ^{Aab}	6.20 ^{Aab}
50TH-50C	8.14 ^{Ab}	4.61 ^{Aab}	0.24 ^{Bb}	5.59 ^{Ab}	3.04 ^{Ab}
20TH-80C	7.47 ^{Ab}	6.14 ^{Aab}	0.38 ^{Bb}	5.84 ^{Ab}	2.39 ^{Ab}
10TH-90C	7.34 ^{Ab}	7.42 ^{Aa}	0.77 ^{Ba}	6.68 ^{Ab}	2.20 ^{Ab}
Pineapple crop waste silage (PS)					
100-PS	8.47 ^{Ba}	1.05 ^{Bb}	0.001 ^{Cc}	4.50 ^{Ba}	3.97 ^{Ba}
90PS-10C	5.92 ^{Bab}	1.08 ^{Bb}	0.06 ^{Cb}	3.32 ^{Bb}	2.72 ^{Bb}
80PS-20C	5.36 ^{Bab}	1.50 ^{Bb}	0.07 ^{Cb}	3.17 ^{Bb}	2.34 ^{Bb}
50PS-50C	4.69 ^{Bab}	1.55 ^{Bb}	0.12 ^{Cb}	2.91 ^{Bb}	2.02 ^{Bb}
20PS-80C	3.35 ^{Bb}	2.40 ^{Bab}	0.15 ^{Cb}	2.49 ^{Bb}	1.10 ^{Bb}
10PS-90C	2.29 ^{Bb}	3.30 ^{Ba}	0.43 ^{Ca}	2.62 ^{Bb}	0.53 ^{Bc}
100-Concentrate (C)	5.77	2.99	0.24	3.99	2.26
Soybean meal	4.87	2.02	0.16	3.18	2.01
Ground Corn	2.30	1.26	0.10	1.62	0.89
SEM	0.369	0.164	0.029	0.250	0.164
p-value Forage	<0.001	0.011	0.007	<0.001	<0.001
p-value Forage-to-concentrate ratio	0.011	0.039	0.021	0.09	0.005
p-value Interaction	0.326	0.288	0.43	0.08	0.157

HAc = acetic acid; HPr = propionic acid; Hbu = butyric acid; CO_2 = carbon dioxide; CH_4 = methane. SEM = standard error of the mean. Means followed by the different letters capital letters (forage) and letters lowercase (forage to concentrate ratio) differ significantly by the Tukey test ($p < 0.05$).

The pH was affected by the inclusion of concentrate ($p < 0.001$), incubation time ($p < 0.001$), and the interaction between them ($p < 0.001$) only for corn silage. There was a sharp drop from 12 h post-incubation in the 10:90 ratio (Figure 1A). However, silage from pineapple crop residues and Tifton hay did not alter the pH ($p > 0.05$) with the inclusion of concentrate; only a time effect was observed ($p < 0.001$) (Figure 1B and Figure 1C,

respectively). As for the redox potential, we observed effects for the inclusion of concentrate ($p < 0.05$), incubation time ($p < 0.05$), and the interaction between them ($p < 0.05$) on corn silage, pineapple crop waste silage, and Tifton hay (Figure 1D, Figure 1E, and Figure 1F, respectively). However, the data behavior varied according to the forage source. We analyzed the concentrations of NH_3-N and noticed effects for the inclusion of concentrate ($p < 0.001$), incubation time ($p < 0.001$), and the interaction between them ($p < 0.001$) on the silages of corn and pineapple crop waste (Figure 1G,H). Conversely, Tifton hay was influenced only by incubation time ($p < 0.001$) (Figure 1I).

