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Effect of *Pituranthos scoparius* essential oils on reducing methanogenesis in cheep: *In Vitro* study

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ABSTRACT

Ruminants produce the methane (CH_4) in large quantities. Its eructation led to both energy losses for animals and a worsening of the greenhouse by its radioactive power. The aim of this study was to investigate the influence of essential oils (EO) extracted from *Pituranthos scoparius* on the methane production and the rumen digestibility.

The extraction of EO was made by steam distillation. *Pituranthos scoparius* essential oils (PS-EO) was investigated for its oil content using the ordinary Gas chromatography–mass spectrometry (GC/MS) analysis. The effect of these EO on the methane production, the digestibility of the dray and organic matter and the protozoa counting was studied in *in vitro* gas production test, using 200 mg of vetch-oat hay in a 60 ml graduated syringes. EO are added at three doses: 50, 100 and 200 µl. Total gas and methane production were recorded at 2, 4, 6, 8, 24, 48 and 72h of incubation.

GC-MS analysis allowed for the identiûcation of 32 compounds as main constituents. The major constituents were myristicin (12.1 %), 7-methoxy-3-methyl-1H-isochromen-1-one (10.6%) 1-cyclohexyliden-2-methylpropene (9.9%), Limonene (8.5%), *p*-Cymene (5.2%) and Thymol (4.8%). The results of this study indicate that EO decreased the methane production after 72 hours of fermentation for the different doses. This decrease the number of protozoa on which the methanogenic archaea was grafted. The results of the digestibility showed that EO deteriorate the digestibility of the dray and organic matter of the oaten vetch hay. The results indicate that the PS-EO had a potential to reduce methanogenesis in the rumen, but further *in vitro* and *in vivo* trials are required to search optimum dose which reduce methane production without adversely changing dietary fermentation and rumen function.

KEYWORDS

Methane;
Essential oils;
In vitro fermentation;
Dgestibility;
Pituranthos scoparius.

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INTRODUCTION

Methane is a gas with very powerful greenhouse effect[7,9]. Its atmospheric concentration has increased from 0.70 to 1.68 ppm during the last two centuries[8] and continues to increase annually by 0.6%[34]. The eructation of methane by ruminants led to both energy loss for animals and a worsening of the greenhouse by its radioactive power. Indeed, microbial fermentation in the rumen lead to the formation of volatile fatty acids and several gases mainly carbon dioxide (CO_2) and methane (CH_4). Methane is the principal route of elimination of hydrogen produced in the rumen during microbial digestion of food[26].

Different strategies are deployed to mitigate methane emissions by ruminants. They are essentially interested to manipulate ruminal fermentation patterns and/or change of the equilibrium of the microbial population. Thus, the use of synthetic molecules regulators of microbial activity (antibiotics, halogenated molecules,...) are used extensively for several years[7,18,24]. However, the new European legislation and the recommendations of consumer protection organizations greatly limited the use of these methods. Recent legislation (1831/2003; EC, 2003) has been introduced within the European Union to prohibit the use of growth-promoting antibiotics in animal feeds which has led to an increased interest of natural substitutes like plants and their extracts[18].

Some of these new additives are the essential oils (EO). These are volatile and aromatic compounds with an oily appearance, which are obtained from plants[10]. Some work *in vitro* (artificial rumen) showed that they are effective in decreasing the amount of methane produced by bacteria in the rumen[4,5,15,17,19,24,30,35].

Pituranthos scoparius is a native plant in North Africa especially in Algeria. It is a widespread plant characterized by much ramified stems almost without leaves and with small fruits. It is a medicinal plant used for many purposes especially to treat asthma by inhaling its vapour.

The aim of this study is to investigate the content of essential oils extracted from *Pituranthos scoparius* (PS-OE) and to explore its anti-methanogenic activity in sheep, as well as an assessment of the ration digestibility and the protozoa counting in absence and in presence of these essential oils.

MATERIAL AND METHODS

Plant material

Aerial parts of *Pituranthos scoparius* were collected on February 2007 in the outskirts of Ghardaia (500 km south of Algiers). The identification of this plant was made on the basis of Quezel and Santa[31] key.

