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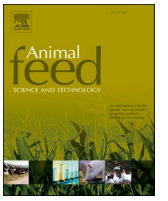

Effect of ellagic and gallic acid on the mitigation of methane production and ammonia formation in an *in vitro* model of short-term rumen fermentation

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ABSTRACT

Ruminant production is an important source of animal proteins for human nutrition. However, ruminants contribute to about 30% of anthropogenic methane (CH_4) emissions worldwide. The reduction of CH_4 emissions could represent an important strategy against climate warming. Tannins can play an important role in the mitigation of CH_4 emissions from ruminants. However, their mode of action is not yet well known. Thus, the present study aimed to gain a better understanding of the effect of ellagic acid (EA) and gallic acid (GA) on rumen fermentation using a model of short-term *in vitro* rumen fermentation. The basal diet (hay) was supplemented with EA and GA in five treatments (mg/g dry matter): i) EA 75, ii) EA 150, iii) GA 75, iv) GA 150 and v) EA 75 + GA 75. After a 24 h incubation, pH, ammonia formation, gas production, short-chain fatty acids (SCFA), *in vitro* organic matter digestibility (IVOMD) and the microbial count were assessed. Total gas production and digestible organic matter (dOM) were decreased after all the treatments, except for GA 75. The treatments EA 150 and EA+GA significantly decreased CH_4 production per unit of dietary DM, dOM, CO_2 and SCFA. Ammonia production was significantly decreased by EA 150 and EA+GA. EA and GA differently affected the relative abundance of selected bacterial taxa in rumen microbiota. To conclude, EA 150 and EA+GA exerted a significant effect on the reduction of CH_4 emissions and ammonia formation, but affecting also the rumen degradability of the diet and the total SCFA production, whereas EA 75 and GA 75 were not effective as EA 150 and EA+GA on CH_4 and ammonia, but were less detrimental on feed degradability and SCFA. Further studies are needed to determine whether the beneficial and detrimental effects of tannins on rumen fermentation can be dissociated.

1. Introduction

Livestock production is the source of animal proteins and plays an essential role in human nutrition worldwide. Through the enteric fermentation processes occurring in rumen, ruminants can efficiently convert low-quality and human-indigestible plant