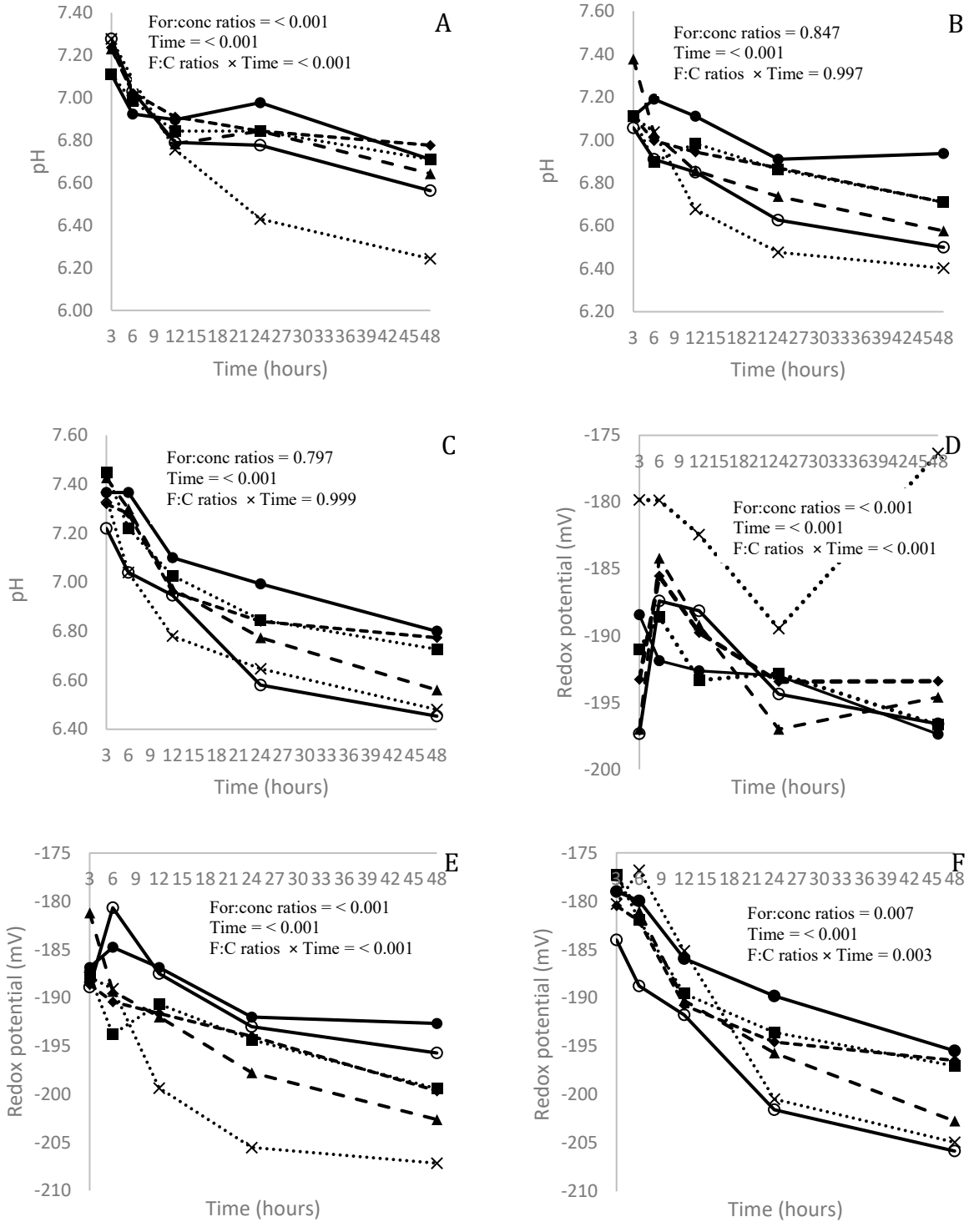

Figure 1. *Cont.*

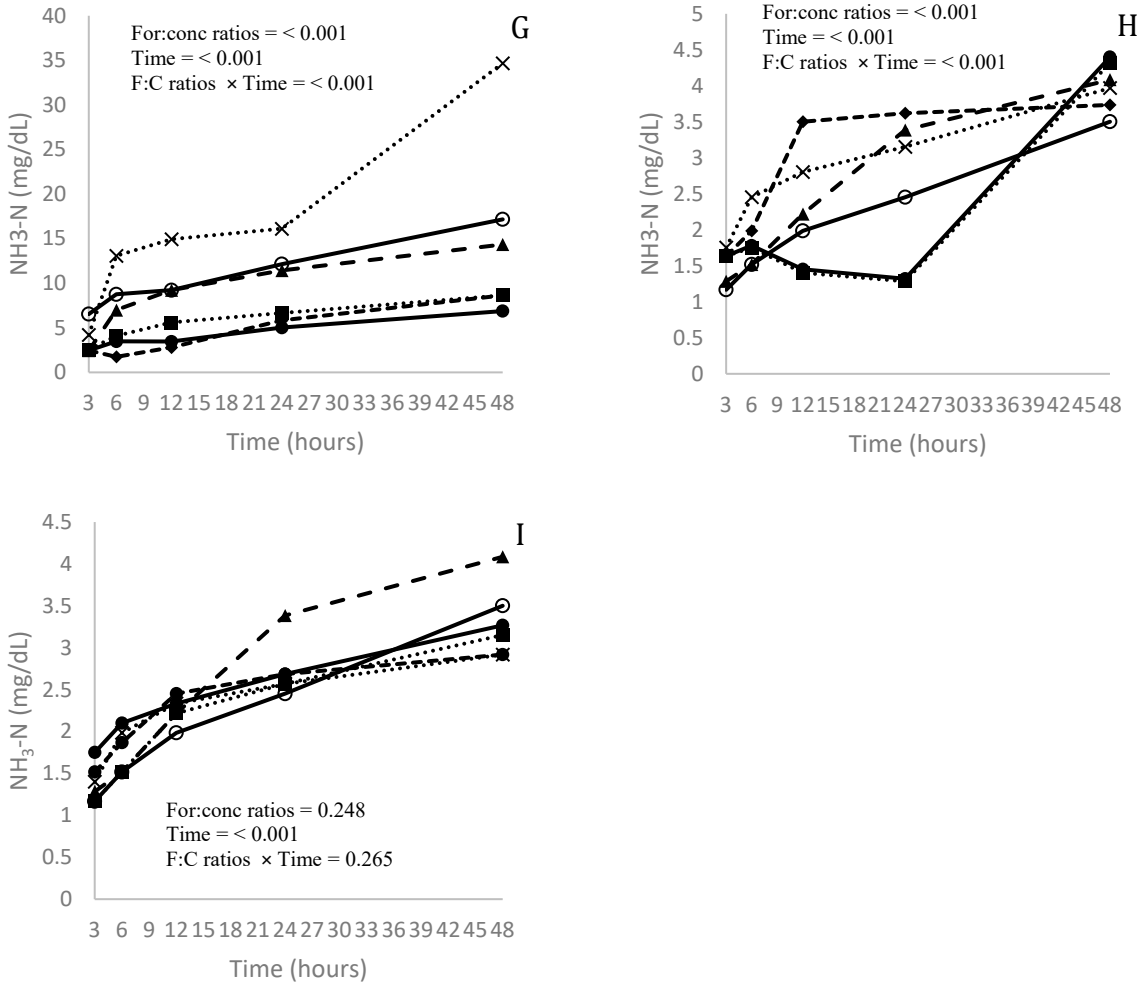

Figure 1. Fermentative profile of corn silage, Tifton hay, and pineapple crop waste silage incubated as single forages and in combinations with concentrate. On panel (**A**) corn silage; (**B**) pineapple crop waste silage; (**C**) Tifton hay; (**D**) corn silage; (**E**) pineapple crop waste silage; (**F**) Tifton hay; (**G**) corn silage; (**H**) pineapple crop waste silage; (**I**) Tifton hay. Forage-to-concentrate ratios: (●) 100% forage; (■) 90:10; (♦) 80:20; (▲) 50:50; (○) 20:80; (×) 10:90.

4. Discussion

Associative effects occur when the apparent digestibility of a feed mixture is different from the weighted sum of the digestibilities of the feeds separately [23,24]. In this sense, the evaluations of the present study pointed out associative effects between forages (corn silage, Tifton hay, and pineapple crop waste silage) and forages and their ratios with concentrate feed on the evaluated parameters.