Animal material

Ruminal fluid was obtained from three Sheep chosen randomly before being sacrificed in the slaughterhouse of Tebessa. Their diet is free and not defined. The rumen fluid was transferred to the laboratory into two prewarmed thermos flasks, preheated to 39 °C, squeezed through four layers of cheesecloth and purged with CO_2.

Extraction of essential oils

1 kg of *Pituranthos scoparius* sample was dried under shade. The extraction of essential oils was made by steam distillation. It was conducted for 3 hours from a mixture of 100 g of plant material and 1.5 L of distilled water. Just after the extraction, the EO have stored in well closed glass bottles at 4°C and in darkness.

Gas chromatography/mass spectrometry (GC/MS)

The oil was analyzed by GC/MS using a Agilent 5973EI mass selective detector coupled with a Agilent GC6890A gas chromatograph, equipped with a cross-linked 5 % PH ME silox-FINAL GALLY PROOF ane HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 μm).

Operating conditions were as follows: carrier gas, helium with a flow rate of 1mL/min; column temperature50 °C for 1 min, 50-150 °C (3 °C/min), 150-250°C (5 °C/min) then isothermal for 5 min. Injector and detector temperatures, 280 ºC; split ratio, 1:50.

The MS operating parameters were as follows: ionization potential, 70 eV; ionization current, 2 A; ion source temperature, 200 ºC; resolution, 1000.

Identification of oil components was achieved based on their retention indices, determined with reference to a homologous series of normal alkanes and by comparison of their mass spectral fragmentation patterns with those reported in the literature and stored on the

MS library (NIST database). The concentration of the identified compounds was computed from the GC peak area without any correction factor.

In vitro gas production study

The estimates of gas production were obtained by the method of Menke and Steingass[25] by incubation in rumen fluid. All incubations were completed in 60 ml calibrated syringes containing 200 mg DM of oaten vetch hay and 30 ml of buffered rumen fluid (10mL rumen fluid and 20mL buffer solution). The piston was fitted precisely and lubricated using a small amount of Vaseline. The needle of the syringe was connected with a silicon rubber tube and closed using a plastic clip. The solution containing buffer solution and the macro and microminerals was prepared the day previous to incubation and stored at 39ºC.

PS-EO were added at doses of 0, 50, 100 and 200 µL in 30 mL of buffered rumen fluid under continuous flushing with CO_2. Triplicate syringes were used for each sampling. The syringes were incubated in a water bath (39ºC) for 72 h. At 2, 4, 6, 8, 24, 48, and 72 h of incubation, the gas production (GP) was recorded and the methane concentration was determined.

Rumen protozoa counts

In this study the protozoa were counted according to the method developed by Ogimoto and Imai[27]. After 24 hours of incubation, 100µl of buffered rumen fluid in each syringe was mixed with 100µl of methylgreen-formaldehyde-saline solution. This solution allows the fixation of cells by formaldehyde and colouring their nuclei by methyl green. The mixture is homogenized and kept in the dark for 30 minutes before counting. 1µl of this mixture was pipetted in Malassez cell. The protozoa were then counted using microscopy (x40). Each sample was counted twice, and if the average of the duplicates differed by more than 10%, the counting were repeated.

Study of the digestibility

(a) Determination of the dry matter digestibility

After 72 hours of fermentation, the content of each syringe is centrifuged at 12000 rpm for 20 min and 4°C. The centrifugation nerve is dried at 60°C for 48 hours. The dry matter (DM) digestibility coefficient is calculated as follows:

D (%) = [IDM-(RDM-RDM) / IDM] * 100

Where: D: Digestibility; IDM: initial dry matter; RDM: residual dry matter after incubation; RDMC: average residual dry matter in the control.

(b) Determination of organic matter digestibility

The DM content of feeds and feces samples was determined by oven-drying at 105°C for 48 h[2] (method 930.15). Ash content of samples was determined after 5h of incineration at 500°C in a muffle furnace, and the organic matter (OM) content was calculated as the difference between 100 and the percentage of ash[2] (method 942.05).

The dry residues were ignited in a muffle furnace at 550ºC for 5 h. The residue obtained after incineration represents the remaining mineral material. The non-degradable organic matter rate corresponding to the difference between the actual dry matter digestibility and mineral matter remaining.

