



Guanidinoacetic acid effects on ruminal fermentation, digestibility and methane production in sheep liquor: Dose-response analysis

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ARTICLE INFO

Keywords:

Guanidinoacetic acid
 Greenhouse gases
 Fermentation kinetics
 Digestibility

ABSTRACT

Guanidinoacetic acid (GAA), a precursor of creatine, is partially degraded in the rumen when unprotected. While GAA has shown promise as a feed additive, its optimal dose for ruminants remains unclear. This study investigated the dose-response effects of GAA on *in vitro* ruminal fermentation and greenhouse gas production in fattened sheep. Rumen inoculum from two Pelibuey sheep (30 kg ± 2 kg) fed a 50:50 forage: concentrate diet was used to test seven GAA doses: 0 (GAA0, control), 500 (GAA0.5), 1000 (GAA1), 1500 (GAA1.5), 2000 (GAA2), 2500 (GAA2.5), and 3000 (GAA3) mg/kg DM (dry matter). A completely randomized design was employed. GAA0.5 produced significantly higher total gas than GAA2. The CH₄ production was similar across doses, except for GAA2.5, which was significantly higher than GAA0 and GAA0.5. GAA exhibited quadratic relationships with total gas production and linear relationships with CH₄ and CO production but no significant effect on H₂S. GAA linearly increased DM digestibility without significantly affecting ruminal fermentation profiles. GAA inclusion strongly influenced crude protein (CP) digestibility and degradability kinetics, with varying effects at different time points. The rate constant for degradation of the insoluble but potentially degradable CP fraction was highest with GAA1. In conclusion, GAA supplementation at GAA0.5 improved total gas production without increasing CH₄ emissions, implying enhanced fermentation efficiency and improved performance. GAA's impact on CP digestibility varied with incubation time. These findings provide insights into developing optimal GAA dose/level supplementation that is effective in fattening sheep, though further *in vivo* studies are warranted to validate these effects and explore long-term impacts on rumen microbial populations and protein utilization.

1. Introduction

The rumen of ruminants is the primary site for fermentation of ingested nutrients. This process, carried out by microorganisms, generates volatile fatty acids (VFA: acetate, propionate, butyrate), which are the animal's primary energy source [1,2]. Additionally, microbial protein serves as an important source of protein and amino acids for the host [3,4].

During ruminal fermentation, by-products such as CO₂ and H₂ are

produced and converted into methane (CH₄) by methanogens [5–7]. Methane production represents an energy loss for the animal (13.3 Mcal/kg CH₄) and contributes to greenhouse gas emissions [8,9].

Maximizing the shift in the flow of metabolic hydrogen (H₂) in the rumen away from CH₄ and toward VFA would increase the efficiency of ruminant production and decrease its environmental impact [10]. Suggestions such as inhibiting methanogenesis to favour microbial biomass production as an alternative hydrogen sink and metabolic hydrogen incorporation into excess NADH have been made [10] but with limited

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<https://doi.org/10.1016/j.jafr.2024.101540>

Received 25 September 2024; Received in revised form 22 November 2024; Accepted 23 November 2024

Available online 26 November 2024

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success. To improve the energetic efficiency of animals while reducing environmental hazards like CH₄ emissions, the rumen microbes thus become the main target [10]. Very efficient animals are noted for less CH₄ and more milk production while consuming less feed owing to their unique set of rumen microbes [11]. Concerted efforts are being made to manipulate rumen fermentation and the rumen microbes, especially those associated with methane and other obnoxious gases [12–14], depending on feed (type and level) and production. The basic principle underlying such strategies is the alteration in ruminal microflora that can be achieved by dietary intervention and the use of additives that selectively affect rumen microbial communities. Ruminant production improvement is possible with rumen fermentation manipulation to increase total VFA and propionate production while decreasing methane emissions [15].

Many feed additives such as antibiotics, ionophores, and defaulting agents (eg. capric acid, lauric acid, and myristic acid against protozoa) have been utilized to mediate rumen fermentation to improve the productivity of ruminants and reduce methanogenesis. Antibiotics are discouraged, though, due to the formation of antimicrobial resistance and the emergence of multi-resistant bacteria that can also endanger human health. Guanidinoacetic acid (GAA), H₂NC(=NH)NHCH₂CO₂H, CAS no. 352-97-6, is an amino acid derived and a direct precursor of creatine [16,17] but, in its unprotected form, can be degraded by rumen microbiota and be used as a nitrogen source [18]. The GAA was used foremost in humans, chickens, and pigs [19]. Several studies have shown that GAA could improve growth performance, promote muscle development, and improve the general health status in monogastric (poultry and pigs) when used as a feed additive [20,21]. Recently, scientists have been interested in studying the effect of supplementing ruminants with GAA. Improved rumen VFA production, microbial growth, growth performance, nutrient digestibility, and enzyme activity at 0.6 and 0.9 g GAA/kg DM have been reported [22]. Improved growth performance and meat quality in lambs [23] at 1 g/kg DM GAA has been established. Moreover, an enhanced antioxidant effect has been noted on the rumen in ruminants [23]. Interestingly, GAA supplementation has been found to affect ruminal fermentation patterns. Including GAA at 0.20, 0.40, 0.60, and 0.80 % led to an increase in microbial protein and propionate, while gas production (GP), pH, and methane were unaffected [24]. The addition of GAA at levels of 0.03, 0.05, 0.07, 0.09, 0.11, 0.13, and 0.15 % to diets of sheep proved effective in improving GP, VFA and microbial protein while propionate increased numerically [25]. Given the variability in responses observed in recent studies, it is necessary to establish the optimal GAA dosage for ruminal fermentation in sheep under feedlot conditions. It was hypothesized that a dose between 0 and 3000 mg/kg optimizes ruminal fermentation without significantly increasing methane production. This *in vitro* experiment aims to determine this optimal GAA dose by evaluating its effects on ruminal gas production, greenhouse gas production, fermentation, and degradation parameters in fattened sheep liquor. By examining a wider range of doses (0–3000 mg/kg), It was expected to provide a more comprehensive understanding of GAA effects and probable mechanisms on ruminal fermentation and gas production, thus contributing to developing more efficient and environmentally sustainable feeding strategies in sheep production systems. Sheep are a main source of meat globally, and they are also used extensively for wool production.

