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Effects of Guanidinoacetic Acid on Ruminal Fermentation and Greenhouse Gas Production Using Fresh Forage and Silage from Different Maize (*Zea mays* L.) Genotypes

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Abstract: Guanidinoacetic acid (GAA) is a feed additive that promotes growth in animals, while maize (*Zea mays* L.) is used for the mitigation of ruminal greenhouse gases. However, it is unknown if GAA affects the efficiency of maize in mitigating gases or if there is synergy between them. Therefore, the objective of this study was to evaluate the in vitro production of total gas, methane (CH_4), carbon monoxide (CO), and hydrogen sulfide (H_2S), ruminal fermentation characteristics, and the CH_4 conversion efficiency of fresh forage and silage of different genotypes (Amarillo, Montesa, Olotillo, Tampiqueño, and Tuxpeño) of maize, with and without the addition of GAA. The silage of the Amarillo genotype without AAG had the highest ($p = 0.01$) total gas production rate and the lowest ($p = 0.044$) delay time before gas production. In addition, at 48 h, the Amarillo silage with GAA increased the production of total gas ($p = 0.0001$) and CH_4, as well as the proportion of CH_4 (mL CH_4 100 mL^{-1} total gas). The Amarillo and Tuxpeño genotype produced more ($p = 0.033$) CO in the first 24 h of incubation, while silage and the addition of GAA only increased ($p = 0.001$) CO at 6 h. The highest ($p = 0.02$) H_2S production was observed with the ensiled Amarillo genotype with GAA. Regarding fermentation characteristics, the silage of the Amarillo and Montesa genotypes presented the highest degradation of dry matter (DMD), short-chain fatty acids (SCFA), and metabolizable energy (ME), and although there was no effect on CH_4 efficiency, the Amarillo and Olotillo genotypes produced more SCFA, ME, and OM per unit of CH_4. It can be concluded that rumen gas production, fermentation characteristics, and CH_4 conversion efficiency are more influenced by the maize genotype and forage condition than by the addition of guanidinoacetic acid, and of the genotypes evaluated, the forage silage from Amarillo showed the best characteristics and efficiency of CH_4.

Keywords: carbon monoxide; guanidinoacetic acid; hydrogen sulfide; methane; rumen fermentation characteristic; ruminants; ruminal gases

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1. Introduction

Ruminant livestock is considered the most efficient in feed conversion compared to non-ruminant livestock [1] because these animals have a digestive system that can take advantage of fibrous forages and transform them into higher-quality products for human nutrition [2,3]. However, during the ruminal fermentation of the forage, they produce gases that, when expelled into the atmosphere, cause the greenhouse effect, thus contributing to global warming [4]. Among these gases is methane (CH_4), a gas that is formed from hydrogen (H_2) and carbon dioxide (CO_2) produced during the fermentation of carbohydrates and which has a higher warming potential than CO_2 [5]. Other no less important gases are carbon monoxide (CO), which is a precursor of ozone in the atmosphere [6], and hydrogen sulfide (H_2S), which serves as an alternate sink for H_2 to reduce CH_4 production [7]. However, H_2S in high concentrations can affect the digestive system and be toxic to animals [8], and although the production of all these gases is inevitable, high amounts indicate a loss of nutrients and energy [9]. Therefore, reducing the production of greenhouse gases (GHG) without causing an imbalance in the rumen and affecting the productivity of the animals is a challenge [10].

A viable alternative to deal with this problem is to manipulate the diet through the selection of forages of high nutritional quality, in terms of highly digestible protein and carbohydrates [11]. In this sense, maize (*Zea mays* L.), as a source of forage, provides fibrous carbohydrates and non-fibrous carbohydrates, such as water-soluble forms and starch [12–14], which is why it is considered to have high energy value for animal nutrition [15] and is used as a strategy to mitigate GHG emissions in ruminants, especially CH_4 [16,17]. In this regard, it has been shown that the silage of whole maize plants reduces the production of ruminal CH_4 [18] since the starch it contains promotes the formation of propionate and decreases the H_2 available to produce CH_4. Furthermore, silage favors post-ruminal digestion [19], which improves performance in cattle and consequently decreases the CH_4 per unit of the animal product [20].

Another strategy that has been implemented is the use of feed additives, which have been shown to positively alter ruminal and post-ruminal metabolism, improving nutrient utilization and animal productivity [21,22] and thereby reducing the intensity of greenhouse gas emissions [23]. In this sense, guanidinoacetic acid (GAA; also called glycinamide), a derivative of arginine and glycine, is a natural precursor of creatine biosynthesis [24]. In turn, creatine is part of the energy and protein metabolism of animals [25–27], which is why GAA has been used as an additive to improve the performance of animals, including ruminants [28–30]. In this regard, it has been reported that in beef cattle, the addition of 0.03 to 0.40% (on DM basis) GAA in the diet increases daily weight gain, dry matter digestibility, and in some cases, feed conversion efficiency, which was reflected by a higher yield and quality of the carcass [31–34], without negative effects on the serum content of arginine, folate, homocysteine, and methionine [34]. In sheep, studies indicated that with the addition of 0.08 to 0.10% (on DM basis) GAA, an effect similar to that observed in beef cattle is obtained, but of a lesser magnitude [35–38]. These improvements were not only reflected by the increase in the carcass yield, but also by the increase in glycogen, intramuscular fat, protein, antioxidant capacity, and moisture retention in the meat [35,39]. In addition, when combined with 0.10% (on DM basis) N-carbamyl glutamate, it improved the distribution of body fat and muscle composition in sheep [40]. Meanwhile, in dairy cattle, it has been reported that the addition of 0.03 to 0.09% (on DM basis) GAA to the diet improves feed efficiency and rumen fermentation, which increases yields and milk components [41]. However, it is unknown if, at the rumen level, the addition of GAA negatively affects the efficiency of maize in mitigating greenhouse gases or if there is synergy between maize forage and GAA. Therefore, the objective of this study was to evaluate the in vitro production of total gas, CH_4, CO, and H_2S, as well as the characteristics of rumen fermentation and the CH_4 conversion efficiency of fresh forage and silage from different maize genotypes, with and without the addition of GAA.

2. Materials and Methods

2.1. Experimental Treatments

The treatments consisted of the evaluation of the fresh forage and silage of four genotypes of maize native to Mexico (Amarillo, Olotillo, Tampiqueño, and Tuxpeño) and a commercial hybrid (Montesa), with and without the addition of 0.3% (on DM basis) guanidinoacetic acid (GAA). The addition level was defined according to the GAA percentages and the results reported in previous studies and corresponded to the average of the doses that presented the best results in both sheep and cattle (beef and dairy). The GAA was acquired from the company Evonik México S.A. de C.V. (Mexico City, Mexico) under the trade name of GuanAMINO® for ruminants, and at the time of evaluation the GAA had a purity of 96%.

2.2. Fresh Forage and Silage Production

Forage production was carried out in the city of Aldama, Tamaulipas, México (22°59′09″ N and 98°10′25″ W, at 190 masl) during the rainy season (May–October) of 2021. On the site, the soil is clay–loam in texture, with a high organic matter content, moderate alkalinity, and low salinity. The climate, according to Köppen's classification, is of the Aw_0 type, which corresponds to the driest of the warm sub-humid [42]. The sowing was carried out at a density of 62,500 plants ha^{-1}, and during the growth and development of the crop, pesticides and herbicides were not applied, since pest and weed control was carried out manually. The harvest was carried out when the grain reached the milky-mass stage, and for this, a minimum of 10 plants from different points of each genotype were selected. These plants were crushed with a forage chopper and manually mixed until homogeneous and, later, samples were obtained for the analysis of the chemical composition, which thereafter was called "fresh forage". Regarding the silages, they were made by placing 5 kg of fresh and chopped forage in black polyethylene plastic bags (30 cm in diameter × 50 cm in height, caliber 500), which were vacuum sealed with the help of a domestic vacuum cleaner and a heat sealer. After 120 days of fermentation, the silages were opened, and "ensiled forage" samples were obtained.

2.3. Chemical Composition

The fresh and ensiled forage of the five genotypes was dehydrated at 60 °C to a constant weight and ground in a hammer mill (Thomas Wiley® Laboratory Mill model 4, Thomas Scientific™, Swedesboro, NJ, USA) with a 2 mm sieve. Organic matter was determined by estimating the ash content using the method of Thiex et al. [43] and subtracting the value obtained from 100. The crude protein (CP) was calculated by multiplying the nitrogen content [44] by the conversion factor of 6.25. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed with the methodology described by Van Soest et al. [45] based on the ANKOM^{200} fiber analyzer (ANKOM Technology Corp., Macedon, NY, USA), while acid detergent lignin was measured through solubilization with a sulfuric acid solution [46]. The ethereal extract (EE) followed the Padmore [47] method, while the non-structural (NSC) and total (TC) carbohydrates were determined with the equations of Mertens [48] and Sniffen et al. [49]:

$$NSC = 100 - (CP + NDF + EE + Ash) \quad (1)$$

$$TC = 100 - (CP + EE + Ash) \quad (2)$$

2.4. In Vitro Incubation

The nutrient solution was prepared following the methodology of Goering and Van Soest [50] and the rumen fluid was obtained from the ruminal content of four male sacrificed sheep with an average weight of 45 kg. These animals were sacrificed in the municipal slaughterhouse of Toluca, State of Mexico, Mexico, and the rumen content was transferred

to the laboratory in a hermetic thermos, where it was filtered with four layers of gauze to extract the rumen liquid. The incubation was carried out in glass vials with a capacity of 160 mL, and to each one, 500 mg of a dehydrated sample of fresh forage and silage of each genotype, 40 mL of nutrient solution, and 10 mL of rumen fluid were added, in addition to 1.5 mg of GAA to 30 vials. The vials were sealed with butyl rubber stoppers and aluminum seals, lightly shaken, and incubated in an incubator (Binder® BD series, BRINDER Inc., Tuttlingen, BaWü, Germany) at 39 °C throughout the evaluation. In total, three incubation runs were performed, and in each one they were incubated in 63 vials, 30 with GAA, 30 without GAA, and 3 blanks (without substrate), since in all cases, samples were incubated in triplicate.

