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# Evaluation of a dietary blend of essential oils and polyphenols on methane emission by ewes

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## ABSTRACT

**Context.** Decreasing enteric methane emissions from small ruminants is important because methane is a greenhouse gas and a major contributor to global warming. **Aim.** The objective of this work was to test the effect of a dietary premix consisting of a blend of essential oils, bioflavonoids and chestnut tannins (EOP, essential oils and polyphenols) on methane emission from dry non-pregnant ewes. **Methods.** Twenty-four dry Sarda ewes were allocated to two homogenous groups: control and treatment groups. Both were fed with a total mixed ration, and the treatment group was supplemented with 1 g/day.ewe of EOP blend. Each animal followed a 20-day adaptation period before methane emission measurements. Methane emissions were measured using a ventilated hood system equipped with a digital gas analyser. **Key results.** The addition of a dietary EOP blend to the total mixed ration did not affect feed intake and nutrient digestibility. Ewes that received the EOP blend had a 13% lower methane yield than ewes that received the control diet (22.4 vs 25.5 g of $CH_4$ per kg of dry matter intake; $P < 0.05$, respectively). The EOP blend had no impact on daily methane emission when calculated as g $CH_4$/ewe or g $CH_4$/kg bodyweight. **Conclusions.** The EOP blend at a daily dose of 1 g/day.ewe decreased methane yield under the experimental conditions described in this work. **Implications.** Feeding an EOP dietary blend to ewes can decrease methane emission. These results were obtained *in vivo* with typical farm conditions, suggesting that a similar response may occur in field conditions.

**Keywords:** digestibility, enteric emissions, essential oils, feed intake, methane, sheep, tannins, ventilated hood.

## Introduction

Decreasing the emission of enteric methane ($CH_4$) by ruminants is crucial, because it represents a major contributor to global warming. Most of the Archaea that reside in the rumen produce $CH_4$ by aerobic catabolism of carbohydrates, and the fermentation byproducts of other microbes in the rumen contribute to $CH_4$ production. Methane production in the rumen fulfils a basic physiological need of these animals by maintaining the redox potential of the ruminal environment (Moss *et al.* 2000; van Zijderveld *et al.* 2010). The fermentation of feed (organic matter [OM]) is responsible for ~90% of the $CH_4$ produced in the rumen (Murray *et al.* 1976), and Archaea reduce $CO_2$ to $CH_4$ using electrons derived from hydrogen (Hook *et al.* 2010; Nolan *et al.* 2010). This represents a loss of 6–10% of gross energy (Johnson and Johnson 1995). Preventing this energy loss can increase feed efficiency and energy use, and decrease production costs at the farm level (Hristov *et al.* 2013).

Many *in vitro* and *in vivo* trials have quantified the $CH_4$ emission potential of feeds, and examined the effects of different bioactive compounds on ruminal fermentation and gas production (Asanuma *et al.* 1999; Eckard *et al.* 2010; Bodas *et al.* 2012; Lee and Beauchemin 2014). A wide range of additives can decrease *in vitro* fermentation and mitigate $CH_4$ production (Pirondini *et al.* 2012). Some of these additives are hydrogen acceptors, such as polyunsaturated fatty acids and nitrates, which decrease the level of electrons needed for $CO_2$ reduction (Lind *et al.* 2021). The mitigating effect of dietary lipids on $CH_4$

production ranges from 12 to 38% when these lipids account for 3.5–7.0% of total dry matter (DM), and this mitigation depends on the amount of fat, fatty acid composition and composition of the basal diet (Patra 2013; Martin *et al.* 2016; Lind *et al.* 2021). A nitrate additive can decrease $CH_4$ production by up to 30% (Feng *et al.* 2020; Lind *et al.* 2021), but its use is limited due to the risk of nitrite toxicity (Lee and Beauchemin 2014). The investigation on natural compounds that can have an inhibitory effect on the enteric $CH_4$ production is then very important, and a huge literature exists on this topic, even if the results are often not univocal. Different classes of bioactive substances can modify metabolism in the rumen, such as polyphenols (Vasta *et al.* 2019) and essential oils (EOs; Belanche *et al.* 2020). Polyphenols (such as tannins) and EOs decrease enteric $CH_4$ production by interfering with the metabolism of microorganisms in the rumen. Recent studies showed that the tannin-mediated decrease in $CH_4$ emission was related to their ability to decrease fibre degradation, which prevents the attachment of microorganisms to plant cell walls, inhibits microbial enzymes and thereby alters the function of ruminal microorganisms (Vasta *et al.* 2019). Recent reviews and research showed that inhibitory effects on fibre degradability were observed with high dosis of tannins or blends with other bioactive compounds, whereas limited amounts reduced emissions without negative effects on digestibility (Lind *et al.* 2021; Foggi *et al.* 2022). Some studies have used flavonoids, the largest class of phenolic compounds, to decrease $CH_4$ emissions by enteric microorganisms, and their effect depends on the chemical structure of the specific flavonoids (Oskoueian *et al.* 2013). Tannins have anti-methanogenic effects, mainly due to their effects on microbial populations in the rumen, because they are toxic to some strains of bacteria, protozoa, fungi and Archaea (Patra and Saxena 2011). The results of *in vitro* and *in vivo* studies have consistently indicated the potential efficacy of tannins in mitigating enteric $CH_4$ emissions from ruminants, and hydrolysed tannins that have little structural variability are especially effective (Aboagye and Beauchemin 2019). Tannins from chestnut (*Castanea sativa L.*) are the most commonly extracted hydrolysable tannins from temperate plants, and these tannins are efficient in decreasing $CH_4$ emissions from ruminants (Hassanat and Benchaar 2013; Aboagye and Beauchemin 2019). However, the effect of this decrease is highly variable, ranging from 4.3% to 70% *in vitro* and from 6.0% to 68% *in vivo* (Aboagye and Beauchemin 2019).

