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The Anti-Methanogenic Activity of Lovastatin in Batch Cultures Using Rumen Inoculum from Sheep, Goats, and Cows

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Abstract: Enteric methanogenesis in ruminants is identified as one of the primary anthropogenic sources of total atmospheric methane. Recent evidence suggests that rumen methanogenesis is significantly suppressed by lovastatin. Nevertheless, it has not been reported whether the methane reduction by lovastatin depends on ruminant livestock type, nor has fiber degradability been examined. The current research aimed to analyze the in vitro effect of lovastatin on the major fermentation end-products, gas production (GP) kinetics, and fiber degradation of a forage-based diet using rumen inoculum from sheep, goats, and cows. The experiment was conducted as a 3 × 3 factorial arrangement of treatments (dose of lovastatin: 0, 80, and 160 mg/L and three inoculum sources) in a completely randomized design. The results suggested that lovastatin did not affect the GP kinetics parameters. The anti-methanogenic properties of lovastatin were variable depending on dose and inoculum source. Lovastatin demonstrated a superior methane-lowering effect in sheep rumen inoculum compared with goat and cow inocula. The total volatile fatty acid (VFA) production was unaffected by lovastatin, but changes in acetate and valerate proportions were registered. Remarkably, lovastatin decreased the NH3-N concentration with goat and sheep inocula and the in vitro neutral fiber detergent (NDF) degradation for all inoculum sources.

Keywords: fermentation; feed additive; fiber degradability; gas production kinetics; livestock; methane

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1. Introduction

Enteric methanogenesis in ruminants is considered one of the largest anthropogenic sources of the total atmospheric methane budget and it is increasing in most regions of the world [1,2]. For instance, from 2000 to 2012, a global emission of 1.6 Tg CH_4/y was estimated to be much higher than in the 1990–1999 period (0.33 Tg CH_4/y) [3]. This fast-rate discharge of CH_4 contributes considerably to global warming and causes atmospheric particulate concentration changes due to alterations in methane sinks, e.g., oxidation of CH_4 by hydroxyl radicals in the troposphere [4]. This issue has become one of the significant environmental impacts of livestock [5–7]. In addition, ruminal production of CH_4 is a loss of gross energy intake in ruminants [8]. Hence, reducing methane emissions from ruminants is an increasingly important area of research and application [9–11].

Recent evidence suggests that lovastatin addition decreased rumen methane production (see the review by Ábrego-García et al. [12]). The latter phenomenon is related to the biosynthesis of membrane lipids in methanogenic archaea [13]. These membranes contain isoprenoid-based lipids (archaeol and caldarchaeol) which are synthesized via the mevalonate pathway where the enzyme hydroxymethylglutaryl-CoA (HMG-CoA) reductase catalyzes the conversion of HMG-CoA to mevalonate [14]. On the other side, lovastatin is a competitive inhibitor of HMG-CoA reductase and thus might disrupt the archaea cell

membrane within the rumen ecosystem [12,13]. The in vitro anti-methanogenic effect of lovastatin has been examined by other researchers [15–17]. However, none of these studies investigated whether lovastatin's methane reduction depends on ruminant livestock type (sheep, goats, and cows), nor has fiber degradability been examined, despite the fact that inoculum from ruminants has dissimilar fermentation profiles regardless of the dietary composition [18].

On the other hand, hydrogenotrophic methanogenesis is the major pathway for hydrogen disposal during the fermentation of feed by the rumen microbiota [19,20]. If this pathway is suppressed, metabolic hydrogen may be elevated, inhibiting enzymatic activity. For instance, the hydrogensome (organelle in anaerobic fungi) regulates an internal redox equilibrium by releasing molecular hydrogen (H_2), but the H_2 accumulation inhibits the anaerobic fungal metabolism [21]. Also, NADH ferredoxin oxidoreductase, the enzyme involved in H_2 formation in bacteria, could be repressed under these conditions [20–22]. Consequently, rumen fermentation might be altered by decreasing fiber degradation [23–25]. This study aimed to analyze the effects of lovastatin on the major fermentation end-products, GP kinetics, and fiber degradability in batch cultures using rumen inoculum from sheep, goats, and cows.

2. Materials and Methods

2.1. Experimental Design and Treatments

Two in vitro trials were performed concurrently to evaluate the effect of lovastatin on ruminal fermentation characteristics and degradability using inoculum from sheep, goats, and cows fed an identical diet. The first trial was conducted to determine CH_4 production, ammonia nitrogen, and the VFA profile. In trial two, the parameters of GP kinetics and degradability were determined. The experiment was performed using a 3 × 3 factorial design with three doses of lovastatin (0, 80, and 160 mg/L) and three inoculum sources (sheep, goats, and cows). The experiment lasted for three runs of 24 and 74 h, each run with nine treatments and blank (no substrate), which were performed in triplicate (analytical replicates). The values of the replicates within each run were averaged and used as experimental replicates for the statistical analysis.

To provide the desired concentration for each treatment, two stocks of lovastatin (powder, >97% purity, FERMIC, SA de CV, Mexico City, Mexico) were prepared by dissolving 72 and 144 mg in 10 mL of 98% ethanol. Immediately, 1 mL of each stock was added into 90 mL of a mixture of reduced mineral solution:rumen fluid to achieve 80 (low dose) and 160 mg/L (high dose). The control without lovastatin (0 mg/L) received the same amount of ethanol.

2.2. Experimental Procedures

Inoculum donor animals were housed at the experimental farm of the Universidad Autonoma Chapingo, Estado de Mexico, Mexico. Rumen fluid inocula were collected from two adult female animals (sheep, goats, and cows). All animals were adapted to a diet with a forage-to-concentrate ratio of 70:30 for a 15 d period (Table 1). The rumen fluid was taken two hours after the morning feeding via an esophageal tube according to the standard length of tubes for the ruminant species [26] and transferred to the laboratory in pre-warmed thermal flasks (39 °C). The animal care was conducted in compliance with the official Mexican standards [27]. The experimental diet was analyzed for dry matter (DM), ash, ether extract, and crude protein using the following AOAC methods [28]: 934.15, 942.05, 920.39, and 976.06, respectively. Also, the fractions of NDF and acid detergent fiber were determined according to Van Soest et al. [29].

Table 1. Ingredients and chemical composition of the experimental diet.