DOM (%) = IOM - (IROM - ROMC) * 100 / IOM

Where: D: Digestibility of organic matter; IOM: (introduced) initial organic matter; IROM: incubated residual organic matter; ROMC: average of the residual organic matter in the control.

Statistical analysis

Data were analyzed by one-way analysis of variance. The differences among means for the all treatments were tested using Dunnett. Calculations was made by STATISTICA program version 6.

RESULTS

Chemical composition of *Pituranthos scoparius* essential oil

GC/MS analysis of the assayed PS-EO led to the identiûcation and quantiûcation of 32 components (TABLE 1), which accounted for 94.8% of the total EO. Sample contains heterogeneous mix of components with a high content of Monoterpenes (42.5%). The major constituents were myristicin (12.1 %), 7-methoxy-3-methyl-1H-isochromen-1-one (10.6 %) and 1-cyclohexyliden-2-methylpropene (9.9%). Percentages of Limonene, *p*-Cymene and Thymol were 8.5%, 5.2% and 4.8% respectively. The lowest content was observed for α-Pyronene, Terpinen-4-ol and Bornyl acetate with

0.1% for each one.

Influence of the PS-EO oils on the *in vitro* gas production kinetic

The addition of the PS-EO decreased the GP for all doses in all times. The magnitude of this decrease is dose-dependent and it is more significant for the dose of 200µL (TABLE 2). The addition of 50µL, 100µL and 200 µL of PS-EO decreased the 72 h GP to 78.33, 73.00 and 43.67 mL respectively in comparison to the control (92.67 mL).

TABLE 1 : Chemical composition of *Pituranthos scoparius* Essential oil (GS-MS)

N°	Compound	%
1	Cyclofenchene	3.6
2	Tricyclene	0.6
3	α-Pinene	2.9
4	β-Pinene	3.7
5	α-Phellanadrene	2.2
6	γ-Terpinene	2.4
7	δ-Carene	0.7
8	*p*-Cymene	5.2
9	Limonene	8.5
10	1-Cyclohexyliden-2-methylpropene	9.9
11	α-Terpinolene	2.7
12	α-Pyronene	0.1
13	Terpinen-4-ol	0.1
14	Bornyl acetate	0.1
15	γ-Elemene	0.2
16	Germacrene D	0.1
17	α-Copaene	1.3
18	*t*-Muurolene	1.1
19	Thymol	4.8
20	4-Acetyl-2-nitroazidobenzene	2.2
21	3,7-Guaiadiene	0.6
22	β-Cubebene	4.6
23	Bicyclogermacene	1.4
24	δ-Cadinene	2.6
25	Myristicin	12.1
26	Spathulenol	0.3
27	Propenal	3.7
28	Muurolol	2.1
29	β-Eudesmol	3.2
30	Butylidene phtalide	0.8
31	7-Methoxy-3-methyl-1*H*-isochromen-1-one	10.6
32	Butylidene dihydro-phtalide	0.4
	% Identification	94.8
	Monoterpene hydrocarbons	42.5
	Oxygen-containing monoterpenes	5
	Sesquiterpene hydrocarbons	12.7
	Oxygen-containing sesquiterpenes	3.8
	Other derivatives	30.8

Influence of the PS-EO on the methane production

Methane concentration was significantly decreased with the addition of PS-EO for all doses at all times compared to the control (TABLE 3). At 72 h after incubation, the PS-EO added with 50µL, 100µL and 200µL reduces methane concentration at 20.67, 13.33 and 08.67 mL respectively compared to the control (41.33 mL) suggesting that PS-EO is a good methane inhibitor.

Digestibility of the dry matter

Whatever the dose used of PS-EO, there is a deterioration of the *in vitro* DM digestibility. This decrease is significant ($p<0.05$) for the highest doses (100 and 200µL). Indeed, using these doses of PS-EO, the *in vitro* DM digestibility was decreased to 63.33% and 41.67% respectively compared to the control (88.33%) (Figure 1).

Digestibility of organic matter

Similarly to the DM digestibility, the results showed that the use of PS-EO is accompanied by a deterioration of the *in vitro* OM digestibility but this effect is significant only for the dose of 200µL ($p>0.05$). Indeed, the *in vitro* OM digestibility recorded in the control was 90.94% whereas the addition of 200µL of PS-EO decreased it to 48.72% (Figure 2).