It was evident that there was an associative effect between forage and the inclusion of concentrate on DM digestibility (Table 2). The inclusion of concentrate increased ($p < 0.001$) DM digestibility regardless of the forage source. For [25], increasing the cell wall digestion rate is essential to improve animal productivity in forage-based diets. However, when the availability of fermentable carbohydrates in the rumen increases, the digestibility of fiber becomes less important for the contribution of digestible energy. Nevertheless, the quality of the forage may influence the intensity of the associative effect that may occur with the addition of a concentrate supplement [26,27]. This fact was observed in our results on NDF digestibility (Table 2). Corn silage showed higher ($p = 0.002$) digestibility of NDF than Tifton hay and pineapple crop waste silage. The inclusion of concentrate decreased ($p = 0.001$) NDF digestibility. Some theories can corroborate this finding, and the first would be the competition between amylolytic and fibrolytic bacteria for essential nutrients, mainly nitrogen. When the supply of starch increases in the rumen, the amylolytic bacteria

benefit more than fibrolytic bacteria because starch has a faster fermentation rate than fiber [28]. The second theory may be related to increased levels of concentrate contributing to dropping ruminal pH (starch fermentation), reducing fibrolytic bacterial activity, and decreasing fiber digestion [29]. The competition for essential nutrients between amylolytic and fibrolytic bacteria may explain why the inhibition effect is more significant for low-quality forage over high-quality forage [30]. This fact can be observed for corn silage and Tifton hay (Table 2). The third theory may be related to the decreased digestion rate and incubation time, or both [31]. When analyzing the energy concentrations, we observed that corn silage presented 20.1 and 9.95% more ($p < 0.001$) ME concentration and 22 and 11.75% ($p < 0.001$) NE concentration than Tifton hay and the silage of the pineapple crop waste, respectively (Table 2). That was probably because of the great availability of soluble carbohydrates (starch) in corn silage. The inclusion of concentrate increased ($p < 0.001$) the ME and NE concentrations. This fact is linked to the increased supply of fermentable carbohydrates (starch, pectin, etc.) due to the disadvantage in producing CH_4.

For [32,33], VFA concentration in the rumen is affected by the forage source, and this fact can be observed in the present study (Table 3). The VFA concentration varied between forage sources, e.g., when analyzing forages without the inclusion of concentrate (100-CS, 100-TH, and 100-PS), acetic acid showed a higher concentration in Tifton hay than in the silages. The propionic acid was higher in the silages than in Tifton hay (Table 3). The availability of carbohydrates can explain this result. Tifton hay has a high amount of potentially degradable fractions, represented by neutral detergent fiber (CB3). In comparison, the silages have a high proportion of soluble carbohydrate fraction (CB1 [starch] and CB2 [pectin], respectively). However, regarding the inclusion of concentrate, we observed that high proportions of forage increased the concentration of acetic acid due to great NDF digestibility (Table 2). Ruminal microorganisms metabolize carbohydrates by converting them mainly into glucose or glucose 1-phosphate, which is subsequently oxidized to pyruvate through the Embden–Meyerhof pathway. Pyruvate can undergo oxidative decarboxylation to acetyl-CoA, which can be converted into acetic acid [34,35]. According to [23,36], the production of acetic acid releases [H^+] that *Methanogenic archaea* can use to reduce CO_2 to CH_4. These arguments corroborate the results of this study (Table 3). Conversely, the inclusion of concentrate increased propionic acid concentrations (Table 3). Propionic acid production is through a competitive pathway of H_2 utilization in the rumen, reducing substrate availability for methanogenesis [13,14,35,36]. Furthermore, increasing the concentrate can alter the fermenting microbial community by promoting the growth of amylolytic bacteria (which ferment starch) and reducing the population of cellulolytic microorganisms (which degrade fiber). Since cellulolytic microorganisms are more closely associated with methane production, their reduction leads to decreased methanogenic activity [6]. Propionic and Butyric acids increased in their concentrations with the inclusion of concentrate, which is the primary propionic acid precursor of glucose for ruminants (Table 3). For [37], when the ratio of acetic and propionic acid (HAc:HPr) decreases, the production of CH_4 reduces, and energy retention may increase. When computing the HAc:HPr ratio, we observed a decrease by including concentrate in the forage sources. For example, we used corn silage; 100-CS was 9.07, and 10CS-90C was 1.38. So, the production of CH_4 was reduced by 63.59%, and ME retention was increased by 32.92%. According to [23], the HAc:HPr ratio in the diet is explained by the metabolic characteristics of fiber and starch digestion, but this explanation is not entirely convincing. Some ruminal bacteria that digest starch can produce propionic acid, and many bacteria that digest fiber produce large amounts of succinate, an intermediate that is eventually converted to propionic acid. On the other hand, pectin fermentation promotes a higher production of acetate and butyrate, which do not use as much hydrogen. This means that more hydrogen is available to methanogenic microorganisms, increasing methane production [35].