Ingredient	g/kg DM
Maize silage	490
Oat straw	210
Ground corn	210
Soybean meal	90
Chemical composition	
Crude protein	113.6
Ether extract	42.2
Ash	71.9
Neutral detergent fiber	461.8
Acid detergent fiber	262.9

2.3. In Vitro 24 h Incubation

Amber serum bottles (125 mL) were used, and the substrate (Table 1) was dried at 55 °C for 24 h, ground through a 1 mm screen, and 0.5 g of sample was placed into the bottles. The reduced mineral solution was prepared according to [30] as follows (concentrations in g/L, unless otherwise stated): K_2HPO_4 0.45, KH_2PO_4 0.45, $(NH_4)_2SO_4$ 0.45, NaCl 0.90, $MgSO_4$ 0.18, $CaCl_2$ 0.12, Na_2CO_3 4, 20 mL/L of a cysteine sulfide solution (2.5 g L-cysteine in 15 mL 2 N NaOH + 2.5 g Na_2S-$9H_2O$ in 100 mL distilled water), and 2 drops of resazurin (0.1%). The rumen fluid was filtered with eight layers of surgical gauze and diluted (1:9 *v*/*v*) with the reduced mineral solution, and 90 mL of this mixture was anaerobically dispensed into the serum bottles. Finally, lovastatin was added to the bottles at 0, 80, and 160 mg/L. A water bath was used to incubate the bottles at 39 °C for 24 h.

2.3.1. Analysis of Gas Profile

The CH_4 production was sampled at 6, 12, 18, and 24 h and determined by a gas chromatograph (Gow-Mac, Model 350, Bethlehem, PA, USA) equipped with a TCD detector and a packed column with Molecular Sieve 5A [31]. A calibration curve was generated using methane as a standard (99% purity). Then, the total GP was determined with a glass syringe (50 mL). The CO_2 production and global warming indicator (GWI = mL CO_2/g DM + (mL CH_4/g DM) × 23/100) were calculated as reported by [32].

2.3.2. Determination of Volatile Fatty Acids and Ammonia Nitrogen Concentrations

The samples for VFA were obtained at 24 h of incubation and were prepared as described elsewhere [31]. A gas chromatograph (PerkinElmer, auto system, Norwalk, CT, USA) equipped with an FID detector and a capillary column (Model ZB-FFAP, Phenomenex, Torrance, CA, USA) was used to determine the total VFA, including the production of acetate, propionate, butyrate, isovalerate, and valerate [16]. Ammonia nitrogen concentration was determined according to McCullough [33]. In brief, 1 mL of sample was mixed with 9 mL of sodium hypochlorite (2.5% *v*/*v*) and centrifuged at 6000 rpm for 10 min. Then, 20 µL of this mixture, 1 mL of phenol, and 1 mL of sodium phenol-nitroprusside solution were added to a 10 mL test tube. The latter mixture was diluted with 5 mL of distilled water and incubated in a water bath at 37 °C for 30 min to read the absorbance in a spectrophotometer (Spectronic Instruments, Inc., Rochester, NY, USA) at 630 nm.

2.4. In Vitro 72 h Incubation

A second set of batch cultures under the described methodology and experimental design was processed to determine the parameters of GP kinetics and degradability (see Section 2.3). A pressure transducer (pressure gauge, 0–1 kg/cm^2, Metron, 6310, Ciudad de México, Mexico) was used to measure the pressure in the headspace of serum bottles before venting at 0, 2, 4, 6, 8, 12, 16, 20, 24, 30, 36, 42, 48, 60, and 72 h. Then, these values were transformed into gas volume [34] and used to estimate the maximum volume (mV, mL/g), delay phase (L, mL/h), and rate (S, h) of GP parameters with the nonlinear regression

procedure (NLIN) of SAS 9.4 software and the following logistic model: V = mV/1 + e [2 − 4 × S (t − L)] as described by Pitt et al. [35], where V = gas volume over time (t), mV = maximum gas volume, S = the GP rate, and L = the duration of the delay phase. Additionally, the gas volume was fractioned to estimate the rapid fermentation fraction (RFF, 0–8 h), medium fermentation fraction (MFF, 8–24 h), slow fermentation fraction (SFF, 24–72 h), and the total fermentable fraction (TFF, 0–72 h) of the incubated substrate based on the recommendations of Miranda-Romero et al. [34]. The in vitro NDF degradation was determined gravimetrically by the difference in the substrate before and after 72 h of incubation [36,37].

2.5. Statistical Analysis

This experiment was conducted as a 3 × 3 factorial arrangement of treatments (lovastatin dose × inoculum source) in a completely randomized design for a total of 9 treatments with three replications using the following model:

$$Yij = \mu + Ai + Bj + (A \times B)ij + eij$$

where Yij = observations, μ = general mean, A = fixed effect of dose of lovastatin, B = fixed effect of inoculum source, (A × B) = interaction of lovastatin dose and inoculum source, and eij = experimental error.

A Tukey's post hoc test was performed to determine the significant differences between treatments at $p < 0.05$. All data were analyzed using the GLM procedure of SAS 9.4 (SAS, Inst. Inc, Cary, NC, USA).

3. Results and Discussion

3.1. Gas Production Kinetics and Methane Production

The results of GP kinetics, methane production, and carbon dioxide of a forage-based diet supplemented with lovastatin are shown in Table 2. The mV (mL/g), S (mL/h), and L (h) parameters were not affected significantly by either lovastatin dose for each source of inoculum (see also Figure 1). This could be attributed to specific inhibition of the methanogenic archaea by lovastatin among rumen microorganisms [12]. Another possible explanation that has received less attention is related to the degradation of lovastatin in the rumen. For instance, Zhao et al. [38] reported that the degradation of lovastatin by human gut microbiota is ca. 30% after fermenting for 24 h, but we speculated that the degradation of lovastatin is highest in the rumen. In this sense, the kinetics of gas production parameters were assessed after 72 h, so, two-thirds of the incubation period could be unaffected by lovastatin, thus minor changes in these parameters were expected. It has been stated that lovastatin did not affect the mV [16,17]. Concerning the effect of lovastatin on S and L parameters, these have not been reported previously. Nonetheless, Osorio-Teran et al. [39] showed similar trends: mV (299 mL/g), S (0.035 mL/h), and L (7.72 h) of the in vitro GP kinetics of a diet containing 40% grain and inoculated with sheep rumen fluid, which supports the results of this investigation.

Table 2. In vitro gas kinetics and methane production of a forage-based diet added with lovastatin using three sources of rumen inoculum.

