# Effects of Plant Secondary Metabolites on Methane Production and Fermentation Parameters in *In vitro* Ruminal Cultures

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**Abstract**

Enteric fermentation process is of concern worldwide for its contribution to global warming. Methanogenesis process represents besides its contribution to greenhouse gases emissions an energy loss to the animal. In the last years, new strategy has been evaluated whether plant secondary metabolites can be used as natural additives to reduce ruminal methane emissions. The present study investigated the effects of trans-cinnamic, caffeic, p-coumaric acids and catechin hydrate, four plant secondary metabolites (PSMs) on methane production and fermentation in *in vitro* ruminal cultures. The four PSMs were added anaerobically in a 6 mM concentration to 100 ml serum bottles containing 500 mg grass hay as substrate, 10 ml rumen fluid collected from a fistulated sheep and 40 ml 141 DSM culture medium. The bottles were incubated at 39℃. The results showed that caffeic ($p = 0.058$) and p-coumaric ($p = 0.052$) acids tended to decrease methane production in comparison to control but the decrease was not statistic significantly at $\alpha = 0.05$. The other two PSMs had no significant effect on methane production. Addition of PSMs did not affected the total gas volume, the pH and VFAs profile ($P>0.05$) in relation to the control (no PSM added). Caffeic and p-coumaric acids in 6 mM concentration showed promising effects for decreasing ruminal methane emissions without affecting ruminal fermentation parameters, further experiments with other concentrations needs to be done.

**Keywords**: greenhouse gases, *in vitro*, methane, plant secondary metabolites, rumen, ruminal fermentation.

## 1. Introduction

In the global worming terms, methane is a particularly potent greenhouse gas (GHG) which has a global potential of 21 times more than carbon dioxide [1], and accounts for 16% of total global GHGs emissions. From anthropogenic sectors arises approximately 70% of methane production and agriculture accounts for about two-third [2], and enteric fermentation, a natural process produced by ruminant animals, is responsible for one-third of methane from agriculture [3]. The enteric methane produced by ruminants has its origin in the rumen [4]. Ruminal digestion of feed by the microorganisms, under anaerobic conditions, results in the production of acetate, propionate and butyrate (volatile fatty acids) which are used by the animal as energy source, and the production of ruminal gases such as $CO_2$ and $CH_4$, eliminated through eructation [4]. Methanogenesis process besides its negative impact on the environment represents a loss of 2-15% of gross energy intake [5] for the animal, leading to an unproductive use of dietary energy [6].

For many years, researchers have tried to develop some strategies in order to manipulate rumen methanogenesis process. The major aim was to

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reduce methane emissions and to improve the efficiency of ruminant production in an ecologically and sustainable way [7]. Techniques to manipulate this process include elimination of protozoa [8, 9], use of antibiotics (such as Monensin) and bacteriocins such as Nisin [10], use of lipids sources (fatty acids, oils, and seeds) [11, 12] organic acids [13] and ionophores [14] or change in dietary composition [15]. Another attempt was immunization and biological control. These techniques were used by Wright and coworkers [7] and implied the production of a vaccine against three rumen methanogens. The results from the study showed a decrease in $CH_4$ production by nearly 8% in Australian sheep. Another vaccine prepared with a different set of methanogens species and tested in other geographical region did not achieve a positive result, maybe because the community of methanogens species differs under different conditions [7]. Cook and coworkers [16] used passive immunization with antibodies produced by laying hens against three common methanogens present in the digestive tract of ruminants. These treatment decreased $CH_4$ production *in vitro*, but the effect was lost after 24 hours of incubation [16].

Plant extracts are a new, safe and inexpensive way to reduce methane emission from ruminants [17] since several plant secondary metabolites have shown antimicrobial activity [18], as they can modify ruminal fermentation in a way that the efficiency of utilization of feed energy is enhanced and methane production is decreased [19]. Plant secondary metabolites are a group of chemicals that only protect the plants against predators, like insects or herbivores and are not involved in the biochemical processes of plant growth or reproduction [18].

In the current study, we examined four phenolic acids: trans-cinnamic, caffeic and p-coumaric acids and catechin hydrate, on methane production and ruminal fermentation in *in vitro* ruminal cultures. These phenolic acids are natural antioxidants and important plant secondary metabolites.

