UNIVERSIDAD AUTÓNOMA DEL ESTADO DE MÉXICO

PROGRAMA DE MAESTRÍA Y DOCTORADO EN CIENCIAS AGROPECUARIAS Y RECURSOS NATURALES

DESARROLLO DE ESTRATEGIAS PARA REDUCIR LA PRODUCCIÓN DE METANO A NIVEL RUMINAL POR EL GANADO BOVINO EN MÉXICO

TESIS

QUE PARA OBTENER EL GRADO DE DOCTOR EN CIENCIAS AGROPECUARIAS Y RECURSOS NATURALES

PRESENTA
M. en C. RAAFAT MAHMOUD MOHAMED GOMAA

Cerrillo Piedras Blancas, Toluca, México; Noviembre de 2016

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D E D I C A T O R I A

A mi familia, mis padres, mi hermana, mi esposa y mis hijos

A mis padres por ser como fueron, porque gracias a ellos yo soy.

Te agradezco padre por dejarme ser, por el amor que me tienes y por estar presente en todas las etapas de mi vida, gracias por mostrarme con tu valentía y tus ganas de vivir el gran sentido de la vida.

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Como las ramas de un árbol crecemos en diferentes direcciones, pero nuestra raíz es una sola, así la vida de cada uno siempre será una parte esencial de la vida del otro, gracias Hermana.

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R E S U M E N

Se ha demostrado que los taninos en el follaje de los árboles y arbustos pueden reducir la producción de metano (CH_4) en el rumen de los bovinos. Se evaluó el potencial de diez especies de arbustivas, nativas del área templada de México, para reducir la producción de metano a nivel ruminal. Se utilizó la técnica de producción de gas *in vitro* para evaluar el efecto de la inclusión de estas plantas a tres niveles (10, 20 y 30%) en una dieta experimental. Dos experimentos independientes se hicieron para controlar la producción de gas metano a lo largo de 24 y 72 h de incubación, la producción de metano se registró después de 24 h. Los resultados mostraron una disminución significativa ($P < 0,05$) en la producción de metano con la inclusión de todas las plantas, se observó la mayor disminución cuando *Amaranthus spinosus*, *Commelina coelestis*, *Tagetes erecta* y *Senna hirsuta* fueron incluidos en comparación con la dieta control. La digestibilidad de la fibra detergente neutra (FDN) después de la incubación no se vio afectada por *Tagetes erecta*, *Amaranthus spinosus*, *Commelina coelestis* mientras *Senna hirsuta* la aumentó ($P < 0.05$). Los resultados sugieren que *Amaranthus spinosus*, *Commelina coelestis*, *Tagetes erecta* y *Senna hirsuta* tienen potencial para reducir las emisiones de metano del ganado bovino.

Palabras clave: Compuestos fenólicos de las plantas; Taninos; Producción de gas *in vitro*; Emisión de metano; Rumiantes.

ABSTRACT

It has been shown that tannins in the foliage of trees and shrubs can reduce methane (CH_4) production in the rumen of cattle. We evaluated the potential of ten shrub plant species, native to temperate Mexico, to reduce methane production in the rumen. We used *in vitro* gas production to evaluate the effect of the inclusion of these plants at three levels (10, 20 and 30%) in an experimental diet. Two independent experiments were done to monitor Gas production throughout 24 and 72 h of incubation, methane production was recorded after 24 h. The results showed a significant decrease ($P<0.05$) in methane production with the inclusion of all plants, the highest decline was observed when *Amaranthus spinosus*, *Commelina coelestis*, *Tagetes erecta* and *Senna hirsuta* included at levels of 20%, 30%, 20% and 20% respectively in comparison to the control diet. The digestibility of the NDF after incubation was not affected by *Tagetes erecta*, *Amaranthus spinosus*, *Commelina coelestis* while *Senna hirsuta* increased it ($P<0.05$). Results suggest that *Amaranthus spinosus*, *Commelina coelestis*, *Tagetes erecta* and *Senna hirsuta* have potential to reduce methane emissions by cattle.

Key Words: Plant phenolics; Tannins; *In vitro* gas production; Methane emission., Ruminant.

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C O N T E N I D O

Í N D I C E D E F I G U R A S

I. INTRODUCCIÓN

Un problema importante que enfrenta nuestro mundo es la emisión de gases de efecto invernadero (GEI) por la actividad antropogénica. Mientras el CO2 recibe una atención considerable como posible causa del calentamiento global, las concentraciones atmosféricas de metano (CH4), clorofluorocarbonos y N_2O se han incrementado notablemente en los últimos 150 años y se pueden contribuir al calentamiento global. El aumento de temperatura en el mundo está teniendo un impacto en muchas especies de plantas y animales en todo el mundo, aunque se espera que los efectos más dramáticos en las próximas décadas, cuando los rendimientos de cultivos y forraje podrían reducirse a causa de sequías y las condiciones meteorológicas extremas (Olesen y Bindi, 2002).

Los rumiantes son animales de pastoreo que tienen una ventaja única de la conversión de material vegetal rica en celulosa no digerible a carne, leche y lana, al mismo tiempo no compiten directamente con los humanos para alimentarse. Sin embargo, grandes cantidades de metano (CH4) se generan en el proceso. El metano es un gas de efecto invernadero (GEI) y está presente de aproximadamente el 6% de pérdida de energía bruta de la dieta. Las Emisiones entéricas pueden calcularse como el producto de la población animal, la cantidad de alimento consumido por los animales y la cantidad de metano (metano / kg MS) producida de cada animal. La cantidad de metano producido se ha observado en un rango de entre 16 y 26 g de CH4 / kg MS consumida según lo reportado en diversos estudios (Munger y Kreuzer 2006).

Las Plantas Tanniferous pueden representar una opción sostenible para la mitigación, sin embargo se necesita un trabajo importante con el fin de encontrar las especies más eficientes. Estas especies tienen que cumplir con dos criterios, uno es reducir la formación de metano en el rumen, y dos, no disminuyen el rendimiento del animal a través de la modificación de la fermentación y la degradación de los piensos en el rumen (Makkar et al., 1993a). La Evaluación *in vivo* de las diferentes especies puede ser costoso y no es factible debido a la gran número de plantas que deben ser evaluados. Por lo tanto, la producción de gas *in vitro* se puede utilizar como prueba de cribado para seleccionar la especie con potencial más grande antes de la evaluación *in vivo*.

Todos estos aspectos de la producción entérica de CH4 han animado a la comunidad científica para encontrar alternativas para mitigar las emisiones de GEI. Las plantas que contienen productos bioactivos tales como aceites esenciales, saponinas y taninos (Wallace et al. 2002) con propiedades antimicrobianas pueden ser explotados en la producción de rumiantes para reducir las emisiones de CH4.

El objetivo del presente estudio fue evaluar la potencialidad de diez plantas del región templado en el estado de México para reducir las emisiones de metano utilizando la técnica de producción de gas *in vitro*.

II. REVISIÓN DE LITERATURA

2.1 Artículo de revisión de literatura en preparación para su envío a una revista científica: "A review on the potentiality of plant secondary metabolites and tannins from tropical plants on mitigating rumen methane production".

2.1.2. Manuscrito

POTENTIALITY OF PLANTS FOR METHANE MITIGATION

A REVIEW ON THE POTENTIAL OF PLANT SECONDARY METABOLITES AND TANNINS FROM TROPICAL PLANTS ON MITIGATING RUMEN METHANE PRODUCTION

Raafat Mahmoud Mohamed Gomaa[1,2], Octavio Alonso Castelán-Ortega[1*], Manuel González-Ronquillo[1], Jorge Arredondo-Ramos[1], Luisa Molina[3]

ABSTRACT

Methane (CH4) is a potent greenhouse gas that is produced by microbial fermentation in the rumen and released to the environment during respiration and eructation. Methane represents a loss of energy for the ruminants and contributes to the emission of greenhouse gases, which have a global warming potential. Increasing atmospheric concentrations of methane have led scientists to examine its sources of origin. Ruminant livestock like cattle can produce 250 to 500L of methane per day. Numerous studies have been carried out in order to investigate the potentiality of plants' secondary metabolites (PSM) to modify rumen fermentation and reduce CH4 production, especially those plants rich in tannins. The objective of the present review is to discuss on the use of tannin rich plants from the tropical regions of the world to mitigate methane emissions by cattle. According with the studies consulted it was concluded that Sorghum silage, *Manihot esculenta, Castanea sativa, Schinopsis sp*. and *Chestnut sumach* could have potential to reduce methane formation in the rumen.

Key words: Enteric methane mitigation, Ruminant animal, Tannins, Phenolic contents, Methane production.

POTENCIALIDAD DE PLANTAS PARA MITIGACIÓN DE METANO

UNA REVISIÓN DEL POTENCIAL DE LOS METABOLISMOS SECUNDARIAS Y TANINOS DE PLANTAS TROPICALES EN LA MITIGACIÓN DE LA PRODUCCIÓN DE METANO EN EL RUMEN

RESUMEN

El metano (CH4) es un potente gas de efecto invernadero que se produce normalmente por la fermentación microbiana en el rumen y liberado al medio ambiente principalmente mediante eructos. El metano representa una pérdida de energía para los rumiantes y contribuye a las emisiones de gases de efecto invernadero que tienen un potencial de calentamiento global. El aumento de las concentraciones atmosféricas de metano han llevado a los científicos a examinar sus fuentes de origen. Rumiantes como el ganado bovino pueden producir de 250 a 500 litros de metano al día. Numerosos estudios se han completado en el uso de metabólicos secundarios de plantas (PSM) como sustituto de aditivos para alimentos químicos ya que algunos de ellos modifican la fermentación ruminal y reducen la producción de CH4, en especial plantas ricas en taninos. Esta revisión describe el uso de plantas ricas en taninos de las regiones tropicales del mundo para mitigar posibles emisiones de metano del ganado bovino. De acuerdo con los estudios consultados

se concluyó que el ensilado de sorgo, *Manihot esculenta, Castanea sativa, Schinopsis sp*. y *Chestnut sumach* podrían tener potencial para reducir la formación de metano en el rumen.

Palabras clave: mitigación entérica metano, Taninos, contenidos fenólicos, Producción de metano.

INTRODUCTION

An important problem facing our world is the emission of green house gases (GHG) by anthropogenic activity. Whilst CO_2 receives substantial attention as a possible cause of global warming, atmospheric concentrations of methane (CH_4), chlorofluorocarbons and N_2O have markedly increased in the last 150 years and may be contributing to global warming. Increased temperature in the globe is having an impact on many plants and animal species worldwide, although the most dramatic effects are expected in the next decades when crop and forage yields might be reduced due to of drought and extreme weather (Olesen and Bindi 2002).

In the case of ruminants the diet composition and intake are the main factors affecting CH_4 production. Ruminants fed forages rich in structural carbohydrates produce more CH_4 than those fed mixed diets containing higher levels of non-structural carbohydrates. The fermentation of the structural carbohydrates of the forage leads to acetate formation, which results in more H_2 release into the rumen environment. The additional H_2 in the rumen environment is used by methanogenic bacteria to reduce the H_2 released by acetate formation to produce CH_4 (Sauvant and Giger-Reverdin 2009). Forages are often the main ingredient in ruminant diets in the tropical region of the world. In these regions, feed resources (i.e., grasses, legumes, tree foliage) differ from those of temperate regions due to their chemical and structural composition and digestibility (Assoumaya *et al*. 2007). It is well known that some tropical forages, such as legumes, shrubs and tree foliages may contain secondary metabolites like condensed tannins and saponins that can alter rumen

methanogenesis (Jouany and Morgavi 2007). It has been reported that ruminants fed tropical forages apparently produce more CH_4 than ruminants fed temperate forages (Bhatta *et al*. 2009). The presence of condensed tannins in tropical legume trees and shrubs foliage has been found to lower CH_4 emissions mainly due to inhibition of methanogens in the rumen (Martin *et al*. 2010).