Item	Sheep			Goats			Cows			SEM	*p* Values		
	Con	L	H	Con	L	H	Con	L	H		I	D	I × D
mV, mL/g	277.7	274.5	251.0	263.6	246.3	250.2	284.1	290.4	274.3	28.37	0.181	0.557	0.926
S, mL/h	0.036	0.034	0.031	0.034	0.036	0.033	0.034	0.035	0.032	0.002	0.896	0.117	0.553
L, h	1.95	1.49	1.16	1.71	1.90	2.1	1.95	1.61	1.36	0.71	0.593	0.709	0.735
CH_4, mL/g DM	44.9 ^{a}	31.2 ^{ab}	20.1 ^{bcd}	27.4 ^{b}	19.2 ^{d}	16.7 ^{cd}	30.2 ^{ab}	18.8 ^{bcd}	23.72 ^{bc}	4.40	0.0002	<0.0001	0.011
CO_2, mL/g DM	216.4 ^{b}	242.6 ^{ab}	205.4 ^{b}	233 ^{c}	225.5 ^{c}	236 ^{c}	270.6 ^{ab}	290.9 ^{a}	267.9 ^{ab}	19.44	<0.0001	0.077	0.765
GWI, CO_2e	1249 ^{a}	961 ^{ab}	575 ^{cde}	724 ^{bc}	537 ^{e}	425 ^{de}	965 ^{ab}	723 ^{bcd}	813 ^{bc}	97.75	<0.0001	<0.0001	0.007

a–e Means in the same row with different superscripts differ ($p < 0.05$). SEM, standard error of the mean; Con, control; L, low (80 mg lovastatin/L); H, high (160 mg lovastatin/L); I, inoculum; D, dose of lovastatin; I × D, inoculum × dose of lovastatin; mV, maximum gas volume; S, rate of gas production; L, delay phase; GWI, global warming indicator.

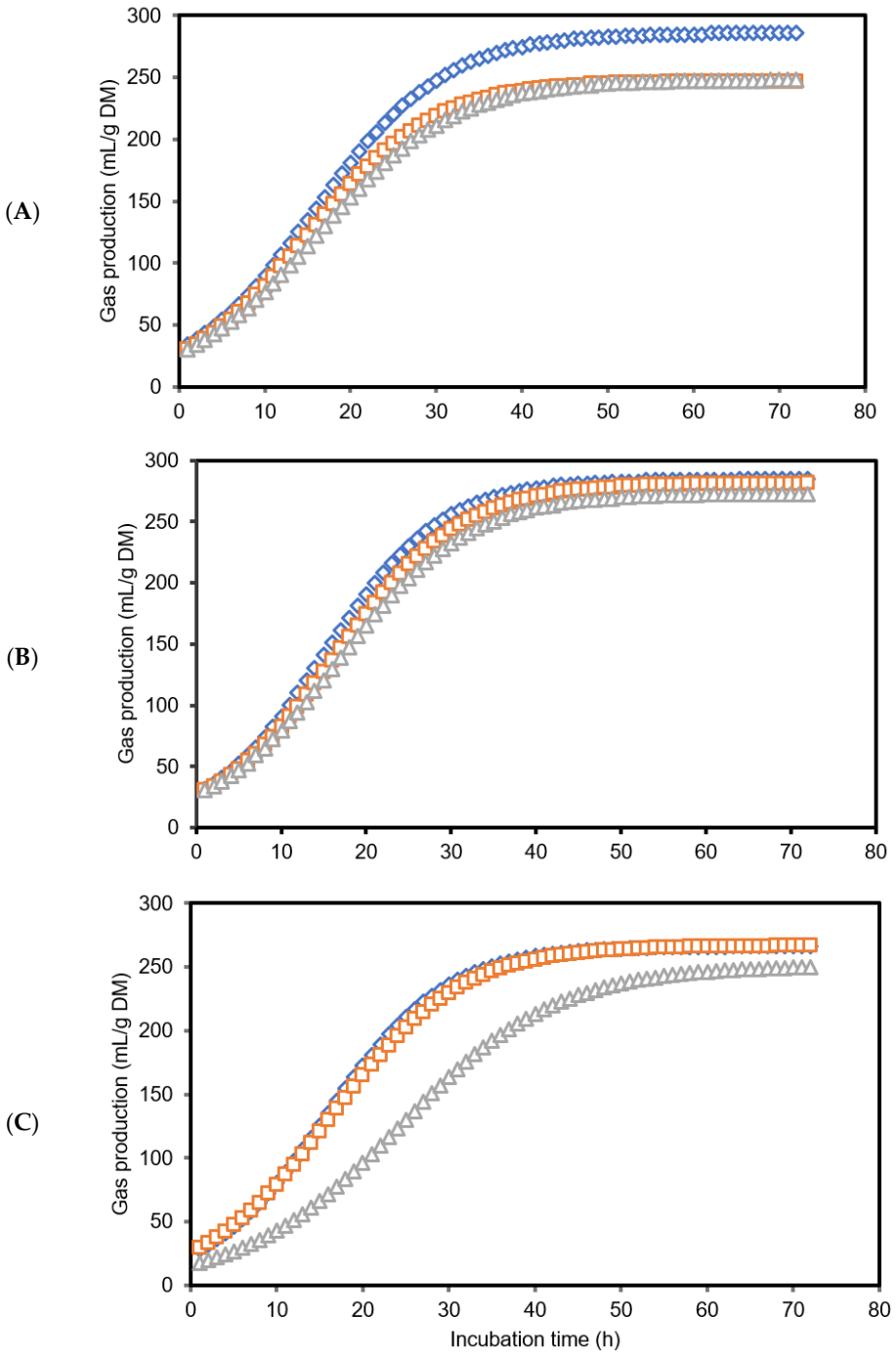

Figure 1. Effects of lovastatin on the gas production kinetics of a forage-based diet. The source of inoculum: (**A**) goats, (**B**) cows, and (**C**) sheep. The dose of lovastatin (mg/L): 0 blue rhombuses, 80 orange squares, and 160 gray triangles.

As mentioned in the Section 2, sheep, goats, and cows were adapted to the same diet, hence, variation in the microbial activity should be due mainly to the inoculum per se [40]. Regarding the methane production (mL/g DM) from small ruminant inocula after 24 h of incubation, it was found that lovastatin inhibited methanogenesis by 55% in sheep spiked with a high dose, while goats with low and high doses demonstrated inhibition of 33 and 39%, respectively.