Essential oils are widely used as a feed additive and an alternative to antibiotics because of their antimicrobial activity (Patra *et al.* 2017). Several studies have shown that EOs modulate rumen metabolism by improving microbial fermentation and decreasing $CH_4$ production due to their inhibition of Archaea (Benchaar *et al.* 2008; Benchaar and Greathead 2011). An advantage of EOs is that they are natural compounds. Several studies have shown that EOs decreased $CH_4$ production and had no negative effects on feed intake or productivity (Belanche *et al.* 2020). However, very few studies have evaluated the effects of EOs and their constituents on $CH_4$ emissions *in vivo*. Specific studies are needed to confirm the effect of EO blends after an initial period of rumen adaptation (Belanche *et al.* 2020).

It is important to use *in vivo* experiments on commercial farms to quantify $CH_4$ emissions from ruminants, because dietary modification may be a relatively simple and effective method to mitigate climate change. Sheep are a valuable animal model for feeding trials, especially for testing the effect of different animal diets on production and metabolic responses. Moreover, considering that the cited compounds (EOs, flavonoids and tannins) can act with different mechanisms as methane production modulators, the use of their mix could be useful, hypothesising a certain synergistic effect. The aim of this study was then to test the ability of a blend of bioactive compounds consisting of tannins, EOs, and bioflavonoids to decrease $CH_4$ emission by ewes with limited negative effects on rumen equilibrium, diet digestibility and animal performance.

## Materials and methods

### Animals and diets

Experiments were conducted at the experimental farm of the Department of Agriculture of the University of Sassari, located in northwest Sardinia (Sassari, Italy; 40°48′41.4″N, 8°17′50.7″E). All procedures involving animals were in full compliance with the regulations of the European Community (86/609) and Italy (DPR 27/1/1992, Animal Protection Regulations of 124 Italy) regarding animal welfare and experimentation. A randomised block design using two parallel groups was established to test the effect of dietary interventions on $CH_4$ emission. Twenty-four adult ewes of the local Sarda dairy breed, who were dry, non-pregnant and had a bodyweight (BW) of 39–70 kg were selected from a larger group of healthy ewes. The sheep were randomly allocated to two homogenous groups that were balanced for BW. Sheep were fed a dry total mixed ration (TMR), which consisted of 70% hay and 30% concentrate (Table 1). The control (CNT) group ($n$ = 12; 52.5 ± 7.13 kg BW) received a basal diet, and the treatment (TRT) group ($n$ = 12; 53.4 ± 10.3 kg BW) received a basal diet supplemented with a bioactive compound premix. The premix contained EOs, bioflavonoids and chestnut tannins (essential oils and polyphenols [EOP]). In particular, the EOP consisted of a coated blend of EOs, mainly from clove (*Syzygium aromaticum*), coriander seed (*Coriandrum sativum*) and geranium (*Pelargonium cucullatum*); tannins from chestnut (*Castanea sativa*); and bioflavonoids from olive (*Olea europea*). The EO:tannins:bioflavonoids ratio was 1:2.5:0.1, and the blend (EOP) was a commercial product (Anavrin; Vetos Europe SAGL, Cadenazzo, Switzerland).

All animals were housed indoors and were exposed to same environmental conditions. In particular, the ewes were

**Table 1.** Ingredients and chemical parameters of the basal diet provided to the control and treatment groups.

| Item[A] | Basal diet |
|---|---|
| Ingredients (g/kg) | |
| Ryegrass hay | 390 |
| Corn meal | 150 |
| Alfalfa hay | 130 |
| Soybean meal 48% | 105 |
| Beet pulp | 82 |
| Wheat straw | 80 |
| Molasses | 56 |
| Mineral premix | 7 |
| Chemical parameters (g/kg) | |
| DM (as fed) | 880 |
| OM (as fed) | 804 |
| CP (on DM) | 150 |
| NDF (on DM) | 446 |
| ADF (on DM) | 321 |
| ADL (on DM) | 65.4 |
| Starch + sugars (on DM) | 223 |
| EE (on DM) | 22.2 |
| Ash (on DM) | 75.6 |
| NFC (on DM) | 306 |