On the other hand, Livestock is assumed to be responsible for the largest part at nearly 80% of total agricultural GHG emissions (Broucek 2014). This is particularly due to methane (CH4) emissions from enteric fermentation and manure handling. The loss of methane to the atmosphere varies based on the ruminant species. Estimates of diet-derived energy losses from methane for dairy cattle, range-cattle, and feedlot cattle vary from 5.5–9.0%, 6.0–7.5%, and 3.5–6.5%, respectively (Hook *et al*. 2010). Production of CH4 has a negative impact on animal productivity, resulting in lost energy ranging from 2% to 12% of the animal's GEI (Gross energy intake) (Ramin *et al*. 2013 ,Haarlem *et al*. 2008).

Additives like microbial and plant secondary metabolites offer a unique opportunity for manipulating ruminal fermentation. Recent research has been greatly focused in exploiting bioactive plant secondary compounds like saponins, tannins, flavonoids, essential oils to improve rumen fermentation such as enhancing protein metabolism, decreasing methane production, reducing nutritional stress like bloat, and improving animal health and productivity (Cottle and Conington 2013). These compounds, called 'phytochemicals' or plant secondary metabolites (PSM), describe non-nutritive plant metabolites, which are essential for plant survival (i.e., protection against herbivores, pests, microorganisms) and proper growth and reproduction. However, recently these phytochemicals have been tested

as natural additives to decrease CH_4 production in ruminants (Patra and Saxena 2011). Therefore, the objective of the present study is to provide an updated review and discussion on the use of tanniferous tropical plants as natural additives to mitigate methane emissions by ruminants.

Methane formation by ruminal microbes. Methane is a key rumen fermentation end product, so understanding why CH_4 is produced in the rumen and its implications to ruminal fermentation is required before proposing any measure to control its production (Janssen, 2010). Methanogenesis is a necessary process because it is a way to maintain low H+ concentrations in the ruminal environment by reducing CO_2 (Bodas *et al.* 2012). The reason of CH_4 formation in the rumen in the oxidation–reduction reactions, which are intrinsic to rumen anaerobic fermentation. These coupled reactions occur at the expense of coenzymes (i.e., as electron carriers) in a complementary process. Nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide are two well-known examples of these coenzymes, but other electron carriers, such as ferrodoxin, are also active in rumen bacteria (Russell and Wallace, 1997). It is noteworthy to consider that the total amount (i.e., oxidized plus reduced forms) of a coenzyme such as NAD/NADH is constant in the bacterial cell. Thus, if all NAD were in the reduced form (NADH), there would be no means for the cell to undergo any other oxidation wherein NAD was required. Substantial amounts of reducing power (i.e., H) arise from catabolism of substrates and bacteria must find ways to dispose of it to attain re-oxidation of the electron carriers.

In contrast to aerobic conditions, cells in anaerobic media do not possess the capability of accomplishing complete oxidation of substrates with ultimate disposal of the reducing

power into O_2, resulting in other strategies (Russell, 2002). One such strategy occurs when bacteria perform relatively simple fermentative routes, where the reducing power first generated is disposed of in final products of the fermentation. This strategy is found in the rumen, such as when succinate or lactate is produced. However, another strategy is of fundamental importance for the rumen, as observed in some of the most characteristic bacteria of rumen microbial fermentation, (i.e., those involved in the production of acetate). Such microbes possess membrane-bound hydrogenases, which are capable of directly oxidizing reduced electron carriers, such as NADH or ferrodoxin, to yield H_2. In the end, substantial amounts of H_2 originate in the rumen as a consequence of this process. Moreover, other rumen microorganisms, such as protozoa and fungi, also produce substantial amounts of H_2 (Russell and Wallace, 1997). However, these NADH-linked membrane-bound hydrogenases are highly sensitive to H_2, being quickly inhibited when H_2 pressure builds up (Russell, 2002). Keeping a low H_2 pressure is critical to maintaining a vigorous fermentation. Thus, H_2 must be disposed of in a fast efficient way. In the rumen, this role is mainly accomplished by the methanogenic Archaea, which efficiently use H_2 to reduce CO_2, producing CH_4 as final product (Moissl-Eichinger and Huber, 2011; Russell and Wallace, 1997), with CH_4 produced being eructated. Transfer of the reducing power to the methanogens is not unique to the rumen, as there are three major types of microorganisms capable of lowering the reducing power in anaerobic microbial consortia, being the methanogens, sulphate reducing bacteria and reductive acetogenic bacteria. The characteristics and conditions of the medium determine the strategy, and the end product, where the reducing power is ultimately disposed.

Formation of CH_4 by ruminal methanogens is autotrophic, and seems to occur mainly from CO_2 and H_2 (Sirohi *et al.* 2010). The origin of H_2 has already been described, but CO_2 is abundant in the rumen as a consequence of decarboxylation of metabolites during fermentation and because of bicarbonate buffering. Part of the CO_2 and H_2 may originate from dehydrogenation of formic acid, completed by other bacteria; but formic acid may also be directly used by several methanogen species. For other microbial consortia, volatile fatty acids (VFA) such as acetic acid are substrates for methanogenesis, but this is irrelevant in the rumen due to the slow growth rate of the species, which is surpassed by their passage rate, thereby precluding their colonization of the rumen (Bodas *et al.* 2012).

Classic microbiological techniques revealed *Methanobrevibacter spp.*, *Methanomicrobium spp.*, *Methanobacterium spp*. and *Methanosarcina spp*. to be the major genera of rumen methanogens (Stewart *et al*., 1997), recent studies based on genetic sequences have revealed greater diversity (Thauer *et al*. 2008). The microbiology of methanogens, including their metabolism and taxonomy, entail areas of study, which are expanding, and the interested reader is referred to such specialized literature. It is nevertheless relevant to note that methanogens (domain Archaea) clearly differ from the other domain, Bacteria, in their metabolism and morphology. The phospholipids layer of the methanogen membrane is mostly composed of glycerol ethers with isoprenoid alcohols, in contrast to the glycerol esters with fatty acids typical of bacteria. These functional and morphological differences suggest that it is plausible to expect that, in a microbial consortium such as the rumen, methanogens may be selectively inhibited or stimulated, via certain manipulations that exploit these differences.

Controlling the production of ruminal methane. Production of CH_4 is an intrinsic process of ruminal fermentation and suppressing or abating its formation is a challenge. Indeed ruminants have evolved over millions of years to utilize cellulose, and polysaccharides, by means of a pregastric fermentation system, which yields CH_4, and there is no advantage to this system to halt CH_4 production (Gill *et al*. 2010).

The rate of CH_4 production by ruminants depends on level of feed intake (Monteny *et al*. 2006), with the fraction of ingested energy lost as CH_4 being reduced with higher feed intake. This effect is partially a consequence of an increased rate of rumen passage, and partially a consequence of the type of VFA produced. Likewise, although CH_4 production increases almost linearly with feed intake, CH_4 emission/unit animal product will be reduced if an improvement in animal productivity is achieved, so it is possibly to have a highly productive animal instead of two normal animals and by that we reduce CH_4 production (Benchaar *et al*. 2001). However, in the present work we will focus on nutritional interventions aimed specifically at controlling the yield of CH_4/unit feed digested, hereafter referred to as the relative yield of CH_4. Despite the rigidity of the rumen relative to suppressing methanogenesis, it is possible to abate the yield of CH_4. There are two main complementary approaches to effectively abate CH_4 formation.

A) The first approach to reducing CH_4 production takes advantage of the reality that not all feed components ferment in the same way in the rumen, thereby yielding different quantities of CH_4/unit carbohydrate fermented. It is often assumed that concentrates yield relatively less CH_4 than forages per MJ of GE intake (Garnsworthy *et al*. 2012).

The relative formation of VFA among feeds and diets determines the amount of excess H_2 in the rumen, which is ultimately converted to CH_4 by methanogenic bacteria. Thus, replacing structural fiber with non-structural carbohydrates shifts VFA patterns from acetic to propionic acid and, therefore, there is an increase in dietary starch at the expense of fiber, which reduces CH_4 losses per mega joule (MJ) of gross energy (GE) intake by redirecting reducing equivalents from CH_4 to propionate and by decreasing fiber digestibility (Benchaar *et al*., 2001). Within forage classes there are differences as well. Relative to concentrates, soluble sugars yield more CH_4 than starch per MJ of GE intake, so that replacing sugars by starch in concentrate feeds causes a 15% reduction in CH_4 production (Mills *et al*. 2001). Rumen pH also drops in diets rich in concentrates -(due to the fast fermenting of concentrates so it be produced a big amount of volatile fatty acids and decrease pH)- facilitating a shift to more production of propionate, which acts as an H_2 sink and, consequently, less CH_4 is produced/unit fermented organic matter (Monteny *et al*. 2006).

However, animal welfare issues must be considered if this strategy is implemented since sub-acute rumen acidosis is a serious production problem in ruminants. Nevertheless, using these principles, this approach would allow choice of feeds, which lead to less CH_4 production. Related to this tactic, the administration of some dietary enzymes or probiotics feed additives may potentially enhance digestion and, as a side effect, reduce rumen CH_4 production. Enzymes such as cellulase and hemicellulase added to the diet of ruminants have demonstrated reduced *in vivo* CH_4 production by 28 and 9%, respectively, possibly by reducing the acetate to propionate ratio (Beauchemin *et al*., 2008).

B) The second approach consists of the use of specific ingredients or additives aimed at specifically reducing production of CH_4. These are compounds, which directly or indirectly inhibit methanogen function. Several chemicals inhibit CH_4 production experimentally.

However, these substances have drawbacks as many cause only a transient decline in CH_4 production, and they are toxic to the host. Some plant secondary metabolites (PSM) and plant extracts fall in this category, e.g. anthraquinones, which are the major secondary compounds in rhubarb (*Rheum rhabarbarum*), directly inhibit methanogens (García-González *et al*., 2010).

Use of some PSM from tropical plants as natural additives to mitigate methane emissions by ruminants. Methane from ruminal fermentation can be decreased by PSM. Ideally, CH_4 should be affected by a primary and selective effect of the PSM through direct inhibition of methanogenic archaea and/or depression of the microbial metabolic processes involved in methanogenesis. In some cases, CH_4 is affected as a result of effects of the phytofactors on ruminal fermentation to redirect processes to metabolic reactions resulting in less CH_4 production (e.g., fermentation towards more formation of propionate will result in less CH_4), or reducing available metabolic H_2 for methanogenesis (e.g., with alternative sinks for disposal of H_2). In a Screening study of 13 plants in continuous cultures of rumen microbes showed that methanogenesis could be decreased between 8% and 14% when flavonoid rich extracts of *Equisetum arvense* and *Salvia officinalis* were added to a 50:50 hay : barley grain diet (Broudiscou *et al*., 2000). Bodas *et al*. (2008) screened more than 450 plant species for effects on CH_4 production and reported a 25% CH_4 depression with the following six plants: *Carduus pycnocephalus, Populus tremula, Prunus avium, Quercus*

robur, *Rheum nobile* and *Salix caprea*, with no adverse effects on feed digestibility, total gas and VFA production.