In the inocula from cows, methanogenesis (CH_4 mL/g DM) decreased significantly by around 37% using low-dose lovastatin ($p < 0.05$). The findings of the current work are consistent with those of [16,17], who proposed that lovastatin and simvastatin decreased

rumen methane production between 35 and 27% for in vitro trials, using cattle ruminal inocula and high- and low-forage diets, respectively. Differences in methane production must be related to the higher carbohydrate degradation to produce methane from microorganisms in the rumen inoculum of sheep than in goat and cow inocula under comparable dietary conditions [41]. In addition, the variation of methane mitigation must be due to the archaeal abundance. It is widely reported that the relative abundance of dominant rumen methanogens (*Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium*) in sheep is higher than in goats and cattle [42] and, as indicated above, lovastatin is an inhibitor of archaea lipid membranes [12,13]. Also, a significant lovastatin dose x inoculum source interaction for the methane variable was observed ($p = 0.011$), suggesting that the anti-methanogenic property of lovastatin was variable depending on the dose and inoculum source utilization. To the best of our knowledge, the current study is the first to report the in vitro effect of lovastatin on rumen methanogenesis across inocula from three ruminant species.

Overall, compared with the control, the amount of CO_2 (mL/g DM) was increased with a low dose in sheep and cows ($p < 0.05$), and no significant lovastatin dose x inoculum interaction was observed ($p > 0.560$). As stated, hydrogenotrophic methanogenesis ($CO_2 + 4[2H] = CH_4 + 2H_2O$) is the main pathway for hydrogen disposal during rumen fermentation [20,43]. It seems that once the CO_2 production rate was higher than CO_2 utilization, the latter was accumulated in the headspace, which agrees with similar in vitro trials where plant bioactive extracts were evaluated for the rumen methanogenesis [44]. As expected, the GWI was lowered by lovastatin treatments ($p < 0.0001$) in each source of inoculum ($p < 0.0001$), and a significant lovastatin dose x inoculum interaction was found ($p < 0.007$). Interestingly, low- and high-dose lovastatin with sheep inoculum reduced ca. 23 and 53% of the GWI, i.e., the impact of gas emissions from the sheep on global warming was much less than goats and cows at similar doses of lovastatin. A general environmental sustainability index for greenhouse gas emissions from livestock serves as a guide to compare methane mitigation strategies and feeding systems. It would be useful to integrate this concept into animal science research for the sustainable development of livestock production [45].

3.2. In Vitro Degradability

Table 3 provides the data on the in vitro fermentation fractions and degradability of a forage-based diet. Low- and high-dose lovastatin decreased the RFF ($p < 0.05$) in cows and no significant differences were observed in sheep and goats. Overall, lovastatin treatments did not significantly affect the MFF and TFF from the diet of sheep, goat, and cow inocula. However, compared with the control, the SFF was slightly increased in sheep with low and high doses ($p < 0.05$), while it decreased in goats only with a low dose by 19% ($p < 0.05$). These fractions are related to the rumen fermentation of dietary monosaccharides (RFF and MFF) and polysaccharides (SFF) [46]. Therefore, we speculated that lovastatin did not reduce the activity of the rumen microorganisms involved in the fermentation of monosaccharides but had an unknown effect on the polysaccharide-fermenting microbes in goats [47].

As for batch trials, variability in the degradation of feeds is mainly attributed to the inoculum donor animal, concentrate composition, and type of forage [48]. Previous studies have reported that lovastatin had no effect on the in vitro DM degradation [17,49,50]. It is worth mentioning that those studies evaluated diets with forage to concentrate ratio of 50:50 as a substrate. It was also reported that lovastatin has no adverse effects on selected cellulolytic and fibrinolytic bacteria [51,52]. However, when a high-forage diet was used, we detected for all inoculum sources that the low- and/or high-dose lovastatin decreased the in vitro NDF degradation compared to the control ($p < 0.05$). The present research has demonstrated that the methanogenesis inhibition by lovastatin is related to a decrease in fiber degradation. Recent evidence suggests that in vitro gaseous H_2 increases when the flow of H_2 to CH_4 is stopped by lovastatin [16,49]. Based on those findings, we

hypothesized that fiber-degrading microbes were affected in response to accumulative metabolic hydrogen in the rumen fluid.

Table 3. Fermentation fractions and degradability of a forage-based diet added with lovastatin using three sources of rumen inoculum.

Item	Sheep			Goats			Cows			SEM	*p* Values		
	Con	L	H	Con	L	H	Con	L	H		I	D	I × D
RFF (mg/g)	123.1 ^{ab}	124.9 ^{ab}	110.2 ^{a}	187.32 ^{b}	197.8 ^{bc}	197.9 ^{bc}	214.0 ^{c}	198.49 ^{bc}	195.01 ^{bc}	38	0.0004	0.917	0.955
MFF (mg/g)	200.5	186.9	165.84	184.74	183.36	175.81	199.93	193.10	179.74	22	0.666	0.153	0.915
SFF (mg/g)	243.3 ^{ab}	268.1 ^{a}	275.4 ^{a}	212.9 ^{ab}	171.16 ^{b}	199.8 ^{ab}	199.4 ^{ab}	211.45 ^{ab}	213.15 ^{ab}	29	0.0003	0.615	0.392
TFF (mg/g)	567.98	579.16	551.20	585.02	552.40	573.54	613.39	602.92	588.71	58	0.382	0.817	0.948
IVNFD (g/kg)	541 ^{a}	433 ^{bc}	477 ^{bc}	483 ^{abc}	420 ^{bc}	403 ^{c}	548 ^{a}	499 ^{ab}	410 ^{c}	24	0.005	0.001	0.020

a–c Means in the same row with different superscripts differ ($p < 0.05$). SEM, standard error of the mean; Con, control; L, low (80 mg lovastatin/L); H, high (160 mg lovastatin/L); I, inoculum; D, dose of lovastatin; I × D, inoculum × dose of lovastatin; RFF, rapid fermentable fraction; MFF, medium fermentable fraction, SFF, slow fermentable fraction; TFF, total fermentation fraction; IVNFDD, in vitro neutral detergent fiber degradation.

3.3. Fermentation Characteristics

The total VFA production was higher for goat inoculum than for sheep and cows ($p = 0.0134$), but there was no effect for the dose of lovastatin ($p = 0.1499$; Table 4). The VFA production, kinetics of GP (Table 2), and fermentable fractions of the feed (Table 3) are associated with each other [53]. The lack of differences in these variables indicated that lovastatin did not considerably alter the fermentative pathways relating to carbohydrate utilization in the rumen ecosystem. In contrast to our study, it is reported that statins reduced the total VFA production [54,55]. Yet, in both studies, statins were obtained via solid-state fermentation by filamentous fungi. This bioprocess commonly generates post-fermented agricultural residues, which are used without pre-treatment as lovastatin carriers for rumen methane mitigation [16,54,55]. It is important to note that during this bioprocess, many organic compounds and secondary metabolites are produced [56]. As a result, the anti-methanogenic effect of the post-fermented agricultural residues as lovastatin carriers can easily be confused.