[A]The dietary supplement in the TRT group consisted of 1 g/day.ewe of the EOs and polyphenols blend (see Materials and methods).
DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, acid detergent lignin; EE, ether extract; NFC, non-fibre carbohydrate.

housed in a pen for 21 days to allow adaptation to the diet, and were then kept in metabolic cages during the experimental phase. The TMR was individually offered to each ewe three times per day (7:00 am, 03:00 pm and 11:00 pm) and remained available for the subsequent 3 h. The weights of feed offered and feed refused were determined for each meal. Refusals were collected each day and analysed to calculate the nutrient intake of each animal. Diets were offered based on the maintenance energy requirement of each ewe, calculated using the Small Ruminant Nutrition System (Tedeschi *et al.* 2010), and the amount of offered feed was based on BW. Each ewe in the TRT group received a dose of the blend (1 g/day), which was mixed with 5 g of cornmeal to facilitate complete ingestion. As a placebo, each ewe in the CNT group received 6 g of cornmeal per day. The daily treatment was split into three portions that were administered at 8-h intervals each day, and were provided as an oral bolus to ensure complete consumption. These treatments were at 7:00 am, 3:00 pm and 11:00 pm (during the main meals). All animals received this diet for a 21-day adaptation period before experimental measurements of $CH_4$ emission.

## Adaptation to cages, digestibility and $CH_4$ measurements

After the initial 21-day adaptation period, the ewes were transferred to individual metabolic cages in the same barn for the following 7 days. The experimental measurements in these metabolic cages included the amount of offered and residual meals, which were used to calculate DM intake (DMI) per meal for each animal, and collection and sampling of faeces. The total feed residuals and total faeces of each ewe were weighed individually during the last 4 days, and the digestibility and $CH_4$ measurements were then performed. In particular, feed residues and faeces were collected each day before each meal; they were separately weighed and mixed, and a 20% sample (total fresh weight) was prepared and was immediately stored at −20°C until chemical analysis. A pool of the total sampled faeces over the 4-day period was used for determination of digestibility. Digestibility calculations were used to measure dietary DM, neutral detergent fibre (NDF) and acid detergent fibre (ADF), each expressed as a percentage of nutrient intake minus nutrient excreted in faeces.

$CH_4$ emissions from individual ewes were measured after the initial 21-day adaptation period. These measurements were performed using an indirect calorimetric system designed for small ruminants that employs ventilated hoods mounted in the metabolic cages. These hoods were managed in parallel (one for a CNT ewe and one for a TRT ewe) at the same time. The same hoods were used for both groups to avoid confounding effects among treatments and hoods. Gas exchange was measured for each animal continuously for 24 h, which included the three 8-h periods after the main meals (7:00 am to 3:00 pm; 3:00 pm to 11:00 pm; and 11:00 pm to 7:00 am).

The ventilated hood system used to measure $CH_4$ emissions and perform indirect calorimetry experiments was described previously (Fernández *et al.* 2012, 2015, 2019; Criscioni *et al.* 2016; Lind *et al.* 2021), and the details of the entire system were described by Lai (2020) and by Lind *et al.* (2021). This system consisted of two parts: (i) two head hoods suspended on the front part of the classic metabolic cage for small ruminants; and (ii) one air sampling system, which contained instruments for air evacuation and sampling from the hoods, and a gas analyser (GMS810; SICK S.p.A., Vimodrone, Italy) that had an internal micro-pump and an auto-calibration system that provides long-term system calibration. All data from the analyser were acquired by software (SOPAS Engineering Tool; SICK S.p.A.).

The ventilated hoods and the open-circuit respiration chambers operate using the same principles, although ventilated hoods are less expensive (Place *et al.* 2011). When using the ventilated hood, the animal was in a metabolic cage that has a separation box for the head (Suzuki *et al.* 2007). The head was separated by a sleeve of waterproof fabric, which allows some movement for the animal and also prevents air leaks (Bhatta and Enishi 2007). The head boxes were equipped with fans to move the main air towards the exhaust pipe. The air filters remove humidity, and all gases are directed into the analyser (Suzuki *et al.* 2007). Airflow was measured, and the $CH_4$ concentration was calculated by measuring the incoming and exhaust levels. Similar to respiration chambers, this method provides continuous measurements of $CH_4$.

## Chemical analysis

The chemical compositions of feed ingredients, feed residues and collected faeces were analysed in duplicate. DM was determined by oven-drying samples at 105°C for 24 h. An Ankom 220 fibre analyser (Ankom™ technology, Fairport, NY, USA) was used to determine NDF and acid detergent lignin, as described by Van Soest *et al.* (1991). The NDF was determined using heat-stable amylase and expressed after excluding residual ash; acid detergent lignin was determined using concentrated sulfuric acid to solubilise the cellulose; crude protein (was determined using the Kjeldahl method (proc. 988.05; AOAC 2000); extract ether was determined using the Soxhlet method (proc. 920.39; AOAC 2005); ash was determined using a muffle at 550°C (proc. 942.05; AOAC 2000); starch was determined using polarimetry (Polax 2 L; Atago®, Tokyo, Japan) according to EC (1999); and non-fibre carbohydrates (g/kg DM) were calculated according (Weiss 1993) as: 100 − (NDF + crude protein + ash + extract ether).