Effect of the EO on protozoa counting

The effect of the PS-EO on the ruminal protozoa counts is presented in Figure 3. According to our results, the PS-EO reduce the protozoa number whatever the dose used but this decrease is not significant. Indeed, the use of 50µL, 100µL and 200µl of PS-EO reduce the protozoa count to 231700 cells/ml, 135000 cells/ml and 85000 cells/ml respectively in comparison to the control (490000 cells/ml).

DISCUSSION

In this study we have evaluate the ability of EO

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TABLE 2 : Effect of PS-EO on the gas production kinetic (ml) ± S.E.M.

Dose of EO	Time after incubation (hours)						
	2	4	6	8	24	48	72
Control	32.00^{a} ± 3.46	41.67^{a} ± 1.53	44.00^{a} ± 1.73	48.00^{a} ± 3.00	71.00^{a} ± 2.65	89.00^{a} ± 2.65	92.67^{a} ± 3.06
50 µl	23.33^{b} ± 3.06	36.67^{a} ± 1.15	43.00^{a} ± 1.73	48.33^{a} ± 2.89	65.33^{b} ± 6.43	74.00^{b} ± 7.00	78.33^{b} ± 8.14
100 µl	22.67^{b} ± 1.15	32.67^{b} ± 1.15	39.00^{a} ± 1.73	43.67^{a} ± 3.79	58.00^{b} ± 4.58	68.33^{b} ± 4.04	73.00^{b} ± 3.61
200 µl	20.67^{b} ± 4.16	28.67^{b} ± 3.06	31.33^{b} ± 4.04	32.00^{b} ± 3.61	38.00^{c} ± 5.00	41.00^{c} ± 7.00	43.67^{c} ± 7.02

a. b. c: column means without a common superscript letter differ significantly (p < 0.05). S.E.M.: standard error mean

TABLE 3 : Effect of PS-EO addition on the methane production kinetic (ml) ± S.E.M

Dose of EO	Time after incubation (hours)						
	2	4	6	8	24	48	72
Control	05.00^{a} ± 3.46	09.67^{a} ± 5.69	10.33^{a} ± 5.51	10.67^{a} ± 6.03	18.00^{a} ± 9.54	41.00 ^{a} ± 19.47	41.33^{a} ± 20.03
50 µl	02.67^{a} ± 0.58	05.67^{a} ± 0.58	06.33^{a} ± 0.58	08.33^{a} ± 4.04	15.00^{a} ± 4.58	19.00^{b} ± 7.55	20.67^{b} ± 10.69
100 µl	02.33 ^{a} ± 0.58	04.33^{a} ± 0.58	04.33^{a} ± 0.58	05.00^{a} ± 1.00	09.67^{b} ± 2.08	12.67^{c} ± 1.53	13.33^{c} ± 2.08
200 µl	02.33^{a} ± 1.53	05.00^{a} ± 1.00	05.67^{b} ± 0.58	06.00^{a} ± 1.00	07.66^{b} ± 2.08	08.33^{c} ± 3.22	08.67^{c} ± 3.79

a, b, c: column means without a common superscript letter differ significantly (p < 0.05). S.E.M.: standard error mean

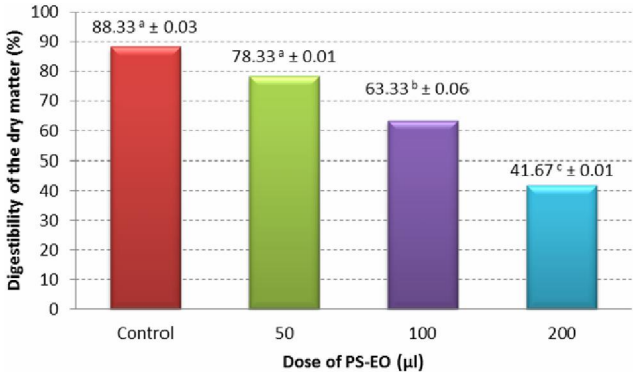

Figure 1 : Effect of the PS-EO on the dry matter digestibility

[a]: values with common superscript letter do not differ (p > 0.05).