2.4.1. Production of Total Gas, Methane (CH_4), Carbon Monoxide (CO), and Hydrogen Sulfide (H_2S)

Total gas production (psi) was measured at different incubation times (from 2 to 48 h after incubation—see figures) using a digital pressure gauge with ±2% accuracy (Manometer model 407910, Extech® Instruments, Nashue, NH, USA), following the technique of Theodorou et al. [51]. In addition, the production of CH_4, CO, and H_2S was evaluated, and for its estimation, the methodology proposed by Acosta et al. [52] was used, which consists of injecting gas extracted from the vials into a portable gas detector (Dräger X-am®, model 2500, Dräger, Lübeck, SH, Germany) using an external pump (Dräger X-am®, Dräger, Lübeck, SH, Germany). At the end of each measurement, the gas accumulated in the upper part of the vials was released with a syringe without a plunger to avoid greater accumulation of gas and the partial dissolution of the gases evaluated [53].

2.4.2. Ruminal Potential of Hydrogen (pH) and Dry Matter Degradability (DMD)

After the in vitro evaluation, the contents of the vials were filtered to retain the residual substrate and collect the liquid in beakers. In the liquid, the pH was measured using a potentiometer with a glass electrode (pH wireless electrode HALO® model HI11102, Hanna® Instruments, Woonsocket, RI, USA), while with the residual substrate, the apparent degradation was estimated using the difference between the weight of the sample at the beginning and the end of incubation; then, it was washed with plenty of water and dehydrated at 60 °C to a constant weight [54].

2.4.3. Calculations

The production kinetics of total gas, CH_4, CO, and H_2S were estimated by adjusting the volume of gases with the NLIN procedure from SAS [55], according to the model proposed by France et al. [56]:

$$y = b \times [1 - e^{-c(t-Lag)}] \quad (3)$$

where

y = volume (mL) of TG, CH_4, CO and H_2S at time t (h).

b = asymptotic TG, CH_4, CO and H_2S production (mL g^{-1} DM).

c = rate TG, CH_4, CO and H_2S production (mL h^{-1}).

Lag = initial delay time before TG, CH_4, CO and H_2S production begins (h).

The metabolic energy (ME; MJ kg^{-1} MS) was estimated according to the equation proposed by Menke et al. [57]:

$$ME = 2.20 + (0.136 \times TGP) + (0.057 \times CP) \quad (4)$$

where

CP = crude protein (g kg^{-1} DM)

TGP = net gas production (mL 200 mg^{-1} DM) at 24 h of incubation.

The total concentrations of short-chain fatty acids (SCFA; mmol 200 mg^{-1} DM) were calculated according to Getachew et al. [58] as:

$$SCFA = (0.0222 \times TGP) - 0.00425 \quad (5)$$

where

TGP = total gas production (mL 200 mg^{-1} DM at 24 h of incubation.

Additionally, the ratio between CH_4 and the SCFA (CH_4:SCFA; mmol $mmol^{-1}$), ME (CH_4:ME; g MJ^{-1}), and OM (CH_4:OM; mL g^{-1}) was calculated.

2.5. Statistical Analysis

The experimental design was completely randomized with a 5 × 2 × 2 factorial arrangement (five maize genotypes × two states of the forage × two levels of guanidinoacetic acid addition) and three repetitions. The data from the three replicates of each treatment in each run were averaged, and the averages obtained were used as the experimental unit of each treatment. Analysis was performed using the GLM procedure of SAS [55] with the following statistical model:

$$Y_{ijk} = \mu + G_i + S_j + A_k + (G \times S)_{ij} + (G \times A)_{ik} + (S \times A)_{jk} + (G \times S \times A)_{ijk} + \varepsilon_{ijk.} \quad (6)$$

where Y_{ijk} is the response variable, μ is the overall mean, G_i is the effect of maize genotype (MG), S_j is the effect of the state of the forage (SF), A_k is the effect of the level of GAA addition, $(G \times S)_{ij}$ is the effect of the interaction between the MG and SF, $(G \times A)_{ik}$ is the effect of the interaction between the MG and the level of addition of GAA, $(S \times A)_{jk}$ is the effect of the interaction between the SF and the level of addition of GAA, and $(G \times S \times A)_{ijk}$ is the effect of the interaction between the MG, the SF, the level of GAA addition, and ε_{ijk}, the experimental error. Tukey's test was used for the comparison of means, and $p < 0.05$ was the significance threshold.

3. Results

The chemical composition changed in all genotypes after silage, which was to be expected, since silage is a method of conservation that is based on the acidification of the forage through the catabolism of carbohydrates and other compounds (Table 1).

Table 1. Chemical composition (%, on DM basis) of the forage fresh and silage of different maize (*Zea mays* L.) genotypes (n = 3).

Maize Genotype and State of the Forage [2]	Component [1]							
	OM	CP	EE	NDF	ADF	ADL	NSC	TC
Amarillo								
Fresh	92.06	10.80	2.39	59.71	31.68	3.84	19.16	78.87
Ensiled	92.76	8.31	3.60	47.56	26.23	3.89	33.29	80.85
Montesa								
Fresh	92.72	10.54	2.59	60.63	30.27	3.67	18.96	79.59
Ensiled	93.26	8.29	3.90	50.35	26.90	4.12	30.72	81.07
Olotillo								
Fresh	92.76	10.29	2.64	66.23	36.37	4.41	13.60	79.83
Ensiled	92.99	8.35	3.80	59.82	36.08	4.89	21.02	80.84
Tampiqueño								
Fresh	92.12	10.49	2.19	61.66	35.05	4.25	17.78	79.45
Ensiled	93.62	8.56	3.40	59.46	32.31	4.86	22.19	81.66
Tuxpeño								
Fresh	92.08	10.26	2.45	58.86	30.52	3.70	20.50	79.36
Ensiled	91.63	9.58	3.60	51.49	28.88	4.21	26.95	78.45

[1] OM: organic matter; CP: crude protein; EE: ether extract; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin; NSC: non-structural carbohydrates; TC: total carbohydrates. [2] The pH of the silages ranged between 3.6 and 3.8.

3.1. Ruminal Gas Total Production

The Amarillo genotype presented the highest ($p = 0.001$) asymptotic total gas production and the shortest ($p = 0.0003$) time in the lag phase and that Montesa at 24 h and Amarillo at 48 h produced more ($p = 0.002$) total gas for DM incubated (Figure 1). In addition, the ensiled forage increased ($p = 0.042$) the asymptotic total gas, the total gas production rate, and the total gas production by DM incubated and, without GAA addition, increased ($p = 0.028$) the rate and production of total gas only at 24 h, with no effect in the rest of the incubation period. For the interactions, it was observed that MG × SF influenced ($p = 0.049$) the parameters and the total gas production; MG × GAA influenced the production rate, asymptotic total gas, and total gas production at 6 h; SF × GAA influenced the total gas production rate; and MG × SF × GAA influenced the total gas production rate, the time in the lag phase, and the total gas production at 6 h by DM degraded. The last interaction showed that the Amarillo genotype silage without GAA obtained the highest total gas production rate ($p = 0.01$) and the shortest ($p = 0.0436$) time in the lag phase and that this did not influence total gas production during incubation for this and the rest of the genotypes, except at 24 h per DM (Table 2).