2. Materials and methods

2.1. Experimental treatments and substrate

This study was carried out in the animal nutrition laboratory of the Faculty of Veterinary Medicine and Animal Science of the Autonomous University of the State of Mexico and the animal nutrition laboratory of the Central Laboratory of the Faculty of Engineering and Sciences of the Autonomous University of Tamaulipas, Mexico. The guanidinoacetic acid (GAA; GuanAMINO®, Evonik Operations GmbH, Germany) used

was 96 % pure. The doses of GAA deployed in this work were based on previous studies [25–27] but considering the degradation rate in the rumen [18] and were 0 (GAA0, control), 500 (GAA0.5), 1000 (GAA1), 1500 (GAA1.5), 2000 (GAA2), 2500 (GAA2.5), and 3000 (GAA3) mg/kg dry matter (DM). As substrate, a diet was formulated to meet the requirements of fattening sheep [28] as shown in Table 1.

Diet samples were dried at 65 °C for 48 hours in a forced air oven and subsequently ground in a Wiley mill (Thomas Wiley® Laboratory Mill model 4, Swedesboro, NJ, USA) with a 1 mm sieve and stored in plastic jars for subsequent chemical composition determination and *in vitro* incubation. Chemical analysis of the diets was performed by near-infrared spectroscopy (NIR) using a spectrophotometer (FT-NIR TANGO®, Bruker Optik GmbH, Germany) equipped with the calibration curves for finished ruminant feeds (AMINONIR® FEEDS, Evonik Operations GmbH, Germany) for determination of the dry matter, ash, crude protein (N content multiplied by 6.25), ether extract, crude fibre, neutral detergent fibre, acid detergent fibre, starch, sugar, phosphorus, and crude energy, and all calibrations are based on official methods [29,30].

2.2. *In vitro* incubations for the experiment 1 and 2

In experiment 1, rumen inoculum was collected from 4 Pelibuey sheep weighing 40 kg ± 2 kg, fed a diet based on ground maize, soybean meal, vitamin-mineral premix, and maize stubble with a forage-to-concentrate ratio of 50:50. The rumen fluid was collected in a thermos flask and immediately transported to the laboratory, filtered through four layers of gauze and stored in a 2000 mL Erlenmeyer flask in a water bath at 39 °C with a constant flow of CO₂. The nutrient medium contained a buffer solution, macro and micro minerals, resazurin, and distilled water [31] in a 9:1 v/v ratio, using a magnetic stirrer, ensuring that this mixture was maintained at 39 °C. Prior to incubation, 1000 mg of substrate were weighed and placed in a 160 mL glass bottle; then, doses of GAA, 90 mL of nutrient solution, and 10 mL of rumen inoculum

Table 1
Ingredients and composition of diets used as substrates.

Ingredients	Inclusion rate, % DM
Corn grain	47.90
Wheat bran	23.00
Corn stover	15.00
Soybean meal	11.50
Vitamin and mineral premix ^a	2.00
Calcium carbonate	0.40
Inert ^b	0.30
Nutrients, % DM^c	
Dry matter, % as is	89.45
Crude protein, %	15.84
Crude fiber, %	11.27
Metabolizable energy, MJ/kg DM ^d	11.35
Gross energy, MJ/kg DM	18.00
Ether extract, %	3.78
Neutral detergent fiber, %	34.23
Acid detergent fiber, %	14.67
Ash, %	6.66
Starch, %	34.08
Sugar, %	4.09
Ca, % ^e	0.80
P, %	0.62

DM, dry matter; ^{d,e} Calculated values from the National Research Council (2007).

^a Vitamin and mineral premix provide the following per-kg: 240 g Ca; 30 g P; 20 g Mg; 80 g Na; 120 g Cl; 5 g K; 5 g S; 5 mg Cr; 4000 mg Mn; 2000 mg Fe; 5000 mg Zn; 100 mg I; 30 mg Se; 60 mg Co; 500000 UI A vitamin; 150000 UI D vitamin; 1000 UI E vitamin.

^b Inert component was bentonite and the inclusion rate of Guanidinoacetic Acid in the diet was at the expense of the inert component.

^c Measurement of nutrients by NIRs with AMINONIR® FEEDS calibration, Evonik Operations GmbH.

were added maintaining a 9:1 v/v ratio. The bottles under CO₂ flow were immediately closed with rubber stoppers and aluminum seals, shaken, and placed in a bath (Forma Scientific Model 2564 Shaker Bath, Division of Mallinckrodt, Inc. OH, USA) at 39 °C [32]. Each treatment, including control (GAA0), was incubated in quadruplicate, and three flasks without substrate were used as blanks to correct the gas measurement. All treatments were handled under identical experimental conditions, using the same batch of rumen inoculum. The temperature was continuously monitored throughout the incubation period, and bottles remained sealed to maintain anaerobic conditions (which was checked by resazurin discoloration). Total gas production values from blank bottles were subtracted from all treatment measurements. Total gas production (GP) was recorded at 2, 4, 6, 8, 10, 12, 15, 19, 24, 24, 30, 48, 60 and 72 hours using a pressure transducer (Manometer Delta OHM® model HD2124.1, Seneca Italy Srl. Italy)- [33].

In experiment 2, the rumen inoculum was obtained from 2 sheep (45 ± 2 kg) slaughtered at the municipal slaughterhouse in Toluca, Mexico State, Mexico. The rumen liquid was placed in a thermos flask and transported to the laboratory, where it was filtered through four layers of gauze and kept at 39 °C until use. The nutrient medium comprised a buffer solution, macro and micro minerals, resazurin, and distilled water [34]. Prior to incubation, the rumen inoculum and the nutrient medium were mixed in a 1:4 ratio (v/v) under constant stirring and kept at 39 °C. For incubation, 500 mg of each diet was weighed and placed in 120 mL glass bottles with the doses of GAA and 50 mL of the nutrient medium mixture with the rumen inoculum. The bottles were immediately closed with rubber stoppers and an aluminum seal and placed in a water bath (BINDER Incubator Model FD115, Germany).