## $CH_4$ calculations

Calculations of enteric emissions of $CH_4$ were performed as described by Pinares-Patiño *et al.* (2012). These calculations used measurements of the head hood wet ventilation rate (Wet VR), net concentration of gas in the dry sample and percentage of gas recovery in the entire system. The calculation started with results from the gas analyser, with each measurement expressed as ppm $CH_4$. The Wet VR was then adjusted to the dry standard temperature and pressure ventilation rate (Dry STP VR). For each measurement, the emission of $CH_4$ was calculated as:

$$CH_4(\text{L/min}) = [\text{Dry STP VR} \times ([CH_4 \text{ ppm}]/1000000)]/[\text{gas recovery rate}] \quad (1)$$

where Dry STP VR has units of L/min and the gas recovery rate is a percentage.

The calculation of Dry STP VR requires data for relative humidity (%), temperature (°C) and pressure (hPa) for each head hood system, and was calculated as:

$$\text{Dry STP VR (L/min)} = [(\text{Air pressure} \times \text{dry gas VR})/(\text{Chamber T} + 273.15) \times 273.15/1013.25 \quad (2)$$

where pressure has units of hPa, Dry gas VR has units of L/min and Chamber T has units of °C.

The Dry gas VR was calculated as:

$$\text{Dry gas VR (L/min)} = \text{Wet VR} \times [(100 - \text{VMR})/100] \quad (3)$$

where Wet VR is the ventilation rate recorded from the flow meters and has units of L/min, and VMR is the volume mixing percentage of moisture

$$\text{Volume mixing ratio (VMR) (\%)} = 100 \times \text{PWP/air pressure} \quad (4)$$

where PWP is the partial water pressure and has units of hPa, and the air pressure has units of hPa.

The partial water pressure was obtained using the Wexler equation:

$$\text{Partial water pressure (hPa)} = (6.1117675 + 0.4439T + 0.014305T^2 + 0.000265T^3 + 0.00000302T^4 + 0.0000000204T^5 + 0.0000000000006388T^6) \times \text{RH}/100 \quad (5)$$

where $T$ is the head hood temperature and has units of °C and RH is the percentage relative humidity of the chamber.

Daily emissions were converted from L/day to g/day using the following equation:

$$1 \text{ g } CH_4 = 1.3962 \text{ L } CH_4$$

## Statistical analysis

Statistical analyses were performed using PROC MIXED in SAS Version 9.0 (SAS Institute, Cary, NC, USA) and the following model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k \times BW + \varepsilon_{ijk} \quad (6)$$

where each $Y_{ijk}$ is a dependent variable (DMI in kg/day, digestibility of DMI and NDF intake, g $CH_4$/day, g $CH_4$/kg DMI, g $CH_4$/kg BW, g $CH_4$/kg DMI and g $CH_4$/kg NDF), $\mu$ is the general mean, $\alpha_i$ is the main treatment effect ($i$ = CNT; TRT), $\beta_j$ is the random effect of a hood ($j$ = 1; 2), $\gamma_k$ is the random effect of the covariate BW and $\varepsilon_{ijkl}$ is the residual term. Data are expressed as means ± s.e.m.s, and the differences of group means were assessed using Tukey's honest significance test, with a $P$-value <0.05 considered significant.

Correlations were computed to assess the relationship of $CH_4$ emission (g $CH_4$/day and g $CH_4$/kg BW) with DMI. The mean concentration of $CH_4$ (ppm) in the emissions was calculated from hourly measurements of animals in each group during a 24-h period.

**Table 2.** Feed intake and nutrient digestibility in the control and treatment groups.

| Parameter | Group | | s.e.m. | Effects and *P*-value[A] | | |
|---|---|---|---|---|---|---|
| | | | | Fixed | Random | |
| | CNT | TRT | | Treatment | Hood | Bodyweight |
| Intake (g/day) | | | | | | |
| DMI | 886 | 974 | 46.3 | 0.29 | 0.68 | 0.06 |
| OMI | 712 | 782 | 37.5 | 0.28 | 0.66 | 0.08 |
| NDFI | 328 | 349 | 33.2 | 0.62 | 0.38 | 0.41 |
| ADFI | 216 | 223 | 21.2 | 0.79 | 0.35 | 0.79 |
| Digestibility | | | | | | |
| DMD (g/kg of DM) | 681 | 666 | 7.21 | 0.74 | 0.41 | 0.08 |
| OMD (g/kg of DM) | 772 | 774 | 7.82 | 0.87 | 0.45 | 0.32 |
| NDFD (g/kg of NDF) | 538 | 551 | 13.7 | 0.32 | 0.41 | 0.46 |
| ADFD (g/kg of ADF) | 479 | 496 | 16.2 | 0.66 | 0.63 | 0.53 |

[A]Least-square means of methane emission in the control (CNT) and treatment (TRT) groups significantly differ when the *P*-value of the fixed effect (treatment) is <0.05. DMI, dry matter intake; OMI, organic matter intake; NDFI, neutral detergent fibre intake; ADFI, acid detergent fibre intake. DMD, dry matter digestibility; OMD, organic matter digestibility; NDFD, neutral detergent fibre digestibility; ADFD, acid detergent fibre digestibility.