Table 4. Volatile fatty acid production and ammonia nitrogen concentration of a forage-based diet added with lovastatin using three sources of rumen inoculum.

Item	Sheep			Goats			Cows			SEM	*p* Values		
	Con	L	H	Con	L	H	Con	L	H		I	D	I × D
VFA production (mmol/24 h)													
Total VFA	3.3 ^{b}	5.2 ^{bc}	5.8 ^{bc}	8.8 ^{ab}	4.9 ^{b}	9.4 ^{ab}	2.8 ^{c}	3.9 ^{bc}	3.7 ^{bc}	0.83	0.0134	0.1499	0.2030
Acetate	2.1 ^{ab}	3.3 ^{ab}	3.8 ^{ab}	6.8 ^{ab}	3.4 ^{ab}	7.1 ^{a}	1.8 ^{b}	2.7 ^{ab}	2.5 ^{ab}	1.34	0.0079	0.1477	0.1472
Propionate	0.79	1.6	1.8	1.2	1.1	2.5	0.67	0.87	0.82	0.89	0.1922	0.1863	0.5485
Butyrate	0.43	0.20	0.19	0.63	0.52	0.37	0.15	0.25	0.26	0.14	0.0725	0.5498	0.5148
Isovalerate	ND	ND	ND	0.10	0.15	0.09	0.03	0.04	0.01	0.04	0.1445	0.3354	0.8795
Valerate	0.03 ^{b}	0.05 ^{b}	0.02 ^{b}	0.06 ^{b}	0.34 ^{a}	0.10 ^{b}	0.03 ^{b}	0.04 ^{b}	0.00 ^{b}	0.024	0.0033	0.0050	0.0171
A/P	2.6	2.1	2.2	5.5	3.0	2.9	2.7	3.6	3.2	1.34	0.3651	0.6845	0.6858
NH_3-N (mg/dL)	1.1 ^{ab}	0.6 ^{b}	0.6 ^{b}	2.2 ^{a}	0.9 ^{ab}	1.3 ^{ab}	0.3 ^{b}	0.6 ^{b}	0.7 ^{ab}	0.47	0.003	0.1541	0.1986

a–c Means in the same row with different lowercase letters showed a significant effect of inoculum (I), dose of lovastatin (D), and the interaction effect I × D ($p < 0.05$). SEM, standard error of the mean; Con, control; L, Low (80 mg lovastatin/L); H, high (160 mg lovastatin/L); VFA, volatile fatty acid; A/P, acetate/propionate ratio; ND, not detected.

The acetate production was increased by low- and high-dose lovastatin using inoculum from cows compared with the control ($p < 0.05$). This indicated that reductive acetogenesis could be a H_2 sink when in vitro methane production was partially inhibited [57]. Although some studies have reported that a decrease in acetate proportion is associated with methane mitigation by lovastatin [15,17], our results are consistent with [49] where lovastatin inhibited the production of methane while increasing acetate proportion compared with the control ($p < 0.05$) using the Rusitec system. When the low-dose lovastatin was added to the culture medium with goat inoculum, the valerate production was increased compared to the control ($p < 0.05$), and no statistical differences were observed with other treatments. Production of valerate involves the consumption of electrons during

the fermentation of feeds and decreases the total amount of molecular hydrogen, which could reduce methanogenesis [58] and explain the higher production of valerate in the goat inoculum treated with lovastatin. In the present study, propionate, butyrate, and isovalerate metabolites, as well as the acetate/propionate ratio, remain unaffected by the lovastatin addition for all inoculum sources. Accordingly, rumen metabolic hydrogen was not redirected to enhance these metabolites when lovastatin inhibited methanogenesis [20].

As far as NH_3-N concentration is concerned, significantly lower concentrations were detected in the low- and high-dose lovastatin compared with the control ($p < 0.05$) in the rumen inoculum of sheep and goats. However, NH_3-N concentration was increased with inocula from cows ($p < 0.05$) using only high-dose lovastatin. Overall, our results concur with those of [49], who indicated that lovastatin (150 mg/L) reduced NH_3-N concentration ($p < 0.05$) in the methanogenesis-inhibited conditions using rumen inoculum from cows. Newbold et al. [59] performed a meta-analysis to assess the role of ciliate protozoa in the rumen ecosystem. They noted a consistent correlation between the rumen protozoa elimination and the reduction of NH_3-N concentration, which is supported by extensive in vitro trials [60]. In this context, fluvastatin, simvastatin, and atorvastatin were effective statins against *Trypanosoma cruzi* and *Acanthamoeba* strains due to the inhibition of the mevalonate pathway, a precursor of ergosterol in some membranes of protozoa [61,62]. Therefore, it is speculated that lovastatin inhibits certain rumen protozoa, which could explain the reduction of the NH_3-N concentration in this work. Unfortunately, there is no report about the role of lovastatin on rumen protozoa.

4. Conclusions

This study provides the first assessment of lovastatin as an anti-methanogenic additive to identify if the ruminant livestock type (inoculum) influences the mitigation of rumen methane production and feed degradability. Lovastatin did not affect the GP kinetics and fermentable fractions of a forage-based diet. The anti-methanogenic property of lovastatin was variable depending on dose and inoculum source but was remarkable in sheep. In this context, the recommended lovastatin dosages for small ruminants and cows were 160 and 80 mg/L, respectively. The total VFA production was unaffected by the dose of lovastatin, but slight changes in acetate and valerate proportions were registered. Overall, lovastatin reduced the NH_3-N concentration and in vitro NDF degradation. The findings of this research should be considered for further research, especially to examine the in vivo effect of lovastatin on rumen methanogenesis, protozoa, and fiber degradability in sheep.