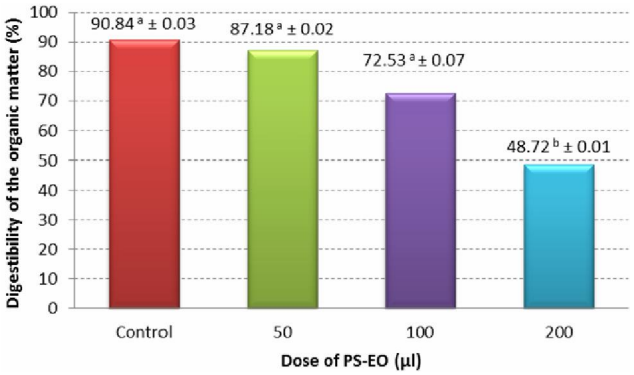

Figure 2 : Effect of the PS-EO on the organic matter digestibility

[a]: values with common superscript letter do not differ (p > 0.05)

extracted from *Pituranthos scoparius* to reduce methanogenosis in sheep which have not been studied for this purpose before in our knowledge. In the current study, the use of PS-EO resulted in a significant decrease of both total gas and methane production especially with highest doses. These results are consistent with those reported in several studies. Indeed, other studies have recently evaluated the capacity of essential oils to mitigate enteric methane production[6,15,17,19,24,30,35]. In these studies, various essential oils have been tested to decrease methanogenesis. In a meta-analysis study conducted in different ruminant species, Khiaosa-ard and Zebeli[23] have found that the effects of essential oils and their bioactive compounds at doses <0.75 g/kg diet DM acted as a potential methane inhibitor in the rumen as a result of decreased acetate to propionate ratio. Durmic et al.[17] have reported a significant reduction (P < 0.05) in methane production was

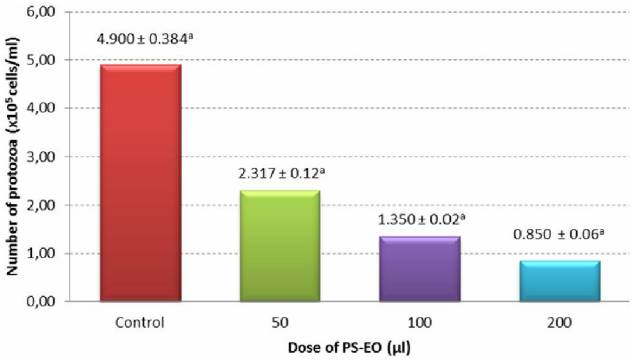

Figure 3 : Effect of the PS-EO on the protozoa number

[a, b]: values with common superscript letter do not differ (p > 0.05)

observed with eight essential oils (up to 75% reduction). Similarly, it was found in Zmora et al.[35] that after 24 hours of incubations, addition of 16.34 and 23.35 mg of *Mentha piperita* L. to the 233.3 mg of substrate significantly decreased methane emission by 41.52 and 15.51%, respectively. Results reported by Jahani - Azizabadi et al.[19] indicated that coriander, cinnamon, red basil, oregano 2, cumin, caraway and dill essential oils caused a significant decrease ($P < 0.05$) in total methane production (1.5, 0.3, 1.0, 1.3, 1.1, 1.1 and 2.0 compared with 2.3 in control as mmol/g DM incubated, respectively). Finely, Origanum and clove oils resulted in lower methane emission compared to control and peppermint oil as reported in Patra and Yu[30].

Many studies reported that the effects of EO differ depending on their chemical components. Several studies on PS-EO composition have been already published, revealing a great variability in its chemical profile (Verite et al., 2004). Furthermore, the composition of PS-EO differs among harvesting seasons and geographical locations[21]. In this study, we have found that the major constituents of PS-EO were myristicin (12.1 %), 7-methoxy-3-methyl-1H-isochromen-1-one (10.6 %) and 1-cyclohexyliden-2-methylpropene (9.9%), limonene (8.5%), *p*-Cymene (5.2%) and thymol (4.8%). Verite et al. (2004) showed that PS-EO revealed menthone, pulegone and neo-menthol as the main constituents (35.9, 23.2 and 9.2% respectively) while Cherrat et al. (2014) found that pulegone is the main component of MP-EO (33.65%) followed by α-pinene (24.29%) and cineole (10.53%).