## Results

Both groups had large variations in animal BW (range 39–70 kg). However, analysis of feed intake parameters in g/day.ewe (Table 2) indicated the two groups had no significant differences in DMI (TRT: 974, CNT: 886 g/day), which corresponded to ~1.8% of BW. The two groups also had no significant differences in the three other intake parameters (OM, NDF and ADF).

We compared the digestibility parameters in the different groups (Table 2). The two groups had no significant difference in digestibility of DM (CNT: 681; TRT: 666 g/kg), NDF (CNT: 538; TRT: 551 g/kg), OM (CNT: 772; TRT: 774 g/kg) or ADF (CNT: 479; TRT: 496 g/kg). These results indicate that the dietary treatment did not affect intake or digestibility.

Analysis of $CH_4$ emissions (Table 3) indicated the two groups had no differences when emission was expressed as g/day.ewe (CNT: 21.7, TRT: 21.4 g/day.ewe) or as g/kg BW (CNT: 0.42, TRT: 0.42 g/kg BW).

However, analysis of plotted records of 24 ewes together indicated there was a significant positive correlation of DMI (g/day.ewe) with $CH_4$ emission (g/day.ewe; Fig. 1). There was also a positive correlation of DMI (g/day.ewe) with $CH_4$ emission (g/kg BW; Fig. 2).

In addition, the TRT group had a lower $CH_4$ yield when expressed as g/kg DMI (22.4 vs 25.5 g $CH_4$/kg DMI, $P < 0.05$) and as g/kg OM intake (27.8 vs 31.7 g $CH_4$/kg OM intake; Table 3). The lower $CH_4$ emission in the TRT group was also evident when $CH_4$ was expressed as g $CH_4$/kg digested OM (36.0 vs 41.2 g $CH_4$/kg digested OM, $P = 0.03$). However, the two groups had no significant differences when $CH_4$ was expressed as g/kg digested DM or as g/kg digested NDF (both $P > 0.05$).

**Table 3.** Methane emission in the control and treatment groups.

| Methane ($CH_4$) emission | Group | | s.e.m. | Effects and *P*-value[A] | | |
|---|---|---|---|---|---|---|
| | | | | Fixed | Random | |
| | CNT | TRT | | Treatment | Hood | Bodyweight |
| $CH_4$ production (g/day) | 21.7 | 21.4 | 0.75 | 0.85 | 0.72 | 0.53 |
| $CH_4$ yield (g/kg of BW) | 0.42 | 0.42 | 0.02 | 0.84 | 0.57 | 0.01 |
| $CH_4$ yield (g/kg of DMI) | 25.5 | 22.4 | 0.82 | 0.04 | 0.78 | 0.03 |
| $CH_4$ yield (g/kg of OMI) | 31.7 | 27.8 | 1.05 | 0.03 | 0.85 | 0.03 |
| $CH_4$ yield (g/kg of digested DM) | 38.3 | 33.0 | 1.42 | 0.06 | 0.59 | 0.07 |
| $CH_4$ yield (g/kg of digested OM) | 41.2 | 36.0 | 1.53 | 0.03 | 0.86 | 0.02 |
| $CH_4$ yield (g/kg of digested NDF) | 125 | 112 | 5.81 | 0.30 | 0.38 | 0.55 |

[A]Least-square means of methane emission in the control (CNT) and treatment (TRT) groups significantly differ when the *P*-value of the fixed effect (Treatment) is <0.05. BW, bodyweight; DMI, dry matter intake; OMI, organic matter intake; DM, dry matter; OM, organic matter; NDF, neutral detergent fibre.

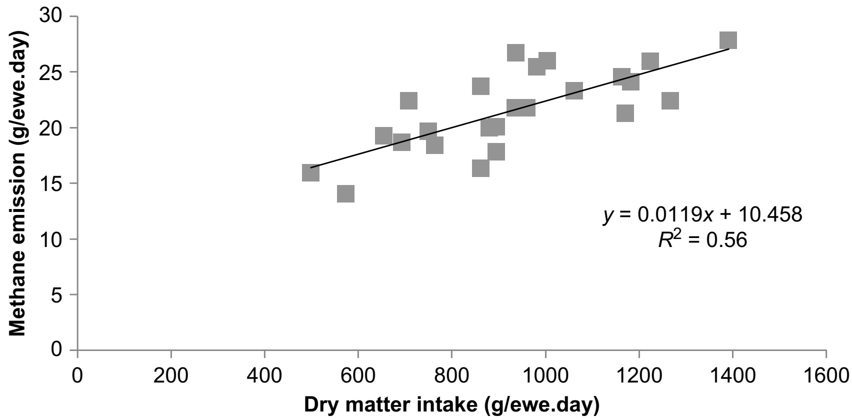


**Fig. 1.** Correlation of dry matter intake (g/day.ewe) with methane emission (g/day.ewe) of plotted records from all 24 ewes.