Each treatment, including control (GAA0), was incubated in triplicate, and three flasks without substrate were used as blanks to correct the gas measurement. All treatments were handled under identical experimental conditions, using the same batch of rumen inoculum. The temperature was continuously monitored throughout the incubation period, and the bottles remained sealed to maintain anaerobic conditions. Gas production values (GP, CH₄, CO, and H₂S) from blank bottles were subtracted from all treatment measurements. The GP was recorded at 2, 4, 12, 24, 27, 30 and 48 hours using a pressure transducer (Manometer model 407910, Extech® Instruments, Nashua, NH, USA). In addition, the production of methane (CH₄), carbon monoxide (CO), and hydrogen sulfide (H₂S) in the headspace of the bottles was quantified using a portable diffusion-based gas detector (Dräger X-am®, model 2500, Dräger, Lübeck, SH, Germany) in the same hours as the GP. After each measurement, in both experiments, the gas accumulated in the headspace of the bottles was released to avoid the dissolution of the gases in the liquid phase and decrease the source of error [35].

2.3. Apparently degraded substrate

In both experiments, after sampling the supernatant for pH determination using a pH meter (Conductronic pH15, Puebla, Mexico), the contents of each bottle were vacuum filtered through sintered glass crucibles (Duran® coarse porosity no. 1, pore size 100–160 µm; Germany). The incubation residues were then dried at 100 °C overnight to estimate the apparent disappearance of DM (ADM). DM degradability at 72 hours of incubation was calculated as the difference between the DM content of the substrate and its non-degradable DM [33].

2.4. Calculation of fermentation kinetics parameters

To estimate the gas production (GP) kinetics parameters in experiment 1 and CH₄, CO, O₂, and H₂S in experiment 2, the results of GP, CH₄, CO, and H₂S (mL/g DM) were fitted using the NLIN option of SAS [36] using a non-linear model [37]:

$$A = b \times (1 - e^{-c(t-Lag)})$$

where *A* is the volume of GP, CH₄, CO, and H₂S at time *t*; *b* the asymptotic production of gas, CH₄, CO, and H₂S (mL/g DM); *c* is the rate of GP, CH₄, CO, and H₂S (/hour), and *Lag* (hour) the lag or colonization time before gas, CH₄, CO and H₂S production starts.

Metabolizable energy (ME) was calculated from the GP of 200 mg of the substrate and the crude protein (CP), ether extract (EE), and ash (CEN) content of the substrate [31]:

$$SCFA(\text{mmol}/(200 \text{ mg DM})) = 0.0222 \text{ GP}24 - 0.00425$$

Where GP24 is the net gas production in mL of 200 mg dry sample after 24 hours of incubation.

The concentration of short-chain fatty acids (SCFA) was calculated from GP24 [38] as follows:

where GP24 is the net gas production at 24 hours (mL/200 mg DM).

Digestible organic matter (DOM) was also calculated from GP24 using the following formula [39]:

$$\text{DOM (\% DM)} = 13.3 \times \text{GP}24 - 0.05\text{PG}24^2 + 511 \times \text{CP} + 76 \times \text{EE} + 91.2$$

where GP is the net gas production in mL of 200 mg dry sample after 24 hours of incubation, all CP and EE values were expressed in g/100 g DM.

2.5. Apparent digestibility of DM and CP

The nylon bag technique [40] was used with methodological modifications proposed by ANKOM Technology Corporation [41] and adaptations consisting of the use of 900 mL glass bottles with an individual bubble Airlock and rubber stopper (Home Brew Ohio, USA) in the lid to allow the release of gases generated during fermentation. ANKOM filter bags (F57, ANKOM Technology Corporation, Macedon, NY, USA) were used, identified with a pencil, and dried at 60 °C to constant weight. 450 mg of dry substrate was weighed and placed in bags with the GAA doses in triplicate for each time evaluated. The bags were then sealed using a heat sealer (Impulse Sealer, ANKOM Technology Corporation, Macedon, NY, USA) and placed in the adapted digestion flasks (16 bags per flask). The ruminal inoculum was obtained via oesophageal route from six growing lambs (Pelibuey 24 ± 0.3 kg body weight) fed a diet based on ground corn, soybean paste, vitamin-mineral premix, and corn stover with a forage concentrate ratio of 50:50.

The collected ruminal fluid was placed in a thermos flask and immediately taken to the laboratory, where it was filtered through four layers of gauze and stored in a 2000 mL Erlenmeyer flask in a water bath at 39 °C with constant CO₂ flow until use. The nutrient medium consisted of a buffer solution, macro and micro minerals, resazurin, and distilled water [31]. An empty bag was inoculated into each flask and served as a blank to correct the data. Subsequently, 800 mL of a 1:4 (v/v) mixture of rumen liquid and nutrient medium was added to each fermentation flask. Seven flasks, one separate flask per GAA dose, were used and placed in a water bath incubator with constant circular agitation (SHELDON MANUFACTURING INC. Cornelius, OR, USA) at 39.2 ± 0.5 °C. The bags were incubated for 48 hours. Bags in triplicate from each treatment were removed from the flasks at 0, 6, 12, 24, and 48 hours, washed with water, and then dehydrated in a forced air oven at 65 °C to constant weight to determine residual DM. Finally, the crude protein content was determined by multiplying the nitrogen content by a factor of 6.25, and the nitrogen (954.01) was measured using the Kjeldahl method [29]. With this data, the apparent digestibility of DM and CP was calculated according to the proposed formulas [41].

2.6. Calculation of degradation kinetics

The DM and CP disappearance data of the seven treatments at the five incubation times of the bags were used to calculate the degradation kinetics using a non-linear model [40]:

$$P = a + b(1 - \exp^{-c \times t})$$

Where P is the potential degradability, t is the incubation time, a an intercept with the y -axis at time zero represents the soluble and fully degradable substrate that rapidly leaves the ANKOM bag, b the difference between the intercept (a) and the asymptote representing the insoluble but potentially degradable fraction of the substrate which is degraded by the microorganisms according to a first order kinetic process, c is the degradation rate constant of the b fraction. Equations were fitted for degradation profiles using the NLIN procedure of SAS 9.4 [36].

The effective degradability of the b -fraction (DEb) of DM and CP was calculated using numerical methods using the Solver function of the Microsoft Office Excel program [42].