The propionic acid concentration increased as concentrate inclusion increased, and acetic acid decreased (Table 3). According to [38], VFA directly affects ruminal pH. Thus,

when analyzing Figure 1A (corn silage), we observed a pH drop ($p < 0.001$) for the 10CS:90C ratio. This can be explained by the acidic excess, which is mainly from soluble carbohydrates. When VFA production exceeds the absorption rate through the ruminal epithelium, the acids may accumulate in the rumen, dropping the pH. It is caused by the change in fermentation parameters resulting in the production of lactic acid, which is considerably stronger (pka = 3.86) than VFA (pka = 4.7 to 4.9), dropping the pH even more [38,39]. However, we did not observe any variation in pH ($p > 0.05$) for the silage of pineapple crop waste and Tifton hay (Figure 1B,C). Another important factor of fermentation is the redox potential (Eh). It is linked to oxygen reduction reactions essential for maintaining all living microorganisms [40]. Despite this, Eh is rarely measured compared with pH. In the present study, we observed an increase in Eh with the advance of incubation hours regardless of forage source and concentrate feed (Figure 1D–F), except for the 10CS:90C ratio. After 24 h of incubation, there was a reduction in Eh, probably due to the increased activity of amylolytic bacteria. The results of our study are within the range of variation (−150 to −260 mV, average for sheep) mentioned by [41,42]. We observed that the inclusion of concentrate did not decrease the Eh. Conversely, Eh values increased regardless of the forage source (Figure 1D–F), except for the 10CS:90C ratio that decreased Eh, which is directly linked to pH. According to [42,43], the reduction in ruminal Eh is associated with high pH and vice versa. When evaluating the pH, we did not observe any value below 6.0 because of the buffer capacity of sodium bicarbonate in the buffer solution, as [44]. Regarding N-NH_3, the inclusion of concentrate increased ($p < 0.001$) N-NH_3 concentration in corn silage (Figure 1G), and the 10CS:90C ratio had a sharp increase. The addition of readily available carbohydrates (starch) in corn silage can produce varying effects on ruminal fermentation. The inclusion of starch sources in a diet can increase DM digestibility. However, it can negatively affect ruminal fermentation, such as a decrease in the total VFA concentration and an increase in the N-NH_3 concentration [45], which was also observed in our study. The N-NH_3 concentration is inversely related to carbohydrate availability [46,47]. However, for [47], fibrous carbohydrate fermenting bacteria exclusively use ammonia as a nitrogen source (N). So, the likely increased growth of these bacteria may have contributed to the decrease in ammonia concentration in the silage of pineapple crop waste and Tifton hay (Figure 1H,I).

5. Conclusions

Corn silage presents higher IVNDFD and about 22% and 11.75% more NE (MJ/kg DM) than Tifton hay and pineapple crop waste silage, respectively. Regarding VFA, Tifton hay has a higher concentration of HAc, whereas corn silage has higher concentrations of HPr and HBu than the others. The production of CH_4 followed the same behavior as the VFA. Different forage sources change the fermentation parameters.

The inclusion of concentrate increases the IVDMD and energy concentration, resulting in a more significant energy contribution to the system. The reduction in HAc concentrations and increase in HPr reduces methanogenesis. The inclusion of concentrate affects fermentation parameters like the forage sources. Thus, the different forage sources and the inclusion of concentrate changed the digestion and fermentation parameters. The amount and availability of soluble carbohydrates affect the final energy production of the system.

Author Contributions: Conceptualization: D.F.B., A.M.F. and T.S.O. Formal analysis: T.S.O. Investigation: D.F.B. and T.S.O. Methodology: M.G.C., I.N.S., J.R.M.J., E.J.L.M.F. and D.F.B. Project administration: T.S.O. Resources: T.S.O. Supervision: T.S.O. and A.M.F. Writing—original draft: D.F.B., E.S.A. and T.S.O. Writing—review and editing: A.M.F. and T.S.O. All authors have read and agreed to the published version of the manuscript.