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polysaccharides into energy and human-edible food, such as meat and milk (Shabat et al., 2016). It is also known that enteric fermentation leads to the physiological CH_4 production, making ruminants the main responsible for greenhouse gas (GHG) emissions among farm animals (Jackson et al., 2021; Vargas et al., 2022). One of the strategies to reduce CH_4 emissions and improve the environmental sustainability of ruminant production relies on tannins as a dietary supplement (Martin et al., 2010; Hassan et al., 2020). Tannins are plant polyphenol secondary metabolites with well-known antimicrobial, anti-inflammatory and antioxidant effects (Huang et al., 2018). Tannins are known to affect rumen fermentation processes through: i) mitigation of CH_4 emissions from enteric fermentation; ii) reduction of ammonia formation; iii) alteration of the rumen degradability of feed, in particular of proteins; and iv) modulation of the rumen microbiota (Jayanegara et al., 2012; Aboagye and Beauchemin, 2019; Vasta et al., 2019; Hassan et al., 2020). Tannins can reduce CH_4 emissions by directly inhibiting rumen methanogens or indirectly modulating the action of methanogen-associated protozoa and fibre-degrading bacteria, for example by increasing the abundance of the bacterial strain *Prevotella*, known to be a competitive H_2 sink for methanogens (Aboagye and Beauchemin, 2019; Aguilar-Marin et al., 2020). Moreover, they can bind dietary proteins and carbohydrates. The formation of protein–tannin complexes protect proteins from rumen degradation, thus leading to higher nitrogen (N) utilisation (Yanza et al., 2021). This results in reduced ruminal ammonia formation and a shift in N excretion from urine to faeces, thus reducing the emission of nitric oxide, which is mainly excreted by urine (Grainger et al., 2009; Aboagye et al., 2018). However, the binding of tannins with carbohydrates (*e.g.* plant fibres) reduces feed palatability and then feed intake. The effect of tannins varies according to the dosage and the animal species considered. The balance between the beneficial and detrimental effects of tannins on rumen fermentation depends mainly on the dosage used but also on the diet composition, the animal species and the source of supplementation (Vasta et al., 2019). The potential of tannins to mitigate CH_4 emissions and ammonia formation in ruminants has been demonstrated both *in vitro* (Getachew et al., 2008; Wei et al., 2018; Terranova et al., 2020; Giller et al., 2021; Foggi et al., 2022) and *in vivo* (Aboagye et al., 2018; Aboagye and Beauchemin, 2019). Most of the studies did not involve the use of single tannin molecules but rather the use of plants or tannin-containing forages, such as woody and herbaceous plants (Terranova et al., 2018; Terranova et al., 2020), *Stevia rebaudiana Bertoni* (Sarnataro et al., 2020), fruit and vegetable pomaces (Giller et al., 2021), quebracho (Getachew et al., 2008; Foggi et al., 2022) and mimosa and chestnut extracts (Bhatta et al., 2009; Hassanat and Benchaar, 2012; Foggi et al., 2022). The two main subgroups of tannins are condensed tannins (CT) and hydrolysable tannins (HT). CT are polymers of flavan-3-ol subunits, whereas HT are water-soluble molecules composed of a glucose core esterified with gallic acid (GA) or ellagic acid (EA). The EA and GA components of HT are the potential bioactive molecules that likely exert the effect on rumen fermentation (Lotfi, 2020). It has been observed that HT are more effective than CT in decreasing CH_4 emissions while maintaining the digestibility of nutrients (Jayanegara et al., 2015; Yanza et al., 2021). Currently, the main knowledge gap about the effect of EA and GA in rumen is their detailed mode of action, their modulating effect on rumen microorganisms, and subsequently their metabolic fate in rumen. Therefore, we decided to investigate the effect of EA and GA, alone or in combination in their pure form, on the modulation of rumen microorganisms and rumen fermentation in a short-term *in vitro* simulation of rumen fermentation using the Hohenheim gas test (HGT). The EA and GA molecules were chosen as major components of HT. Also, EA-secondary metabolites, such as urolithins, are considered to be the responsible molecules exerting the modulating effect in the rumen. The doses of supplementation were chosen because of i) the significant results of *in vitro* studies showing reduced CH_4 emissions up to 15–20% DM of tannins inclusion (Getachew et al., 2008; Bhatta et al., 2009; Hassanat and Benchaar, 2012; Terranova et al., 2018; Terranova et al., 2020; Giller et al., 2021) and ii) the average high level of HT (around 60–80% DM) in plant extracts such as chestnut, quebracho or mimosa (Hassanat and Benchaar, 2012; Min et al., 2015; Lotfi, 2020). The aim of the study was multiple: first, to observe at what extent these high doses of supplementation could positively influence rumen fermentation and rumen microorganisms, in particular regarding CH_4 emissions, without deeply altering the rumen degradability of feed; second, to assess how EA and GA could interact in their modulating effect on rumen fermentation; last, to observe how EA and GA could modulate the microbial microorganisms in rumen.

2. Materials and methods

2.1. Incubated materials

Rye grass-based hay (first cut, > 90% grass content) was used as a control and basal diet for the incubation of the supplements. It was ground using a centrifugal mill (Model ZM 200, Retsch GmbH, Hann, Germany) to pass through a 1 mm sieve. The chemical composition of the hay was (g/kg DM) 925 organic matter (OM), 75.1 ash, 112 crude protein (CP), 540 neutral detergent fibre (NDF), 295 acid detergent fibre (ADF) and 24.1 ether extract (EE). The EA and GA were purchased from Sigma-Aldrich (St. Louis, MO, USA). The purity level was $\geq$ 95% for EA and $\geq$ 98.5% for GA. For incubation, 200 mg DM of hay were used and supplemented with EA and GA in pure form in the following concentrations (solid phase). Five different treatments were evaluated in this study (mg/g of DM): i) EA 75, ii) EA 150, iii) GA 75, iv) GA 150 and v) EA 75 + GA 75. Rumen fluid (pure) and rumen fluid with hay were added as a blank and a control.

2.2. Animal ethics

Animal manipulation was performed according to the Swiss guidelines for animal welfare procedure and was approved by the animal ethics committee of the Cantonal Veterinary Office of Zurich with approval number ZH113/18.

2.3. Rumen fluid collection and in vitro incubation

Animals were fed a total mixed ration (TMR). Each animal was fed with 17.8 kg/DM/day. The TMR was composed of (% DM) grass silage (48%), maize silage (20%), sugar beet pulp (17%), hay (8%), concentrate (8%) and mineral supplement (0.2%). The experiment was conducted during October and November 2021. For each run, rumen fluid was collected directly before the morning feeding from one of four in total fistulated, lactating Original Brown Swiss cows. The pH of the freshly taken rumen fluids always ranged between 5.9 and 6.9.

