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ANIMAL PRODUCTION

Pithecellobium dulce, Tagetes erecta and *Cosmos bipinnatus* on reducing enteric methane emission by dairy cows

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ABSTRACT: *The aim of the present research was to evaluate the effect of Pithecellobium dulce, Tagetes erecta and Cosmos bipinnatus on methane emission, milk yield and dry matter intake in dairy cattle. A 4×4 Latin square experimental design was employed, using four multiparous Holstein cows of 553±72.4kg body weight, at mid lactation and average milk yield of 17.3±3kg/day. The experiment lasted 92 days, divided into four experimental periods of 23 days each. All cows had free access to maize and alfalfa silage in a 50:50 proportion, 4kg of concentrate/day and ad libitum access to water. Treatments consisted in supplementation of 0.5kg/day of the experimental plants, with one control treatment without supplementation. Each cow received one of each treatment in turn during one of the four periods. The C. bipinnatus reduced methane production by 16% (P<0,05) in comparison with the control diet. Milk production, milk composition and dry matter intake were not affected (p>0 0.05) by the use of C. bipinnatus or any other plant species. Supplementation at low doses of C. bipinnatus showed a reduction in ruminal methane production in dairy cows.*
Key words: *methane, cattle, climate change, mitigation, tannins.*

Pithecellobium dulce, Tagetes erecta e *Cosmos bipinnatus* na redução de emissão de metano por vacas leiteiras

RESUMO: *O objetivo deste estudo é avaliar o efeito do Pithecellobium dulce, Tagetes erecta e o Cosmos bipinnatus na emissão do metano, a produção de leite e de ingestão diária de matéria seca pelo gado. Um desenho experimental do quadrado latino 4x4 foi usado, com quatro vacas da raça Holandesa, multíparas, de 553±72,4kg, no seu segundo terço da lactação e rendimento médio de leitedo de 17,3±3kg/dia. O experimento durou 92 dias e foi dividido em quatro períodos de 23 dias cada um. Das vacas tinham acesso livre a milho e silagem de alfafa, numa proporção de 50:50, com 4kg de concentrado por dia, com acesso a água ad libitum. Os tratamentos consistiram na ingestão de 0,5kg por dia, com as plantas experimentais; as vacas no tratamento de controle não receberam nenhuma planta, porém, elas receberamum tratamento, em cada um dos quatro períodos. O C. bipinnatus reduziu significativamente a produção de metano em 16% (P<0,05), em comparação com a dieta controle. A produção de leite, sua composição e o consumo de matéria seca não foram afetados (P>0,05) pelo uso de C.bipinnatus ou outras espécies de plantas. A ingestão com doses baixas de C. bipinnatus, que é uma planta arbustiva com um centéudo moderado de taninos, mostrou ter potencial para reduzir a produção de metano ruminal em vacas leiteiras.*
Palavras-chave: *metano, gado, mudança climática, mitigação, taninos.*

INTRODUCTION

The main sources of anthropogenic methane (CH_4) are the production of ruminants for meat and milk production, the extraction and use of fossil fuels, and rice production. Agriculture contributes to approximately 13% of the total global emission of greenhouse gases (GHG) (IPCC, 2014), of which cattle are responsible for 53% (CHARMLEY et al., 2016). Thus, several studies, mostly *in vitro* experiments, have been conducted on the effect of tanniferous plants on reducing rumen CH_4 production, in order to find natural alternatives to mitigate the environmental impact generated by the emissions of this GHG by the cattle industry (CUARTAS et al., 2014). However, results in literature are contradictory, because in some cases a small methane reduction has been observed whereas in others a large reduction potential was identified. For example, NAUMANN et al. (2013) observed a small CH_4 reduction of 3.2% and 0.5% in an experiment conducted to evaluate the *in vitro* effect of two types of *Acacia angustissima* – the South Texas ecotype and Cross timbers ecotype – with a condensed tannin content of 8.4% and 8.9%, respectively. Similarly, in a study conducted by BHATTA et al. (2009) in order