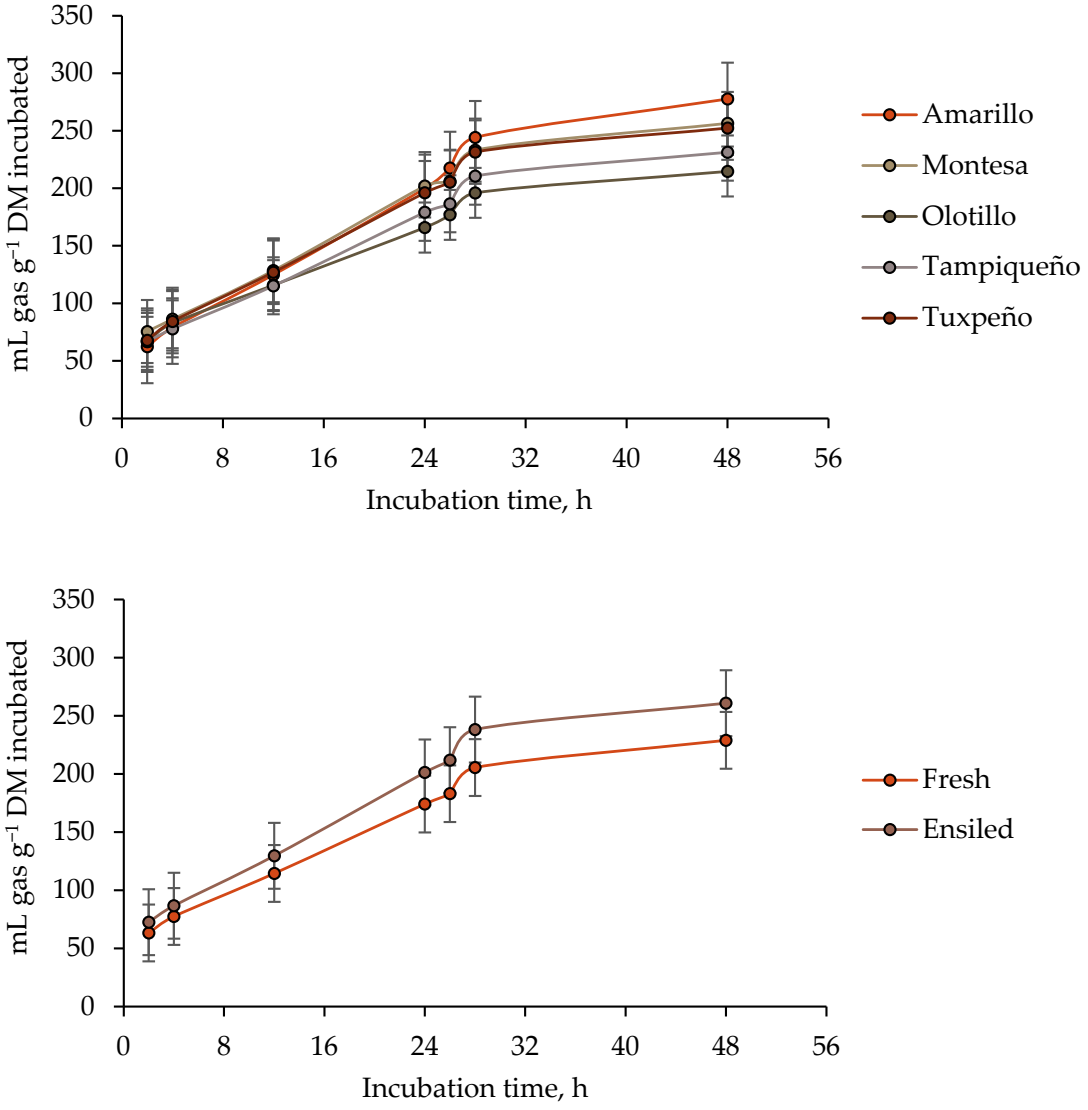

Figure 1. *Cont.*

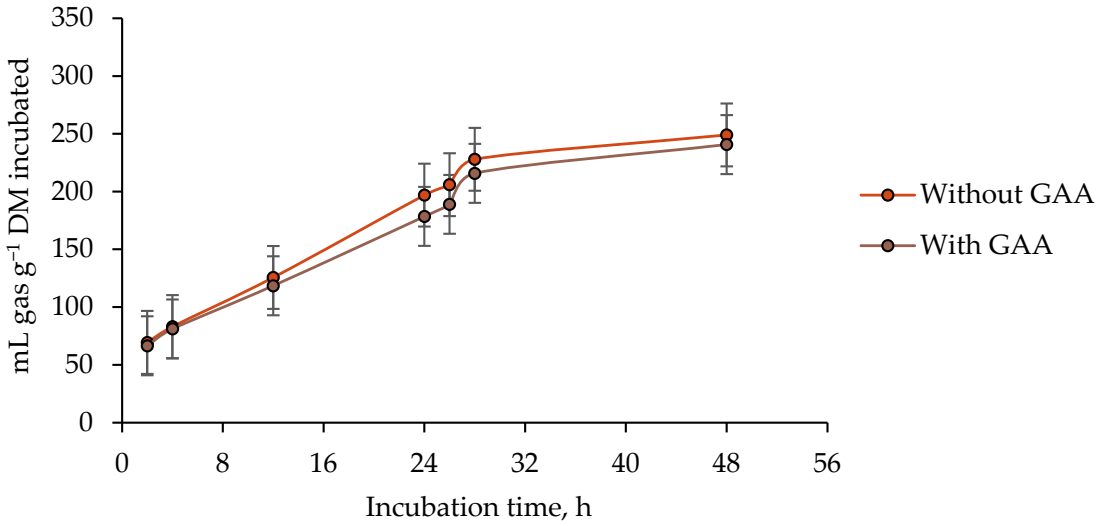

Figure 1. Ruminal total gas production kinetics with fresh forage and silage of different maize (*Zea mays* L.) genotypes at different incubation times, with and without the addition of guanidinoacetic acid (GAA).

Table 2. Ruminal total gas kinetics and production, at 4, 24, and 48 h of incubation of fresh forage and silage of different genotypes of maize (*Zea mays* L.), with (+) and without (−) the addition of guanidinoacetic acid.

Maize Genotype	State of the Forage (SF)	Guanidinoacetic Acid (GAA)	Total Gas Production					
			Kinetics Parameters [1]			mL gas g^{-1} DM Incubated		
			b	***c***	***Lag***	**4 h**	**24 h**	**48 h**
Amarillo	Fresh	−	234.85	0.0245	5.85	68.04	160.52	219.71
		+	277.20	0.0170	14.37	85.72	168.94	227.60
	Ensiled	−	307.45	0.0310	2.30	76.40	228.45	295.09
		+	414.90	0.0230	2.63	86.04	241.63	368.05
		SEM [2]	29.32	0.0021	1.93	7.22	16.89	23.86
		SF	0.0230	0.0396	0.0166	0.5800	0.0141	0.0106
		GAA	0.0630	0.0203	0.0834	0.1313	0.5575	0.1655
		SF × GAA	0.3292	0.9100	0.1007	0.6068	0.8947	0.2445
Montesa	Fresh	−	230.90	0.0255	9.71	82.65	185.99	218.70
		+	230.35	0.0225	8.61	69.31	161.27	210.39
	Ensiled	−	327.70	0.0260	6.86	96.63	249.36	306.00
		+	329.75	0.0200	10.33	96.63	211.29	290.70
		SEM [2]	30.35	0.0020	2.20	3.40	13.70	24.86
		SF	0.0319	0.6483	0.8094	0.0037	0.0144	0.0280
		GAA	0.9815	0.0911	0.6182	0.1216	0.0838	0.6596
		SF × GAA	0.9679	0.5012	0.3577	0.1216	0.6518	0.8949
Olotillo	Fresh	−	226.60	0.0210	14.79	90.91	170.16	209.33
		+	189.60	0.0150	20.98	82.76	121.45	165.92
	Ensiled	−	257.95	0.0195	10.62	76.93	164.44	225.64
		+	236.70	0.0285	7.23	82.23	190.59	226.28
		SEM [2]	9.51	0.0013	1.34	2.09	8.73	10.65
		SF	0.0146	0.0093	0.0026	0.0254	0.0221	0.0228
		GAA	0.0376	0.3046	0.3549	0.5313	0.2661	0.1151
		SF × GAA	0.4542	0.0042	0.0232	0.0322	0.0128	0.1075

Table 2. *Cont.*

Maize Genotype	State of the Forage (SF)	Guanidinoacetic Acid (GAA)	Total Gas Production					
			Kinetics Parameters [1]			mL gas g^{-1} DM Incubated		
			b	***c***	***Lag***	**4 h**	**24 h**	**48 h**
Tampiqueño	Fresh	−	261.10	0.0250	6.34	73.12	184.56	243.74
		+	278.10	0.0220	4.77	63.38	167.62	243.43
	Ensiled	−	253.70	0.0235	12.19	97.37	200.44	239.14
		+	214.40	0.0230	11.50	77.57	163.97	199.12
		SEM [2]	21.56	0.0017	2.35	3.02	14.58	20.96
		SF	0.1745	0.8887	0.0555	0.0031	0.6964	0.3081
		GAA	0.6323	0.3556	0.6561	0.0081	0.1409	0.3905
		SF × GAA	0.2616	0.4975	0.8595	0.1708	0.5396	0.3972
Tuxpeño	Fresh	−	307.40	0.0270	5.05	83.50	229.71	290.59
		+	281.10	0.0245	5.57	75.98	191.54	259.63
	Ensiled	−	263.45	0.0225	9.98	85.72	196.47	243.16
		+	228.20	0.0230	11.42	91.86	167.09	216.11
		SEM [2]	14.71	0.0008	0.60	7.08	11.28	14.28
		SF	0.0302	0.0192	0.0008	0.2704	0.0628	0.0334
		GAA	0.1047	0.2746	0.1769	0.9271	0.0402	0.1121
		SF × GAA	0.7762	0.1306	0.4848	0.3897	0.7167	0.8976
Pooled SEM [2]			22.60	0.0016	1.80	5.05	13.34	19.72
p value								
Maize genotype			0.0009	0.0828	0.0003	0.1336	0.0015	0.0008
State of the forage			0.0052	0.0416	0.1893	0.0006	0.0002	0.0017
Guanidinoacetic acid			0.9284	0.0015	0.1041	0.3907	0.0057	0.3528
Maize genotype × State of the forage			0.0001	0.0014	<0.0001	0.0051	0.0003	<0.0001
Maize genotype × Guanidinoacetic acid			0.0210	0.0081	0.3362	0.0120	0.1436	0.1317
State of the forage × Guanidinoacetic acid			0.8589	0.0315	0.1729	0.3343	0.3629	0.4607
Maize genotype × State of the forage × Guanidinoacetic acid			0.4613	0.0099	0.0436	0.2396	0.1358	0.3773

[1] *b* is the asymptotic gas total production (mL gas g^{-1} DM); *c* is the rate gas total production (mL gas h^{-1}); *Lag* is the initial delay before gas total production begins (h). [2] SEM, standard error of the mean.