Within 1 h of collection, the rumen fluid was transported in a preheated thermos flask to the laboratory and filtered through four layers of gauze. Two samples of pure rumen fluid were collected to measure pH and ammonia and to perform a microbial count (bacteria and protozoa). Then, rumen fluid was mixed with a preheated (39 °C) reduced buffer solution in a 1:2 ratio according to the protocol of Menke and Steingass (1988).

Scaled glass syringes with two outlets, one for rumen fluid and one for gas analysis, were the experimental units used for incubation (Soliva and Hess, 2007). The glass syringes were filled with the solid phase and then with 30 ml of the rumen fluid–buffer solution (Suppl. Figure 1). The solid phase was composed of the basal diet alone (control) or the basal diet in combination with EA and GA alone or combined. Additionally, a standard hay (purchased from the Institute of Animal Nutrition, University of Hohenheim, Stuttgart, Germany) and blanks (rumen fluid only) were incubated.

2.4. Sample collection and analysis

Syringes were incubated for 24 h at 39 °C. After 24 h, the syringes were taken out of the incubator and the volume of total gas production was recorded. Fermentation was stopped by removing the liquid phase and leaving the gas phase inside the syringes for later analysis. Ammonia and pH were measured in the liquid phase samples using a potentiometer (ammonia: model 713, Metrohm, Herisau, Switzerland; pH: model 913; Metrohm, Herisau, Switzerland) equipped with electrodes. Then, 4 ml of liquid phase per syringe were mixed with H_2SO_4 50% (m/v) to stabilise the samples and frozen at −20 °C for later SCFA analysis. For microbial count, the liquid phase samples were mixed with a solution of 6% formaldehyde in a 1:1 ratio for protozoa and 4% formaldehyde in a 1:100 ratio for bacteria. Gas samples of 150 μl were collected using a Hamilton syringe and injected in a gas chromatograph (GC-TCD 6890 N, Agilent Technologies, Wilmington, NC, USA) equipped with a thermal conductivity detector to measure the CH_4 and CO_2 concentrations.

The SCFA profile in rumen fluid samples was determined by HPLC. Samples were filtered and analysed for total SCFA production using a liquid chromatography (Ultimate 3000, Thermo Fisher Scientific, Reinach, Switzerland) with an exchange ion column (Nucleogel ION 300 OA 300 × 7.8 mm) and equipped with a refractive index detector (RefractoMax 521, Thermo Fisher Scientific, Reinach, Switzerland). To calculate the IVOMD, we used Menke and Steingass (1988)'s standard equation: IVOMD (g/kg) = 14.88 + 0.8893 × total gas production (ml/200 mg DM) + 0.0448 × crude protein (g/kg DM) + 0.0651 × ash (g/kg DM).

In total, four runs were performed, testing each of the treatments in triplicate (n = 12 replicates per treatment).

2.5. Real-time qPCR analysis

The liquid phase samples collected after the incubation were kept at −80 °C for further DNA extraction and quantitative PCR (qPCR). The DNA was extracted using QIAMP Fast DNA Stool Mini Kit (Qiagen, Hombrechtikon, Switzerland), as described by Böttger et al. (2019), with minor modifications. In brief, 2 ml of liquid phase samples were centrifuged at 6500g for 30 min at 4 °C. Then, the pellet was resuspended in 1.5 ml of Inhibitex buffer (provided with the kit) and heated at 90 °C for 5 min. The tubes were allowed to return to room temperature before 15 s vortexing and further centrifugation at 16,000g for 1 min. Later, 200 μl of the supernatant were

Table 1
List of primers used for real-time qPCR.

Target	Primers	Sequences (5'−3')	Size (bp)
16v3	Forward	CCTACGGGAGGCAGCAG	193
	Reverse	ATTACCGCGGCTGCTGG	
Butyvibrio fibrisolvens	Forward	ACCGCATAAGCGCACGGA	65
	Reverse	CGGGTCCATCTTGTACCGATAAAT	
Fibrinobacter succinogenes	Forward	GTTCGGAATTACTGGGCGTAAA	121
	Reverse	CGCCGTCCCCTGAACTATC	
Ruminococcus albus	Forward	CCCTAAAAGCAGTCTTAGTTCG	176
	Reverse	CCTCCTTGCGGTTAGAACA	
Ruminococcus flavefaciens	Forward	TGTCCCAGTTCAGATTGCAG	171
	Reverse	GGCGTCCTCATTGCTGTTAG	
Selenomonas ruminantium	Forward	TGCTAATACCGAATGTTG	237
	Reverse	GCTTTCGCCCATTGCGGA	
Total Prevotella	Forward	CCAGCCAAGTAGCGTGCA	152
	Reverse	TGGACCTTCCGTATTACCGC	
Total Brevibacteria	Forward	TATTCACCGCGCGATTGTGAC	190
	Reverse	ACGACGGTAGGTCCGTATGC	