Funding: Rio de Janeiro—FAPERJ (RJ, Brazil), grant numbers E-26/010.100974/2018 and E-26/010.00-2458/2019 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Raw experimental data are available upon request to the corresponding author.

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

References

1. Matthews, C.; Crispie, F.; Lewis, E.; Reid, M.; O'Tolle, P.W.; Cotter, P.D. The rumen microbiome: A crucial consideration when optimising milk and meat production and nitrogen utilisation efficiency. *Gut Microbes* **2019**, *10*, 115–132. [CrossRef] [PubMed]
2. Kingston-Smith, A.H.; Marshall, A.H.; Moorby, J.M. Breeding for genetic improvement of forage plants in relation to increasing animal production with reduced environmental footprint. *Animal* **2013**, *7*, 79–88. [CrossRef] [PubMed]
3. Russell, J.B.; Mantovani, H.C. The bacteriocins of ruminal bactéria and their potential as an alternative to antibiotics. *J. Mol. Microbiol. Biotechnol.* **2002**, *4*, 347–355.
4. Johnson, K.; Johnson, D. Methane emissions from cattle. *J. Anim. Sci.* **1995**, *73*, 2483–2492. [CrossRef] [PubMed]
5. Tseten, T.; Sanjorjo, R.A.; Kwon, M.; Kim, S.W. Strategies to mitigate enteric methane emissions from ruminant animals. *J. Microbiol. Biotechnol.* **2022**, *28*, 269–277. [CrossRef] [PubMed]
6. Kataria, R.P. Use of feed additives for reducing greenhouse gas emissions from dairy farms. *Microbiol. Res.* **2015**, *6*, 6120. [CrossRef]
7. Devries, T.J.; Schwaiger, T.; Beauchemin, K.A.; Penner, G.B. Impact of severity of ruminal acidosis on feedsorting behaviour of beef cattle. *Anim. Prod. Sci.* **2014**, *54*, 1238–1242. [CrossRef]
8. Messana, J.D.; Carvalho, A.L.E.G.F.; Ribeiro, A.F.; Fiorentini, G.; Castagnino, P.S.; Granja-Salcedo, Y.T.; Pires, A.V.; Berchielli, T.T. Effects of different sources of forage in high-concentrate diets on fermentation parameters, ruminal biohydrogenation and microbiota in Nellore feedlot steers. *J. Agric. Sci.* **2016**, *154*, 928–941. [CrossRef]
9. Nagaraja, T.G.; Titgemeyer, E.C. Ruminal acidosis in beef cattle: The current microbiological and nutritional outlook. *J. Dairy Sci.* **2007**, *90*, E17–E38. [CrossRef]
10. Hino, T.; Russel, J.B. Effect of reducing equivalent disposal and NADH/NAD on deamination of amino acids by intact rumen microorganisms and their cell extracts. *Appl. Environ. Microbiol.* **1985**, *50*, 1368–1374. [CrossRef]
11. Yang, W.Z.; Beauchemin, K.A. Physically effective fiber: Method of determination and effects on chewing, ruminal acidosis, and digestion by dairy cows. *J. Dairy Sci.* **2006**, *89*, 2618–2633. [CrossRef] [PubMed]