Carduus pycnocephalus were selected for further testing on the basis of consistency of effects on CH_4 production (i.e., 20% decrease). *Carduus* leaves were effective in decreasing CH_4 *in vitro* when added to both forage and concentrate diets, and this effect was not due to tannin or saponin active moieties (Goel *et al*. 2008). García-González *et al*. (2008a) examined more than 150 plants, herbs and spices to assess their potential to modify ruminal fermentation and decrease *in vitro* CH_4 production, identifying rhizomes and roots of *Rheum officinale* (rhubarb), bark of *Frangula alnus* (frangula) and bulb of *Allium sativum* (garlic) as the most effective candidates methane was decreased by 20%. Effects of these plants on CH_4 production were confirmed in subsequent dose response assays (García-González *et al*., 2008b). Based on the effects of rhubarb and frangula on other fermentation end-products, and on the diminished calculated H recovery, it was clear that both plants have a direct effect on methanogens due to their content of anthraquinone derivatives (García-González *et al*. 2008b).

Anthraquinones (also known as anthraquinonoids) are a class of naturally occurring phenolic compounds based on the 9, 10-anthraquinone skeleton (Akinjogunla *et al*. 2010). Kung *et al*. 2003 evaluated the effects of adding 9,10 anthraquinone, a known inhibitor of methanogenesis and sulfate reduction, on blood metabolites, digestibility, and distribution of gas in sheep. In an 8-wk study, feeding up to 66 ppm (dry matter basis) of 9,10 anthraquinone had no adverse effects on blood metabolites including indicators of normal enzyme function, mineral concentrations, and hematological measurements. Feeding 500

ppm of 9, 10 anthraquinone to sheep resulted in a decrease ($P < 0.07$) in the concentration of methane, but an increase ($P < 0.05$) in hydrogen concentration of ruminal gas throughout the 19 d of feeding. There was no indication of ruminal adaptation throughout this time.

In an extensive *in vitro* screening of various leaves, seeds and fruits of tropical multi-purpose shrubs and trees, leaves of medicinal plants and residues of leguminous food-feed crops, it was observed that seeds and fruits of *Albizia rhizonse* and *Sapindus saponaria*, and leaves of *Samanea saman* and different *Acacia* and *Sesbania* species had the lowest methanogenic potential (Soliva *et al*. 2008). Jayanegara *et al*. (2011a) reported CH_4 mitigating effects with tropical plants rich in phenolic compounds, such as *Swietenia mahagoni*, *Acacia villosa*, *Eugenia aquea*, *Myristica fragrans* and *Clidemia hirta*, concluding, surprisingly, that the most interesting plants would be those containing non-tannin phenols these plants are *Swietenia mahagoni*, *Acacia villosa*, *Eugenia aquea*, *Myristica fragrans* and *Clidemia hirta* reduced CH_4 production by more than 20%. Kamra *et al*. (2008) screened 93 plant extracts for their potential to inhibit *in vitro* methanogenesis and ciliate protozoa using buffalo rumen liquor, and reported that 20 extracts abated CH_4 production by more than 25%, accompanied by a sharp decline in methanogen numbers. Some of the plant species showing a more pronounced effect were rich in saponins (*Sapindus mukorossi*), tannins (*Terminalia chebula*, *Populus deltoids*, *Mangifera indica* and *Psidium guajava*) or essential oils (*Syzygium aromaticum*, *A. sativum*). Consistent with other studies, Kamra *et al*. (2008) reported some anti-methanogenic activity of *E. arvense*, *Lotus corniculatus*, *Rheum palmatum*, *S. officinalis*, *S. saponaria*, *Uncaria gambir* and *Y. schidigera* with 20% methane reduction. Durmic *et al*. (2010) evaluated 128 Australian

woody perennial plants for their potential to induce favorable metabolic pathways in the rumen, and reported that CH4 production was reduced by 30% with the plant species *Cullen australasicum, Enchylaena tomentosa, Eremophila longifolia, Maireana astrotricha* and *Templetonia retusa*. Although these species have not been widely used as forages because of their low biomass productivity and nutritive value, they may be considered as options to reduce CH_4 emissions for grazing ruminants.

In a screening of medicinal plant essential oils and garlic oil, CH_4 was diminished by 15% with coriander, cinnamon, red basil, oregano, cumin, caraway and dill essential oils (Jahani-Azizabadi *et al*. 2011). Abdalla *et al*. (2012) evaluated *in vitro* the effects of several tannin-rich plants, essential oils and biodiesel co-products on CH_4 formation, and found that plant material from *Mimosa caesalpiniaefolia* and essential oils of eucalyptus (*Eucalyptus melliodora*) and black seed (*Nigila sativa*) showed potential to abate CH_4 emission from ruminants by 15%, although effects were attributed to a decrease in fermentable substrate rather than to a direct effect on methanogenesis.

From this screening studies it can be reported that there are a large number of plant species with a potential to decrease CH_4 production when used as feed additives, as supplementary feed or as feed for grazing ruminants. In most cases, their beneficial effects have been observed *in vitro*, but *in vivo* studies are required to confirm the potential of the candidates, and their feasibility as feed additives or ingredients in ruminant diets. This effect is most likely due to their PSM content, which would affect rumen microbes and fermentation although, in most studies, the specific PSM responsible for effects on CH_4 have not been identified. However, based on the main PSM in plants, which show a depression of CH_4

production, the main phyto-factors responsible for this effect would be tannins, saponins, essential oils, organosulphur compounds and other phytochemicals.

Tannins. Tannins represent an important class of plant secondary metabolites and are produced by the plants in their intermediary metabolism. Chemically, they are polyphenolic compounds with varying molecular weights, and they have the ability to bind natural polymers such as proteins and carbohydrates (Mueller-Harvey 2006).

Tannins reduce methane due to their inhibitory effect upon methanogens, protozoa and other hydrogen-producing microbes (Patra and Saxena, 2010; Tavendale *et al.* 2005). Temperate plants rich in tannins such as *Lotus pedunculatus* have been shown to reduce methane production by up to 30% (Woodward *et al.* 2004) and can replace other forages in the diet. In the hot and arid regions of the world many legumes, particularly foliage from leguminous trees, are rich in tannins and they represent a valuable feed resource in some countries. However, the use of forages rich in tannins as a major feed component seems difficult to implement in arid and subtropical conditions but in production systems where the use of supplements is possible the utilization of tannin containing extracts could be a viable alternative. And in many situations, tannin extracts have been proven effective for reducing methane production. Jayanegara *et al.* (2011a) reported that noticeable effects of tannins are observed at levels higher than 20 g/kg feed.

Methane production from ruminal fermentation has been decreased up to 50% in response to tannin or plant extracts containing these polyphenolic compounds (Patra and Saxena 2010, Goel and Makkar 2012) and some authors have suggested that the molecular weight

of condensed tannins has a direct impact on CH_4 production with the impact more pronounced at higher molecular weight (Huang *et al*. 2011). However, there are also studies with no effects of tannins on CH_4 production. Even so, tannins are recognized as compounds with a high capacity to reduce CH_4 production in the rumen (Morgavi *et al*. 2012).

In Table 1 it is obviously the effect of the plants on methanogenesis at in vitro level, the highest inhibation on methanogenesis was with 96.5% in *Castanea sativa* (leaves) among other Mongolian plants in the study of Jayanegara *et al*. 2011b, among the positive extract the extracts of *Schinopsis sp*. and *Chestnut sumach* of the studies of Min *et al.* 2005 and Jayanegara *et al.* 2015a with a percentage of 78% and 36.7% respectively.

In Table 2 it can be observed the negative effect of Sorghum silage on methanogenesis in steers 70% lower methane produced in the study of De oliveira *et al.* 2007, on the other hand with sheep the use of *Manihot esculenta* plant was effective on methanogenesis lowered the methane production with 68.5% in the study of Rira *et al.* 2015.

From the screening studies it can be concluded that tannins has negative effect on methanogenesis. Several authors agree (Tavendale *et al*. 2005, Patra and Saxena 2011) that tanniferous plants reduce methane production due to their antimicrobial properties, for example Jayanegara *et al.* (2015a) found that all tannins decreased methane concentration either linearly or quadratically, also reported that the magnitude of decrease was greater for the hydrolysable tannins containing plants than for those plants rich in condensed tannins.

The mode of action of tannins has not been completely described. Tannins may be antimicrobial compounds, inhibiting some ruminal microorganisms. In particular, studies have shown that tannins may inhibit, through bactericidal or bacteriostatic activities, growth or activity of rumen methanogens (Tavendale *et al*. 2005, Liu *et al*. 2011) probably by binding proteins and enzymes of the microbial cell.

Milk Production. Bell *et al*. (2011) suggests that non-grazing low forage feeding system result in the lowest enteric CH_4 emissions.kg^{-1} energy corrected milk, with about 13% less enteric CH_4 compared to a high forage feeding system at the same farm. Body weight and milk yield accounted for significant proportions of variation in CH_4 emissions. Both parameters were positively related to methane concentrations (Garnsworthy *et al*. 2012).

Danielsson *et al.* (2012) evaluated dairy cows fed a diet with forage: concentrate ratio of 500:500 or 900:100 $g.kg^{-1}$ of DM of total DMI. Mean CH_4 yields did not differ between diets, being 16.9 and 20.2 $g.kg^{-1}$ DMI for the 500:500 and 900:100 diets, respectively. Methane productions were 267 and 339 $g.day^{-1}.cow^{-1}$, respectively. Muñoz *et al*. (2012) found at the DMI of 17.5 $kg.d^{-1}$ and milk yield of 22.9 $kg.d^{-1}$ CH_4 measured by sulfur hexafluoride technique of 469 $g.d^{-1}$ (292 - 647), and CH4 measured by respiration chamber 422 $g.d^{-1}$ (275 - 577), They calculated ratios during measuring by respiration chamber technique CH_4: DMI of 24.3 $g.kg^{-1}$ (14.1 - 29.2) and CH_4: milk yield of 19.9 $g.kg^{-1}$ (6.9 - 54.2).

Pedreira *et al*. (2009) recorded methane production from lactating and dry cows and heifers on pasture under tropical conditions, using the tracer gas technique that Holstein produced

more CH_4 (299.3 $g.d^{-1}$) than the Crossbred (264.2 $g.d^{-1}$). Lactating cows produced more CH_4 (353.8 $g.d^{-1}$) than dry cows (268.8 $g.d^{-1}$) and heifers (222.6 $g.d^{-1}$). Dairy cows emit approximately 430 $g.d^{-1}$ at peak lactation down to 250 g.d−1 as milk yield declines (Eckard *et al*. 2010, Cottle and Conington 2013). Holstein cows produced less CH_4 per unit of dry matter intake (19.1 $g.kg^{-1}$) than the Crossbred (22.0 $g.kg^{-1}$). Methane emission by heifers grazing fertilized pasture was higher (222.6 $g.day^{-1}$) than that of heifers on unfertilized pasture (179.2 $g.day^{-1}$) (Pedreira *et al*. 2009).

Beef production. Laubach *et al*. (2008) worked with steers of average LW 325 kg based on the animal-scale method, the average CH_4 emission rate over 9 days 161 ± 20 $g.day^{-1}$. There was a significant difference between two contrasting diets (Lucerne silage diet, cereal, Lucerne, and straw mixed ration) in daily CH_4 production, with mean methane production of 124.3 $g.day^{-1}$ and 169.8 $g.day^{-1}$ (Vlaming *et al*. 2008). On average, mature beef cows emit CH_4 from 240 $g.day^{-1}$ to 350 $g.day^{-1}$ (Eckard *et al*. 2010, Cottle and Conington 2013). Huarte *et al*. (2010) found that the CH_4 emission rates corresponding to values of 190 $g.day^{-1}$ per beef cattle head.