2.7. Statistical analysis

The design of the three experiments followed a completely randomized design under controlled laboratory conditions. Prior to analysis, data were tested for normality using the Shapiro-Wilk test ($P > 0.05$) and homogeneity of variance using Bartlett's test ($P > 0.05$). The data from the four replicates for total gas production and three replicates for CH_4 , CO, H_2S , apparent digestibility, and degradation kinetics were averaged for each treatment in each run, serving as the experimental unit. The analysis was conducted using the GLM procedure in SAS [36] with the following statistical model:

$$Y_{ijk} = \mu + A_i + \epsilon_{ijk}$$

Where: Y_{ijk} is the response variable, μ is the general mean, A_i is the effect of GAA levels ($i = 0$ (control), 500, 1000, 1500, 2000, 2500, and 3000 mg/kg), and ϵ_{ijk} is the experimental error, assumed to be normally distributed with mean zero and constant variance. Treatment means were compared using Tukey's HSD test with significance set at $P \leq 0.05$. Both linear and quadratic effects of increasing GAA levels were evaluated, and their respective P -values were reported. For variables showing significant quadratic effects, the optimal dose was calculated by taking the first derivative of the quadratic equation and setting it equal to zero.

3. Results

3.1. Ruminant total gas and CH_4 production and kinetics

The effect of guanidinoacetic acid (GAA) at different levels (0, 500,

1000, 1500, 2000, 2500, 3000 mg GAA/kg dietary DM) on ruminal total gas and CH_4 production and kinetics of high concentrate diet using ruminal contents of sheep as a source of inoculum is as presented in Table 2. The GAA inclusion was similar across the treatments at 6 h, except that the inclusion at 500 was significantly higher than at 2000 mg/kg DM. The CH_4 production rate was identical with the inclusion of 0, 500, 1000, 1500, 2000, and 3000 but higher with GAA2.5 compared to GAA0 and GAA0.5. There was a quadratic ($P = 0.0033$) relationship between the doses of GAA and asymptotic total gas. A similar relationship was also exhibited at 48 hours of gas production. However, linear ($P = 0.0294$) and quadratic ($P = 0.0292$) relationships were observed in the total gas kinetics lag. However, a linear ($P = 0.0405$) relationship existed between GAA and CH_4 . The inclusion of GAA at 2500 consistently had higher total gas CH_4 (mL/g DM) for a high concentrate diet than others (Fig. 1).

3.2. Ruminant CO and H_2S production and kinetics

The results of the effect of guanidinoacetic acid (GAA) at different levels (0, 500, 1000, 1500, 2000, 2500, 3000 mg GAA/kg dietary DM) on ruminal CO and H_2S production and kinetics of high concentrate diet using ruminal contents of sheep as a source of inoculum is as shown in Table 3. The different treatments had no significant effect on all the parameters under consideration. However, a linear ($P = 0.0115$) relationship existed between CO (mL/g DM) with the inclusion of GAA at 24 hours. The inclusion of GAA at 2500 consistently had higher CO and H_2S (mL/g DM) for a high-concentrate diet than others (Fig. 1).

3.3. Ruminant fermentation profile

The results of the effect of GAA at different levels (0, 500, 1000, 1500, 2000, 2500, 3000 mg GAA/kg dietary DM) on ruminal fermentation profile and CH_4 conversion efficiency of high concentrate diet using ruminal contents of sheep as a source of inoculum is presented in Table 4. Although the inclusion of GAA had no significant effect on the ruminal fermentation profile in this study, there was a linear ($P = 0.0196$) relationship between GAA and DMD.

3.4. Dry matter digestibility and degradability

Table 5 presents the results of the effect of guanidinoacetic acid (GAA) at different levels (0, 500, 1000, 1500, 2000, 2500, 3000 mg

Table 2

Effect of guanidino acetic acid (GAA) at different levels (0, 500, 1000, 1500, 2000, 2500, 3000 mg GAA/kg dietary DM) on ruminal total gas and CH_4 production and kinetics of high concentrate diet using ruminal contents of sheep as a source of inoculum.

Item		Doses of GAA, mg/kg DM							SEM ^b	P value	
		0	500	1000	1500	2000	2500	3000		Linear	Quadratic
Total gas kinetics ^a	<i>b</i>	322.30	316.75	296.58	283.25	282.60	288.38	325.60	12.16	0.3553	0.0033
	<i>c</i>	0.033	0.033	0.033	0.030	0.033	0.035	0.030	0.002	0.8307	0.9017
	<i>Lag</i>	3.56	3.21	3.36	3.38	3.45	3.62	3.78	0.128	0.0294	0.0292
Gas production (mL/g DM)	6 h	19.54 ^{a,b}	23.58 ^a	19.82 ^{a,b}	19.09 ^{a,b}	17.34 ^b	18.69 ^{a,b}	20.26 ^{a,b}	1.116	0.1023	0.2850
	24 h	163.58	159.54	153.56	152.57	146.08	160.59	166.96	7.969	0.9112	0.0629
	48 h	244.64	239.37	223.38	217.86	213.86	226.45	247.27	9.547	0.5921	0.0047
CH_4 kinetics ^a	<i>b</i>	27.58	36.40	32.66	46.41	34.13	67.14	45.92	10.98	0.2456	0.7519
	<i>c</i>	0.11 ^b	0.11 ^b	0.13 ^{a,b}	0.13 ^{a,b}	0.13 ^{a,b}	0.15 ^a	0.13 ^{a,b}	0.006	0.0528	0.6503
	<i>Lag</i>	14.03	13.89	13.45	14.12	13.50	14.18	13.93	0.311	0.8544	0.1196
CH_4 production (mL/g DM)	6 h	0.00	0.00	0.00	0.13	0.00	0.00	0.24	0.104	0.2046	0.4673
	24 h	3.27	3.55	3.81	3.64	4.45	5.36	3.78	0.636	0.1079	0.4974
	48 h	27.52	36.33	32.73	46.42	34.15	67.22	45.97	10.972	0.0605	0.8537
CH_4 (mL CH_4 /100 mL gas)	6 h	0.00	0.00	0.00	0.17	0.00	0.00	0.25	0.114	0.2325	0.5840
	24 h	2.63	3.13	2.50	3.00	2.88	3.13	2.88	0.334	0.5348	0.8412
	48 h	12.29	15.38	14.92	18.25	14.21	21.13	19.71	2.767	0.0405	0.9884

^a b = asymptotic total gas or CH_4 production (mL/g DM); c = rate of total gas or CH_4 production (mL/hour); Lag = initial delay before total gas or CH_4 production begins (hour).