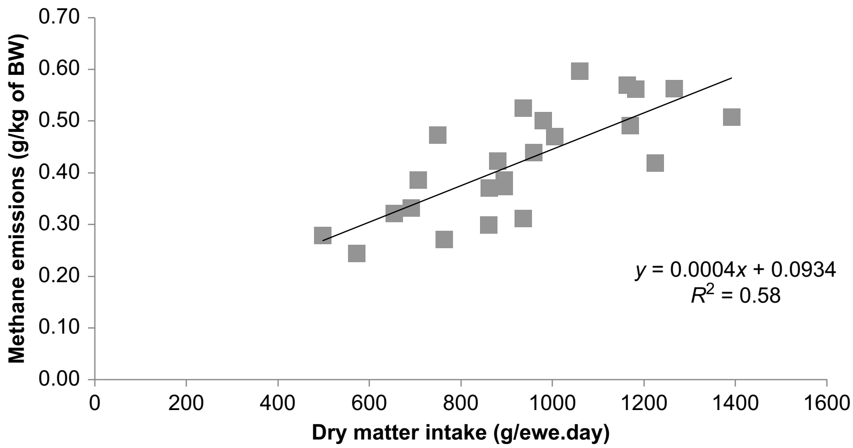


**Fig. 2.** Correlation of dry matter intake (g/day.ewe) with methane emission (g/kg bodyweight) of plotted records from all 24 ewes.

## Discussion

In the current study, the reduction of methane emission was observed in sheep supplemented with a blend of EOs, flavonoids and chestnut tannins. This result was consistent with previous studies, which reported that these classes of compounds inhibited methanogenesis (Vasta *et al* 2019; Lind *et al.* 2021). In particular, the emissions of the TRT group were lower (−13%) than those observed in the CNT group when expressed per unit of DMI (g $CH_4$/kg DMI). More specifically, our observation is consistent with the effect of EO blends fed to dairy cows under comparable conditions (12.9%) reviewed by Belanche *et al.* (2020), and with the results of experiments performed in respiration chambers (11.0%) reported by Klop *et al.* (2017). Yatoo *et al.* (2018) studied buffaloes, and found that an EO blend decreased the $CH_4$ yield by 14% per kg DMI and digested DM during a 6-month feeding period, and that there were no negative effects on intake or digestibility of DM and nutrients relative to the control diet. A meta-analysis of 21 refereed publications involving sheep (Torres *et al.* 2020) found that dietary EOs did not affect feed intake or decrease daily $CH_4$ emission. Cobellis *et al.* (2016) concluded that the most consistent results regarding $CH_4$ emission were observed when the source of EOs was thyme, oregano, cinnamon and garlic or their principal components (thymol, carvacrol, cinnamaldehyde and allicin, respectively).

It appears important to consider the biological reasons for the decreased $CH_4$ yield in ewes that received bioactive compounds. Our results demonstrated an anti-methanogenic effect of dietary polyphenols. Previous research reported contrary results regarding the effect of dietary polyphenols of $CH_4$ emission from ruminants. For example, one study reported that chestnut tannins inhibited $CH_4$ emission in sheep and typically had no adverse effects on growth performance (Liu *et al.* 2011). However, another study reported that dietary tannins had no effect on $CH_4$ emission from sheep (Wischer *et al.* 2014).

The effect of the dietary supplementation of EOs on $CH_4$ emission could be attributable to its modulation of microbial activity in the rumen (Oh *et al.* 1967; Vasta *et al.* 2019). Cobellis *et al.* (2016) concluded that dietary EOs decreased the population of protozoa by 16%. A meta-analysis of 21 studies of sheep concluded that dietary EOs led to an altered fermentation pattern, with a 0.59% molar increase of propionate and a 1.0% molar decrease of acetate (Torres *et al.* 2020).

Foggi *et al.* (2022) performed *in vitro* testing of 48 treatments of tannins alone, EOs alone, and tannins and EOs in combination, and found that tannins were mostly responsible for the decreased production of $CH_4$ and ammonia, but that blends had the greatest effect. The same authors also found that the tested supplements had minimal effects on protozoa, but led to significantly decreased fibre digestibility relative to the control diet.

Our results for daily $CH_4$ emission (average 21.6 g/day, range 19.0–26.0 g/day) were similar to the values reported in previous studies of sheep (24.1 ± 7.6 g/day), even though these previous studies used different experimental procedures, examined different breeds and used different levels of DMI (Pinares-Patiño *et al.* 2003; Santoso *et al.* 2007; Knight *et al.* 2008; Bhatt *et al.* 2019). Our results correspond to ~9 kg of $CH_4$ per ewe per year, in line with the estimates of the IPCC (2019; 9.0 kg/head for dry ewes) and Vermorel *et al.* (2008; 9.3 kg/head for dry ewes). In contrast, when emissions are expressed as g $CH_4$/day.animal, there could be confounding effects due to individual differences in DMI and animal BW. DMI is a major determinant of $CH_4$ emissions (Blaxter and Clapperton 1965; Charmley *et al.* 2016; Bond *et al.* 2019), and our results confirmed the positive relationship between DMI and $CH_4$ emission (Figs 1 and 2).