3.2. Ruminal Methane (CH_4) Production

Although MG did not affect the parameters and the production of CH_4 throughout the incubation, it was observed that the silage increased ($p = 0.014$) the production rate and the production of CH_4 per DM incubated at 6 h and that a lack of GAA addition also increased ($p = 0.002$) the production of CH_4 at 6 h (Figure 2). In the interactions, it was observed that MG × SF affected ($p = 0.029$) the CH_4 asymptote and CH_4 production throughout the incubation, except at 6 h for CH_4 per DM incubated. In contrast, the MG × GAA interaction only affected CH_4 production at 6 h, while SF × GAA did not affect the parameters and CH_4 production. MG × SF × GAA influenced ($p = 0.023$) the production rate, time in the lag phase, and CH_4 production per DM incubated at 6 h, although this did not impact the production of CH_4 in the rest of the incubation period.

The result also showed that the Amarillo genotype, the silage forage, and a lack of GAA addition resulted in the highest ($p = 0.033$) proportion of CH_4 at 6 h but did not affect that at 24 and 48 h, as well as that at 6 h between genotypes. The interaction of MG × SF showed an effect ($p = 0.028$) on the proportion and of CH_4 throughout the incubation, except for at 6 h with the proportion of CH_4. Furthermore, MG × GAA and MG × SF × GAA only affected ($p = 0.012$) the proportion of CH_4 at 6 h, and SF × GAA did not influence any parameter (Table 3).

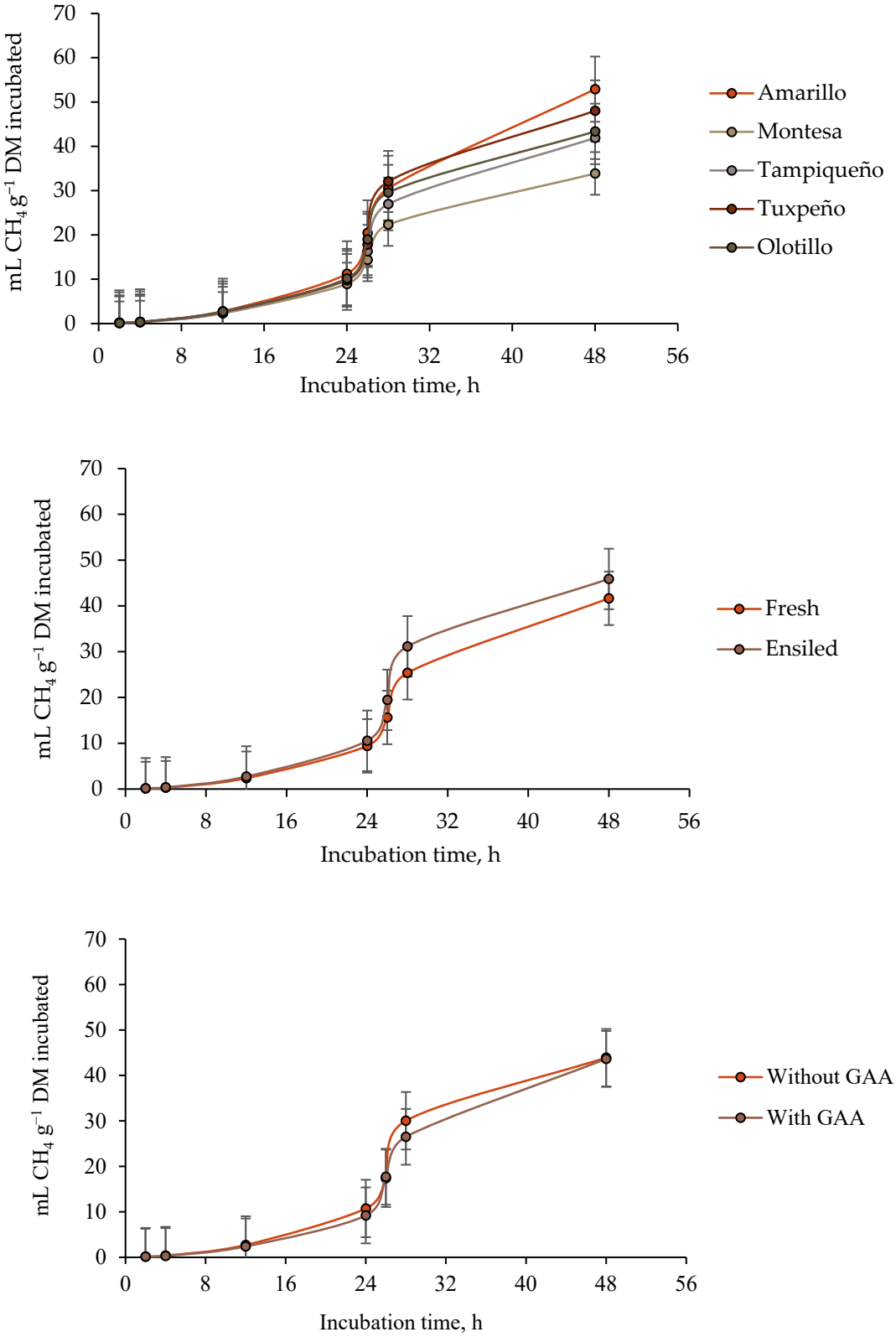

Figure 2. Ruminal methane (CH_4) production of fresh forage and silage of different maize (*Zea mays* L.) genotypes at different incubation times, with and without the addition of guanidinoacetic acid (GAA).

Table 3. Ruminal methane (CH_4) kinetics and production, at 4, 24, and 48 h of incubation of fresh forage and silage of different genotypes of maize (*Zea mays* L.), with (+) and without (−) the addition of guanidinoacetic acid.

Maize Genotype	State of the Forage (SF)	Guanidinoacetic Acid (GAA)	CH_4 Production								
			Kinetics Parameters [1]			mL gas g^{-1} DM Incubated			mL gas 100 mL^{-1} Gas		
			b	*c*	*Lag*	4 h	24 h	48 h	4 h	24 h	48 h
Amarillo	Fresh	−	38.27	0.1255	12.52	0.3400	8.97	38.13	0.5000	5.50	17.25
		+	27.52	0.0650	9.86	0.3315	4.36	36.85	0.3750	2.56	16.19
	Ensiled	−	58.92	0.1170	11.43	0.3820	19.18	58.55	0.5000	8.50	20.00
		+	78.71	0.0960	12.72	0.4300	12.44	78.10	0.5000	5.31	21.44
		SEM [2]	5.20	0.0276	1.77	0.0749	2.89	3.60	0.0625	1.62	1.79
		SF	0.0023	0.7044	0.6420	0.4016	0.0338	0.0010	0.3739	0.1505	0.0890
		GAA	0.4338	0.2138	0.7192	0.8051	0.1201	0.0641	0.3739	0.1316	0.9216
		SF × GAA	0.0426	0.5137	0.3262	0.7253	0.7302	0.0444	0.3739	0.9422	0.5233
Montesa	Fresh	−	42.97	0.1225	11.81	0.4135	12.46	42.86	0.5000	6.63	19.50
		+	22.70	0.0480	4.87	0.2540	9.65	21.70	0.3750	6.06	10.35
	Ensiled	−	36.45	0.1330	12.24	0.4830	7.17	36.29	0.5000	2.88	11.63
		+	34.94	0.0880	11.98	0.2415	6.51	34.69	0.2500	3.06	11.44
		SEM [2]	10.81	0.0222	1.25	0.0365	1.79	10.78	0.0625	0.93	2.82
		SF	0.8045	0.3191	0.0396	0.4787	0.0778	0.7803	0.3739	0.0220	0.2953
		GAA	0.3707	0.0546	0.0453	0.0054	0.3861	0.3506	0.0399	0.8498	0.1733
		SF × GAA	0.4345	0.5430	0.0560	0.3244	0.5803	0.4157	0.3739	0.7068	0.1874
Olotillo	Fresh	−	21.71	0.0760	10.70	0.2270	4.47	19.86	0.2500	2.63	9.50
		+	25.89	0.1185	12.37	0.4140	5.56	25.89	0.5000	4.56	15.56
	Ensiled	−	58.90	0.1565	13.79	0.3850	8.40	58.88	0.5000	4.94	25.56
		+	64.43	0.1280	11.52	0.4110	21.67	64.09	0.5000	11.31	28.19
		SEM [2]	11.37	0.0203	1.01	0.0102	3.08	11.40	0.0000	1.58	4.03
		SF	0.0291	0.0910	0.3261	0.0016	0.0313	0.0276	<0.0001	0.0453	0.0235
		GAA	0.6915	0.7477	0.7798	0.0005	0.0800	0.6476	<0.0001	0.0579	0.3413
		SF × GAA	0.9555	0.1553	0.1216	0.0014	0.1192	0.9734	<0.0001	0.2322	0.6914
Tampiqueño	Fresh	−	64.20	0.1090	12.29	0.3655	18.38	63.78	0.5000	9.25	24.63
		+	43.67	0.0955	11.80	0.1585	9.37	43.30	0.2500	5.50	17.75
	Ensiled	−	33.17	0.1085	10.91	0.4870	6.92	32.84	0.5000	3.44	13.31
		+	27.72	0.1485	13.34	0.1940	4.41	27.75	0.2500	2.69	13.94
		SEM [2]	18.90	0.0278	1.73	0.0137	6.82	18.78	0.0000	3.19	5.82
		SF	0.2817	0.3979	0.9644	0.0046	0.2953	0.2835	<0.0001	0.2478	0.2639
		GAA	0.5296	0.6581	0.6047	<0.0001	0.4462	0.5334	<0.0001	0.5195	0.6200
		SF × GAA	0.7103	0.3898	0.4482	0.0347	0.6587	0.7029	<0.0001	0.6627	0.5547
Tuxpeño	Fresh	−	60.04	0.0845	12.02	0.4175	15.65	59.33	0.5000	7.00	20.75
		+	64.88	0.1665	14.25	0.1900	5.44	64.97	0.2500	2.87	24.62
	Ensiled	−	29.10	0.1860	13.75	0.4285	5.96	28.66	0.5000	3.06	11.81
		+	39.27	0.1210	11.39	0.4590	12.89	39.14	0.5000	7.75	18.12
		SEM [2]	16.99	0.0085	0.16	0.0277	4.91	17.05	0.0000	2.34	5.83
		SF	0.1714	0.0303	0.0223	0.0072	0.8306	0.1728	<0.0001	0.8512	0.2563
		GAA	0.6815	0.3750	0.6958	0.0237	0.7547	0.6612	<0.0001	0.9103	0.4318
		SF × GAA	0.8828	0.0010	0.0001	0.0096	0.1558	0.8940	<0.0001	0.1333	0.8447
Pooled SEM [2]			13.56	0.0224	1.32	0.0400	4.28	13.43	0.0395	2.08	4.37
p value											
Maize genotype			0.4842	0.1032	0.1094	0.1104	0.9620	0.3673	0.0330	0.9465	0.2759
State of the forage			0.4214	0.0135	0.0882	0.0003	0.5637	0.4893	0.0104	0.9683	0.9733
Guanidinoacetic acid			0.8197	0.1676	0.2278	0.0001	0.4347	0.9645	<0.0001	0.8217	0.8531
Maize genotype × State of the forage			0.0041	0.8829	0.2206	0.4349	0.0228	0.0052	0.0119	0.0280	0.0086
Maize genotype × Guanidinoacetic acid			0.7263	0.1011	0.1933	<0.0001	0.2354	0.6304	<0.0001	0.1635	0.4339
State of the forage × Guanidinoacetic acid			0.2549	0.3519	0.4050	0.9362	0.0760	0.3314	1.0000	0.0869	0.3685
Maize genotype × State of the forage × Guanidinoacetic acid			0.9455	0.0196	0.0233	0.0115	0.5353	0.9716	0.0020	0.5793	0.8646