CONCLUSIONS

It can be concluded that Sorghum silage, *Manihot esculenta,* at *in vivo* level and *Castanea sativa, Schinopsis sp*. and *Chestnut sumach* at *in vitro* level have a great potential negative effect on methanogenesis, these plants must be produced in a wide level in order to be used in the worldwide and to do various studies on other animal spices in various climate in other countries.

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Table 1. Effect of tanniferous plants on methane mitigation at *in vitro* level.

Experimental method	Basal diet	Plants	Level (g kg^{-1} DM)	Tannin presentation	Effect of methane mitigation in Comparison to control	Reference
In vitro	Wheat forage	*Schinopsis sp.*, (commercial condensed tannins)	0 - 20	extract	Positive with 78%	Min *et al.* 2005
In vitro	_____	*Terminalia sp., Castanea sp., Schinopsis sp., Acacia sp.*	52.7 - 185	extract	Positive with 5.5%	Bhatta *et al*. 2009
In vitro	_____	Various Mongolian plants	4 - 209.3	(Plant) Non-extract	Positive with 25%	Jayanegara *et al.* 2009
In vitro	_____	Various	2 - 220	(Plant)	Positive	Jayanegara

		Mongolian plants		Non-extract	with 57% in *Swietenia mahagoni*	*et al.* 2011a
In vitro	_____	Various Mongolian plants	0 - 79	(Plant) Non-extract	Positive with 96.5% in *Sambucus nigra*	Jayanegara *et al.* 2011b
In vitro	TMR devoid of tannins	*Autocarpus integrifolis, Azardirachta indica, Ficus bengalensis*	0 - 300	(Plant) Non-extract	Positive with 61.5%	Bhatta *et al.* 2014
In vitro	4 roughages	*Acacia molissima*	50	Extract	Positive with 17%	Bueno *et al*. 2015
In vitro	Hay: concentrate 70:30	*Chestnut sumach, mimosa, quebracho*	3.8	Extract	Positive with 36.7%	Jayanegara *et al.* 2015a
In vitro	Hay: concentrate 70:30	*Chestnut, sumach, mimosa, quebracho*	3.8	Extract	Positive with 27%	Jayanegara *et al.* 2015b

Table 2. Effect of tannins on methane mitigation at *in vivo* level.

Animal species	Basal diet	Plants	Level (g kg^{-1} DM)	Tannin presentation	Adaptation period (days)	Effect of methane mitigation in Comparison to control	Reference
Sheep	Hay: concentrate	*Castanea* sp.	1, 2	Extract	12	Negative - increased CH_4 with 33%	Sliwinski *et al.*2002
Cattle	_____	*Lolium perenne*, *Hedysarium coronarium*	0, 27.2	(Plant) Non-extract	_____	Similar to control with 98%	Woodward *et al.* 2002
Cattle	_____	*Lolium perenne*, *Lotus corniculatus*	0, 26.5	(Plant) Non-extract	7	Positive with 23%	Woodward *et al.* 2004
Cattle	Barley silage, concentr-ate	*Schinopsis* sp.	0 - 18.2	Extract	23	Similar to control with 99%	Beauchemin *et al.* 2007
Cattle	_____	Sorghum bicolor silage	0.2 - 1	Non-extract	10	Positive with 70%	De oliveira *et al.* 2007
Cattle	Reygrass pasture, corn grain	*Acacia mearnsii*	0 - 18	Extract	35	Positive with 29%	Grainger *et al.* 2009
Cattle	Rice	*Sapindus*	0, 4.8	(Plant)	14	Positive	Poungch

	straw, concentrate	*saponaria* fruit and *Garcinia mangostana* peel		Non-extract		with 68.8%	ompu *et al*. 2009
Sheep	*Dichanthium* spp.	*Gliricidia sepium*, *Leucaena leucocephala*, and *Manihot esculenta*	39, 75 and 92	(Plant) Non-extract	_____	Positive with 68.5%	Rira *et al.* 2015

III. JUSTIFICACIÓN

Es importante reducir las emisiones de metano pues éste es un gas de efecto invernadero, el cual tiene efectos negativos sobre el clima mundial. como aumentar la temperatura de la tierra y eso aumentara el nivel del mar, y eso afecta a todos los seres vivos.

El uso de las plantas ricas en taninos podría ser empleado para reducir las emisiones de metano a nivel ruminal, con una manera natural y efectiva.

La reducción de las emisiones de CH4 representarían un ahorro del 10% de las pérdidas de energía en la producción de metano de los piensos de rumiantes (IPCC 2007).

IV. HIPÓTESIS

El uso de las plantas ricas en taninos podría ser útil para reducir las emisiones de metano a nivel ruminal debido a los efectos de los taninos sobre las bacterias metanogénicas del rumen.

V. OBJETIVOS

5.1 OBJETIVO GENERAL

Desarrollar estrategias nutritivas, con base en las mediciones *in vitro*, para reducir el metano producido por la fermentación entérica del ganado bovino en México.

5.2 OBJETIVOS PARTICULARES

Evaluar la composición química de cada especie de las diez plantas

Evaluar el efecto de las plantas ricos en taninos sobre el producción del metano usando la técnica del producción de gas *in vitro*.

Determinar el efecto de diferentes niveles de substitución (10, 20 y 30%) del forraje basal de la dieta experimental con las plantas experimengtales sobre la producción de metano.Identificar las plantas que reduzcan la producción de metano sin afectar la digestibilidad de la dieta.

VI. MATERIALES Y MÉTODOS

El estudio se llevó a cabo en el laboratorio de Nutrición animal de la Facultad de Medicina Veterinaria y Zootecnia de la Universidad Autónoma del Estado de México, el cumple con las normas éticas y bienestar animal establecidas por la institución.

6.1 Colecta del material vegetal y preparación de las muestras

Las especies de plantas utilizadas en el estudio fueron *Amaranthus spinosus, Cosmos bipinnatus, Commelina coelestis, Eupatorium glabratum, Galinsoga parviflora, Ipomoea orizabensis, Jaegeria hirta, Senna hirsuta, Tagetes erecta* y *Tagetes filifolia*. Muestras de plantas fueron recolectadas en septiembre de 2013 desde seis lugares diferentes seleccionados al azar en la región templada del Estado de México, que se encuentra a 19 ° 17' 32" N y 99 ° 39' 14" W y a 2663 metros sobre el nivel del mar. Para cada especie, se recogieron y se cortaron en trozos pequeños (3-5 cm) seis kilogramos de materia vegetal fresco (uno para cada ubicación). Las muestras fueron luego secados a 50 ° C durante 48 h usando un horno de aire forzado con el fin de evitar la degradación enzimática de los compuestos fenólicos presentes en la materia vegetal (Makkar *et al*., 1993b). Una vez seco, se molieron 400 g de materia vegetal en un molino Lab-Willey Grinder (código de RSU-342- EN; 10.122.740) y se tamizaron a través de un tamízde 2 mm de poro. El material molido se mezcló bien y después aproximadamente 100 g fueron sub-muestreada, nuevamente molidos y pasados por un tamiz de pantalla de 0.5-mm. Estas sub-muestras finamente molidas se utilizaron en el análisis de taninos, mientras que el resto del material fue utilizado para el análisis *in vitro* de la producción de gas.

6.2 Análisis bromatológico

La materia seca (MS) se determinó mediante el secado de las muestras a 135 ºC durante 2 h. (AOAC, 1995, ID 930.15). La materia orgánica (MO) se calculó como el peso perdido en la muestra después de fue INCINERADA a 600 ºC (AOAC, 1995, ID 942.05). Fibra detergente neutro (FDN) y fibra detergente ácida (FDA) se determinaron utilizando la técnica de la tecnología de fibra ANKOM (Robinson *et al.,* 1999) sin el uso de alfa-amilasa. Proteína bruta (CP) se determinó por el método de Kjeldahl (AOAC, 1995, ID 984,13).

6.3 Experimento 1. Evaluación *in vitro* de cinco plantas taniferas

El trabajo experimental se realizó en el laboratorio de nutrición de la Facultad de Medicina Veterinaria y Zootecnia de la Universidad Autónoma del Estado de México.

Las plantas fueron *Amaranthus spinosus, Cosmos bipinnatus, Commelina coelestis, Eupatorium glabratum* y *Galinsoga parviflora*.

Tratamientos

La dieta de control estaba compuesta por 64.6% raigrás (*Lolium perenne*), 20.8% de grano de maíz (*Zea mays*), 8.3% de harina de colza (*Brassica napus*) y el 6.3% el rastrojo de maíz. En los tratamientos experimentales 10, 20 y 30% de raigrás fue reemplazado por cantidades equivalentes de las plantas de ensayo.

6.4 Experimento 2. Evaluación de cinco plantas taniferas a 24 y 72 h de incubación

El trabajo experimental se realizó en de la Facultad de Medicina Veterinaria y Zootecnia de la Universidad Autónoma del Estado de México.

Las plantas fueron *Ipomoea orizabensis, Jaegeria hirta, Senna hirsuta, Tagetes erecta* y *Tagetes filifolia*.

Tratamientos

La dieta de control estaba compuesto por 64.6% raigrás (*Lolium perenne*), 20.8% de grano de maíz (*Zea mays*), 8.3% de harina de colza (*Brassica napus*) y el 6.3% el rastrojo de maíz. En los tratamientos experimentales 10, 20 y 30% de raigrás fue reemplazado por cantidades equivalentes de las plantas de ensayo.

6.5 La producción de gas *in vitro*

La técnica de producción de gas (GP) *in vitro* se determinó siguiendo la técnica de Theodorou *et al.* (1994). El liquido ruminal se recogió antes del alimentación de la mañana dedos -ruminalmente fistulados y no lactantes y no grávidas- vacas Holstein. Todas las vacas tenían acceso libre a agua fresca, las vacas fueron alimentadas con la misma dieta experimental y pastaban en una pradera de raigrás de 6:00 a 16:00 h todos los días. Muestras de 0,999 g de peso del control y dietas experimentales se colocaron en botellas de vidrio de 125 ml, y se añadieron aproximadamente 90 ml de liquido ruminal tamponado a cada botella. Una vez cerrado, las botellas se agitaron suavemente y se colocaron en un baño de agua a 39 ºC. Se completaron seis corridas independientes. Las tres primeras corridas se incubaron durante 24 h con el fin de simular el tiempo de residencia normal de

alimento en el rumen, y se utilizaron para medir la producción de metano a 24 h post-incubación. Las otras tres corridas se incubaron durante 72 h. Un total de 288 botellas fueron utilizadas y todas las incubaciones se completaron por triplicado dentro del plazo.

Mediciones de producción de gas fueron tomadas por hora (hasta 8 horas después de la incubación), a continuación, cada cuatro (12- 28), ocho (36-60) y 72 h después de la incubación. Después de 24 y 72 h, el residuo de incubación se analizó para determinar la digestibilidad de la materia seca (DMS), la materia orgánica (DMO) y la digestibilidad de la fibra neutra detergente (dFDN) contenido utilizando la técnica de la tecnología de fibra ANKOM (Robinson *et al*., 1999).