Previous studies of $CH_4$ yield showed that expression of emission as g $CH_4$/kg DMI or as g $CH_4$/kg OMI was most appropriate (Blaxter and Clapperton 1965; Fernández *et al.* 2012; Patra *et al.* 2016). These phenotypic measures distinguish differences among groups and eliminate the effects of individual differences in intake (Pinares-Patiño *et al.* 2011*a*, 2011*b*). In fact, it refers to the amount of substrate available for fermentation in the rumen, as well as hydrogen formation. The value we observed (23.9 g $CH_4$/kg DMI) was within the range observed by Bhatt *et al.* (2019), which was 21.4–30.3 g $CH_4$/kg DMI in sheep fed TMR provided as feed block or mash. This previous study found these yields were negatively associated with DMI (1255 and 926 g/day of TMR per ewe, respectively; Bhatt *et al.* 2019). Our values were also in line with those measured in sheep that were fed a hay- and silage-based diet (21.9 to 25.2 g $CH_4$/kg DMI; Santoso *et al.* 2007), and in sheep that were fed a diet with a different forage-to-concentrate ratio (23.8 g $CH_4$/kg of DMI; Knight *et al.* 2008). It confirmed the effect of bioactive compounds, such as blends of EOs and polyphenols, used in low doses to reduce methane emissions (Lind *et al.* 2021).

Our finding of a strong relationship between $CH_4$ emission and DMI is also reflected in the pattern of daily $CH_4$ emissions measured during a 24-h period for ewes in the two experimental groups (Fig. 3). On average, the TRT group had greater $CH_4$ concentrations after the first and third daily meals, but the CNT group had greater $CH_4$ concentrations after the second meal. Although we did not examine the biological reasons for these results, it is possible that feeding behaviour had an effect, although we did not record feeding behaviour in this study. In addition, our two groups had no significant differences in terms of total daily emissions (Table 2). In both groups, analysis of the daily patterns of $CH_4$ concentration indicated peaks during the three daily mealtimes (07:00 am, 3:00 pm and 11:00 pm). Previous studies reported similar patterns from *in vivo* observations in sheep and cattle (Crompton *et al.* 2011; Lai 2020).

Additional studies are needed to assess the effect of different EO doses, and different types of EOs and extracts on $CH_4$ emission (Calsamiglia *et al.* 2007). In the present work, we administered a dose of 1 g/day of an EOP blend to each ewe, similar to the dose given to dairy cows in previous studies (Klop *et al.* 2017; Belanche *et al.* 2020). A previous study of sheep found that 0.4 g/day.kg of DMI of

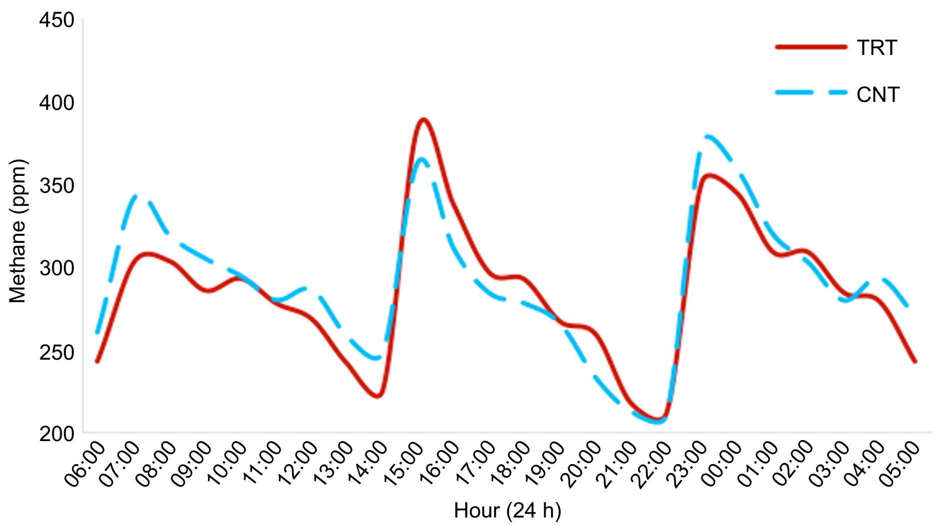


**Fig. 3.** Mean methane concentration (ppm) of emissions from ewes in the TRT and CNT groups based on hourly measurements for 24 h. The daily meals were provided at 07:00, 15:00 and 23:00.

micro-encapsulated EO led to a 28% decrease in $CH_4$ emission (Soltan *et al.* 2018); these same authors reported a 35% decrease in $CH_4$ emission when the dose was 0.2 g/day.kg of DMI, higher than the values of the present study. Thus, our direct oral administration of 1 g/day.ewe of EOP powder appeared to have lower efficiency than micro-encapsulated EO.