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References

1. Yusuf, R.O.; Noor, Z.Z.; Abba, A.H.; Hassan, M.A.A.; Din, M.F.M. Methane Emission by Sectors: A Comprehensive Review of Emission Sources and Mitigation Methods. *Renew. Sust. Energy Rev.* **2012**, *16*, 5059–5070. [CrossRef]
2. Stavert, A.R.; Saunois, M.; Canadell, J.G.; Poulter, B.; Jackson, R.B.; Regnier, P.; Lauerwald, R.; Raymond, P.A.; Allen, G.H.; Patra, P.K.; et al. Regional Trends and Drivers of the Global Methane Budget. *Glob. Change Biol.* **2012**, *28*, 182–200. [CrossRef] [PubMed]
3. Chang, J.; Peng, S.; Ciais, P.; Saunois, M.; Dangal, S.R.C.; Herrero, M.; Havlík, P.; Tian, H.; Bousquet, P. Revisiting Enteric Methane Emissions from Domestic Ruminants and their $\delta 13CCH_4$ Source Signature. *Nat. Commun.* **2019**, *10*, 3420. [CrossRef]
4. Van, A.A. Methane. A review. *J. Integr. Environ. Sci.* **2012**, *9*, 5–30.
5. Min, B.R.; Solaiman, S.; Waldrip, H.M.; Parker, D.; Todd, R.W.; Brauer, D. Dietary Mitigation of Enteric Methane Emissions from Ruminants: A Review of Plant Tannins Mitigation Options. *Anim. Nutr.* **2020**, *6*, 231–246. [CrossRef]
6. Scoones, I. Livestock, Methane, and Climate Change: The Politics of Global Assessments. *Interdiscip. Rev. Clim. Change* **2022**, *14*, e790. [CrossRef] [PubMed]
7. Zubir, M.A.; Bong, C.P.C.; Ishak, S.A.; Ishak, S.A.; Ho, W.S.; Hashim, H. The Trends and Projections of Greenhouse Gas Emission by the Livestock Sector in Malaysia. *Clean Technol. Environ. Policy* **2022**, *24*, 363–377. [CrossRef]
8. Johnson, K.A.; Johnson, D.E. Methane Emissions from Cattle. *J. Anim. Sci.* **1995**, *73*, 2483–2492. [CrossRef] [PubMed]
9. Haque, M.N. Dietary manipulation: A sustainable way to mitigate methane emissions from ruminants. *J. Sci. Biotechnol.* **2018**, *60*, 15. [CrossRef]
10. Jose, S.; Dollinger, J. Silvopasture: A Sustainable Livestock Production System. *Agrofor. Syst.* **2019**, *93*, 1–9. [CrossRef]
11. Honan, M.; Feng, X.; Tricarico, J.M.; Kebreab, E. Feed Additives as a Strategic Approach to Reduce Enteric Methane Production in Cattle: Modes of Action, Effectiveness and Safety. *Anim. Prod. Sci.* **2021**, *62*, 1303–1317. [CrossRef]
12. Ábrego-García, A.; Poggi-Varaldo, H.M.; Robles-González, V.; Ponce-Noyola, T.; Calva-Calva, G.; Ríos-Leal, E.; Estrada-Bárcenas, D.; Mendoza-Vargas, A. Lovastatin as a Supplement to Mitigate Rumen Methanogenesis: An Overview. *J. Sci. Biotechnol.* **2021**, *12*, 131.
13. Gottlieb, K.; Wacher, V.; Sliman, J.; Pimentel, M. Review Article: Inhibition of Methanogenic Archaea by Statins as a Targeted Management Strategy for Constipation and Related Disorders. *Aliment. Pharmacol. Ther.* **2016**, *4*, 197–212. [CrossRef] [PubMed]
14. Tobert, J. Lovastatin and Beyond: The History of the HMG-Coa Reductase Inhibitors. *Nat. Rev. Drug. Discov.* **2003**, *2*, 517–526. [CrossRef] [PubMed]
15. Khonkhaeng, B.; Cherdthong, A. Improving Nutritive Value of Purple Field Corn Residue and Rice Straw by Culturing with White-Rot Fungi. *J. Fungi* **2020**, *6*, 69. [CrossRef]
16. Ábrego-García, A.; Poggi-Varaldo, H.M.; Mendoza-Vargas, A.; Mercado-Valle, F.G.; Rios-Leal, E.; Ponce-Noyola, T.; Calva-Calva, G. Effects of Fermented Oat Straw as a Lovastatin-Carrier on In Vitro Methane Production and Rumen Microbiota. *Front. Energy* **2021**, *9*, 630701.
17. Joch, M.; Vadroňová, M.; Výborná, A.; Jochová, K. Inhibition of In Vitro Rumen Methane Production by Three Statins. *Ann. Anim. Sci.* **2022**, *22*, 271–282. [CrossRef]
18. Bueno, I.C.S.; Brandi, R.A.; Franzolin, R.; Benetel, G.; Fagundes, G.M.; Abdalla, A.L.; Louvandini, H.; Muir, J.P. In Vitro Methane Production and Tolerance to Condensed Tannins in Five Ruminant Species. *Anim. Feed. Sci. Technol.* **2015**, *205*, 1–9. [CrossRef]
19. Martinez-Fernandez, G.; Denman, S.E.; Yang, C.; Cheung, J.; Mitsumori, M.; McSweeney, C.S. Methane Inhibition Alters the Microbial Community, Hydrogen Flow, and Fermentation Response in the Rumen of Cattle. *Front. Microbiol.* **2016**, *7*, 1122. [CrossRef]
20. Leahy, S.C.; Janssen, P.H.; Attwood, G.T.; Mackie, R.I.; McAllister, T.A.; Kelly, W.J. Electron Flow: Key to Mitigating Ruminant Methanogenesis. *Trends Microbiol.* **2022**, *30*, 209–212. [CrossRef]
21. Ma, J.; Zhong, P.; Li, Y.; Sun, Z.; Sun, X.; Aung, M.; Hao, L.; Cheng, Y.; Zhu, W. Hydrogenosome, Pairing Anaerobic Fungi and H2-Utilizing Microorganisms Based on Metabolic Ties to Facilitate Biomass Utilization. *J. Fungi* **2022**, *8*, 338. [CrossRef] [PubMed]
22. Ungerfeld, E.M. Metabolic Hydrogen Flows in Rumen Fermentation: Principles and Possibilities of Interventions. *Front. Microbiol.* **2020**, *11*, 589. [CrossRef] [PubMed]
23. Jayanegara, A.; Goel, G.; Makkar, H.P.S.; Becker, K. Divergence between Purified Hydrolysable and Condensed Tannin Effects on Methane Emission, Rumen Fermentation and Microbial Population In Vitro. *Anim. Feed. Sci. Technol.* **2015**, *209*, 60–68. [CrossRef]
24. Schilde, M.; von Soosten, D.; Hüther, L.; Kersten, S.; Meyer, U.; Zeyner, A.; Dänicke, S. Dose–Response Effects of 3-Nitrooxypropanol Combined with Low- And High-Concentrate Feed Proportions in the Dairy Cow Ration on Fermentation Parameters in a Rumen Simulation Technique. *Animals* **2021**, *11*, 1784. [CrossRef] [PubMed]
25. Wang, R.; Wang, M.; Lin, B.; Ma, Z.Y.; Ungerfeld, E.M.; Wu, T.T.; Wen, J.N.; Zhang, X.M.; Deng, J.P.; Tan, Z.L. Association of Fibre Degradation with Ruminal Dissolved Hydrogen in Growing Beef Bulls Fed with Two Types of Forages. *Br. J. Nutr.* **2021**, *125*, 601–610. [CrossRef]
26. Ramos-Morales, E.; Arco-Pérez, A.; Martín-García, A.I.; Yáñez-Ruiz, D.R.; Frutos, P.; Hervás, G. Use of stomach tubing as an alternative to rumen cannulation to study ruminal fermentation and microbiota in sheep and goats. *Anim. Feed. Sci. Technol.* **2014**, *198*, 57–66. [CrossRef]
27. SAGARPA. *Norma Oficial Mexicana NOM-062-ZOO-1999. Especificaciones Técnicas para la Producción, Cuidado y Uso de los Animales de Laboratorio. 2001. Secretaria de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación*; Diario Oficial de la Federación: Ciudad de México, Mexico, 2001.