6.6 Medición de metano

Con una jeringa hermética, muestras de gas se obtuvieron de cada botella a las 24 h post-incubación. Después se registró el volumen de gas, y la muestra retiró para el análisis de metano, el gas restante fue liberado y la botella volvió al baño de agua.

Contenido de metano se determinó mediante la inyección de 1 ml de gas a un cromatógrafo de gases Perkin Elmer (modelo: Clarus serie 500) equipado con un detector de ionización de llama (FID). La separación se consiguió usando una columna capilar Parcela Elite-Q (Perkin Elmer) empaquetada con una fase estacionaria Carboxen TM-1000 60/80 de malla. Se utilizó nitrógeno como gas acarreador con un caudal de 30 ml / min, una temperatura del horno isotérmica de 50 ºC, y la temperatura del inyector fue de 250 ºC. La curva de calibración usando una ecuación de regresión se completó con el estándar de CH4 (99,99% de ALTECH).

6.7 Medición de fenoles y taninos

La extracción de taninos y medición de fenoles totales y taninos contenidos fenoles se determinó utilizando el método Folin-Ciocalteu y taninos se midieron utilizando polivinilpolipirrolidona (PVPP) como se describe por Makkar *et al.* (1993b).

6.8 Análisis estadístico

Un diseño completamente al azar con arreglo factorial se utilizó en el que tanto las plantas utilizadas y sus niveles de inclusión fueron los tratamientos. El modelo lineal general fue:

$Yij = \mu + Ti + Bj + (Ti * Bj) + eij$

Donde,

Yij = variable independiente

μ = media general,

Ti = efecto de la planta (i = 1 ... 5)

Bj = efecto del nivel de inclusión (j = 1 ... 4)
(Ti * Bj) = Efecto de la interacción entre las especies de plantas y el nivel de inclusión
eij = en español en la inclusión nivel i del tratamiento j.
Los resultados se analizaron con el comando de Modelo general lineal en Minitab v14 (2013), utilizando ANOVA y prueba de Tukey.

VII. RESULTADOS

7.1 Artículo aceptado con correcciones mayores en la revista Ecosistemas y Recursos Agropecuarios: "Effect of five shrub mexican tanniferous plants on in vitro rumen methane production".

7.1.2 Correo de confirmación

UNIVERSIDAD JUÁREZ AUTÓNOMA DE TABASCO
"ESTUDIO EN LA DUDA, ACCIÓN EN LA FE"

Ecosistemas y Recursos Agropecuarios
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Revision No. 01
F-DDCT-24

Dictamen

Villahermosa, Tabasco a 16 de octubre de 2016

Artículo Científico --- **"EFFECT OF FIVE SHRUB MEXICAN TANNIFEROUS PLANTS ON IN VITRO RUMEN METHANE PRODUCTION"**

Registro --- **1160 ERA**

Autores --- Raafat Mahmoud Mohamed Gomaa, Manuel González-Ronquillo, Jorge Arredondo-Ramos, Luisa T. Molina, Octavio Alonso Castelán-Ortega

Dictamen --- **Sigue el Proceso Editorial**

Comentarios --- El cuerpo arbitral de la Revista ERA ha evaluado su manuscrito y han declarado que el manuscrito fue corregido por lo que en su forma actual puede seguir con el proceso editorial.

Los comentarios de los árbitros son:

Revisor 1

Revisor 2
The document may be published in the journal. however to continue the arbitration process it's advisable that the authors perform corrections derived of the first round; or otherwise justify the rejection of any suggestion (Revisar archivo adjunto).

Revisor 3
The only changes they made from the first draft were in table 3, 4 and 5 and no further discussion was made. So the same recommendations explained in the first draft are hardly recommended.

Revisor 4.
El manuscrito es importante para la búsqueda de recursos locales que contienen metabolitos secundarios y su efecto sobre la reducción de las emisiones de metano.
Es importante hacer las pruebas bajo condiciones in vivo con el mejor nivel de las arbustivas encontrado en este trabajo, ya que existen pocos estudios in vivo utilizando estas arbustivas.

UNIVERSIDAD JUÁREZ AUTÓNOMA DE TABASCO
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Revision No. 01
F-DDCT-24

Se solicita a los autores envíen sus correcciones un plazo no mayor **de 15 días naturales** (de lo contrario el articulo causara baja del sistema), así mismo, deberán de enviar una carta de revisión (Revision Note), donde den respuesta detallada a los comentarios de los árbitros, observando en particular cada observación hecha por los árbitros y / o editor, y la descripción de todos los cambios. En caso de no estar de acuerdo con cualquier comentario (s), explique por qué. En caso de que el Editor Asociado o un revisor hayan suministrado una lista detallada de cambios pequeños, por favor utilice el tipo de color rojo en el texto para indicar los cambios que ha realizado.

Dr. Alfonso J. Chay-Canul
Editor Asociado
c.c.p. Archivo

Vo. Bo. 20/oct/2016

Vo. Bo.

7.1.3 Manuscrito 2

-Tanniferous plants and methane production

EFFECT OF FIVE SHRUB MEXICAN TANNIFEROUS PLANTS ON IN VITRO RUMEN METHANE PRODUCTION

EFECTO DE CINCO PLANTAS ARBUSTIVAS TANIFERAS MEXICANAS SOBRE LA PRODUCCIÓN DE METANO IN VITRO

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Abstract

It has been shown that tannins in the foliage of trees and shrubs can reduce methane (CH_4) production in the rumen of cattle. The aim of the present paper was to evaluate the potential of five browse tanniferous plant species, native to temperate central Mexico, to reduce methane production in the rumen. It was used *in vitro* gas production and gas chromatography to evaluate the effect of the inclusion of these plants at three levels (10, 20 and 30%) in an experimental diet. Gas production was monitored throughout 72 h of

incubation and methane production was recorded after 24 h. The results showed that a significant decrease in methane production was observed with all plants at 10% inclusion level ($P<0.001$), and that the highest reduction (>26%) was observed with *Amaranthus spinosus* and *Commelina coelestis* at inclusion of 20% and 30% respectively in comparison to the control diet. However, diet degradability was negatively affected at these inclusion levels. It was concluded that all the tested plants reduced methane production at all inclusion levels, however the best tradeoff between methane reduction and tannins' associated negative effects on diet degradation was achieved at the 10% inclusion level.

Key Words: Plant phenolics; Tannins; *In vitro* gas production; Methane emission.

Resumen

Se ha demostrado que los taninos en el follaje de los árboles y arbustos pueden reducir la producción de metano (CH_4) en el rumen de los bovinos. El objetivo del presente trabajo fue evaluar el potencial de cinco especies de plantas arbustivas, nativas de la región de clima templado del centro de México, para reducir la producción de metano en el rumen. La técnica de producción de gas *in vitro* asociada con cromatografía de gases fueron utilizadas para evaluar el efecto de la inclusión de estas plantas a tres niveles (10, 20 y 30%) en una dieta experimental. La producción de gas se monitoreó a lo largo de 72 h de incubación y la producción de metano se registró después de 24 h. Los resultados mostraron una disminución significativa en la producción de metano con todas las especies de plantas a un nivel de inclusión de 10% ($P<0.001$), mientras que la reducción más alta (> 26%) se observó con *Amaranthus spinosus* y *Commelina coelestis* a niveles de inclusión de

20% y 30%, respectivamente, en comparación con la dieta control. Sin embargo, la degradabilidad de la dieta se ve afectada negativamente a estos niveles de inclusión. Se concluyó que todas las plantas evaluadas en todos los niveles de inclusión redujeron la producción de metano, sin embargo el mejor balance entre la reducción en la producción de metano y los efectos negativos sobre la degradación de la dieta asociados a los taninos se alcanzó a un nivel de inclusión del 10%.

Palabras clave: Compuestos fenólicos de plantas; Taninos; Producción de gas *in vitro*; Emisión de metano.

INTRODUCTION

The accumulation of greenhouse gas emissions (GHG) such as (CO_2), methane (CH_4) and nitrous oxide (N_2O) in earth's atmosphere has led to predictions that global surface temperatures will increase between one and six degrees Celsius during the 21st century (IPCC 2007). According with Arbre *et al*. (2016) contribution of livestock to anthropogenic GHG emissions is estimated at 14.5% of total emissions, of which 39% is due to methane (CH_4) produced by enteric fermentation in ruminants. Approximately 6% of cattle dietary gross energy intake is lost to the atmosphere in the form of methane (De Ramus *et al*. 2003). Hence, mitigating methane emissions could improve sustainability and profitability of cattle enterprises (Vanlierde *et al*. 2016). Historically, the inhibition of enteric methanogenesis has been approached in regard to livestock nutrition. More recently the reduction of CH_4 formation has garnered interest in relation to reducing GHG emissions. Previous studies have reported that CH_4 production can be reduced by the addition of plant

extracts containing tannins to livestock feed (Patra *et al*. 2006; Patra *et al*. 2010). A meta-analysis by Jayanegara *et al*. (2012) showed that condensed and hydrolysable tannins may reduce CH_4 production. Tannins may affect methanogenesis by directly inhibiting the growth, development and activity of methanogens, or act indirectly by reducing the number of protozoa associated with the methanogens (Makkar *et al*. 1993a). Tanniferous plants can represent a sustainable and environmentally friendly option for mitigation, however a significant screening work is needed in order to find the most efficient species. These species have to meet two criteria, one is to reduce CH_4 formation in the rumen, and two, do not diminish animal performance through modification of feedstuffs' fermentation and degradation in the rumen. *In vivo* evaluation of different species can be expensive and not feasible due to the large number of plants that need to be evaluated. So, *in vitro* gas production can be used as screening test to select the species with larger potential before *in vivo* evaluation. The objectives of the present study were to 1) evaluate the potential use of five shrub Mexican tanniferous plants to reduce methane emission using the *in vitro* gas production technique; and 2) to evaluate their effect on rumen fermentation and degradation parameters.

MATERIALS AND METHODS

The study was conducted at the animal nutrition laboratory of the Faculty of Veterinary Medicine of the Autonomous University of the State of Mexico, and complied with the ethical standards set by the institution.

Sample collection and preparation. The browse plant species used in the study were *Amaranthus spinosus*, *Cosmos bipinnatus*, *Commelina coelestis*, *Eupatorium glabratum* and *Galinsoga parviflora*. Plant samples were collected from six different locations randomly selected in the temperate region of the State of Mexico, located at 19° 17′ 32″ N and 99° 39′ 14″ W and at 2663 meters above sea level. For each species, six kilograms of fresh plant matter (one for each location) were collected and cut into small pieces (3-5 cm). Samples were then dried at 50°C for 48 h using a forced air oven in order to prevent enzymatic degradation of the phenolic compounds present in the plant matter (Makkar *et al*. 1993b). Once dry, 400 g of plant matter were ground in a Lab-Willey Grinder (code MSW-342- IN; 10122740) and sieved through a 2-mm screen. The grounded material was mixed well and then 100 g were sub-sampled, reground and passed through a 0.5-mm screen sieve. These finely ground subsamples were used in tannin analysis while the rest of the material was used for *in vitro* gas production analysis.

Treatments. The control diet was composed of 64.6% ryegrass (*Lolium perenne*), 20.8% corn grain (*Zea mays*), 8.3% canola meal (*Brassica napus*) and 6.3% corn stover. In the experimental treatments 10, 20 and 30% of ryegrass was replaced by equivalent amounts of the test plants.