12. Russell, J.B.; Sniffen, C.J.; Van Soest, P.J. Effect of carbohydrate limitation on degradation and utilization of casein by mixed rumen bacteria. *J. Dairy Sci.* **1983**, *66*, 763–775. [CrossRef]
13. Van Soest, P.J. *Nutritional Ecology of the Ruminant*, 2nd ed.; Cornell University Press: Ithaca, NY, USA, 1994.
14. Patra, A.; Park, T.; Kim, M.; Yu, Z. Rumen methanogens and mitigation of methane emission by anti-methanogenic compounds and substances. *J. Anim. Sci. Biotechnol.* **2017**, *8*, 13. [CrossRef] [PubMed]
15. AOAC—Association of Official Analytical Chemistry. *Official Methods of Analysis*, 21st ed.; AOAC International: Gaithersburg, MD, USA, 2019.
16. Mertens, D.R. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: Collaborative study. *J. AOAC Int.* **2002**, *517*, 1217–1240.
17. Möller, J. Gravimetric determination of acid detergent fiber and lignin in feed: Interlaboratory study. *J. AOAC Int.* **2009**, *92*, 74–90. [CrossRef]
18. Yáñez-Ruiz, D.R.; Bannink, A.; Dijkstra, J.; Kebreab, E.; Morgavi, D.P.; O'Kiely, P.; Reynolds, C.K.; Schwarm, A.; Shingfield, K.J.; Yu, Z.; et al. Design, implementation and interpretation of in vitro batch culture experiments to assess enteric methane mitigation in ruminants—A review. *Anim. Feed Sci. Technol.* **2016**, *216*, 1–18. [CrossRef]
19. McDougall, E.I. The composition and output of sheep's saliva. *Biochem. J.* **1948**, *43*, 99–109. [CrossRef]
20. Tilley, J.M.A.; Terry, R.A. A two-stage technique for the in vitro digestion of forage crops. *Grass Forage Sci.* **1963**, *18*, 104–111. [CrossRef]
21. Menke, K.H.; Steingass, H. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Anim. Res. Dev.* **1988**, *28*, 7–55.
22. Fenner, H. Method for determinning total volatile bases in rumen fluid by steam distillation. *J. Dairy Sci.* **1965**, *48*, 249–251. [CrossRef] [PubMed]
23. Hungate, R.E. Hydrogen as an intermediate in the rumen fermentation. *Arch. Mikrobiol.* **1967**, *59*, 158–164. [CrossRef] [PubMed]
24. Niderkorn, V.; Baumont, R. Associative effects between forages on feed intake and digestion in ruminants. *Animal* **2009**, *3*, 951–960. [CrossRef] [PubMed]
25. Getachew, G.; Robinson, P.H.; DePeters, E.J.; Taylor, S.J. Relationships between chemical composition, dry matter degradation and in vitro gas production of several ruminant feeds. *Anim. Feed Sci. Technol.* **2004**, *111*, 57–71. [CrossRef]
26. Mould, F.L.; Orskov, E.R.; Mann, S.O. Associative effects of mixed feeds. I. Effects of type and level of supplementation and the influence of the rumen pH on cellulolysis in vivo and dry matter digestion of various roughages. *Anim. Feed Sci. Technol.* **1983**, *10*, 15–30. [CrossRef]