[1] *b* is the asymptotic CH_4 production (mL gas g^{-1} DM); *c* is the rate CH_4 production (mL gas h^{-1}); *Lag* is the initial delay before CH_4 production begins (h). [2] SEM, standard error of the mean.

3.3. Ruminal Carbon Monoxide (CO) Production

It was observed that MG, SF, GAA, and their interactions did not have an impact on the parameters of CO production, but that Amarillo and Tuxpeño produced more ($p = 0.033$) CO per DM incubated and degraded at 24 and 6 h, respectively. Moreover, the silage and the

addition of GAA increased (p = 0.001) CO at 6 h. For the interactions, it was found that MG × SF affected (p = 0.025) the production of CO during the entire incubation and that MG × GAA only influenced (p = 0.02) the production of CO by DM incubated at 6 and 24 h, while the rest of the interactions did not show a significant effect on the production of CO (Figure 3 and Table 4).

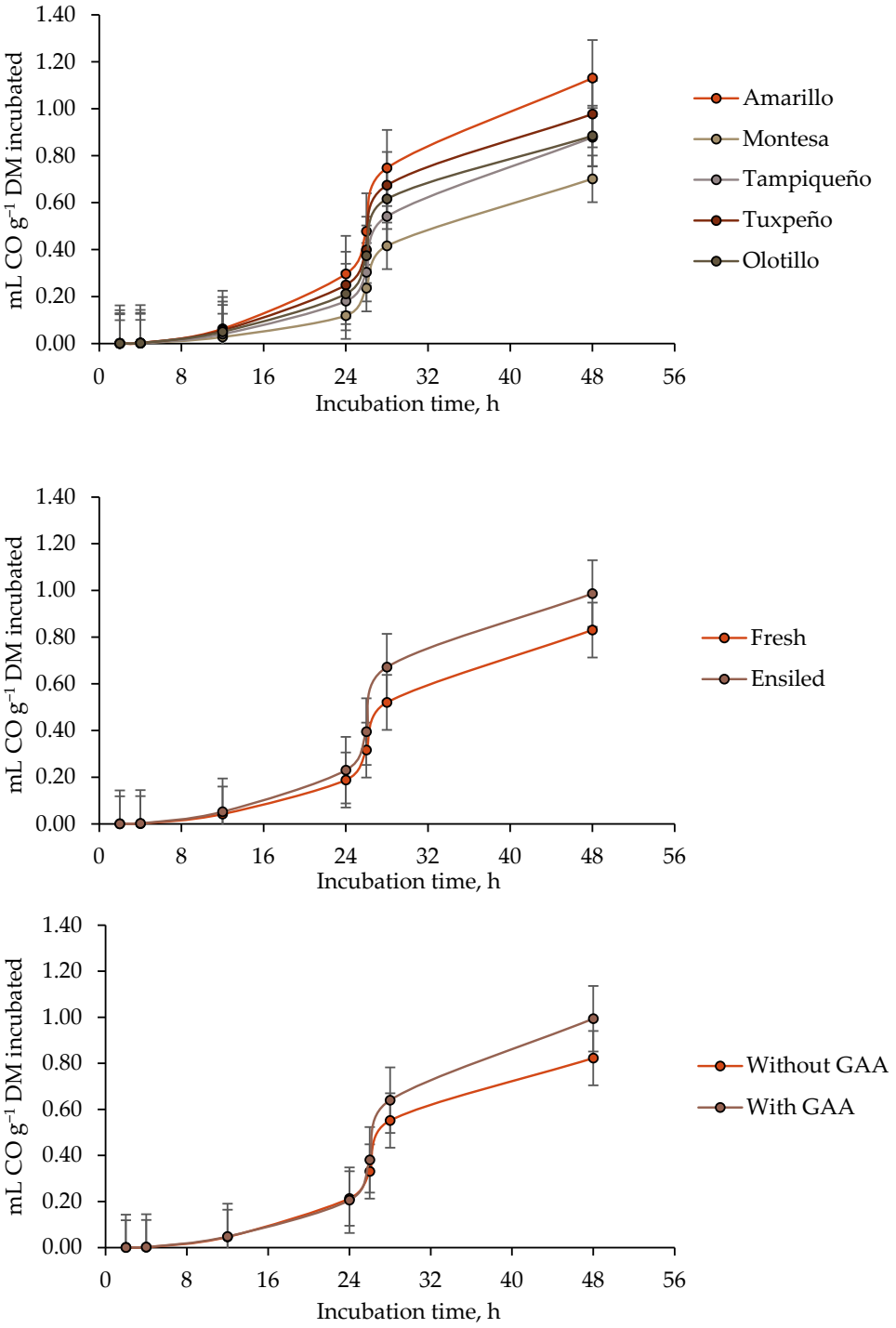

Figure 3. Ruminal carbon monoxide (CO) production of fresh forage and silage of different maize (*Zea mays* L.) genotypes at different incubation times, with and without the addition of guanidinoacetic acid (GAA).

Table 4. Ruminal carbon monoxide (CO) Kinetics and production, at 4, 24, and 48 h of incubation of fresh forage and silage of different genotypes of maize (*Zea mays* L.), with (+) and without (−) the addition of guanidinoacetic acid.