## 2. Materials and methods

### *In vitro* batch cultures incubation

An adult rumen cannulated sheep fed grass hay was used as a donor for rumen fluid, which was collected before morning feeding. The rumen fluid was collected in a thermos flask, carried to the laboratory, filtered through sterile sieve and kept in an anaerobic chamber. The substrate used for the batch cultures was chopped grass hay, the basal diet for the cannulated sheep.

The four phenolic acids were prepared by solubilization in sodium phosphate buffer with pH 6.7 and stored at 4°C not more than 12 hours.

*In vitro* incubations were carried out in 100 ml serum bottles with the following: 500 mg substrate, 10 ml filtered rumen fluid, 40 ml medium (141 DSM Medium for Methanogens with some modifications: $Na_2S$ x $9H_2O$ was substitued with Cysteine-HCl x $H_2O$ and Na-acetate was eliminated from the medium preparation) and trans-cinnamic, caffeic, p-coumaric acids and catechin hydrate in 6 mM concentration. The bottles were sealed with a rubber stopper and placed in an incubator at 39℃. Three replicates were incubated for testing the effect of plant secondary metabolites (500 mg substrate, 10 ml rumen fluid, 40 ml medium and trans-cinnamic, caffeic, p-coumaric acids and catechin hydrate in 6 mM), along with blank (only 500 mg substrate and 40 ml medium) and controls (500 mg of substrate, 10 ml rumen fluid and 40 ml medium).

### Analyses

After 24 hours of incubation, the following parameters were analyzed: cumulative gas volume, gas composition, pH and volatile fatty acids concentration. A sample from the head space gas was transferred to a 20 ml GC vial for the methane analysis by gas chromatography and the incubated inoculums were sub sampled for pH analysis and for VFAs (acetate, propionate, n-butyrate, izo-butyrate, izo-valerate) analyses by HPLC (SHIMADZU HPLC with HiPlex Agilent column and $H_2SO_4$ 5mM as eluent).

The results from the experiment were subjected to one-way analysis of variance (ANOVA), option in Statistica 12 StatSoft.

## 3. Results and discussion

The effects of the four plant secondary metabolites used in our studies on cumulative gas volume, methane production, and volatile fatty acids profile and on the pH are represented in Figure 1, Figure 2 and respectively in Table 1.

Addition of caffeic and p-coumaric acids had no significant effect on cumulative gas volume (Figure 1) and tended to decrease methane production (Figure 2) in comparison to control but the decrease was not statistically significant (P= 0.058, respectively 0.052). Trans-cinnamic acid and catechin hydrate presented no significant effect on total gas volume (Figure 1) and on methane production (Figure 2).

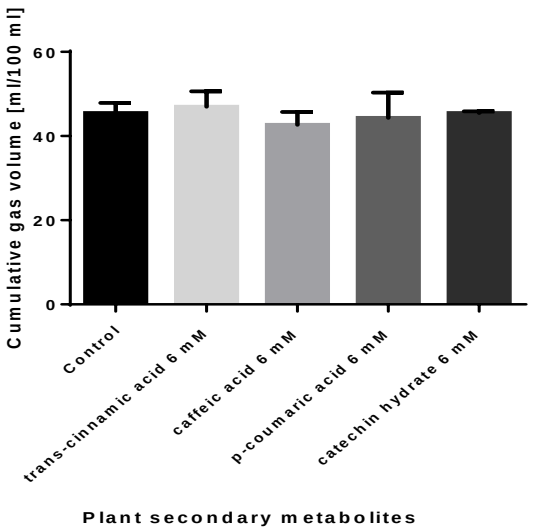


**Figure 1.** Effect of plant secondary metabolites on methane production [ml/100 ml] in comparison to control after 24 h of incubation (P > 0.05)