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to evaluate the effect of different tannins (in their pure form) on *in vitro* CH_4 production, it was observed that hydrolysable tannins reduced CH_4 production by only 0.6%, while a mixture of hydrolysable and condensed tannins reduced it by 5.5%. Unfortunately, there are few studies where the effects of entire tanniferous plants on rumen methano genesis in cattle and animal performance have been evaluated *in vivo* (PATRA & SAXENA 2010). For example, BEAUCHEMIN et al. (2007) conducted an experiment to determine if 1% and 2% of dietary DM as condensed quebracho tannin extract could be used to reduce enteric CH_4 emissions in cattle. They used six spayed Angus heifers and 6 Angus steers and concluded that feeding with up to 2% of dietary DM as quebracho tannin extract failed to reduce enteric methane emissions from growing cattle. No information was reported in the literature on the *in vivo* anti-methanogenic properties of *Tagetes erecta*, *Cosmos bipinnatus* and *Pithecellobium dulce*. However, our group conducted several *in vitro* studies to evaluate the potential of *Tagetes erecta* for reducing CH_4 production; for example, ANDRADE et al. (2012) observed a reduction of 39% in comparison with a control diet. Similarly, MAHMOUD et al. (2016) reported a 35% reduction ($p<0.05$) in methane production in comparison with a control diet when only 10% dietary dry matter (DM) of *T. erecta* was included in the diet. Likewise, a reduction of 11% ($P<0,001$) in methane production was also reported by MAHMOUD et al. (2017) with the inclusion of only 10% of dietary DM of *C. bipinnatus* in the experimental diet. These studies also suggested that the anti-methanogenic capacity of *T. erecta* and *C. bipinnatus* is not linear but quadratic, and that the best effect was observed at low inclusion levels. Therefore, it was hypothesized that *T. erecta, C. bipinnatus* and *P. dulce* could reduce *in vivo* methane production and that this effect could be detected at low inclusion levels without affecting animal productive performance. The objective of the present study was to evaluate the effect of supplementation of *Tagetes erecta, Cosmos bipinnatus* and *Pithecellobium dulce* on reducing enteric methane emission, and on milk production and dry matter intake in dairy cattle.

MATERIALS AND METHODS

Area of study

The experiment was conducted at the Laboratory for Research on Livestock, Environment and Renewable Energies of the Faculty of Veterinary Medicine and Animal Science of the Universidad Autónoma del Estado de México, located in Toluca, State of Mexico, Mexico at 19°27'N and 98° 38'W and 2600m.a.s.l.

Experimental design:

A 4×4 Latin square experimental design was employed, using four multiparous Holstein cows of 553±72.4kg of body weight, at mid lactation and average milk yield of 18±3kg/day. The experiment lasted 92 days, which were divided into four experimental periods of 23 days each. All cows had free access to maize-alfalfa silage in a 50:50 proportion, 4kg of concentrate/day and *ad libitum* access to water. The concentrate was composed of 48.7% corn, 20% soya bean, 14.8% canola bean, 14.7% wheat bran and 1.8% of mineral premix. The four treatments consisted of the supplementation of 0.5kg DM/day/cow for each of the three experimental plants plus the control, which was the diet alone without the addition of experimental plants. Each cow received each treatment in turn, once in each of the four periods. Before the beginning of the trial, the experimental plants were dried over a period of 8 weeks away from sunlight to prevent denaturalization of phenolic compounds as indicated in MAKKAR et al. (1993). Once dried they were ground and incorporated into the concentrate to ensure that the cows would eat the entire daily ration provided, as the aim of the experiment was to evaluate the effect of the entire plants and not only the effect of the phenolic compounds within them. Samples of the experimental plants were collected for later chemical analysis in the laboratory. For each 23-day experimental period, 15 days were used for diet adaptation and the remaining to measure methane production, dry matter intake and digestibility of the diet. The cows were confined in individual pens of 4×4m, equipped with drinker and trough, throughout the experiment. Milk yield was weighed daily during the entire 23-day period and milk composition was evaluated daily during the measuring period of eight days. A Lactichek ™-01 (Rapi Read, Page & Pedersen International Ltd. Hopkinton, Massachusetts) analyzer was used to determine milk composition: fat, lactose, protein, non-fat milk solids. Body weight was measured once at the beginning and once at the end of each experimental period. We used adult cows and the body weight was recorded only. Dry matter intake (DMI) was determined by weighing the silage and concentrate offered in the morning and collecting and weighing the rejected feed the next morning. The difference between the offered feed minus the rejected feed was the daily DMI. Digestibility of the dry matter (DMD) was calculated as shown in equation

(1). Samples of silage were taken every week for later chemical analyses in the laboratory.

DMD(%)=((DMI-FAe)/DMI)×100 Eq. (1)

Where: DMI=dry matter intake in kg ofDM,FAe=feces weight in kg of DM.