Note: 16v3: V3 region of 16 S rRNA.

used for DNA extraction following the kit's procedure. The concentration of the DNA extracts was measured with spectrophotometry by using NanoDrop 1000 (Witec AG, Luzern, Switzerland). The quality of the extracted DNA was assessed by capillary electrophoresis using Fragment Analyzer (Agilent technologies, Basel, Switzerland). Before the qPCR, the DNA extracts were diluted at a final concentration of 4 ng/µl with RNase-free water. A reference sample was generated using a mixture of DNA derived from five different random samples. The real-time qPCR was performed to measure the relative abundance of several bacterial taxa commonly present in rumen fluid and important for the rumen fermentation processes (Tapio et al., 2017; Lan and Yang, 2019). The PCR was performed using the KAPA SYBR FAST Universal Kit (Roche, Basel, Switzerland). The primers (Table 1) were used at a final concentration of 200 nmol/l with a PCRmax real-time PCR device (PCRmax, Staffordshire, UK). The amplification profile included an activation step of 5 min at 95 °C followed by 40 cycles of a two-step amplification step (5 s at 95 °C and 20 s at 60 °C). The percentage of each bacterial taxa considered in relation to the total bacterial 16 S ribosomal DNA (determined by amplification with 16v3 primers) was calculated for the reference sample using the formula previously described (Brinkhaus et al., 2016). For all the other samples, an induction fold was calculated relative to the abundance in the reference sample using a ΔΔCt method with efficiency correction (Pfaffl, 2001) and the EcoStudy software (PCRmax, Staffordshire, UK). The induction fold was then multiplied by the percentage calculated for the reference sample.

2.6. Statistical analysis

Data were analysed by ANOVA with RStudio software (version 4.0.5) using linear mixed-effects regression (Lme4) models (Bates et al., 2015). Residuals were checked for normality and homoscedasticity. If the data were not normally distributed, they were analysed with the non-parametric Kruskal–Wallis test. Multiple comparisons were performed using the pairwise Wilcoxon comparison *post hoc* test with the Benjamini–Hochberg *P*-value correction method. The effect of the treatment was used as a fixed factor, whereas the effect of the run was used as a random factor. Differences were considered significant if $P < 0.05$. Data were reported as least squares means and pooled standard error of the mean (SEM).

3. Results

3.1. Effects on rumen fermentation parameters

The pH of the liquid phase samples after incubation was on average 7 ± 0.6 with no significant differences between the control and treatments ($P > 0.05$, data not shown). As reported in Table 2, total gas production per unit of dietary DM, IVOMD and the amount of OM digested in 24 h (dOM) were decreased by 10% on average with all the treatments ($P < 0.001$), except for GA 75. The gas chromatography results showed that CH_4 production per unit of dietary DM significantly decreased with EA 150 (–20%, $P < 0.001$) and EA+GA (–25%, $P < 0.001$) treatments. These two treatments significantly reduced CH_4 production per unit of dOM (both –15%, $P < 0.001$) and CH_4-to-SCFA ratio (both –25%, $P < 0.001$). CO_2 production per unit of dietary DM significantly decreased ($P < 0.001$) with all the treatments (except for GA 75), but the rate of decrease was lower if compared to the decrease of CH_4 production. The latter was also confirmed by the results of the CH_4/CO_2 ratio ($P < 0.001$). Ammonia production per unit of dietary DM significantly decreased after the treatments EA 150 (–13%, $P < 0.001$) and EA+GA (–20%, $P < 0.001$). As seen above, EA 150 and EA+GA were the most effective treatments for all results of the study.

Total SCFA production decreased by approximately 10% with all the treatments ($P < 0.001$). However, total SCFA production decreased to a lesser extent than CH_4/DM, as can be inferred by the production of CH_4 per moles of total SCFA produced (CH_4/SCFA, Table 2), which was decreased by 24% ($P < 0.001$) by EA 150 and EA+GA. Furthermore, slight differences were observed for the ruminal SCFA profile, excluding acetic, isovaleric and valeric acid ($P > 0.05$) (Table 3).

4. Effects on selected rumen bacteria

The treatments did not affect the bacterial and the protozoal count ($P > 0.05$) (Suppl. Figure 2). The results of the quantitative real-

Table 2
Comparison of selected rumen fermentation parameters between control and treatments.