^b SEM = standard error of the mean.

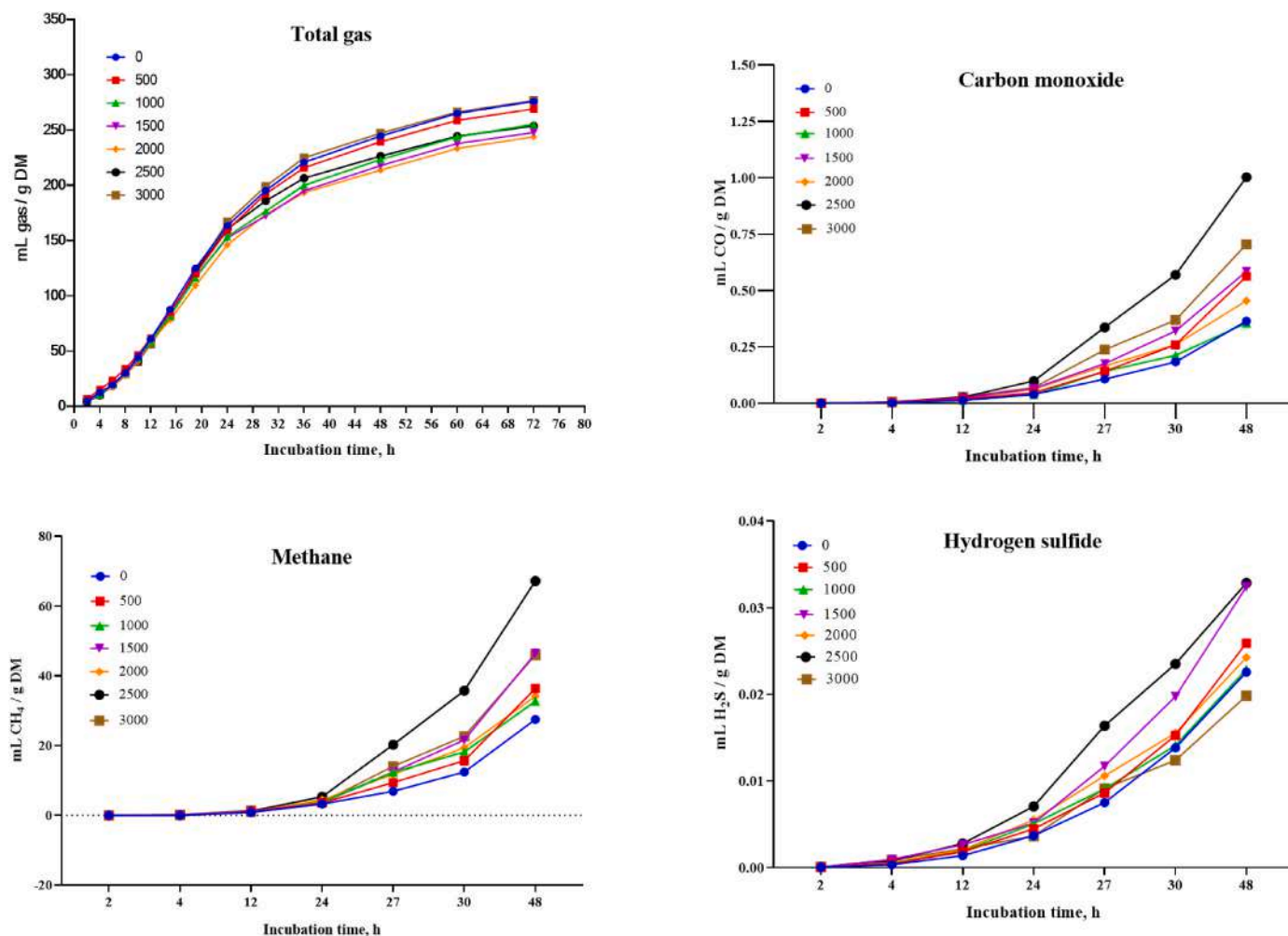


Fig. 1. Ruminal total gas production, methane, carbon monoxide, and hydrogen sulfide production (mL/g DM) of high concentrate diet with different levels (0, 500, 1000, 1500, 2000, 2500, 3000 mg GAA/kg dietary DM) using ruminal contents of sheep as a source of inoculum.

Table 3

Effect of guanidino acetic acid (GAA) at different levels (0, 500, 1000, 1500, 2000, 2500, 3000 mg GAA/kg dietary DM) on ruminal CO and H₂S production and kinetics of high concentrate diet using ruminal contents of sheep as a source of inoculum.

Item		Dosis of GAA, mg/kg DM							SEM ^b	P value	
		0	500	1000	1500	2000	2500	3000		Linear	Quadratic
CO kinetics ^a	<i>b</i>	0.81	1.22	1.25	0.52	0.81	2.87	3.29	0.986	0.0688	0.1969
	<i>c</i>	0.0033	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0013	0.1558	0.1709
	<i>Lag</i>	5.37	6.77	6.03	6.33	4.40	7.65	6.68	1.123	0.5066	0.7319
CO (mL/g DM)	6 h	0.002	0.003	0.003	0.004	0.003	0.003	0.007	0.002	0.1050	0.4512
	24 h	0.04	0.05	0.04	0.07	0.06	0.10	0.07	0.014	0.0115	0.8856
	48 h	0.36	0.56	0.36	0.59	0.45	1.00	0.71	0.202	0.0826	0.7596
H ₂ S kinetics ^a	<i>b</i>	0.01	0.01	0.17	0.02	0.01	0.02	0.01	0.059	0.7071	0.3719
	<i>c</i>	0.000	0.000	0.000	0.007	0.007	0.000	0.000	0.004	0.7289	0.1750
	<i>Lag</i>	7.84	8.27	4.76	5.75	5.40	7.34	6.69	1.719	0.6138	0.2442
H ₂ S (mL/g DM)	6 h	0.0003	0.0005	0.0005	0.0006	0.0006	0.0008	0.0009	0.0003	0.1536	0.7758
	24 h	0.004	0.004	0.005	0.005	0.006	0.007	0.004	0.001	0.4337	0.2144
	48 h	0.023	0.026	0.023	0.032	0.024	0.033	0.020	0.007	0.8555	0.3871

^a *b* = asymptotic CO or H₂S production (mL/g DM); *c* = rate of CO or H₂S production (mL/h); *Lag* = initial delay before CO or H₂S production begins (hour).