Chemical composition of plants. Dry matter (DM) was determined by drying the samples at 135 ºC for 2 h. (AOAC, 1995, ID 930.15). Organic matter (OM) was calculated as weight lost at sample ignition at 600 ºC (AOAC, 1995, ID 942.05). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the ANKOM fiber

technology technique (Robinson *et al*., 1999) without using alpha amylase. Crude protein (CP) was determined by Kjeldahl method (AOAC, 1995, ID 984.13).

Extraction of tannins and measurement of total phenolics and tannins content. Phenols content was determined using the Folin-Ciocalteu method and tannins were measured using polyvinylpolypyrrolidone (PVPP) as described by Makkar *et al*. (1993b). Tannic acid (Sigma Aldrich CAS Number 1401-55-4) was used to make the calibration curve.

***In vitro* gas production.** Gas production (GP) was determined following Theodorou *et al*. (1994) technique. For every experiment fresh rumen fluid was collected before the morning feeding from two ruminally-fistulated, non-lactating and non-gravid Holstein cows. All cows had access to fresh water at all times, were fed the same experimental diet, and grazed on a ryegrass sward from 6:00 until 16:00 h daily. Samples weighing 0.999 g of the control and experimental diets were placed in 125 ml serum glass bottles, and approximately 90 ml of buffered rumen fluid was added to each bottle. Once closed, the bottles were gently shaken and placed in a water bath at 39 ºC. Six independent runs were completed with 48 bottles per run where 45 bottles were used for species and levels within species, and three more bottles for the control diet. All runs included blanks and the treatment bottles were corrected against the blanks. The first three runs were incubated for 24 h in order to simulate normal residence time of feed in the rumen, and were used to measure methane production at 24 h post-incubation. The other three runs were incubated for 72 h. A total of 288 bottles were used and all incubations were completed in triplicate within the run. Gas production measurements, for the samples incubated 72 h, were taken hourly (up to 8 hours

post incubation), then every four (12- 28), eight (36-60) and 72 h post incubation. After 24 and 72 h, the incubation residue, respectively, was analyzed for digestibility of dry matter (DMD), organic matter (OMD) and the digestibility of neutral-detergent fiber (DNDF) content using the ANKOM fiber technology technique (Robinson *et al*. 1999).

Methane measurement. Using a gas-tight syringe, gas samples were collected from each bottle at 24 h post-incubation. After the volume of gas was recorded, and the sample removed for methane analysis, the remaining gas was released. CH_4 content was determined by injecting 1 ml of gas into Perkin Elmer gas chromatograph (model: Clarus 500 series) equipped with a flame ionized detector (FID). Separation was achieved using an Elite-Q Plot Capillary Column (Perkin Elmer) packed with a 60/80 mesh carboxen^{TM}-1000 stationary phase. Nitrogen was used as the carrier gas with a flow rate of 30 ml/min, an isothermal oven temperature of 50 ºC, and the injector temperature was 250 ºC. The calibration curve using a regression equation was completed with standard CH_4 (99.99% from ALTECH).

Experimental design and analysis of results. A complete randomized design with a 5 X 4 factorial arrangement was used in which both the plants and their inclusion levels are factors. The general linear model was:

$$Y_{ij} = \mu + T_i + B_j + (T_i * B_j) + e_{ij}$$

Where,

Y_{ij}= independent variable

μ= general media

T*i*= effect of plant (*i*=1…5)

B*j*= effect of the level of inclusion (*j*=1…4)

(T*i* * B*j*)= Effect of the interaction between plant species and the level of inclusion

eij= True error contented in the *i* level inclusion of *j* treatment.

Results were analyzed with the use of the general linear model command of Minitab v14 (2013), using ANOVA. The GP curves were not fitted to mathematical models in order to determine fermentation kinetics because the aim of the work was not to describe the effect of tanniferous plants on diet's kinetics, but their effect on reducing methane production in the rumen. We used a simpler, original and intuitive approach as in the Menke *et al.* (1979) original technique; we wanted to know how much gas was produced from the feed sample as an indirect indication of the extent of digestion (fermentation) of the diet in the rumen (bottles). We also wanted to take samples of the gas produced and then analyze them by gas chromatography in order to know how much methane was produced.

RESULTS

Table 1 shows the chemical composition of the experimental diet and plant species, the highest CP content was observed in *A. spinosus*, followed by *C. coelestis*. It can also be observed that the NDF content for all plant species is moderate. Table 2 shows the content (g kg^{-1} DM) of total phenols, non-tannin phenols, total tannins and condensed tannins for all the experimental plants used. It can be observed that the highest concentration of total tannins was in *C. bipinnatus*, and the lowest in *E. glabratium*. Highest concentration of condensed tannins was observed also in *C. bipinatus*, followed by *A. spinosus* and C. *coelestis* both with the same content. Table 3 shows the CH_4 production after 24 h of

fermentation and the cumulative volume of gas produced at 24 and 72 h of fermentation for the five species at the different inclusion levels. The cumulative gas produced after 72 h of incubation for the five species at all inclusion levels, follows the same trend as does the gas produced at 24 h of incubation; the highest volume of gas produced was observed with *C. coelestis* at 20% inclusion level ($p<0.001$).

The inclusion of all plant species ($P<0.001$) at all inclusion levels resulted in a significant reduction ($p<0.002$) of methane produced when the linear comparison model is used. The highest reduction in methane production (26.2%) was observed when *C. coelestis* was included at a level of 30% ($p<0.002$) followed by including *A. spinosus* at 20% (26.0% less CH_4 produced). The other three plants also significantly reduced ($p<0.002$) CH_4 production by 20% when included at 30% level.

Table 4 shows the digestibility (g kg^{-1} DM) of the DM, OM and NDF at 24 h of incubation for all species at all inclusion levels. The DMD was negatively affected by all species at all inclusion levels ($P<0.001$), e.g. *A. spinosus* at 10% and 20%, *C. bipinnatus* at 10% and 30%, *C. coelestis* at 30% and *G. parviflora* at 10% and 20 levels. The inclusion of all species at all levels resulted in a significant ($P<0.001$) reduction of OMD too. However, OMD was not affected when using *C. bipinnatus* at 20% inclusion level ($p< 0.001$). This may be an important indicator that this plant may decrease methane production *in vivo* but not the overall digestive ability of cattle. The DNDF was positively affected by the inclusion of all plant species at inclusion level of 10% at 24 h of incubation, but at higher levels of inclusion the DNDF decreased ($p<0.001$). Table 5 shows the digestibility (g kg^{-1} DM) of the DM, OM and NDF after 72 h of incubation for all species at all inclusion

levels. It was observed a similar trend as in Table 4, since the inclusion of all plants at all levels resulted in a significant reduction of CH_4 production and all the degradation parameters ($p<0.001$). The Figure 1 shows the effect of interaction between plant species and the inclusion levels on methane production at 24 h ($p<0.001$), it can be observed that the response is not lineal, but has a quadratic effect. All plants reduced the methane production at 10% of inclusion but at 20% of inclusion only *Amaranthus spinosus, Cosmos bipinnatus, Eupatorium glabratum* and *Galinsoga parviflora* kept methane production low. However, a significant interaction can be observed in level three for *A. spinosus* ($P<0.001$), *C. bipinnatus* ($P<0.01$) *and C. coelestis* ($P<0.001$) because lines of these species are not parallel implying that the effect of the species upon CH_4 production depends up on the inclusion level. Moreover, the inclusion level effect explains more than two and a half times the total variance than that explained by the species.

DISCUSSION

Tannin content. In the present study, we found significantly lower levels of methane released (per unit of apparently fermented organic matter) with increasing levels of most tannin-rich plants added to the experimental diet. Tannins were frequently observed to reduce structural carbohydrate degradation by reducing the number of cellulolytic microbes in rumen fluid (Singleton 1981), inhibiting cellulase enzyme (Leinmüller *et al*. 1991; Makkar 1993a), preventing adhesion of microbes onto food particles (Leinmüller and Menke 1990) and/or suppressing degradation by the formation of complexes with cellulose (McSweeney *et al.* 2001). Still, no information is given by these authors as to whether there is a difference between the effects of hydrolysable and condensed tannins, but Leinmüller

et al. (1991) considered hydrolysable tannins as being less adverse in this respect. It is worth mentioning that the effect of tannins on DNDF was not the same for all species at 10% inclusion level, for example the DNDF for *A. spinosus,* C. *bipinnatus* and *G. parviflora* increased at this level. This suggest that at low inclusion levels these plants may reduce methane production without significantly affecting the DNDF.

Methane production. Results of the present study show that methane was consistently reduced by all five tanniferous plants, and that the highest reduction was observed by the inclusion of *C. coelestis* and *A. spinosus* at 30 and 20% of inclusion level respectively. The same effect of tannin-rich plants was observed by Rodríguez *et al*. (2011) who reported reduced methane production with the addition of plant extract containing tannins. The concentration of condensed tannins in both plants species may have also exerted a negative effect on methane production because *C.coelestis* and *A. spinosus* showed the second highest concentrations of condensed tannins just behind *C.bipinatus*. These results are in line with Tavendale *et al.* (2005) who demonstrated by the first time that condensed tannins can inhibit methane production by inhibiting the growth of rumen methanogens and by their indirect effects via reduced hydrogen production (possible by reducing fiber degradation). Our results are also in line with Tan *et al*. (2011) who found that a relatively low level of 15 mg of condensed tannins/500 mg DM reduced methane production by 47% with only 7% reduction in degradation of feed DM. No information was found in the literature reviewed on the effect of the plants analysed in the present work on methane production, so no further discussion was possible.

Gas production. Gas production is generally recognized as a more sensitive approach for detection of differences in substrate fermentation using *in vitro* systems than DM disappearance (Xu *et al.* 2010). Our results show that GP was negatively affected by most levels of inclusion of tanniferous plants in the experimental diet. This effect could be attributed to the antimicrobial properties of tannins and phenolic compounds in the experimental plants, which in turn cause a reduction in gas production (Francis *et al*. 2002). However, the less severe effect on GP was observe at 10% inclusion level suggesting that the best tradeoff between methane reduction and reduction of diet degradation can be achieved at low inclusion levels of these plants.

Interaction plant species x inclusion rate. The significant interaction observed between inclusion level and plant species for methane and gas production, indicate that the behaviors of both variables are the result of the combined effect between the plant species and, particularly, the inclusion level of it in the diet. This effect is clearly seen in the case of *C. bipinnatus and C. coelestis* where, at low inclusion level the effect on methane production is moderate, however at the highest inclusion rate methane production is drastically reduced due to the effect of tannins, specially condensed ones since both plants have a high content of these secondary metabolites. Unfortunately, methane reduction was accompanied by a significant reduction in DMD and OMD, which may prevent the inclusion of these plants at high rates in *in vivo* experiments.

CONCLUSION

It is concluded that all the tested plants reduced methane production at all inclusion levels, however the best tradeoff between methane reduction and negative effects on diet

degradation was achieved at the lowest inclusion level. The impact of this inhibition on ruminant whole animal energetic needs to be evaluated *in vivo* in order to use these plants in sustainable methane mitigation strategies provided they show the same effects as *in vitro*.

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Tables and figures

Table 1: Chemical composition of the experimental diet, ingredients of diet and plant species (g kg^{-1} DM).

ID of samples	DM	OM	CP	Ash	NDF	ADF
Diet	909	810	160	103	439	181
Amaranthus spinosus	930	760	184	166	448	220
Cosmos bipinnatus	901	810	84	88	481	327
Commelina coelestis	926	780	93	146	550	360
Ebatorium glabratium	928	824	100	104	370	209
Galinosa parviflora	918	809	109	107	488	306

key DM = dry matter, OM = organic matter, CP = crud protein, NDF = neutral detergent fiber, ADF = Acid detergent fiber

Table 2: Phenols and tannins content of the experimental plant species (g kg^{-1} DM).