Item	CTR	EA 75	EA 150	GA 75	GA 150	EA+GA	SEM	*P*-value
Total gas/DM (ml/g)	52.3^{c}	46.5^{ab}	45.3^{a}	51.1^{c}	47.5^{b}	46.6^{ab}	0.7	$2.2 * 10^{-16}$
IVOMD (g/kg)	63.5^{c}	58.4^{ab}	57.3^{a}	62.5^{c}	59.3^{b}	58.5^{ab}	0.6	$2.2 * 10^{-16}$
dOM (mg/24 h)	117.5^{c}	108.0^{ab}	106.1^{a}	115.6^{c}	109.7^{b}	108.1^{ab}	1.1	$2.2 * 10^{-16}$
CH_4/DM (ml/g)	30.6^{d}	26.7^{b}	24.5^{a}	29.1^{c}	25.9^{b}	23.7^{a}	2.2	$2.2 * 10^{-16}$
CO_2/DM (ml/g)	170.4^{bc}	162.5^{ab}	155.8^{a}	176.3^{c}	161.1^{a}	157.1^{a}	10.0	$2.2 * 10^{-16}$
CH_4/dOM (ml/g)	66.4^{d}	59.7^{b}	56.9^{a}	61.3^{c}	59.2^{b}	55.9^{a}	2.6	$5.9 * 10^{-4}$
CH_4/CO_2 (ml/l)	178.6^{d}	164.1^{c}	156.5^{b}	164.5^{c}	160.1^{c}	151.6^{a}	8.4	$2.2 * 10^{-16}$
CH_4/SCFA (mmol/ml)	110.2^{d}	90.8^{b}	84.6^{a}	99.4^{c}	90.6^{b}	83.9^{a}	3.8	$2.2 * 10^{-16}$
Ammonia (mmol/l)	11.7^{c}	12.1^{c}	10.1^{a}	12.3^{c}	10.9^{b}	9.5^{a}	1.9	$2.2 * 10^{-16}$

Note: Values are presented as mean and standard error of the mean (SEM). Numbers with different superscripts (^{a,b,c,d}) in a row differ significantly. CTR = control; DM = dry matter; EA = ellagic acid; GA = gallic acid; IVOMD = *in vitro* organic matter digestibility; dOM = digestible organic matter.

Table 3
Comparison of total SCFA production and of the relative abundance of single SCFA species.

Item (n_i/n_{tot})	CTR	EA 75	EA 150	GA 75	GA 150	EA+GA	SEM	*P*-value
Total SCFA	99.98^{c}	92.99^{b}	86.93^{a}	93.02^{b}	86.94^{a}	86.95^{a}	0.02	1.9 * 10^{-11}
Acetic acid	64.94	65.09	65.06	65.96	65.78	65.88	0.86	4.9 * 10^{-1}
Propionic acid	20.44^{c}	20.22^{bc}	20.41^{c}	19.64^{a}	20.09^{abc}	19.80^{ab}	0.30	9.2 * 10^{-8}
Isobutyric acid	0.89^{c}	0.84^{ab}	0.82^{a}	0.85^{b}	0.84^{ab}	0.81^{a}	0.11	2.2 * 10^{-16}
Butyric acid	10.72^{ab}	10.94^{c}	10.87^{bc}	10.61^{a}	10.61^{a}	10.77^{ab}	0.45	2.4 * 10^{-12}
Isovaleric acid	1.48	1.37	1.32	1.41	1.40	1.32	0.27	2.4 * 10^{-1}
Valeric acid	1.53	1.53	1.54	1.70	1.71	1.51	0.08	8.6 * 10^{-2}

Note: Values are expressed as molar fraction (n_i/n_{tot}) = mol single gas/mol total SCFA. Values are presented as mean and standard error of the mean (SEM). Numbers with different superscripts (^{a,b,c,d}) in a row differ significantly. CTR = control; EA = ellagic acid; GA = gallic acid; SCFA = short chain fatty acids.

time qPCR showed that EA and GA differently modulated the relative abundance of the bacterial taxa evaluated. Indeed, *Butyvibrio fibrisolvens* and *Ruminococcus albus* are two cellulolytic bacteria, and their relative abundance was not significantly modulated by the treatments with EA and GA ($P > 0.05$). Instead, there were significant differences in the relative abundance of the cellulolytic *Ruminococcus flavefaciens*, the hemicellulolytic *Total Prevotella* and the amylolytic *Selenomonas ruminantium* between the control and treatments. In particular, EA 75 significantly decreased the abundance of *R. flavefacien*s, whereas EA 150 significantly increased the abundance of *S. ruminantium* and *Total Prevotella.* Furthermore, the relative abundance of the methanogenic *Total Brevibacteria* was not significantly altered by the treatments ($P > 0.05$).