27. Mould, F.L.; Orskov, E.R.; Gauld, S.A. Associative effects of mixed feeds. II. The effect of dietary addition of bicarbonate salts on the voluntary intake and digestibility of diets containing various proportions of hay and barley. *Anim. Feed Sci. Technol.* **1983**, *10*, 31–47. [CrossRef]
28. Dixon, R.M.; Stockdale, C.R. Associative effects between forages and grains: Consequences for feed utilisation. *Aust. J. Agric. Res.* **1999**, *50*, 757–774. [CrossRef]
29. El-Shazly, K.; Dehority, B.A.; Johnson, R.R. Effect of starch on the digestion of cellulose in vitro and in vivo by rumen microorganisms. *J. Anim. Sci.* **1961**, *20*, 268–273.
30. Burroughs, W.; Gall, L.S.; Gerlaugh, B.H.; Bethke, R.M. The influence of casein upon roughages digestion in cattle with rumen bacteriological studies. *J. Anim. Sci.* **1950**, *9*, 214–220. [CrossRef]
31. Nousiainen, J.; Rinne, M.; Huhtanen, P. A meta-analysis of feed digestion in dairy cows. 1. The effects of forage and concentrate factors on total diet digestibility. *J. Anim. Sci.* **2009**, *92*, 5019–5030. [CrossRef]
32. Alstrup, L.; Søegaard, K.; Weisbjerg, M.R. Effects of maturity and harvest season of grass-clover silage and of forage-to-concentrate ratio on milk production of dairy cows. *J. Dairy Sci.* **2016**, *99*, 328–340. [CrossRef]
33. Bharanidharan, R.; Arokiyaraj, S.; Kim, E.B.; Lee, C.H.; Woo, Y.W.; Na, Y.; Kim, K.H. Ruminal methane emissions, metabolic, and microbial profile of Holstein steers fed forage and concentrate, separately or as a total mixed ration. *PLoS ONE* **2018**, *13*, e0202446. [CrossRef] [PubMed]
34. Russell, J.B. *Rumen Microbiology and Its Role in Ruminant Nutrition*; Cornell University: Ithaca, NY, USA, 2002.
35. Ungerfeld, E.M. Shifts in metabolic hydrogen sinks in the methanogenesis-inhibited ruminal fermentation: A meta-analysis. *Front. Microbiol.* **2015**, *6*, 37. [CrossRef] [PubMed]
36. Janssen, P.H. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Anim. Feed Sci. Technol.* **2010**, *160*, 1–22. [CrossRef]
37. Wolin, M.J. A theoretical rumen fermentation balance. *J. Dairy Sci.* **1960**, *43*, 1452–1459. [CrossRef]
38. Penner, G.B.; Taniguchi, M.; Guan, L.L.; Beauchemin, K.A.; Oba, M. Effect of dietary forage to concentrate ratio on volatile fatty acid absorption and the expression of genes related to volatile fatty acid absorption and metabolism in ruminal tissue. *J. Dairy Sci.* **2009**, *92*, 2767–2781. [CrossRef]
39. Owens, F.N.; Secrist, D.S.; Hill, W.J.; Gill, D.R. Acidosis in cattle: A review. *J. Anim. Sci.* **1998**, *76*, 275–286. [CrossRef]
40. Husson, O. Redox potential (Eh) and pH as drivers of soil/plant/microorganism systems: A transdisciplinary overview pointing to integrative opportunities for agronomy. *Plant Soil* **2013**, *362*, 389–417. [CrossRef]
41. Barry, T.N.; Thompson, A.; Armstrong, D.G. Rumen fermentation studies on two contrasting diets. 1. Some characteristics of the in vivo fermentation, with special reference to the composition of the gas phase, oxidation/reduction state and volatile fatty acid proportions. *J. Agric. Sci.* **1977**, *89*, 183–195. [CrossRef]
42. Huang, Y.; Marden, J.P.; Julien, C.; Bayourthe, C. Redox potential: Na intrinsic parameter of the rumen enviroment. *J. Anim. Physiol. Anim. Nutr.* **2018**, *102*, 393–402. [CrossRef]
43. Marden, J.P.; Julien, C.; Monteils, V.; Auclair, E.; Moncoulon, R.; Bayourthe, C. How does live yeast differ from sodium bicarbonate to stabilize ruminal pH in high yielding dairy cows? *J. Dairy Sci.* **2008**, *91*, 3528–3535. [CrossRef]
44. Kohn, R.A.; Dunlap, T.F. Calculation of the buffering capacity of bicarbonate in the rumen and in vitro. *J. Anim. Sci.* **1998**, *76*, 1702–1709. [CrossRef] [PubMed]
45. Heldt, J.S.; Cochran, R.C.; Stokka, G.L.; Farmer, C.G.; Mathis, C.P.; Titgemeyer, E.C.; Nagaraja, T.G. Effects of different supplemental sugars and starch fed in combination with degradable intake protein in low-quality forage use by beef steers. *J. Anim. Sci.* **1999**, *77*, 2793–2802. [CrossRef] [PubMed]
46. Heldt, J.S.; Cochran, R.C.; Mathis, C.P.; Woods, B.C.; Olson, K.C.; Titgemeyer, E.C.; Nagaraja, T.G.; Vanzant, E.S.; Johnson, D.E. Effects of level and source of carbohydrate and level of degradable protein on intake and digestion of low-quality tallgrass-prairie hay by beef steers. *J. Anim. Sci.* **1999**, *77*, 2846–2854. [CrossRef] [PubMed]
47. Russell, J.B.; O'Connor, J.D.; Fox, D.G.; Van Soest, P.J.; Sniffen, C.J. A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. *J. Anim. Sci.* **1992**, *70*, 3551–3561. [CrossRef] [PubMed]