The literature suggests that essential oils mitigate methanogenesis mainly by a direct toxic effect on methanogens[7,15]. It has been suggested that essential oils cause changes in the archaeal communities, or in the activity of methanogens, to reduce the rate of CH_4 production by rumen archaea[28]. Several studies, most of them *in vitro*, have been published on effects of essential oils components on rumen microbial fermentation[6,11]. Many of the component secondary metabolites in essential oils (Thymol, Carvacrol, Eugenol, Cinnamaldehyde…) exhibit different antimicrobial, antifungal and antiprotozoal activity[1,7,10,12,13,16,18,22,24] witch influence directly the methane production. Oxygenated monoterpenes, particularly monoterpene alcohols and aldehydes, strongly inhibit growth and metabolism of rumen microorganisms[6]. Rossi (1995) found that thymol, eugenol, vanillin, and limonene witch are the main components on an organic carrier reduce volatile fatty acids to a maximum of 10%.

The antimicrobial action of EO is due to their potential to be intruded into the bacterial cell membrane and disintegrate its structures which causes ion leakage. Essential oils have a high affinity for microbial cell membranes their lipophilic nature. The functional groups of essential oils interact with membrane components[20] and the normal ion transport across the membrane is altered. The membrane becomes disrupted and microbial enzymes are inactivated[28,29] which compromise viability and activity of the ruminal microorganisms.

Clearly essential oils are able to manipulate rumen fermentation. The reported effects are likely to be due to selective pressures exerted on different microbial populations, resulting in different bacterial numbers and subsequently different activities which influence directly the nutrient digestibility. Results of this study have shown that PS-EO deteriorate both DM and OM digestibility. This finding is in agreement with many other studies using other oils. Indeed, Sallam et al. (2012) reported that highest levels of *Achillea santolina*, *Artemisia judaica*, and *Mentha microphylla* EO produce a significant reduction in true degradation of dry matter and organic matter. However, Zomora et al.[35] found that *Mentha piperita* supplementation exerted no effect on the dry matter digestibility after 24 hours of incubations. A reduction in the digestibility even if accompanied by reductions in CH_4 production is not interesting. It would generally be viewed to be nutritionally unfavourable,

and can be associated with nonspecific depressed ruminal fermentation with less efficient rumen fermentation.

Protozoa have a prominent position supported by their close physical association with methanogens, which favours H_2 transfer from one to the other. A strong positive interaction was found between protozoal numbers and methane emissions[26]. In the present study, protozoa counts were reduced by approximately 20 to 50% depending on doses of PS-EO compared to the control. In the literature, it appears clearly that the effect of essential oils on rumen protozoa varies. Sallam et al. (2011) found that highest levels of *Achillea santolina*, *Artemisia judaica*, and *Mentha microphylla* EO produce a significant reduction in protozoa count. Similarly, Ando et al.[1] have shown that feeding 200 g/d of dried peppermint (*Mentha piperita* L.) to cannulated steers significantly decreased protozoa numbers by approximately 50%. However, Benchaar et al.[3] reported that there was no effect of feeding 750 mg/d of blends EO to dairy cattle on protozoal numbers determined *in vivo*. Although the mechanism of action has not been clearly elucidated, it may be related to the lipophilic nature of compounds such as anethol which facilitates permeation of essential oil across the protozoal membrane[12].

CONCLUSION

It can be concluded from the present results that PS-EO significantly influenced total gas and methane production. This effect is accompanied by a reduction of the dry and organic matter digestibility and a strong toxicity towards the ruminal protozoa. The antimicrobial effect of the EO components is at the origin of this effect.

Rumen is a complex ecosystem in which the different microorganisms interfere with each other, and whose determination is essential for the understanding of many phenomena taking place in the rumen. Our work must be completed by:

- further *in vitro* and *in vivo* trials are required to search optimum dose which reduce methane production without adversely changing dietary fermentation and rumen function.
- A determination quantitative and qualitative of AGV products during fermentation.

Toxicity to ruminants, users and consumers, palatability and effects on organoleptic quality of animal products require further research to ensure that these EO can be safely used in livestock production which allow us to advise farmers on using plants rich on essential oils in the diet of ruminants in order to combat the emission of CH_4, performs the ruminants productivity and preserve the environment.

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