5. Discussion

Tannins can reduce the environmental impact of ruminant production in terms of CH_4 emissions and ammonia production (Orzuna-Orzuna et al., 2021). However, the impact of tannins on feed intake and nutrient utilisation is well known (Yanza et al., 2021). In this study, we assessed the effect of EA and GA on rumen fermentation using HGT. This is a valid method to determine the effect of plant extracts or single molecules on rumen fermentation and offers a higher level of standardisation and reproducibility when compared to the *in vivo* condition (Jayanegara et al., 2012). Furthermore, since the duration of the experimental trial is 24 h, HGT is considered a short-term and fast approach, if compared to other long-term rumen simulation techniques, such as Rusitec (Deitmers et al., 2022). Here, we used single molecules of tannins rather than tannin plant extracts because plant extracts contain different tannin molecules, thus making difficult to assess which component exerts a specific effect. In this study, EA and GA alone or in combination at high doses were used to simulate a diet including natural extracts rich in hydrolysable tannins.

6. Gas production, digestibility of the diet and SCFA production

All the treatments, except for GA 75, decreased total gas production. The main reduction was exerted on CH_4 rather than CO_2, as observed with the CH_4/CO_2 ratio (Table 2). EA 150 and EA+GA exerted the most effective reduction of CH_4 production per unit of DM, dOM and total SCFA, whereas GA alone exerted a weaker effect than EA. So far, few studies have reported the effective CH_4-mitigating activity of EA (Wei et al., 2018), whereas the literature on GA is more consistent. Aboagye et al. (2019) reported a mild CH_4 decrease exerted by GA 15 mg/g DM when supplemented to beef cattle fed with a standard diet containing alfalfa silage. Wei et al. (2019) observed that adding GA up to 40 mg/g DM linearly decreased CH_4 production in a short-term *in vitro* rumen fermentation, whereas the GA effect was weaker when performing a long-term *in vitro* fermentation. Significant results were also observed using tannin plant extracts. Bhatta et al. (2009) observed reduced CH_4 emissions using chestnut, quebracho and mimosa extracts (alone or in combination) supplemented up to 250 mg/g DM. Similar results were also obtained by Hassanat and Benchaar (2012), who reported that quebracho extract supplemented at 100, 150 and 200 mg/g DM decreased CH_4 emissions by 23%, 34% and 40%, respectively, when compared to the control. Furthermore, Foggi et al. (2022) observed a reduced CH_4 production up to 15 mg/g DM by using chestnut and quebracho extracts, alone or in combination, at 20 mg/g DM.

With the exception of the total SCFA and CO_2, in our study, EA 15 was more effective than GA 15 for all the parameters evaluated, whereas the co-treatment likely improved the effect of GA in terms of total gas production, CH_4 emissions and ammonia formation (Table 2). In a single study (Lotfi et al., 2020), it was reported that EA can be converted to GA in the rumen. Since EA can be potentially converted into GA, it can be hypothesized that GA is the responsible of the effect on rumen fermentation, even though overall action of EA is more effective than GA. In any case, the metabolic pathway linking EA and GA should be further investigated.

The lower level of ammonia formation could be related to the complex formation between tannins and proteins, subtracting N from rumen degradation and increasing its ritention (Aboagye and Beauchemin, 2019). In Terranova et al. (2018), several plant substrates supplemented at 167 mg/g DM reduced both CH_4 emissions and ammonia formation using HGT. The reduced CH_4 emissions and ammonia formation were also observed when tannin-rich plant extracts were *in vitro* supplemented to a high-forage and a high-concentrate diet (Jayanegara et al., 2020). The significant reduction of ammonia formation observed in this study (Table 2) could be also given by the average low CP content of hay used as a standard diet.

Concomitantly, tannin supplementation also influences the fermentability of a standard diet with potential consequences for SCFA production as well (Aboagye and Beauchemin, 2019). In this study, EA and GA caused an 10% average reduction of total SCFA

production (Table 3). Furthermore, the CH_4/SCFA ratio was significantly reduced (Table 2). As discussed for the CH_4/CO_2 ratio, the percentage reduction was lower for CH_4 than for total SCFA. Several plant extracts supplemented at 167 mg/g DM showed an average 10% reduced total SCFA production (Terranova et al., 2018). Tannin plant extracts supplemented at around 80 mg/g DM reduced the production of total SCFA by 14% (Jayanegara et al., 2015). When HT-source plant extracts were used as supplements, there was a negative correlation between increasing the HT-extract concentration and the total SCFA production (Bhatta et al., 2009; Hassanat and Benchaar, 2013; Foggi et al., 2022). Interestingly, Getachew et al. (2008) observed that GA supplemented at 50 and 100 mg/g DM did not impair the total SCFA production.