Maize Genotype	State of the Forage (SF)	Guanidinoacetic Acid (GAA)	CO production					
			Kinetics Parameters [1]			mL gas g^{-1} DM Incubated		
			b	*c*	*Lag*	4 h	24 h	48 h
Amarillo	Fresh	−	6.1784	0.0272	4.90	0.0008	0.2536	0.7511
		+	3.9989	0.0003	2.90	0.0019	0.0817	0.6090
	Ensiled	−	3.1119	0.0008	7.55	0.0019	0.5838	1.4391
		+	3.1728	0.0020	5.81	0.0030	0.2685	1.7249
		SEM [2]	1.3634	0.0047	2.15	0.0002	0.0316	0.1129
		SF	0.2266	0.0570	0.2662	0.0085	0.0012	0.0013
		GAA	0.4806	0.0508	0.4337	0.0100	0.0015	0.5592
		SF × GAA	0.4574	0.0393	0.9551	0.9198	0.0855	0.1311
Montesa	Fresh	−	6.3918	0.0003	4.20	0.0010	0.1599	0.6604
		+	2.2404	0.0003	5.10	0.0019	0.1214	0.5415
	Ensiled	−	0.6923	0.0171	4.31	0.0021	0.1026	0.7018
		+	0.9613	0.0109	6.25	0.0030	0.0928	0.9027
		SEM [2]	1.1929	0.0100	2.35	0.0001	0.0387	0.3252
		SF	0.0430	0.2416	0.8012	0.0002	0.3297	0.5694
		GAA	0.1790	0.7695	0.5781	0.0003	0.5668	0.9056
		SF × GAA	0.1376	0.7722	0.8359	0.5614	0.7298	0.6487
Olotillo	Fresh	−	1.4100	0.0004	5.07	0.0021	0.0785	0.2683
		+	0.2768	0.0446	7.95	0.0022	0.1266	0.5658
	Ensiled	−	1.8519	0.0008	2.40	0.0019	0.1801	1.1317
		+	4.3147	0.0151	7.28	0.0028	0.4182	1.4497
		SEM [2]	1.9449	0.0228	0.99	0.0003	0.0493	0.0703
		SF	0.3136	0.5590	0.1679	0.6041	0.0163	0.0002
		GAA	0.7497	0.2699	0.0174	0.1343	0.0439	0.0119
		SF × GAA	0.4076	0.5477	0.3717	0.2084	0.1260	0.8917
Tampiqueño	Fresh	−	2.5560	0.0009	2.74	0.0011	0.2323	1.0656
		+	0.9572	0.0004	5.90	0.0015	0.2012	1.1168
	Ensiled	−	1.0592	0.0034	7.11	0.0030	0.0990	0.6514
		+	1.5083	0.0010	6.14	0.0023	0.1915	0.6809
		SEM [2]	0.6003	0.0014	1.04	0.0003	0.0787	0.2833
		SF	0.4750	0.3266	0.0902	0.0132	0.4149	0.2079
		GAA	0.3925	0.3473	0.3490	0.6728	0.7167	0.8937
		SF × GAA	0.1633	0.5122	0.1177	0.1707	0.4761	0.9712
Tuxpeño	Fresh	−	2.3052	0.0052	5.75	0.0018	0.2971	1.0811
		+	3.1465	0.0037	6.15	0.0021	0.3330	1.6444
	Ensiled	−	3.3266	0.0001	6.55	0.0027	0.1452	0.4791
		+	5.0119	0.0171	4.20	0.0027	0.2238	0.7056
		SEM [2]	1.8381	0.0024	0.97	0.0003	0.1306	0.3833
		SF	0.4762	0.1614	0.5864	0.0870	0.3742	0.1148
		GAA	0.5297	0.0319	0.3731	0.6540	0.6839	0.3610
		SF × GAA	0.8297	0.0185	0.2305	0.6540	0.8779	0.6830
Pooled SEM [2]			1.4699	0.0114	1.62	0.0003	0.0751	0.2648
p value								
Maize genotype			0.1204	0.5711	0.9685	0.1154	0.0330	0.2576
State of the forage			0.5062	0.7723	0.3500	<0.0001	0.2251	0.2018
Guanidinoacetic acid			0.6217	0.4514	0.3390	0.0004	0.8290	0.1638
Maize genotype × State of the forage			0.0687	0.3949	0.2884	0.0254	0.0043	0.0005
Maize genotype × Guanidinoacetic acid			0.5531	0.1415	0.1461	0.0179	0.0198	0.8052
State of the forage × Guanidinoacetic acid			0.0592	0.8699	0.6269	0.5256	0.4799	0.7331
Maize genotype × State of the forage × Guanidinoacetic acid			0.9205	0.4370	0.6375	0.1880	0.6021	0.8569

[1] *b* is the asymptotic gas production (ppm CO g^{-1} DM); *c* is the rate gas production (ppm CO h^{-1}); *Lag* is the initial delay before gas production begins (h). [2] SEM, standard error of the mean.

3.4. Ruminal Hydrogen Sulfide (H_2S) Production

It was found that MG, SF, GAA, and their interactions did not have an impact on the H_2S production parameters, except for silage, which increased ($p = 0.023$) the time in the lag phase. In this regard, the highest ($p = 0.02$) H_2S production was observed with the Amarillo

genotype, the ensiled forage, and with the addition of GAA. In the interactions, MG × SF affected (p = 0.0001) H_2S production at 48 h and MG × GAA influenced (p = 0.041) that throughout the incubation. In addition, for SF × GAA, no significant effect was observed, and MG × SF × GAA showed that Montesa and Amarillo silage, with and without GAA, produced the highest and lowest (p = 0.002) amount of H_2S, respectively (Figure 4 and Table 5).

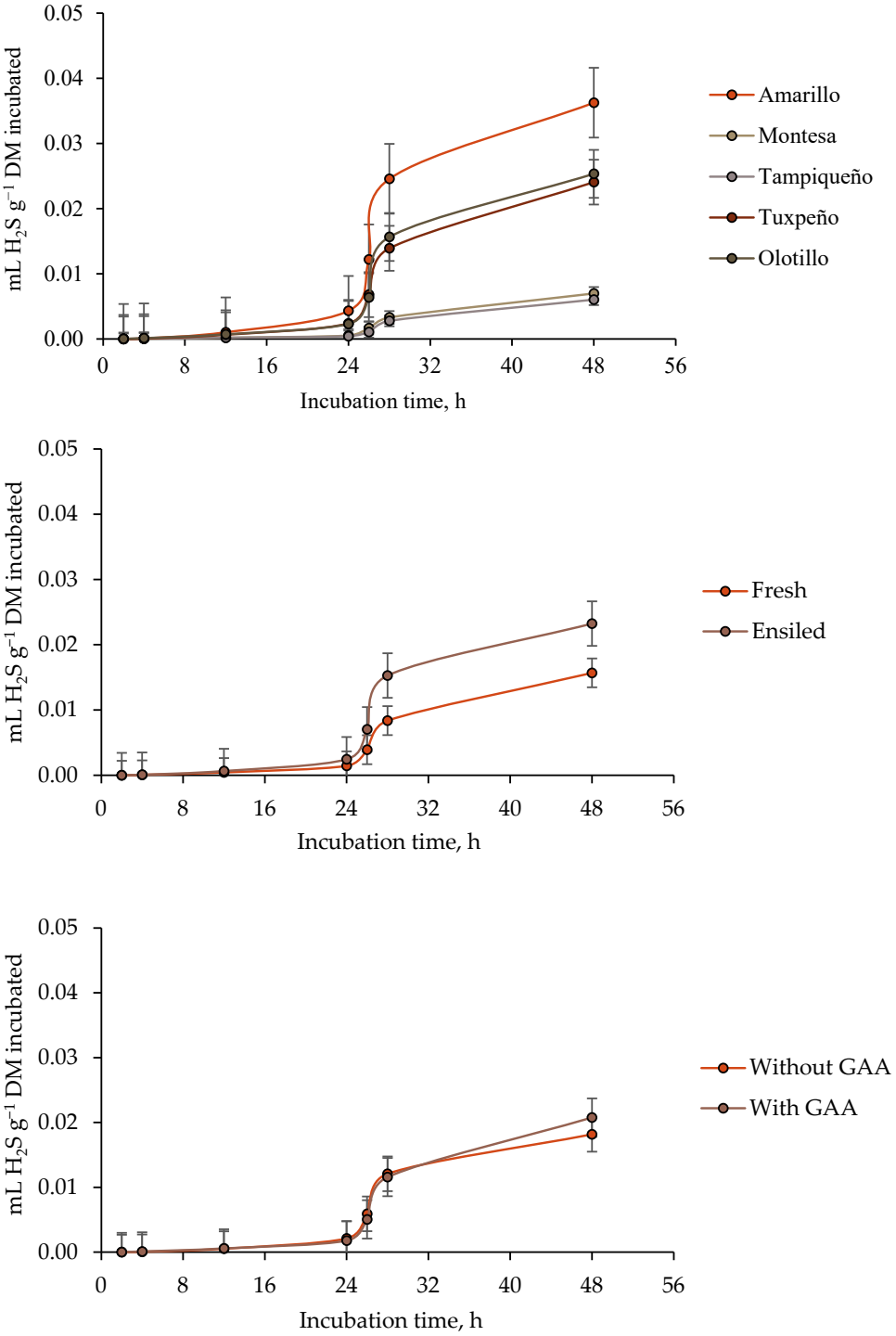

Figure 4. Ruminal hydrogen sulfide (H_2S) production of fresh forage and silage of different maize (*Zea mays* L.) genotypes at different incubation times, with and without the addition of guanidinoacetic acid (GAA).

Table 5. Ruminal hydrogen sulfide (H_2S) kinetics and production, at 4, 24, and 48 h of incubation of fresh forage and silage of different genotypes of maize (*Zea mays* L.), with (+) and without, (−) the addition of guanidinoacetic acid.