Methane production measurement:

The CH_4 production was measured with a respiration chamber of the head-box type as described previously in PEDRAZA-BELTRÁN et al. (2016). The CH_4 analyzer that was used measures emissions highly accurately over a wide range by combining noise amplification and signal processing with a computerized controller and digital filtering to provide a maximum resolution of 0.0001% to 0.01%. Before each assay two calibrations of this instrument were performed: a zero calibration using high-purity nitrogen (N_2) (Praxair Inc., Mexico), and a calibration against a reference gas, which is also known as a span gas. The N_2 in the zero calibration was first passed through a drying unit to remove moisture and then through the analyzer at a flow rate of 0.3L/min to obtain a reading close to zero. The span calibration was performed using a known CH_4 concentration gas mixture (1000ppm of CH_4 in high-purity N_2). The span gas passed through the analyzer (0.3L/min) to obtain a reading corresponding to the concentration of CH_4 in the span gas. Every assay started at 10:00h, the mass flow generator was set at 480L/min, the analyzer was set to measure CH_4 concentration every second and then the chamber was closed. The CH_4 emissions were measured for 24-h period. The cows were removed from the chamber for milking at 6:00h and 15:00h, and each milking lasted 1.5h, subsequently they were returned to the chamber. The diet was weighed before the beginning of the assay, and all animals were given the same amount at the same time (9:00h and 16:00h). The next morning, the orts were removed and weighed to calculate DMI. Diet samples were collected and kept in a freezer until laboratory analysis. Feces were collected and weighed at the end of each measurement. A sample of approximately 1kg of feces was obtained and kept frozen until laboratory analysis. Four assays were completed in each experimental period.

Chemical analysis of the feed

Silage, concentrate feed and stool samples were dried in a forced air oven at 60°C for 72h until constant weight was obtained, then later ground and passed through a 1-mm sieve. The DM and organic matter (OM) contents were determined according to the procedures of the Official Methods of Analysis (AOAC, 1997). The nitrogen content in the silage and concentrate was determined by the Kjeldhal method (AOAC, 1997), and subsequently multiplied by a factor of 6.25 to obtain the protein content. The neutral detergent fiber (NDF), acid detergent fiber (ADF) and lignin contents were determined by the method of VAN SOEST et al. (1991) using an ANKOM A200 fiber analyzer (Technology Corporation, Fairport, NY, USA). The DM content in the silage was corrected as in HAIGH (1995) to include the volatile solids in the DM. The concentration of total phenols in the experimental plants was determined by the Folin-Ciocalteu method and the tannin content by the polyvinylpolypyrrolidone method as described in MAKKAR (1993). Table 1 shows the chemical composition of silage and concentrates, and the phenol and tannin content of the experimental plants.

Analysis of results

The experimental variables were analyzed with an analysis of variance for a Latin square experimental design as shown in equation 2. In order to eliminate Type II error (KAPS & LAMBERSON 2009) the Fisher's Least Significant Difference (LSD) test between control and treatment means was also carried out. The analytical procedures were carried out using the *lmer* function of the lme4 package (BATES et al., 2015) in R software (R core team, 2016). Post hoc pairwise comparison was carried out using the Tukey HSD test using the *lsmeans* function in the lsmeans package (LENTH, 2016).

$Y_{ijk}=\mu+TX_i+Per_j+Cow_k+\varepsilon ijk$ Eq. (2)

where Y_{ijk} is the individual observation, μ is the overall mean, TX_i is the fixed effect of treatment (i =1, 2, 3, and 4), Per_j is the effect of period (j=1, 2, 3, and 4; treated as a random effect), Cow_k is the effect of cow (k=1, 2, 3, and 4; treated as a random effect) and ε_{ijk} is the residual error term. A multiple correlation analysis between all variables was also performed in order to find associations that help in explaining CH_4 production. The corr plot routine from the corr plot v.0.77 package (WEI & VILIAM 2016) of the R software v.3.2.2. was used (R, CORE TEAM, 2011).

RESULTS

Table 2 shows the effect of the experimental plants on DMI, DMD, milk yield and CH_4 production. Results show that no differences (P>0,05) were observed between the control and the treatments for all variables except for CH_4 production. The treatment with *C. bipinnatus* reduced CH_4 production

Table 1 - Chemical composition of the silage and concentrate; and chemical composition, total phenols and total tannin concentration of the experimental plants (all in g/Kg of DM, except DM, which is g/Kg).