^b SEM = standard error of the mean.

GAA/kg dietary DM) on ruminal fermentation profile and CH₄ conversion efficiency of high concentrate diet using ruminal contents of sheep as a source of inoculum. The inclusion of GAA did not significantly affect the apparent digestibility and degradability kinetics of DM under consideration, except for D12. At D12, similar values were obtained for

GAA0, GAA0.5, GAA1, GAA1.5, and GAA2, except that the value obtained for GAA2 was significantly higher than those of GAA2.5 and GAA3.

Table 4

Effect of guanidinoacetic acid (GAA) at different levels (0, 500, 1000, 1500, 2000, 2500, 3000 mg GAA/kg dietary DM) on ruminal fermentation profile of high concentrate diet using ruminal contents of sheep as a source of inoculum.

Item	Dosis of GAA, mg/kg diet							SEM ^b	P value	
	0	500	1000	1500	2000	2500	3000		Linear	Quadratic
Fermentation profile										
pH	7.11	7.20	7.16	7.30	7.15	7.17	7.21	0.081	0.8034	0.6809
DMD	85.42	85.56	85.52	86.32	85.53	85.81	86.36	0.248	0.0196	0.8224
DOM	55.62	54.78	53.54	53.54	52.05	55.05	56.33	1.631	0.8939	0.0629
SCFA	3.85	3.75	3.61	3.59	3.43	3.78	3.93	0.190	0.9112	0.0629
ME	9.47	9.34	9.15	9.12	8.92	9.37	9.57	0.250	0.9139	0.0647

^a pH = ruminal pH; DMD = dry matter degradability (%); DOM = digestible organic matter (%); SCFA = short-chain fatty acids (mmol/g DM); ME = the metabolizable energy (MJ/kg DM 24 hours).

^b SEM = standard error of the mean.

Table 5

Effect of guanidinoacetic acid (GAA) at different levels (0, 500, 1000, 1500, 2000, 2500, 3000 mg GAA/kg dietary DM) on apparent digestibility and degradability kinetics of dry matter (DM) at different hours of incubation of high concentrate diet using ruminal contents of sheep as a source of inoculum.

Item	Dosis of GAA, mg/kgDM							SEM ^a	P value	
	0	500	1000	1500	2000	2500	3000		Linear	Quadratic
Digestibility (D), % DM										
D06	36.22	34.23	35.97	34.83	38.11	38.35	36.53	1.280	0.1170	0.8533
D12	47.18 ^{a,b}	46.05 ^{a,b}	46.72 ^{a,b}	45.96 ^{a,b}	48.30 ^a	45.14 ^b	45.27 ^b	0.605	0.0839	0.2521
D24	59.42	65.23	58.39	62.92	59.71	64.42	64.48	1.899	0.1609	0.4503
D48	78.66	78.91	74.61	78.90	79.08	79.27	77.74	1.145	0.6956	0.6205
Degradability kinetics ^b										
a	24.08	25.00	24.82	24.03	24.70	25.34	23.97	0.899	0.9627	0.5986
b	70.72	70.68	62.33	72.36	70.11	69.66	66.85	3.981	0.7850	0.9772
a+b	94.81	95.67	87.15	96.39	94.82	95.01	90.82	4.526	0.8177	0.9371
c	0.03	0.03	0.04	0.03	0.03	0.04	0.04	0.004	0.4011	0.8700
DEb	29.34	29.17	28.76	29.54	29.35	29.42	29.44	0.941	0.7857	0.8722

^a SEM = standard error of the mean.

^b a = soluble and completely degradable DM (g/100 g DM), b = insoluble fraction but potentially degradable DM (g/100 g substrate), a+b = is the DM potentially degradable (g/100 g DM), c = the rate constant for the degradation of b fraction (%/hour), DEb = effective degradability of the b fraction (g/100 g DM).

3.5. Crude protein digestibility and degradability

The effect of GAA at different levels (0, 500, 1000, 1500, 2000, 2500, 3000 mg GAA/kg dietary DM) on apparent digestibility and degradability kinetics of crude protein (CP) at different hours of incubation of high concentrate diet using ruminal contents of sheep as a source of inoculum is as shown on Table 6. On D06, the inclusion of GAA at 0, 500, 1000, 1500, 2000, and 3000 had similar effects, except that GAA at 2500 was significantly higher than the value obtained for 0 and 1500

inclusions. The inclusion of GAA at 2000 mg was higher than that at 3000 compared to others at D12. At D48, GAA at 500 mg had higher crude protein digestibility than others. The soluble and completely degradable crude protein was similar with GAA inclusion at 0, 500, 1000, 2000, 2500, and 3000, except that GAA1.5 was significantly lower than GAA0, GAA2, and GAA2.5. A significant quadratic relationship was observed between protein digestibility at 12 hours and the inclusion of GAA, where the dose that maximized digestibility was 1437.13 mg/kg. A quadratic relationship existed between the soluble and completely

Table 6

Effect of guanidinoacetic acid (GAA) at different levels (0, 500, 1000, 1500, 2000, 2500, 3000 mg GAA/kg dietary DM) on apparent digestibility and degradability kinetics of crude protein (CP) at different hours of incubation of high concentrate diet using ruminal contents of sheep as a source of inoculum.