Sample	*Amaranthus spinosus*	*Cosmos bipinnatus*	*Commelina coelestis*	*Ebatorium glabratium*	*Galinosa parviflora*
Total phenols	48.4	90.7	32.3	29.5	60.4
Non-tannins phenols	10.9	19.0	11.2	11.3	17.3
Total tannins	37.5	71.7	21.1	18.2	43.2
Condensed tannins	5.5	8.4	5.4	0.6	1.0

Table 3: Effect of the inclusion of five tanniferous plant species on methane production at 24 h and cumulative gas production at 24 and 72 h at 24 h of incubation.

	Species						P value					
							Lineal			Quadratic		
Inclusion level	***Amaranthus spinosus***	***Cosmos bipinnatus***	***Commelina coelestis***	***Ebatorium glabratium***	***Galinosa parviflora***	$\overline{X}$ for level	S	L	S×L	S	L	S×L
Methane production (ml of CH_4 g^{-1} DM)												
0%	65.6	65.6	65.6	65.6	65.6	65.6	0.001	0.001	0.001	0.001	0.002	0.001
10%	55.8	58	56.1	56.9	54.3	56.2						
20%	48.5	59.2	63.5	57.6	55.4	56.8						
30%	58.3	52	48.4	52.3	52.9	52.7						
$\overline{X}$ for species	57	58.7	58.3	58.1	57							
SED							0.54	0.49				
LSD							1.1	1.0				
Gas production at 24 h(ml g^{-1} DM)												
0%	177.9	177.9	177.9	177.9	177.9	177.9	0.001	0.001	0.001	0.001	0.07	0.001
10%	170.2	180.1	186.3	168.5	183.2	177.6						
20%	149.8	183.4	186.7	170.5	181.4	174.3						
30%	177.9	164.4	160.9	163.9	175.8	168.5						
$\overline{X}$ for species	168.9	176.4	177.9	170.2	179.5							
SED							1.7	1.53				
LSD							3.4	3.1				
Gas production at 72 h(ml g^{-1} DM)												
0%	265.1	265.1	265.1	265.1	265.1	265	0.001	0.001	0.001	0.001	0.06	0.001
10%	246.9	258.4	270.9	249.7	256.5	256.4						
20%	221.8	264.5	276.7	250.1	251.7	252.9						
30%	268.4	250.8	249	241.6	248	251.5						
$\overline{X}$ for species	250.5	259.6	265.4	251.6	255.3							

SED	2.2	1.9
LSD	4.4	3.9

SED= standard error of the difference, LSD=Least significant difference at P<0.05. S=species, L=level.

Table 4: Effect of the inclusion of five tanniferous plant species on the digestibility of dry matter, organic matter and NDF after 24 h of incubation (g kg^{-1} DM).

	Plant species						*P* value					
							Lineal			Quadratic		
Inclusion level	*Amaranthus spinosus*	*Cosmos bipinnatus*	*Commelina coelestis*	*Ebatorium glabratium*	*Galinosa parviflora*	$\bar{X}$ for level	S	L	S×L	S	L	S×L
Digestibility of dry matter												
0%	739	739	739	739	739	739	0.001	0.001	0.001	0.01	0.001	0.001
10%	673	683	719	701	628	681						
20%	686	732	726	717	680	708						
30%	723	649	621	705	744	688						
$\bar{X}$ for species	705	700	701	715	697							
SED							1.4	1.3				
LSD							2.8	2.5				
Digestibility of organic matter												
0%	742	742	742	742	742	742	0.001	0.001	0.001	0.01	0.001	0.01
10%	679	693	728	710	638	689						
20%	690	740	731	729	692	716						
30%	728	659	631	716	753	697						
$\bar{X}$ for species	710	708	708	724	706							
SED							1.6	1.4				
LSD							3.2	2.8				
Digestibility of NDF												
0%	657	657	657	657	657	657	0.001	0.001	0.001	0.01	0.001	0.2

10%	680	725	655	665	715	688		
20%	656	686	680	634	652	661		
30%	643	651	624	582	662	632		
$\overline{X}$ for species	659	679	654	634	671			
SED							5.5	4.9
LSD							11.1	9.9

SED is stander error of difference, LSD is least significant difference at P<0.05. S=species, L=level.

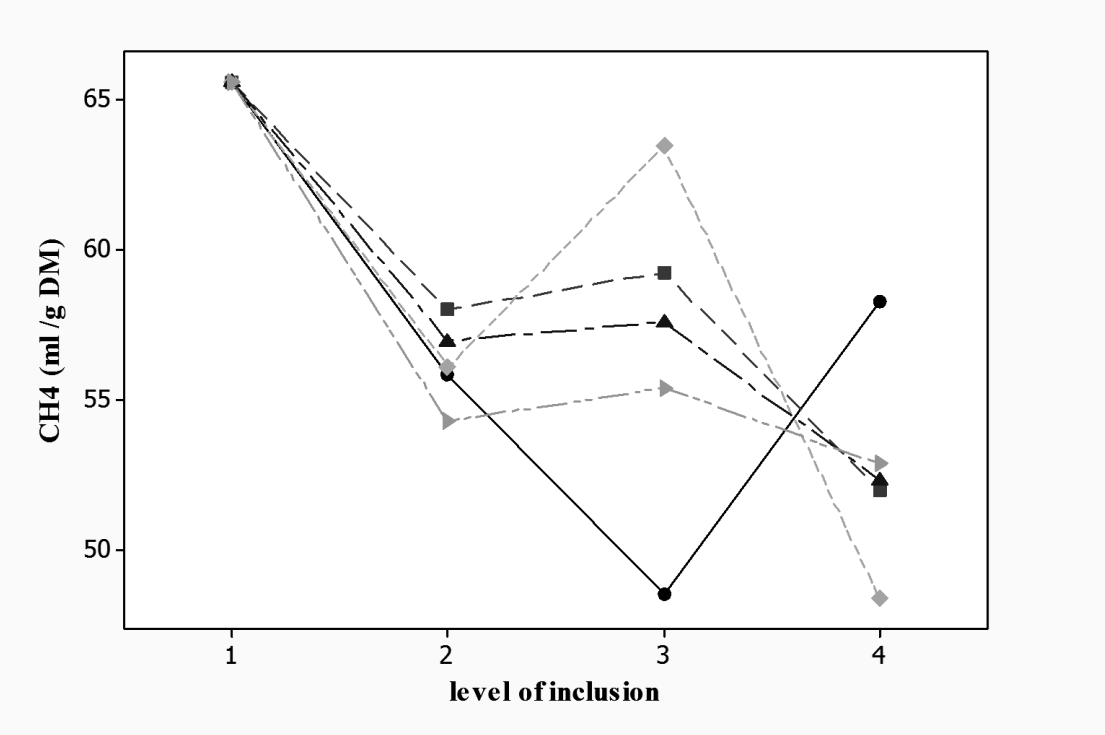
Table 5: Effect of the inclusion levels of the five plant species on digestibility percentages of dry matter, organic matter and NDF after 72 h of incubation (g kg^{-1} DM).

Inclusion level	Plant species						*P* value					
							Lineal			Quadratic		
	Amaranthus spinosus	*Cosmos bipinnatus*	*Commelina coelestis*	*Ebatorium glabratium*	*Galinosa parviflora*	$\overline{X}$ for level	S	L	S×L	S	L	S×L
Digestibility of dry matter												
0%	814	814	814	814	814	814	0.001	0.001	0.1	0.01	0.08	0.1
10%	823	794	793	769	839	804						
20%	812	817	799	794	839	812						
30%	808	788	766	765	819	789						
$\overline{X}$ for species	814	803	793	786	828							
SED							3.9	3.6				
LSD							7.9	7.3				
Digestibility of organic matter												
0%	821	821	821	821	821	820	0.001	0.001	0.001	0.01	0.001	0.04
10%	843	799	797	778	847	813						
20%	824	821	801	808	847	820						
30%	821	799	774	773	833	800						
$\overline{X}$ for species	827	810	798	795	837							
SED							4.9	4.3				
LSD							9.9	8.7				
Digestibility of NDF												
0%	755	755	755	755	755	754	0.001	0.001	0.001	0.01	0.001	0.05
10%	779	728	726	698	774	740						
20%	704	715	719	667	748	710						

30%	685	658	674	579	742	667		
$\bar{X}$ species	730	713	718	674	754			
SED							4.1	3.6
LSD							8.3	7.3

SED is stander error of difference, LSD is least significant difference at P<0.05. S=species, L=level.

Figure 1. Interaction plot for methane production at 24 h of incubation for the five bush tanniferous species at the different inclusion levels.



Key: species: *Amaranthus spinosus* (●) , *Cosmos bipinnatus* (■), *Commelina coelestis* (◇) , *Ebatorium glabratium* (▲) , *Galinosa parviflora* (▷).

Level : 1= 0%, 2=10%, 3=20%, 4= 30% of inclusion.

7.2 Artículo enviado a la revista Animal production science: "**Reduction of methane production from ruminant livestock using tropical tanniferous plants: a sustainable option for mitigation**".

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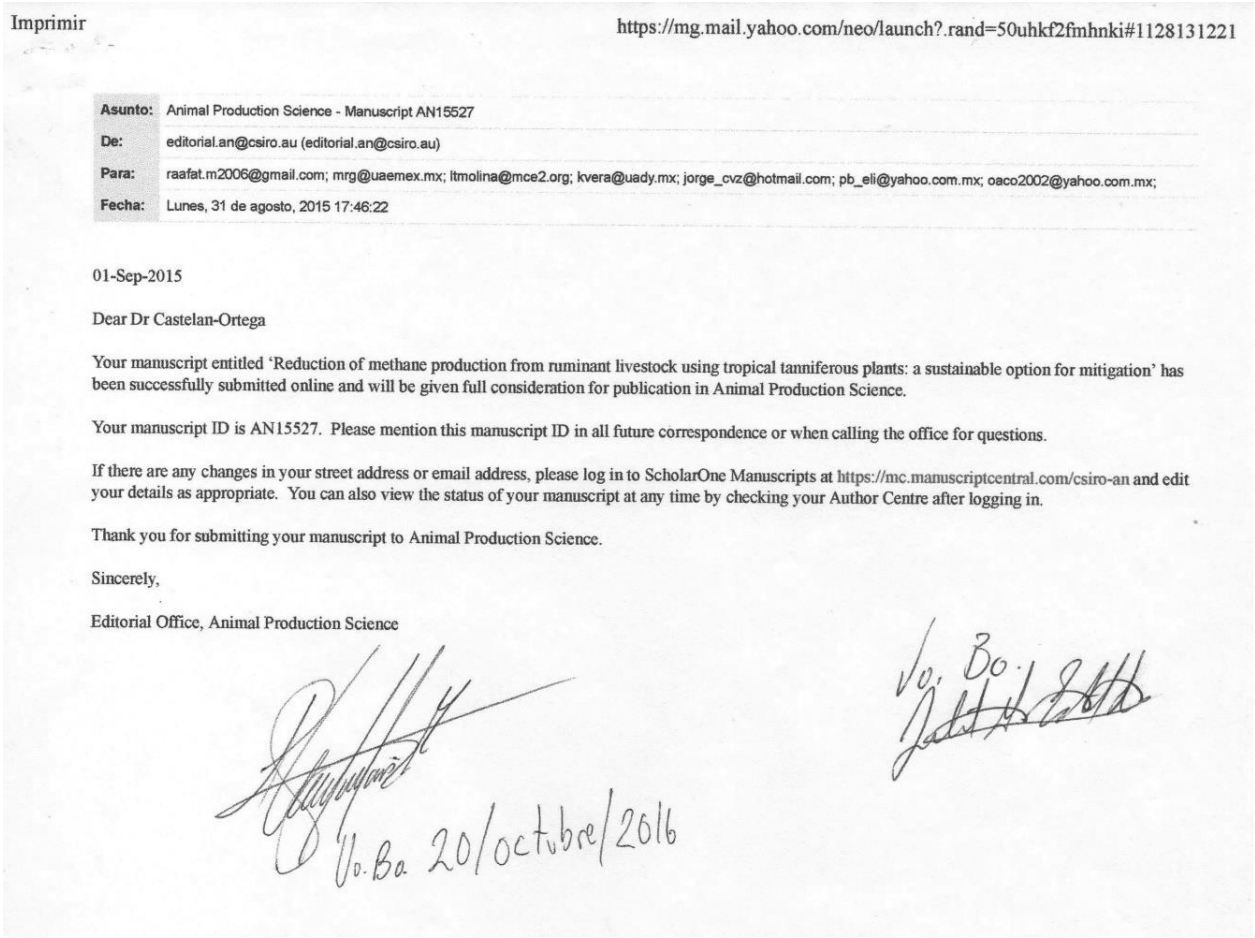