Maize Genotype	State of the Forage (SF)	Guanidinoacetic Acid (GAA)	H_2S Production					
			Kinetics Parameters [1]			mL gas g^{-1} DM Incubated		
			b	*c*	*Lag*	4 h	24 h	48 h
Amarillo	Fresh	−	0.0236	0.0002	5.90	0.0001	0.0058	0.0458
		+	0.0041	1.6232	6.04	0.0001	0.0011	0.0068
	Ensiled	−	0.0590	0.0015	3.80	0.0001	0.0093	0.0768
		+	0.0057	0.1531	5.22	0.0001	0.0011	0.0157
		SEM [2]	0.0060	0.7424	1.84	0.0000	0.0002	0.0035
		SF	0.0361	0.3785	0.4698	<0.0001	0.0008	0.0049
		GAA	0.0037	0.2980	0.6930	<0.0001	<0.0001	0.0001
		SF × GAA	0.0475	0.3778	0.7464	<0.0001	0.0008	0.0358
Montesa	Fresh	−	0.4036	0.0027	3.40	0.0000	0.0007	0.0125
		+	0.0682	0.0195	2.43	0.0001	0.0005	0.0052
	Ensiled	−	0.0030	0.1610	5.34	0.0000	0.0005	0.0057
		+	0.0319	0.0099	4.43	0.0001	0.0006	0.0047
		SEM [2]	0.1507	0.0224	1.31	0.0000	0.0001	0.0005
		SF	0.2208	0.0295	0.2057	<0.0001	0.6213	0.0022
		GAA	0.3667	0.0401	0.5115	<0.0001	0.6213	0.0013
		SF × GAA	0.2932	0.0200	0.9843	<0.0001	0.3453	0.0038
Olotillo	Fresh	−	0.0049	0.1863	3.59	0.0000	0.0017	0.0062
		+	0.0171	0.0001	1.35	0.0001	0.0029	0.0356
	Ensiled	−	0.0214	0.3001	3.93	0.0001	0.0011	0.0060
		+	0.0239	0.0006	9.65	0.0003	0.0027	0.0482
		SEM [2]	0.0120	0.1502	0.53	0.0000	0.0003	0.0015
		SF	0.3873	0.7228	0.0012	0.0075	0.2931	0.0128
		GAA	0.5759	0.1812	0.0300	0.0075	0.0084	<0.0001
		SF × GAA	0.7092	0.7251	0.0017	0.3739	0.5275	0.0115
Tampiqueño	Fresh	−	0.0255	0.0009	5.53	0.0002	0.0010	0.0072
		+	0.0608	0.0862	4.39	0.0000	0.0004	0.0085
	Ensiled	−	0.0391	0.0044	6.88	0.0000	0.0002	0.0045
		+	0.0461	0.0086	6.12	0.0001	0.0002	0.0041
		SEM [2]	0.0296	0.0417	3.26	0.0001	0.0004	0.0009
		SF	0.9848	0.4242	0.6605	0.7556	0.3046	0.0171
		GAA	0.5148	0.3434	0.7856	0.7556	0.4766	0.6489
		SF × GAA	0.6579	0.3857	0.9565	0.1709	0.4766	0.4059
Tuxpeño	Fresh	−	0.0144	0.0725	1.65	0.0000	0.0003	0.0072
		+	0.0318	0.2500	2.64	0.0001	0.0005	0.0221
	Ensiled	−	0.1363	0.0067	4.91	0.0001	0.0008	0.0100
		+	0.0258	0.0070	8.25	0.0001	0.0081	0.0570
		SEM [2]	0.0267	0.1271	1.82	0.0000	0.0029	0.0055
		SF	0.0958	0.2913	0.0714	0.3739	0.2343	0.0264
		GAA	0.1565	0.5228	0.3000	0.0399	0.2650	0.0048
		SF × GAA	0.0748	0.5242	0.5525	0.3739	0.2818	0.0430
Pooled SEM [2]			0.0700	0.3441	1.96	0.0000	0.0013	0.0030
p value								
Maize genotype			0.2186	0.4336	0.7079	0.1138	0.0026	<0.0001
State of the forage			0.4128	0.3142	0.0230	0.1473	0.1077	<0.0001
Guanidinoacetic acid			0.1994	0.3665	0.5312	0.0034	0.5732	0.0716
Maize genotype × State of the forage			0.0788	0.4591	0.2350	0.0751	0.1115	<0.0001
Maize genotype × Guanidinoacetic acid			0.4496	0.2123	0.6985	0.0411	0.0004	<0.0001
State of the forage × Guanidinoacetic acid			0.6044	0.2061	0.1862	0.1473	0.4167	0.0561
Maize genotype × State of the forage × Guanidinoacetic acid			0.1569	0.5650	0.6160	0.0751	0.1102	<0.0001

[1] *b* is the asymptotic H_2S production (ppm gas g^{-1} DM); *c* is the rate H_2S production (ppm gas h^{-1}); *Lag* is the initial delay before H_2S production begins (h). [2] SEM, standard error of the mean.

3.5. Fermentation Characteristics and CH_4 Conversion Efficiency

The results showed that MG, SF, GAA, and their interactions influenced (p = 0.03) the fermentation characteristics and did not affect the CH_4 conversion efficiency, except for the MG × SF interaction (p = 0.028). It was observed that the Amarillo genotype was

similar to Montesa and that both were associated with the lowest pH and degradation of dry matter (DMD), short-chain fatty acids (SCFA), and the highest metabolizable energy (ME), while Olotillo resulted in the highest pH and the lowest values ($p = 0.0015$) of DMD, SCFA, and ME. In addition, the ensiled forage resulted in the lowest ($p = 0.03$) pH and the highest DMD and SCFA levels and the same ME as the fresh forage, while, without the addition of GAA, the SCFA levels and ME increased ($p = 0.0057$) without causing variations in the pH and DMD. The MG × SF interaction affected ($p = 0.0028$) the pH, DMD, SCFA levels, and ME, as well as the CH_4 conversion efficiency, while the others only influenced ($p = 0.022$) the DMD. In particular, the MG × SF × GAA interaction showed that the ensiled forage of all the genotypes and the addition of GAA increased the DMD, except for with the Tampiqueño genotype, where an opposite response was observed (Table 6).

Table 6. Fermentation characteristics and methane (CH_4) conversion efficiency with fresh forage and silage of different genotypes of maize (*Zea mays* L.), with (+) and without (−) the addition of guanidinoacetic acid.

Maize Genotype	State of the Forage (SF)	Guanidinoacetic Acid (GAA)	Fermentation Characteristics [1]				CH_4 Conversion Efficiency [2]		
			pH	DMD	SCFA	ME	CH_4:SCFA	CH_4:ME	CH_4:OM
Amarillo	Fresh	−	7.04	44.09	3.54	5.50	72.20	7.54	9.75
		+	7.09	37.94	3.73	5.59	33.63	3.61	4.74
	Ensiled	−	6.88	50.07	5.05	5.93	111.39	15.10	20.68
		+	6.93	51.55	5.34	6.08	69.61	9.60	13.41
		SEM [3]	0.09	1.38	0.38	0.19	21.24	2.48	3.12
		SF	0.1619	0.0021	0.0141	0.0739	0.1515	0.0521	0.0347
		GAA	0.6100	0.1650	0.5582	0.5576	0.1316	0.1296	0.1201
		SF × GAA	1.0000	0.0502	0.8950	0.8952	0.9434	0.7671	0.7352
Montesa	Fresh	−	7.14	34.12	4.11	5.75	86.90	10.01	13.44
		+	7.15	33.88	3.56	5.47	79.59	8.23	10.40
	Ensiled	−	6.95	50.88	5.51	6.17	37.66	5.40	7.69
		+	6.97	51.30	4.67	5.74	40.15	5.25	6.98
		SEM [3]	0.06	1.11	0.30	0.16	12.18	1.38	1.93
		SF	0.0365	0.0001	0.0144	0.0946	0.0220	0.0511	0.0758
		GAA	0.8426	0.9393	0.0837	0.0839	0.8528	0.5227	0.3865
		SF × GAA	0.9050	0.7813	0.6526	0.6532	0.7083	0.5867	0.5796
Olotillo	Fresh	−	7.38	33.60	3.76	5.54	34.45	3.75	4.82
		+	7.25	35.91	2.68	4.98	60.01	5.18	6.00
	Ensiled	−	7.10	40.03	3.63	5.21	64.80	7.39	9.03
		+	7.09	44.53	4.21	5.51	148.36	18.26	23.31
		SEM [3]	0.03	0.91	0.19	0.10	20.69	2.53	3.31
		SF	0.0030	0.0011	0.0221	0.3828	0.0455	0.0299	0.0314
		GAA	0.1167	0.0198	0.2665	0.2647	0.0577	0.0719	0.0800
		SF × GAA	0.1383	0.2931	0.0128	0.0128	0.2336	0.1357	0.1193
Tampiqueño	Fresh	−	7.28	39.98	4.08	5.73	121.30	14.47	19.95
		+	7.20	35.66	3.70	5.54	72.18	7.82	10.17
	Ensiled	−	7.06	44.24	4.43	5.65	45.07	5.68	7.39
		+	7.08	41.22	3.62	5.23	35.28	3.92	4.71
		SEM [3]	0.09	0.91	0.32	0.17	41.82	5.24	7.41
		SF	0.1232	0.0057	0.6963	0.3103	0.2476	0.2922	0.2908
		GAA	0.7483	0.0158	0.1406	0.1410	0.5200	0.4672	0.4476
		SF × GAA	0.5627	0.5153	0.5396	0.5409	0.6627	0.6649	0.6567
Tuxpeño	Fresh	−	7.09	41.14	5.08	6.21	91.74	11.84	17.00
		+	7.10	34.13	4.23	5.78	37.71	4.39	5.91
	Ensiled	−	7.02	39.36	4.34	5.74	40.16	4.84	6.51
		+	7.10	42.61	3.69	5.41	101.72	11.10	14.07
		SEM [3]	0.05	1.23	0.25	0.13	30.73	3.81	5.33
		SF	0.4839	0.0526	0.0627	0.0306	0.8496	0.9713	0.8375
		GAA	0.3778	0.2007	0.0401	0.0402	0.9084	0.8827	0.7572
		SF × GAA	0.4282	0.0140	0.7168	0.7172	0.1332	0.1462	0.1553
Pooled SEM [3]			0.07	1.12	0.30	0.15	27.28	3.36	4.64