Variables	Doses of GAA, mg/kg DM							SEM ^a	P-value	
	0	500	1000	1500	2000	2500	3000		Linear	Quadratic
Digestibility (D), % crude protein										
D06	40.84 ^b	42.14 ^{a,b}	41.96 ^{a,b}	38.34 ^b	44.06 ^{a,b}	47.37 ^a	42.76 ^{a,b}	1.187	0.0112	0.5527
D12	49.08 ^{a,b}	50.72 ^{a,b}	51.42 ^{a,b}	51.88 ^{a,b}	53.19 ^a	49.78 ^{a,b}	48.2 ^b	1.001	0.6141	0.0019
D24	56.32	63.00	55.86	65.70	58.22	62.94	67.06	2.792	0.0352	0.6495
D48	75.94 ^{cd}	99.86 ^a	67.81 ^d	83.05 ^{bc}	90.33 ^b	89.41 ^b	79.16 ^c	1.711	0.2339	0.0671
Degradability kinetics ^b										
a	34.25 ^a	30.37 ^{a,b}	28.76 ^{a,b}	26.57 ^b	34.92 ^a	34.38 ^a	32.76 ^{a,b}	1.307	0.183	0.007
b	60.71 ^a	66.57 ^a	45.10 ^b	62.28 ^a	62.24 ^a	59.69 ^a	58.51 ^{a,b}	2.970	0.840	0.374
a+b	94.96 ^a	96.95 ^a	73.86 ^b	88.84 ^{a,b}	97.16 ^a	94.06 ^a	91.27 ^a	3.433	0.727	0.066
c	0.03 ^{a,b}	0.03 ^{a,b}	0.06 ^a	0.04 ^{a,b}	0.02 ^b	0.02 ^b	0.03 ^{a,b}	0.006	0.446	0.112
DEb	25.19 ^{a,b}	28.14 ^a	23.09 ^b	28.93 ^a	26.28 ^{a,b}	26.12 ^{a,b}	24.77 ^{a,b}	0.956	0.684	0.133

^a SEM = standard error of the mean.

^b a = soluble and completely degradable CP (g/100 g CP), b = insoluble fraction but potentially degradable CP (g/100 g substrate), a+b = is the CP potentially degradable (g/100 g CP), c = the rate constant for the degradation of b fraction (%/hour), DEb = effective degradability of the b fraction (g/100 g CP).

degradable crude protein and GAA inclusion with the dose of GAA that minimized the protein degradation situated in 1305.56 mg/kg. The insoluble fraction but potentially degradable CP for GAA1 was lower than other inclusions except for GAA3, which was similar. The GAA1 and GAA1.5 had similar values, except that GAA1 had potentially degradable CP significantly lower than others. The rate constant for the degradation of the insoluble fraction but potentially degradable crude protein was significantly higher ($P < 0.05$) with GAA1 compared to GAA2 and GAA2.5. The effective degradation of the insoluble fraction but potentially degradable CP was significantly lower with the inclusion of GAA at 1000 compared with inclusion at 500 and 1500.

4. Discussion

4.1. Ruminant total gas and CH₄ production kinetics

Total gas production and the rate of gas production have been noted as benchmarks to measure the degradability of feed and the functionality and adaptability of microbial communities to substrate diets [43]. After the 6 hours of fermentation, the increase in the total gas production with GAA0.5 inclusion in the diet than at a higher dose (2000) could be attributed to the stimulatory effect of the bioactive compounds of GAA on the microbial communities at that dose, which increased microbial access and degradation of the feed compared to an inhibitory effect with an increase in dosage and its attendant decreased microbial access and feed degradation. This agrees with the findings of Alvarado-Ramírez et al. [44], which reported that a higher GAA dosage decreased total gas production, production rate of gas production, and asymptotic gas. The mechanism behind the decreased gas production is attributed to the proportion of short-chain fatty acids (SCFA) that GAA favours, which is propionate, and this occasioned the lowered gas production in comparison to that from acetate and butyrate [45,46]. The lower dose (GAA0.5) effect could also be attributed to the fact that GAA might have increased the activity of fibrinolytic enzymes, alpha-amylase, and protease [45], as well as the populations of microbial communities that favoured degradations of the substrate and in turn elicited high gas production.

Lower CH₄ generation by GAA0.5 implied increased feed degradation efficiency, considering that this was accompanied by increased gas production and, by extension, feed degradation. This means the amount of energy that could have been lost in the form of CH₄ could be channeled for productive purposes with a reduced impact on the environment. The high CH₄ production rate adduced to the higher additive dose (GAA2.5) could be because of creating an environment that favoured the quick adhesion and colonization of the substrate by methanogens [47]. This implies the stimulation of the CH₄-producing microbes, resulting in an increased rate of CH₄ generation by increased GAA dosage.

The quadratic relationship between doses of GAA and asymptotic total gas agrees with those of the Agricultural Research Council [44], which reported that adding GAA to the diet elicited a decrease in the asymptotic production of GP. With the increase in GAA doses, there was a subsequent decrease in the asymptotic values, except at GAA, which was 2500 and 3000 mg. A similar trend was seen at 48 hours of gas production, where an increase in GAA doses decreased gas production. The reason for this could be that GAA at higher doses perhaps does not favour enzymatic activity and increase in the populations of fibrolytic bacteria hence the lowering of gas volumes at 48 hours [26].

This study's lag phase of total gas kinetics showed linearity with GAA doses. There was an increase in the lag phase as the dosage of GAA increased. This effect can be attributed to the time that ruminal microorganisms are required to adapt to the presence of GAA, as it is susceptible to degradability when not rumen-protected, and it could be a source of nutrients (energy and nitrogen) for microbes to synthesize their proteins [45]. However, a quadratic relationship in the lag also played out with increased GAA doses, eliciting a decrease in adhesion and colonization of substrates. This infers that the low doses had a

stimulatory effect on the microbes, allowing them to quickly adapt to the diet and elicit a rapid fermentation process.

The high production of CH₄ with the inclusion of GAA at 2500 can be attributed to the higher total gas production that was observed since feeds that present high total gas production generally also show a higher production of gas CH₄ [43]. In addition, it is possible that this dose of GAA caused an inhibitory effect on sulfur-reducing bacteria, and apparently this metabolic pathway could not function as an alternate H₂ sink [26].

4.2. Ruminant CO and H₂S production and kinetics

Several factors are noted to influence CO production, among which are microbial activity and the fermentative capacity of the ruminal microbe communities, as the type of degraded nutritive constituents [44]. Carbon monoxide has been described as an intermediate product of DM degradation; hence its production is associated with incomplete feed degradation or lower microbial activity in the rumen [48]. However, GAA inclusion barely influenced the CO and H₂S production of carbohydrate digestion of the substrate.