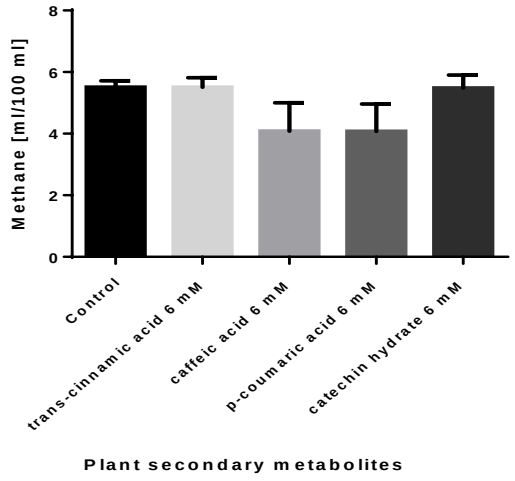


**Figure 2.** Effect of plant secondary metabolites on cumulative gas volume [ml/100 ml] in comparison to control after 24 h of incubation (P > 0.05)

The four PSMs tended to decrease the total VFAs production (Table 1), although the differences were not statistically significant (P>0.05). The pH of the incubations was not affected by these compounds and remained in the same range as in control.

Some of the methane inhibitors such as chemicals can have adverse effects on ruminal fermentation and on animal physiology and metabolism [18]. Has been reported that methane production decreased in response to plant secondary metabolites such as tannins which reduced methane emissions in sheep and cattle [20], or saponins which decreased ruminal methanogenesis *in vitro* studies [21, 22]. Some authors consider that the mode of action for these compounds is related to their anti-protozoal activity [13, 9] and that they can inhibit fiber degradation [12].

The inhibition in fiber degradation will shift VFAs composition away from acetate and hence less production of hydrogen and less methane formation [24]. Anti-protozoal effect of phenolic compounds can decrease methane production since in rumen a portion of methanogens is attached to protozoa [2, 23]. Janayegara [2] reported that the effect of phenolic acid on ruminal methane production depends on the source and concentration applied. In his experiment with phenolic acids, the order of phenolic acids to decrease methane production was caffeic>p-coumaric>feluric>cinnamic acids [2].

In the current study, addition of caffeic and p-coumaric acids tended to decrease methane production but not statistically significant at α=5 (95% confidence) and the other compounds presented no significant effect. The other ruminal fermentation parameters were not affected by these phenolic acids. It is known that rumen microorganisms can adapt in the presence of phenolic compounds and this may result in a small decrease in methane production. One mechanism of defense that organisms active in fiber degradation can have is hydrogenation of the more toxic phenolic compounds to a less toxic form [2]. Another explanation is that from rumen fluid, by non-specific absorption to microbial surfaces or by specific uptake and utilization by some rumen microbes, the phenolic acids added may be lost [2]. In this way, a decrease in methane production cannot occur.

**Table 1.** Effects of trans-cinnamic, caffeic and p-coumaric acids and catechin hydrate on rumen fermentation parameters *in vitro* after 24 h incubation

| Rumen parameters | Control | trans-Cinnamic acid 6 mM | Caffeic acid 6 mM | p-Coumaric acid 6 mM | Catechin hydrate 6 mM | SEM |
|---|---|---|---|---|---|---|
| Total VFA (mg/l) | 610.6 | 579.3 | 533.1 | 577.1 | 611.4 | 12.983 |
| Acetate (mg/l) | 1764.4 | 1705.3 | 1654.3 | 1720.9 | 1800.5 | 22.881 |
| Propionate (mg/l) | 644.4 | 565.0 | 511.9 | 567.9 | 821.6 | 8.646 |
| n-Butyrate (mg/l) | 549.7 | 561.7 | 546.9 | 571.3 | 366.3 | 21.926 |
| izo-Butyrate (mg/l) | 23.1 | 12.4 | 6.3 | 6.3 | 13.5 | 5.119 |
| izo-Valerate (mg/l) | 71.4 | 52.3 | 46.1 | 19.3 | 54.9 | 6.248 |
| pH | 6.3 | 6.3 | 6.3 | 6.4 | 6.4 | 0.025 |

SEM- standard error of the mean

## 4. Conclusions

Addition of plant secondary metabolites to *in vitro* ruminal cultures did not affected the total gas volume, the pH and the profile of volatile fatty acids. Caffeic and p-coumaric acids tended to decrease methane production but it was not statistically significant at $\alpha = 5$. This fact suggested that caffeic and p-coumaric acids may have the potential possibility for use as a strategy to decrease methane emission from ruminants. The other two compounds can be used in other studies but maybe another concentration is needed. Further research is required to understand the ruminal fermentation and the effects of plant secondary metabolites on ruminal methanogenesis.

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