Furthermore, only GA 75 did not impair dOM and IVOMD (Table 3). The reduced digestibility of the organic matter could derive from the binding between tannins and macromolecules, such dietary carbohydrates, thus subtracting them from the degradation in rumen. In all the studies cited above, there was a linear correlation between a reduction in gas production, dOM, IVOMD (or *in vitro* dry matter digestibility), CH_4 emissions and total SCFA production. Hence, tannin supplementation influences rumen fermentation with a cascade effect. This explains why GA 75 did not impair dOM and IVOMD, since this treatment was less effective for the other parameters as well. Total SCFA production and OM digestibility are related to diet digestibility and nutrient availability. Therefore, their reduced level in response to EA and GA could partially explain the mitigation of CH_4 production observed in this study.

7. Microbial count and quantification of selected rumen bacteria

The bacterial and protozoal count did not reveal any quantitative differences between the control and the treatments (Suppl. Figure 2). This is in line with the literature, where it has been reported that the variation of protozoa abundance is not associated with CH_4 emissions (Guyader et al., 2014) and the level of dietary tannins (Jayanegara et al., 2012). However, some studies have reported reduced CH_4 emissions and a reduced level of protozoa after *in vitro* supplementation with dietary tannins, thus underlining the "subordinate" role of protozoa in methanogenesis, along with the major role of methanogen bacteria (Bhatta et al., 2009; Morgavi et al., 2010; Sarnataro et al., 2020). In our study, a similar total microbial count between the control and the treatments also confirmed that the concentrations used were not toxic for the ruminal bacterial community. Despite there being no quantitative difference, the relative abundance of *R. flavefaciens, S. ruminantium* and *Prevotella* was modulated by EA and GA (Table 4). Surprisingly, only EA 75 decreased the abundance of *R. flavefaciens*. Contrastingly, a higher concentration of EA not only did not affect *R. flavefaciens* but also increased the relative abundance of *S. ruminantium* and *Prevotella*. A higher abundance of *Prevotella* was correlated with lower CH_4 emissions *in vivo* (Aguilar-Marin et al., 2020). It is possible that propionate, the main fermentation product of *Prevotella*, is an H_2 sink and can compete with methanogen bacteria for the use of H_2 (Aguilar-Marin et al., 2020; Pereira et al., 2022). H_2 is used by methanogens to reduce CO_2 to CH_4 (Aboagye and Beauchemin, 2019). *R. flavefaciens* is one of the predominant cellulolytic rumen bacteria (Miron et al., 2001), and its ability to produce acetic and formic acids, H_2 and CO_2 is well known (Latham and Wolin, 1977). Therefore, the reduced CH_4 production in EA groups could be explained by the increased abundance of the hemicellulolytic *Total Prevotella* and decreased abundance of the cellulolytic *R. flavefaciens*. Even if not significant, a numerical difference in the relative abundance of *R. flavefaciens* was observed between the control and EA 15. Moreover, EA showed a stronger modulating effect on the ruminal bacterial community than GA.

Tannins can interact with the extracellular enzymes secreted and the cell walls of bacteria, leading to membrane disruption, detrimental effects on microbial metabolism and deprivation of substrates for microbial growth (Patra and Saxena, 2011). However, several species of tannin-tolerating bacteria have been identified (Patra and Saxena, 2011). The increased abundance of *S. ruminantium* in the EA 150 group could be explained by the metabolic characteristics of such bacteria. Members of *S. ruminantium* species are able to grow on tannic acid or condensed tannin as a sole energy source but cannot grow on GA as an energy source (Skene and Brooker, 1995). This could explain why the abundance of the taxa only increased in the EA group and not in GA-treated samples. *S. ruminantium* mainly ferments carbohydrates to lactate, propionate, acetate and CO_2. Its ability to produce H_2 seems limited, but it can strongly increase in the presence of methanogenic bacteria (Scheifinger et al., 1975). Similar to *Prevotella*, *S. ruminantium* was associated with a higher production of propionate (Asanuma et al., 1999). Also, *Prevotella* species have been found to tolerate the presence of tannins in the culture medium (Patra and Saxena, 2009), justifying the effects of EA on *Prevotella* abundance. Even in short-term *in vitro* trials, the contrasting effects of tannins against bacteria differ for their ability to tolerate the presence of these phenolic compounds.