28. AOAC. *Association of Official Analytical Chemists. Official Methods of Analysis of the AOAC*, 15th ed.; Association of Official Analytical Chemists: Arlington, VA, USA, 1990.
29. Van Soest, P.J.; Robertson, J.B.; Lewis, B.A. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597. [CrossRef] [PubMed]
30. Ferraro, S.M.; Mendoza, G.D.; Miranda, R.L.A.; Gutiérrez, C.G. In Vitro Gas Production and Ruminal Fermentation of Glycerol, Propylene Glycol and Molasses. *Anim. Feed. Sci. Technol.* **2009**, *154*, 112–118. [CrossRef]
31. Ábrego-García, A.; Héctor, M.P.V.; Robles, G.V.S.; Rios, L.E.; Ponce, N.T.; Calva, C.G.; Estrada, B.D.A.; Mendoza, V.A. In Vitro Inhibition of Rumen Methanogenesis of a High-Grain Diet Using Lovastatin. *Rev. Int. Contam. Ambient.* **2022**, *38*, 491–500.
32. Martínez-Hernández, B.E.; Salvador-Flores, O.; Miranda-Romero, L.A. Indicador de Calentamiento Global a Partir de la Fermentación Ruminal de Alimentos con diferentes Niveles de Energía y Proteína. *Pastos Forrajes* **2020**, *42*, 285–289.
33. McCullough, H. The Determination of Ammonia in Whole Blood by a Direct Colorimetric Method. *Clin. Chim. Acta* **1967**, *17*, 297–304. [CrossRef] [PubMed]
34. Miranda-Romero, L.A.; Tirado-González, D.N.; Tirado-Estrada, G.; Améndola-Massiotti, R.; Sandoval-González, L.; Ramírez-Valverde, R.; Salem, A.Z.M. Quantifying Non-fibrous Carbohydrates, Acid Detergent Fiber and Cellulose of Forage Through an In Vitro Gas Production Technique. *J. Sci. Food. Agric.* **2020**, *100*, 3099–3110. [CrossRef] [PubMed]
35. Pitt, R.E.; Cross, T.L.; Pell, A.N.; Schofield, P.; Doane, P.H. Use of In Vitro Gas Production Models in Ruminal Kinetics. *Math. Biosci.* **1999**, *159*, 145–163. [CrossRef] [PubMed]
36. Roman-Garcia, Y.; Denton, B.L.; Mitchell, K.E.; Lee, C.; Socha, M.T.; Firkins, J.L. Conditions Stimulating Neutral Detergent Fiber Degradation by Dosing Branched-Chain Volatile Fatty Acids. I: Comparison with Branched-Chain Amino Acids and Forage Source in Ruminal Batch Cultures. *J. Dairy Sci.* **2021**, *104*, 6739–6755. [CrossRef] [PubMed]
37. Chávez-Espinoza, M.; Bernal-Barragán, H.; Vásquez-Aguilar, N.C.; Cantu-Silva, I.; Cotera-Correa, M.; Estrada-Castillón, A.E.; González-Rodríguez, H.; Sarquis-Ramírez, J.I. Cell-Wall Composition and Digestibility of Five Native Shrubs of the Tamaulipan Thornscrub in Northeastern Mexico. *Trop. Subtrop.* **2021**, *24*, 15. [CrossRef]
38. Zhao, C.; Hu, Y.; Chen, H.; Li, B.; Cao, L.; Xia, J.; Yin, Y. An in vitro evaluation of the effects of different statins on the structure and function of human gut bacterial community. *PLoS ONE* **2020**, *15*, e0230200. [CrossRef]
39. Osorio-Teran, A.I.; Mendoza-Martínez, G.D.; Miranda-Romero, L.A.; Martínez-Gomez, D.; Hernández-García, P.A.; Martínez-García, J.A. Effect of Calcium Propionate and Monensin on In Vitro Digestibility and Gas Production. *Rev. Bras. De Zootec.* **2017**, *46*, 348–353. [CrossRef]
40. Yáñez-Ruiz, D.R.; Bannink, A.; Dijkstra, J.; Kebreab, E.; Morgavi, D.P.; O'Kiely, P.; Reynolds, C.K.; Schwarm, A.; Shingfield, K.J.; Yu, Z.; et al. Design, Implementation and Interpretation of In Vitro Batch Culture Experiments to Assess Enteric Methane Mitigation in Ruminants—A Review. *Anim. Feed. Sci. Technol.* **2016**, *216*, 1–18. [CrossRef]
41. Langda, S.; Zhang, C.; Zhang, K.; Gui, B.; Ji, D.; Deji, C.; Cuoji, A.; Wang, X.; Wu, Y. Diversity and Composition of Rumen Bacteria, Fungi, and Protozoa in Goats and Sheep Living in the Same High-Altitude Pasture. *Animals* **2020**, *10*, 186. [CrossRef]
42. Henderson, G.; Cox, F.; Ganesh, S.; Jonker, A.; Young, W.; Janssen, P.H. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* **2015**, *5*, 14567. [CrossRef]
43. Wang, K.; Xiong, B.; Zhao, X. Could Propionate Formation Be Used to Reduce Enteric Methane Emission in Ruminants? *Sci. Total Environ.* **2022**, *855*, 158867. [CrossRef] [PubMed]
44. Ahmed, E.; Fukuma, N.; Hanada, M.; Nishida, T. The Efficacy of Plant-Based Bioactives Supplementation to Different Proportion of Concentrate Diets on Methane Production and Rumen Fermentation Characteristics In Vitro. *Animals* **2021**, *11*, 1029. [CrossRef] [PubMed]
45. Lashof, D.A.; Ahuja, D.R. Relative Contributions of Greenhouse Gas Emissions to Global Warming. *Nature* **1990**, *344*, 529–531. [CrossRef]
46. Albores-Moreno, S.; Alayon-Gamboa, J.A.; Miranda-Romero, L.A.; Jiménez-Ferrer, G.; Ku-Vera, J.C.; Vargas-Villamil, L. Nutritional Composition, In Vitro Degradation and Potential Fermentation of Tree Species Grazed by Ruminants in Secondary Vegetation (Acahual) of Deciduous Forest. *J. Anim. Plant Sci.* **2018**, *28*, 1263–1275.
47. Zhang, L.; Chung, J.; Jiang, Q.; Sun, R.; Zhang, J.; Zhong, Y.; Ren, N. Characteristics of Rumen Microorganisms Involved in Anaerobic Degradation of Cellulose at Various pH Values. *RSC Adv.* **2017**, *7*, 40303–40310. [CrossRef]
48. Prusty, S.; Kundu, S.; Kumar, V. Nutrient Utilization and Methane Emissions in Murrah Buffalo Calves Fed on Diets with Different Methanogenic Potential. *Livest. Sci.* **2017**, *202*, 89–95. [CrossRef]
49. Soliva, C.R.; Amelchanka, S.L.; Duval, S.M.; Kreuzer, M. Ruminal Methane Inhibition Potential of Various Pure Compounds in Comparison with Garlic Oil as Determined with a Rumen Simulation Technique (Rusitec). *Br. J. Nutr.* **2011**, *106*, 114–122. [CrossRef]
50. O'Brien, M.; Navarro-Villa, A.; Purcell, P.J.; Boland, T.M.; O'Kiely, p. Reducing In Vitro Rumen Methanogenesis for Two Contrasting Diets Using a Series of Inclusion Rates of Different Additives. *Anim. Prod. Sci.* **2013**, *54*, 141–157. [CrossRef]
51. Miller, T.L.; Wolin, M.J. Inhibition of Growth of Methane-Producing Bacteria of the Ruminant Forestomach by Hydroxymethylglutaryl-Scoa Reductase Inhibitors. *J. Dairy Sci.* **2001**, *84*, 1445–1448. [CrossRef]
52. Mohd, A.P.; Jahromi, M.F.; Ariff, M.O.; Ebrahimi, M.; Candyrine, S.C.L.; Liang, J.B. Aspergillus Terreus Treated Rice Straw Suppresses Methane Production and Enhances Feed Digestibility in Goats. *Trop. Anim. Health Prod.* **2018**, *50*, 565–571. [CrossRef]