Table 6. *Cont.*

Maize Genotype	State of the Forage (SF)	Guanidinoacetic Acid (GAA)	Fermentation Characteristics [1]				CH_4 Conversion Efficiency [2]		
			pH	DMD	SCFA	ME	CH_4:SCFA	CH_4:ME	CH_4:OM
p value									
Maize genotype			0.0015	<0.0001	0.0015	0.0006	0.9460	0.9558	0.9604
State of the forage			0.0301	<0.0001	0.0002	0.4089	0.9709	0.5263	0.5823
Guanidinoacetic acid			0.9475	0.0954	0.0057	0.0057	0.8244	0.5705	0.4344
Maize genotype × State of the forage			0.0022	<0.0001	0.0003	0.0028	0.0280	0.0212	0.0233
Maize genotype × Guanidinoacetic acid			0.6926	0.0024	0.1436	0.1439	0.1630	0.1965	0.2400
State of the forage × Guanidinoacetic acid			0.8693	0.0003	0.3626	0.3626	0.0871	0.0761	0.0756
Maize genotype × State of the forage × Guanidinoacetic acid			0.7568	0.0220	0.1358	0.1364	0.5794	0.5269	0.5363

[1] pH is the ruminal pH; DMD is dry matter degradability (%); SCFAs are short-chain fatty acids (mmol g^{-1} DM); ME is the metabolizable energy at 24 h (MJ kg^{-1} DM). [2] CH_4:ME is the methane:metabolizable energy ratio (g MJ^{-1}); CH_4:OM is the methane:organic matter ratio (mL g^{-1}); CH_4:SCFA is the methane:short-chain fatty acid ratio at 24 h (mmol $mmol^{-1}$). [3] SEM, standard error of the mean.

4. Discussion

4.1. Ruminal Gas Total Production

Total gas production is generally associated with greater feed degradability [52] and in turn, with the chemical composition, especially with the concentration of rapidly fermenting carbohydrates [59]. In this study, the highest total gas production and the shortest time in the lag phase were obtained with the Amarillo genotype and the ensiled forage, which was associated with a higher concentration of non-structural carbohydrates in both the genotype and the silage. In this regard, Kholif et al. [60] reported that silage increases non-structural carbohydrates and that this favors the concentration of water-soluble carbohydrates, which represent additional energy for the rumen microbiota and are reflected by greater microbial activity. This coincides with what has been reported in other investigations where they found that in fibrous forages, including maize forage, silage serves as a treatment to reduce structural carbohydrates and that this allows rumen microbes to adhere more easily to the forage and proliferate more rapidly [61]. Contrary to this, forages with a high content of structural carbohydrates promote low rumen fermentation and, therefore, lower total gas production per unit of DM incubated [62]. This statement was corroborated in this study since the Olotillo genotype was associated with the highest percentage of structural carbohydrates, compared to the rest of the genotypes, and showed the longest time in the lag phase and the lowest rate and production of asymptotic total gas. However, it is interesting to note that although the Tuxpeño genotype did not result in more non-structural carbohydrates compared to those with the Amarillo genotype, it was the most efficient in what can be called "degradation efficiency", since it produced more total gas per unit of DM with the highest total gas production rate. This could be caused by a balance between structural (NDF, ADF, and ADL) and non-structural carbohydrates in this genotype, which allowed ruminal microorganisms to improve digestion and nutrient extraction. Regardless of the state of the forage, in some genotypes, the addition of GAA caused a slight reduction in the production rate and total gas production, which can be attributed to possible momentary inhibition of the growth of some rumen microorganisms while they adapted, since GAA is susceptible to microbial degradation when supplied through the diet and is not protected ruminally [63,64], as in the case of this study.

4.2. Ruminal Methane (CH_4) Production

Considering that, in this study, the Amarillo genotype was the one that produced the highest asymptotic gas total, it can be assumed that the CH_4 may be split into a larger part of the gas that was produced during digestion. The interaction of genotypes and status showed that both genotype and forage status played an important role in reducing CH_4 production. Silage is a conservation method that is based on a fermentation process in an anaerobic environment; thus, it is possible some methanogenic activity would have occurred during this process, resulting in reduced CH_4 production during storage incubation [65]. Zhu et al. [66] reported that lactic acid bacteria are important during the

silage process since they are the predominant microorganisms in the silage and are in charge of catabolizing carbohydrates for pH reduction [67]. Likewise, this microbe can reduce CH_4 due to bioactive compounds, such as b-hydroxy-propionaldehyde (reuterin), an antimicrobial compound resistant to protease (PRA-1), which it produces [68–70]. Along the same lines, analysis of another genotype showed that the fresh forage produced less CH_4 than the ensiled state, which demonstrated that it is possible that during incubation some genotypes produce fermentation products/metabolites that favor the formation or production of CH_4 among themselves. However, it is important to mention that with some genotypes, the addition of GAA decreased the production of CH_4, which is consistent because GAA decreases the population of protozoa and methanogens [41], ruminal microorganisms associated with the production of CH_4. Furthermore, it has been shown that GAA increases propionate formation and decreases the acetate:propionate ratio [34,36], which translates to a lower availability of metabolic H_2 for CH_4 production.

4.3. Ruminal Carbon Monoxide (CO) Production

CO is an intermediate product of DM degradation, so its production is associated with incomplete feed degradation or lower microbial activity in the rumen [71]. In addition, it plays an important role in the biological cycle of bacteria and archaea since it represents an essential metabolite and acts as a regulator of some important metabolic pathways [72]. Considering that in this study the trend observed in CH_4 production with the genotypes, forage states, and with and without the addition of GAA was similar to that obtained for CO, the variations in production can be attributed to the constant oxidation of CO to carbon dioxide (CO_2) and hydroxide (H_2) caused by rumen microorganisms, such as methanogenic bacteria and acetogens, for the production of CH_4 and acetate [73–75]. In addition, it could be assumed that the addition of GAA inhibits the activity of methanogens, and, for this reason, there was a higher accumulated production of CO at 48 h of incubation, since there was no rapid oxidation of CO.

4.4. Ruminal Hydrogen Sulfide (H_2S) Production

H_2S plays a crucial role in the physiological function and maintenance of the gastrointestinal tract of animals [71], as well as in the reduction in ruminal CH_4 production. It has been reported that during the degradation process, amino acids containing sulfur (S) groups, including methionine and cysteine, are catabolized by rumen microbes to produce H_2S [76]. Under this assumption, the high production of H_2S with the ensiled forage of the Amarillo genotype may be associated with the concentration and degradation of the amino acids that comprise it, especially those amino acids that contain sulfur [77]. Meanwhile, the low H_2S production with the Montesa silage may be attributable to the addition of GAA, since there was an interaction between the genotype and the addition of GAA, and it has been reported that the use of additives may influence the reduction in H_2S production in the rumen [78].

4.5. Fermentation Characteristics and CH_4 Conversion Efficiency

This study showed that the genotype and the state of the forage are the factors that most affect the ruminal fermentation profile and that the silages of the Amarillo and Montesa genotypes were the ones that resulted in the lowest pH and the highest dry matter degradation (DMD), short-chain fatty acids (SCFA), and metabolizable energy (ME). The reduction in pH is rational, taking into account that all forage after silage contains more easily fermentable carbohydrates than in its fresh state [60] and that this favors a decrease in the pH [79]. Consequently, this propitiates a favorable environment for the proliferation of fibrinolytic bacteria, which require a pH between 5.5 and 7.0 [80], thereby increasing microbiological activity and increasing forage degradation. In the particular case of the Amarillo genotype, the DMD of the ensiled forage can also be attributed to the shorter time in the lag phase and the high rate of degradation that resulted, since this helped the forage to ferment for longer. In addition, it has been reported that SCFAs are directly proportional

to the degradation, and therefore, the greater the degradation of the forage, the higher the SCFA levels will be, which increases the availability of energy [81,82]. On the other hand, it has been reported that the addition of GAA increases the population of some rumen microbes [34,36], and thus, this cannot be ruled out as the cause of the increase in SCFA and ME in the ensiled forage with the Amarillo and Olotillo genotypes. Similarly, although there was no significant effect of the addition of GAA on the efficiency of CH_4, it was observed that efficiency increased quantitatively with all genotypes with GAA, since regardless of the state of the forage, the majority decreased CH_4 per unit from SCFA, ME, and OM. However, the state of the forage did affect the efficiency with the Olotillo and Amarillo genotypes; with Olotillo, this can be attributed to the low degradability caused by the high concentration of the detergent acid lignin, while with Amarillo, it was believed to be due to the increase in DMD, which increases gas production [83].

5. Conclusions

It is concluded that the production of total gas, CH_4, CO, and H_2S, in addition to the ruminal fermentation characteristics and the CH_4 conversion efficiency are more influenced by the maize genotype and the state of the forage than by the addition of GAA. Of the genotypes evaluated, Amarillo resulted in the lowest incubation pH and the highest DMD, SCFA levels, and ME, and in both forage states, the addition of GAA improved SCFA levels, ME, and the CH_4 conversion efficiency, especially in silage, where the DMD also increased. Therefore, it is suggested that GAA be used as an additive and/or nutritional supplement in ruminant diets that include fresh forage or maize silage, since it does not negatively alter the fermentation profile and the production of ruminal greenhouse gases.

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Institutional Review Board Statement: This study did not require ethical review and approval from any Educational Institution because the rumen fluid was extracted from the rumen content of animals that were slaughtered in the municipal slaughterhouse of Toluca, State of Mexico, Mexico. However, it is important to mention that said place is legally regulated by the official Mexican norm NOM-033-ZOO-1995, which establishes the methods for the humane slaughter of domestic and wild animals.

Informed Consent Statement: Not applicable.

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