EA and GA did not significantly alter the relative abundance of *Brevibacteria*, one of the major methanogen taxa present in rumen

Table 4
Relative abundance of bacterial taxa in rumen fluid after *in vitro* fermentation.

Bacteria (mRNA induction fold)	Treatment						SEM	*P*-value
	CTR	EA 75	EA 150	GA 75	GA 150	EA+GA		
Butyvibrio fibrisolvens	1.00	0.87	0.85	0.92	0.88	0.97	0.07	$2.7 * 10^{-2}$
Fibrobacter succinogenes	1.00^{ab}	0.91^{ab}	1.07^{b}	0.84^{a}	0.98^{ab}	0.92^{ab}	0.14	$1.1 * 10^{-2}$
Ruminococcus albus	1.00	0.91	0.95	0.92	1.01	0.94	0.50	$3.6 * 10^{-1}$
Ruminococcus flavefaciens	1.00^{b}	0.77^{a}	0.84^{ab}	0.84^{ab}	1.00^{b}	0.98^{ab}	0.11	$6.0 * 10^{-4}$
Selenomonas ruminantium	1.00^{a}	1.45^{ab}	1.60^{b}	1.36^{ab}	1.24^{ab}	1.29^{ab}	0.45	$1.7 * 10^{-3}$
Total Brevibacteria	1.00	1.04	1.01	1.05	1.13	1.23	0.31	$5.2 * 10^{-2}$
Total Prevotella	1.00^{a}	1.08^{ab}	1.15^{b}	1.04^{ab}	0.98^{a}	1.06^{ab}	0.10	$1.3 * 10^{-2}$

Note: CTR = control; EA = ellagic acid; GA = gallic acid. Numbers with different superscripts (^{a,b,c,d}) in a row differ significantly.

(Table 4). Therefore, the obtained results suggested that a reduction in CH_4 production was achieved by modulating the relative abundance of fibre-degrading bacteria, such as *R. flavefaciens, Prevotella* and *S. ruminantium*, rather than a direct modulation of *Brevibacteria*. Indeed, fibre-degrading bacteria are H_2 producers; therefore, they indirectly sustain the methanogenic process because methanogens use H_2 to reduce CO_2 to CH_4 (Aboagye and Beauchemin, 2019). It is possible that EA and GA impaired the action of fibre-degrading bacteria, thus reducing CH_4 production.

Given the complexity of the rumen microbial community, it is necessary to further investigate the effects of EA and GA on the molecular processes occurring during rumen fermentation. In addition, it would be interesting to test if in a long-term *in vitro* rumen fermentation, a shift in the rumen microbiota from low to high tannin-tolerant bacteria could result in a more adapted ecosystem able to limit the detrimental effects of EA and GA observed in this study.

8. Conclusion and implications

The EA 150 and EA+GA treatments altered the relative abundance of selected rumen microorganisms, lowering CH_4 production and ammonia formation but also affecting the total SCFA production and the *in vitro* digestibility of OM. The novelty of this work stands in how EA and GA influenced rumen fermentation. The effects of EA and GA were similar at 75 mg/g DM, but at 150 mg/g DM EA showed a stronger effect than GA. Furthermore, the EA+GA treatment showed similar effect to EA 150, suggesting a potential mutual effect of EA and GA that need to be further investigated. To conclude, the reduced CH_4 production was a consequence of the altered mechanisms of H_2 production by EA and GA. These results could lead the way to further analyses on the long-term effects of EA and GA on rumen fermentation. Further, a metagenomic investigation of the perturbation of the rumen microbial community by EA and GA could deepen and strengthen our current findings, to assess whether the decreased CH_4 emissions and ammonia formation and the decreased digestibility could be dissociated.

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CRediT authorship contribution statement

Michele Manoni: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Melissa Terranova:** Methodology, Supervision, Validation, Writing – review & editing. **Sergej Amelchanka:** Methodology, Supervision, Validation, Writing – review & editing. **Luciano Pinotti:** Supervision, Validation. **Paolo Silacci:** Visualisation, Writing – review & editing, Methodology, Project administration. **Marco Tretola:** Methodology, Data curation, Software, Supervision, Writing – review & editing, Project administration.

Declaration of Competing Interest

The authors declare no conflicts of interest in carrying out this work.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2023.115791.

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