The linear relationship showed that with an increase in GAA doses, CO production increased, and this agrees with the report Vazquez-Mendoza et al. [26]. From our findings, it appears the production of CO at GAA2.5 was positively related to the production of CH₄, which may show that methanogens maintained a synergy with other microorganisms that produce CO, since, in the presence of water, acetogens, and methanogens oxidize CO to form CO₂ and H₂ and subsequently produce CH₄ [49,50]. Increased GAA inclusion numerically increased H₂S production with increased inclusion of GAA at 6 hours, and it appears that the inclusion of GAA caused an inhibitory effect or an unfavorable environment for the activity of Sulfur sulfur-reducing bacteria [26]. The dose at 2500 of GAA appeared to have elicited higher values for CO and H₂S, which implies that the dose had a stimulatory role on the microbial communities that influenced gas production.

4.3. Ruminant fermentation profile

An increase in the dosages of GAA increased DMD, and this agrees with the findings of Vazquez-Mendoza et al. [26]. It has been reported that the inclusion of GAA in the diet increases the rumen microbial population and the degradability of substrate nutrients [22,51,52] through its stimulatory effect.

The pH of this study was in the range of 7.11–7.30, which was alkaline hence the reason for the low production of H₂S could suffice. The production of H₂S has been noted to require an acidic rumen environment, preferably with a pH range of 5.5–6.5 [53].

4.4. Dry matter digestibility and degradability

It has been reported that in beef cattle, the addition of 0.03–0.40 % (on a DM basis) GAA in the diet increases daily weight gain and dry matter digestibility [22,51]. Moreover, the inconsistent DM digestibility observed in this study was likely related to enhanced enzyme activities and bacterial populations [52] also, GAA addition at an appropriate concentration improves fiber digestibility [24], which could have been responsible for higher digestibility on D12 with GAA2 compared to GAA2.5 and GAA3.

4.5. Crude protein digestibility and degradability

The increase in CP digestibility of GAA2.5 at D06 compared to the control and GAA1.5 could be attributed to the direct impact of the bioactive compound of GAA on the feed within the time frame. This is consistent with Li et al. [22] who reported that higher GAA inclusion resulted in higher CP digestibility with a linear relationship between the digestibility of CP and other nutrients with increased additions of GAA.

The altered response of CP digestibility was indicative that CP degradation in the rumen was stimulated by dietary GAA supplementation [54].

Adding higher doses of GAA across the digestibility (D06 – D48) did not explicitly show better results, which may be related to methyl donor limitation [54] as there appears to be an inversely proportional relationship between GAA inclusion and CP digestibility with time. As the CP digestibility increases, the dose level reduces with increased incubation duration. As observed at D06, GAA2.5 had better digestibility, but with increased incubation duration (from D12 to D48), it improved with reduced inclusion levels (GAA2 to GAA0.5). The increased digestibility on D06 by GAA2.5 could be attributed to the initial stimulatory effect of the sufficiently dosed bioactive components of GAA on the microbial community within a short time, which may become toxic with increased duration of incubation. In contrast, the increased digestibility at D48 by GAA0.5 was more effective in the long run due to a more sustainable stimulatory effect.

Soluble protein is assumed to be immediately available for utilization by rumen microbes and chemically defined as protein fractions dissolved in a borate phosphate buffer [55]. Insoluble and potentially degradable protein is not as immediately available for utilization as soluble protein but is fractionally degraded with time. Not all potentially degradable proteins may be rumen-degraded due to lignin-bound proteins, passage rates, and feeding management [56]. The higher rate constant could be attributed to rapid degradability [57] caused by the stimulatory effects of the additive at that dosage [58]. Moreover, the dosage might have created an environment that favoured the adhesion and colonization of the insoluble fraction but potentially degradable CP by microbes thereby increasing the rate constant [49]. The rate and extent of protein degradability of different feed ingredients varies considerably with protein source and animal species, which ultimately determines the incorporation of feed ingredients in the diets of ruminants [59]. It is suggested that there should be enough soluble, readily fermentable protein to support microbial growth and fermentation in the rumen for optimum utilization of roughages and a source of less fermentable protein, which can pass directly to the abomasum and small intestine for a normal proteolytic digestion and absorption process for optimal performance in high-producing ruminants [56].

From the preceding, it can be inferred that the varied results obtained for the degradability kinetics could be attributed to the stimulatory role or otherwise of the varied level of GAA inclusion on the microbial community, effect on feed, and the duration of incubation.

5. Conclusions

This study examined the effects of GAA supplementation on *in vitro* ruminal fermentation, greenhouse gas production, and crude protein (CP) digestibility in fattened sheep. GAA0.5 improved total gas production without significantly increasing CH₄ emissions, while higher doses increased CH₄, CO, and H₂S production. GAA's impact on CP digestibility varied with incubation time. The longer the duration of incubation (6–48 hours), the lower the dosage of GAA required for increased CP digestibility. GAA1 exhibited the highest rate constant for the insoluble but potentially degradable CP fraction degradation. These findings suggest that GAA can modulate ruminal protein degradation kinetics, potentially improving protein utilization efficiency in sheep. However, the *in vitro* nature of this study limits its generalizability. Future research should focus on *in vivo* validation, long-term effects on rumen microbial populations, and interactions with different dietary protein sources. While GAA shows promise for enhancing ruminal fermentation and protein degradation at lower doses, its application in ruminant nutrition requires careful consideration to balance benefits against increased greenhouse gas emissions and optimize protein utilization across different ruminal digestion time points.

CRedit authorship contribution statement

Oscar Vicente Vazquez-Mendoza: Writing – original draft, Validation, Formal analysis, Conceptualization. **Mona M.M.Y. Elghandour:** Investigation and data validation. **Maria A. Mariezcurrena Berasain:** Investigation and data validation. **Udoh A. Inyang:** Wring the draft. **Akaninyene Jack:** Wring the draft. **Maximilian Lackner:** Writing – review & editing. **Abdelfattah Z.M. Salem:** Supervision, Funding acquisition, data validation, Writing – review & editing.

Ethical statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to, and the appropriate ethical review committee approval has been received. The authors confirm that they have followed local and EU standards for the protection of animals used for scientific purposes.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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