Item	Concentrate	Silage	*Pithecellobium dulce*	*Cosmos bipinnatus*	*Tagetes erecta*
DM	888.5	367.2	931.5	928.1	931.5
OM	826.4	890.8	852.8	821.7	846.6
CP	221.1	108.4	90.5	115.2	137.2
NDF	340.7	517.8	527.1	470.5	458.7
ADF	123.5	390.3	468.3	387.3	403.8
Lignin	40.2	102.2	184.0	96.2	76.4
Hemicellulose	217.2	127.5	58.7	83.2	54.9
Cellulose	83.3	288.1	284.3	291.1	327.4
Total phenols	-	-	49.2	80.6	99.4
Total tannins	-	-	22.8	69.7	78.1

OM organic matter, CP crude protein, NDF neutral detergent fiber, ADF acid detergent fiber.

by 98.2L/day (<16%) relative to the control (P<0,05), whereas *P. dulce* and *T. erecta* showed no effect on CH_4 production (P>0,05). The LSD between the control and *P. dulce* was 86.9L/day (P<0,05). All the productive parameters, such as body weight, milk yield, DMI and DMD were not affected by any of the plants tested at the level they were supplemented. Percentages of each plant in relation to the total dry matter intake for the treatments of control, *P. dulce*, *C. bipinnatus* and *T. erecta* were 3.5, 2.6, 3.6 and 3.1%, respectively. Milk composition was also unaffected (P>0,05) by the experimental plants, as shown in Table 3. The multiple correlation analysis showed positive associations between CH_4 L/day and DMI (r=0,5, P<0,05), and between DMD and CH_4 L/day (r=0.6, P<0,01). In contrast, a negative association (r=-0.64, P<0,001) was observed between milk yield and CH_4 in L/Kg of milk. No other association was observed among the studied variables. A large numerical difference of 22.4L was observed between the control and *P. dulce* treatments for the variable CH_4, L/kg of DMD; however, this was not significant since the LSD is 53 L (P>0,05).

DISCUSSION

The CH_4 production for individual cows in the control treatment was within the range reported for animals of similar body live weight and intake (NIU et al., 2016). On the other hand, the percentage

Table 2 - Effect of supplementation with *P. dulce*, *C. bipinnatus* and *T. erecta* on methane production, dry matter intake, dry matter digestibility, live weight and milk yield.

	----------Treatment----------					
Item	Control	*Pithecellobium dulce*	*Cosmos bipinnatus*	*Tagetes erecta*	S.E.	P value
Live weight, Kg	568±63	583±57	576±68	569±69	5.6	0.31
DMI, kg DM/d	14.7±3	19.7±6	14.5±3	16.8±4	1.9	0.28
DMD, %	55.6±6	62.8±9	58.8±16	62.5±12	7.3	0.71
CH_4, L/d	613.5±97[a]	635.5±105[a]	515.3±41[b]	541.5±49[ab]	25.1	0.04
Milk, Kg/d	17.3±3	17.4±3	16.9±3	15.7±3	0.95	0.6
CH_4 , L/Kg of DMI	43±11	34±8	36.7±7	33.3±7	4.8	0.41
CH_4, L/Kg of DMD	76.9±29	54.5±20	64.8±25	55.4±25	15.3	0.7
CH_4, L/Kg of milk	36.9±9	38±12	30.8±3	34.9±5	2.63	0.35

±= standard deviation, S.E.= standard error, DMI= dry matter intake, DMD= dry matter digestibility, values in the same row with different superscripts indicate differences p<0.05.

Table 3 - Effect of supplementation with *P. dulce*, *C. bipinnatus* and *T. erecta* on milk composition (%).

	----------Treatment----------					
Item	Control	*Pithecellobium dulce*	*Cosmos bipinnatus*	*Tagetes erecta*	S.E.	P value
Fat	4.2±0.70	3.7±0.10	3.9±0.86	4.0±0.55	0.18	0.41
Protein	3.4±0.08	3.3±0.07	3.4±0.16	3.4±0.08	0.03	0.19
Non-fat solids	9.0±0.20	8.8±0.20	9.1±0.41	9.0±0.21	0.09	0.15
Density	31.2±0.36	30.7±0.82	31.8±1.10	31.4±0.49	0.30	0.18

±= standard deviation. S.E.= standard error.