Another important consideration regarding the dose of a dietary supplement is the level of polyphenols, tannins in particular. Polyphenols and tannins have beneficial effects on ruminants at low doses (Correddu *et al.* 2019; Nudda *et al.* 2019) and moderate doses (Vasta *et al.* 2019; Correddu *et al.* 2020), although high doses can decrease intake, digestion and absorption of nutrients, and negatively affect overall animal performance (Kumar and Singh 1984). Yatoo *et al.* (2018) observed no difference in $CH_4$ production by water buffaloes that were given a dose of EO blend in which 5.5 kg of DMI was supplemented with 0.15 or 0.30 mL of EO/kg DMI. However, another study reported higher DM digestibility of DM, NDF and ADF in sheep that were fed with 4 g EO/day compared with control sheep that did not receive EO (Jiao *et al.* 2021).

Another important result of this investigation is related to the feed intake and nutrient digestibility. It should be noted that we provided the ewes in each group with a diet that was slightly greater than the estimated maintenance requirement, based on calculating DMI as a function of BW. Previous *in vitro* and *in vivo* studies found that intake and digestibility were unaffected by EOs supplementations in sheep (Soltan *et al.* 2018) and dairy cows (Belanche *et al.* 2020). Effects on intake and digestibility of EOs, polyphenols and their blends were observed with high doses of bioactive compounds. In particular, tannins are reported to be astringent and decrease palatability with negative effects on intake when administered at a high dietary level (Correddu *et al.* 2020). Also, negative effects of EOs on feed intake are generally related to palatability problems (Busquet *et al.* 2003). The lack of effect of the dietary treatment on the feed intake and nutrient digestibility observed in this study could be related to the moderate dose of EOs used. Indeed, previous works reported that high levels of EOs can negatively affect ruminal fibre degradation in sheep and cows (Tager and Krause 2011; Lin *et al.* 2013). Differences in digestibility were also not significant, suggesting that observed variability, both in intake and DM digestibility, could be related to individual effects of BW (Table 2). It should be also considered that contrasting results have been reported on the effects of EOs and polyphenols in ruminant diets, because the effects depend on the molecular weight and chemical structures of the considered compounds, their proportions in the diet, and their interaction with the other dietary ingredients (Correddu *et al.* 2020; Bešlo *et al.* 2022).

Finally, we suggest that further studies of this topic examine small ruminants to test the effects of different EO blends and tannins on $CH_4$ emissions, determine the optimal dose of different EO blends, and assess the effect of the duration of the rumen adaptation period needed to achieve the greatest effect. The effect of the adaptation period on response to the EO blend is an important topic, and the present study must be considered a short-term study, because the total duration of our experiments was only 28 days (including the 21-day adaptation period). Several previous studies demonstrated that EO blends led to decreased $CH_4$ emissions, and that treatment duration had major effects on the magnitude and consistency of this effect. For example, cows that were fed an EO blend for 10 weeks after an adaptation period had decreased DMI and DM digestibility; but *in vitro* tests of the rumen fluid from these cows indicated no effect on $CH_4$ production, although there was a decreased $CH_4$ yield from the first to the last week of the trial (Klop *et al.* 2017). These authors suggested that *in vivo* trials are needed to provide more definitive conclusions. We used an adaptation period of 21 days in the present study, slightly shorter than the 4 weeks suggested as optimal by Belanche *et al.* (2020) in their meta-analysis of *in vivo* studies that examined the effect of the Agolin Ruminant® EO blend. Belanche *et al.* (2020) performed short-term *in vivo* studies and reported only a 2.3% decrease in $CH_4$ emission, with inconsistent results. In particular, the $CH_4$ yield, when expressed as g $CH_4$/DMI, was not significantly affected when all short-term and long-term supplementation studies were considered. However, analysis of the long-term studies reported consistent anti-methanogenic effects, with an 8.8% decrease in $CH_4$ production, a 12.9% decrease in $CH_4$ yield, and a 9.9% decrease in emission intensity per kg of fat- and protein-corrected milk. These results were obtained without any adverse effects on feed intake, milk production or feed conversion efficiency. A study of dairy cattle reported that 6 weeks of an EO-supplemented diet decreased daily $CH_4$ emissions by 15% and decreased the level of $CH_4$ relative to DMI (g $CH_4$/kg DMI) by 14% (Castro-Montoya *et al.* 2015), in line with the results of the present study.

## Conclusions

In conclusion, the dietary EOP blend used in this study indicated that a low dose (1 g/day.ewe) led to significant decreases in the production of $CH_4$ by ewes when calculated as g $CH_4$/kg DMI, g $CH_4$/kg OMI and g $CH_4$/kg digested OM, and had no impact on intake or digestibility. The blend was evaluated under experimental conditions that, from a nutritional point of view, can be considered similar to what observed in sheep farms and after an adaptation period of 20 days, thus comparable decreases in methane production are expected with respective applications at a large scale.

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