53. Getachew, G.; Robinson, P.H.; DePeters, E.J.; Taylor, S.J. Relationships between Chemical Composition, Dry Matter Degradation and In Vitro Gas Production of Several Ruminant Feeds. *Anim. Feed. Sci. Technol.* **2004**, *111*, 57–71. [CrossRef]
54. Morgavi, D.P.; Martin, C.; Boudra, H. Fungal Secondary Metabolites from *Monascus* Spp. Reduce Rumen Methane Production In Vitro and In Vivo. *J. Anim. Sci.* **2013**, *91*, 848–860. [CrossRef] [PubMed]
55. Ramírez-Restrepo, C.A.; O'neill, C.J.; López-Villalobos, N.; Padmanabha, J.; McSweeney, C. Tropical Cattle Methane Emissions: The Role of Natural Statins Supplementation. *Anim. Prod. Sci.* **2014**, *54*, 1294–1299. [CrossRef]
56. Roasa, J.; De Villa, R.; Mine, Y.; Tsao, R. Phenolics of Cereal, Pulse and Oilseed Processing By-Products and Potential Effects of Solid-State Fermentation on Their Bioaccessibility, Bioavailability and Health Benefits: A Review. *Trends Food Sci. Technol.* **2021**, *116*, 954–974. [CrossRef]
57. Lan, W.; Yang, C. Ruminal Methane Production: Associated Microorganisms and The Potential of Applying Hydrogen-Utilizing Bacteria for Mitigation. *Sci. Total Environ.* **2019**, *654*, 1270–1283. [CrossRef] [PubMed]
58. Janssen, P.H. Influence of Hydrogen on Rumen Methane Formation and Fermentation Balances through Microbial Growth Kinetics and Fermentation Thermodynamics. *Anim. Feed. Sci. Technol.* **2010**, *160*, 1–22. [CrossRef]
59. Newbold, C.J.; de la Fuente, G.; Belanche, A.; Ramos-Morales, E.; McEwan, N.R. The Role of Ciliate Protozoa in the Rumen. *Front. Microbiol.* **2015**, *6*, 1313. [CrossRef]
60. Ayemele, A.G.; Ma, L.; Park, T.; Xu, J.; Yu, Z.; Bu, D. Giant milkweed (*Calotropis gigantea*): A New Plant Resource to Inhibit Protozoa and Decrease Ammoniagenesis of Rumen Microbiota In Vitro Without Impairing Fermentation. *Sci. Total Environ.* **2020**, *743*, 140665. [CrossRef]
61. Melo, L.; Caldas, I.S.; Azevedo, M.A.; Gonçalves, K.R.; da Silva do Nascimento, A.F.; Figueiredo, V.P.; de Figueiredo Diniz, L.; de Lima, W.G.; Torres, R.M.; Bahia, M.T.; et al. Low Doses of Simvastatin Therapy Ameliorate Cardiac Inflammatory Remodeling in Trypanosoma Cruzi-Infected Dogs. *Am. J. Trop. Med. Hyg.* **2011**, *84*, 325–331. [CrossRef]
62. Martín-Navarro, C.M.; Lorenzo-Morales, J.; Machin, R.P.; López-Arencibia, A.; García-Castellano, J.M.; de Fuentes, I.; Loftus, B.; Maciver, S.K.; Valladares, B.; Piñero, J.E. Inhibition of 3-Hydroxy-3-Methylglutaryl–Coenzyme A Reductase and Application of Statins as A Novel Effective Therapeutic Approach Against Acanthamoeba Infections. *Antimicrob. Agents Chemother.* **2013**, *57*, 375–381. [CrossRef]