of CH_4 reduction was achieved due to the inclusion of *C. bipinnatus* and was comparable to the values reported in similar studies, even though the percentage of plants in the diet and the tannin content, 2.6% and 69.7g/kg of plant's DM respectively, were lower than in most published studies. This confirmed our hypothesis that the supplementation of small quantities of this experimental plant can reduce *in vivo* methane emission by adult cows. The inclusion rate of experimental plants in our research ranged from 2.6 to 3.1% of the average total DMI, whereas in similar studies the supplementation rate is significantly higher. For example, PIÑEIRO et al. (2017) evaluated the effect of increasing levels of *Leucaena leucocephala* (0, 20, 40, 60 and 80%) on methane production in heifers fed with a low-quality diet. The DM intake in their work ranged from 7 to 7.15kg of DM/day, meaning that the intake of *L. leucocephala* reached up to 5.72kg of DM/day at an 80% inclusion level. In other studies, the tanniferous plant constituted 100% of the diet, as in in WOODWARD et al. (2002), where dairy cows were fed only with *Hedysarum coronarium* with no effect on daily CH_4 production. The reduction in methanogenesis observed for *C. bipinnatus* may be attributed to the higher content of total tannins than *P. dulce*, although *T. erecta* has more tannins than *C. bipinnatus*. This suggested that it may be the type of tannins, particularly condensed tannins that may be responsible for the reduction in methane emissions. For example, it was reported by MAHMOUD et al. (2017) that the percentage of condensed tannins in the total tannin content of *C. bipinnatus* (same plant, same region) is 11.7%, whereas that for *T. erecta* is only 1.2%. This is in line with IVES et al. (2015), who evaluated the effect of condensed tannins from an extract of *Acacia molissima* (50g/Kg of diet DM) on *in vitro* CH_4 production in five different species of ruminants and found that tannins from *A. molissima* reduced CH_4 production by up to 12% in Holstein cows. Similarly, MOREIRA et al. (2013) tested the effect of condensed tannins from *Leucaena leucocephala, Styzolobium aterrimum* and *Mimosa caesalpiniaefolia* on CH_4 production in sheep, and reported that condensed tannins (40g/Kg of diet DMI) of *L. leucocephala* reduced CH_4 production by 25%, *S. aterrimum* by only 1%, while M. *caesalpiniaefolia* increased it by 7%. However, they had to increase the inclusion level of *L. leucocephala* and *S. aterrimum* by up to 82% and 69%, respectively, in order to achieve the reduction reported. Furthermore, at the inclusion level employed by MOREIRA et al. (2013), it is difficult to elucidate if the mitigation effect can be attributed to the condensed tannins in the legumes alone, as they stated, to other secondary metabolites in the plants (VERCOE 2015), or to the chemical composition of the plants themselves. This is because it is well established that diets with a high content of legumes produce less CH_4, due to their low fiber content, than diets rich in grasses (ARNDT et al., 2014). TAVENDALE et al. (2005), who conducted an *in vitro* experiment to evaluate the CH_4-reducing potential of *Lotus pedunculatus* and *Medicago sativa*, also reported a discrete reduction in CH_4 production. They observed that *M. sativa* reduced CH_4 production by 5% despite the fact that it only had 0.2g/kg of DM of condensed tannins, whereas *L. penduncula*tus, with a higher concentration of condensed tannins (107g/kg of DM), had no effect on CH_4 production. The approximate content of condense tannins of the diet with *C. bipinnatus* was 0.28g/Kg of diet DM, which is very similar to that reported by TAVENDALE et al. (2005). Indeed, it seems that the CH_4 reduction potential of tannins is not dose dependent, because high doses do not always lead to less CH_4 production (BHATTA et al., 2009); whereas "low doses, as in the present study, seem to produce similar effects to those reported by the previous authors. This pattern of response was elegantly demonstrated by MAHMOUD et al. (2017) in an *in vitro* experiment that established that the response is not linear, but quadratic, and that there is an interaction (P<0,01) between the plants species and the level of inclusion in the diet for CH_4 production. The positive associations between CH_4 L/day and DMI (r=0.5, P<0,05) and between DMD and CH_4 L/day have been reported before, because intake is the most important variable that determines CH_4 production; similarly, more CH_4 will be produced in response to more degraded substrate (HALES et al., 2013). In contrast, the negative association observed between milk yield and CH_4 in L/Kg of milk has been reported before by GARG et al. (2013) where increased milk yield per cow was associated with lower methane production per kilogram of milk produced. In fact, intensification of milk production is considered an option for mitigating CH_4 production in dairy systems (GERBER et al., 2011).

CONCLUSION

It can be concluded that supplementation at low doses with *C. bipinnatus* has the potential to reduce ruminal methane production in dairy cows without affecting animal production parameters. More research is needed in order to determine whether higher levels of *C. bipinnatus* may lead to further reduction in rumen methanogenesis. Also more research is needed for higher supplementation doses of *T. erecta* because it showed a trend towards reducing methanogenesis.

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DECLARATION OF CONFLICTING OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

All the experimental procedures were approved by the Committee on Bio-ethics and Animal Welfare of the Universidad Autónoma del Estado de México on the 24th of April 2015.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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