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7.2.3 Manuscrito 3

Reduction of methane production from ruminant livestock using tropical tanniferous plants: a sustainable option for mitigation

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Short title: Reduction of methane production from ruminants

ABSTRACT

Ruminant livestock contributes to the greenhouse effects via the production of large amounts of methane. So, there is a critical need to find sustainable ways to mitigate methane production by ruminants. We evaluated the potential of five plant species, native

to temperate Mexico, to reduce methane production in the rumen. We used in *vitro* gas production to evaluate the effect of the inclusion of these plants at three levels, 10, 20 and 30%, in an experimental diet. Two independent experiments were done to monitor Gas production throughout 24 and 72 h of incubation, methane production was recorded after 24 h. The results showed a significant decrease ($P<0.05$) in methane production with the inclusion of all plants, the highest decline was observed when *T. erecta* (<41.8%) and *S. hirsuta* (<23.3%) when included at 20% level ($P<0.05$) in comparison with the control diet. The NDFD was not affected by *Tagetes erecta*, while *Senna hirsuta* increased it ($P<0.05$). The impact of this inhibition on ruminant whole animal energetic should be evaluated *in vivo*.

Key words: Enteric methane production, Plant Phenolic contents, Ruminant, Tanniferous plants.

Introduction

Green house gas (GHG) emissions are particularly high in the case of ruminant livestock farming because of methane production through enteric fermentation (Pitesky et al., 2009). The issue of GHG emissions in livestock farms has been addressed in a number of studies that focus mainly in dairy cow and cattle farms (Weiske et al., 2006; Olesen et al., 2006). Grazing ruminants have the unique advantage of converting otherwise indigestible cellulose-rich plant material into meat, milk, wool, and leather, whilst not competing directly with humans for food. However, large amounts of methane (CH_4) are generated in the process. Methane is GHG) and present approximately 6% of dietary gross energy losing

as methane. Enteric emissions may be calculated as the product of animal population, the quantity of food consumed per animal and the methane yield (methane/kg DMI) of each animal. Methane yield has been observed to range between 16 and 26 g CH_4/kg DMI in global studies (Munger and Kreuzer 2006).

The sustainable animal diets (StAnD) was developed by FAO (2014), which consider the use of native resources in animal feeding, should not compete with human food and reduce de GHG emissions, and diminish the environmental degradation, grand loss of biodiversity, in the sense the use of native plants is an option in ruminant feeding. The objective of the present study was to evaluate the potentiality of five Mexican regional plants to reduce methane emission using the *in vitro* gas production technique.

Materials and methods

The study was carried out at the Animal nutrition laboratory of the Facultad de Medicina Veterinaria y Zootecnia , of the Universidad Autonoma del Estado de Mexico, and complied with the ethical standards set by the institution.

Sample collection and preparation

The plant species used in this study were *Ipomoea orizabensis, Jaegeria hirta, Senna hirsuta, Tagetes erecta* and *Tagetes filifolia*. Plant samples were collected from different places randomly in the temperate region of the State of Mexico, located at 19° 17′ 32″ N and 99° 39′ 14″ W and at 2663 meters above sea level. For each species, five kilograms of fresh plant matter were collected and cut into small pieces (3-5 cm). Samples were then dried at 50–52°C for 48 hours using a forced air oven in order to prevent enzymatic

degradation of the phenolic compounds present in the plant matter (Makkar *et al*., 1993). Once dry, approximately 400 g of each plant were ground it in a Lab using a Willey Grinders (code MSW-342- IN;10122740) and sieved through a 2-mm screen. The ground material, 100 g were sub sampled, reground and passed through a 0.5-mm screen sieve, used for tannin content analysis, while the rest of the material was used for *in vitro* gas production and chemical composition analysis.

Experimental diets

The control diet was composed (As dry matter, DM) of 64.6% ryegrass (*Lolium perenne*), 20.8% corn grain (*Zea mays*), 8.3% canola meal (*Brassica napus*) and 6.3% corn stover; the diet was formulated using the AFRC (1993) system. In the experimental treatments include it 10, 20 and 30% of ryegrass was replaced by equivalent amounts of the test plants.

Chemical composition of plants

Dry matter (DM) was determined by drying the samples at 135 ºC for 2 h. (AOAC, 1995, ID 930.15). Organic matter (OM) was calculated as weight lost at sample ignition at 600 ºC (AOAC, 1995, ID 942.05). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the ANKOM fiber technology (Robinson *et al*., 1999) without using alpha amylase. Crude protein (CP) was determined by Kjeldahl method (AOAC, 1995, ID 984.13).

In vitro gas production

Gas production was determined following Theodorou *et al*. (1994) technique. Rumen fluid was collected before the morning feeding from three ruminally-fistulated, non-lactating and

non-gravid Holstein cows (LW 550 ± 30 kg), the three ruminal samples were blended in the laboratory to assure an uniform composition of the fluid. All cows had access to fresh water at all times, animals were fed with the same experimental control diet, and grazed on a ryegrass sward from 6:00 until 16:00 h daily.

Plant samples and diets were weighing 0.999 mg and placed in 125 ml serum glass bottles, 10 ml of ruminal liquid and 90 mL of buffer solution was added to each bottle. Once closed, the bottles were gently shaken and placed in a water bath at 39 ºC. Six independent runs were completed. The first three runs were incubated for 24 h in order to simulate normal residence time of feed in the rumen, and were used to measure methane production at 24 h post-incubation as described in Lopez and Newbold (2007). The other three runs were incubated and cumulative gas production (ml/g DM) was recorded at 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, 20, 24, 28, 36, 44, 52, 60 and 72 h post incubation at 39 °C. . A total of 288 bottles were used and all incubations were completed in triplicate within the run.

After 24 and 72 h, the incubation residue was analyzed for apparently digested substrates of dry matter (ADS), organic matter (OMD) and the digestibility of neutral-detergent fiber (NDFD) content using the ANKOM fiber technology technique (Robinson *et al*., 1999).

Methane measurement

Using a gas-tight syringe, 1 ml representative gas samples were collected from each bottle at 24 h post-incubation. After the volume of gas was recorded, and the sample removed for methane analysis, the remaining gas was released and the residues subjected to digestibility

analyses. Methane content was determined by injecting 1 ml gas samples into a Perkin Elmer gas chromatograph (model: Clarus 500 series) equipped with a flame ionized detector (FID). Separation was achieved using an Elite-Q Plot Capillary Column (Perkin Elmer) packed with a 60/80 mesh carboxen^{TM}-1000 stationary phase. Nitrogen was used as the carrier gas with a flow rate of 30 ml/min, an isothermal oven temperature of 50 ºC, and the injector temperature was 250 ºC (Lopez and Newbold 2007). The calibration curve using a regression equation was completed with a methane standard (99.99% from ALTECH).

Extraction of tannins and measurement of total phenolics and tannins content

Phenols content was determined using the Folin-Ciocalteu method (Makkar *et al*.1993). Tannins were measured using poly vinyl poly pyrrolidone (PVPP), which binds tannin-phenolics (Makkar *et al*., 1993).

Calculations

To estimate kinetic parameters of GP results (ml/g DM) were fitted using the NLIN option of SAS (2002) according to the Krishnamoorthy et al. (1990) using the model:

$GPg = b\ (1-e^{-ct})$ (eq.1)

Were GP= Gas production (ml gas/ g DM inicial); b= total gas production (ml gas/ g DM initial); c= degradation rate compared with the time (hours); t= time (h).

Metabolizable energy (ME, MJ/kg DM) were estimated according to AFRC et al. (1993) as:

$$ME = 0.0157*DOMD\ (g/kg\ DM) \qquad (eq.2)$$

where: DOMD is dry matter digestibility after 72 h of incubation.

Gas yields (GY_{24}) was calculated as the volume of gas (ml gas/g DM) produced after 24 h of incubation divided by the amount of ADS (g) as:

$$\text{Gas yields } (GY_{24}) = \text{ml gas/g DM/g ADS} \qquad (eq.3)$$

Short chain fatty acids concentration (SCFA) was calculated according to Getachew et al. (2002) as:

$$SCFA\ (mmol/200\ mg\ DM) = 0.0222\ GP - 0.00425 \qquad (eq.4)$$

where: GP is the 24 h net gas production (ml/200 mg DM).

Microbial biomass production (MP) was calculated according to Blümmel et al. (1997) as:

$$MP\ (mg/g\ DM) = mg\ ADS - (ml\ gas \times 2.2\ mg/ml) \qquad (eq.5)$$

Where: 2.2 mg/ml is a stoichiometric factor which expresses mg of C, H and O required for the SCFA gas associated with production of one ml of gas (Blümmel et al., 1997).

2.6. Statistical analyses

The chemical composition of the plant species and the control diet was subjected in a complete randomized design.

$$Y_{ijk} = \mu + S_i + \varepsilon_{ij}$$

where: Y_{ijk} represents every observation of the i^{th} plant feed species, S_i (i=6) the plant feed species and diet effect and ε_{ij} is the experimental error.

The *in vitro* ruminal GP and fermentation parameters were analyzed as a 5 × 4 factorial experiment (*i.e*., five feed species (random effect) and four doses of plant species (fixed effect) according to a randomized block design using the PROC MIXED procedure of Minitab v. 14 (2013). Data of each of the three runs within the same sample were averaged. Mean values of each individual sample within each species (three samples of each) were used as the experimental unit (Udén et al., 2012), and the statistical model was:

$$Y_{ijk} = \mu + S_i + Z_j + S_i*Z_j + \varepsilon_{ijk}$$

where: Y_{ijk} represents every observation of the i^{th} plant feed species when incubated in the j^{th} plant species levels (forage feed species), S_i (i=1-5) the plant feed species effect, Z_j is the plant levels effect, S_i*Z_j is the interaction between plant feed species and plant levels**,** and ε_{ijk} is the experimental error. Linear and quadratic polynomial contrasts were used to examine responses of feeds to increasing addition levels of the plant species. Tukey's test was used for the multiple comparisons among mean values for the five feeds species.

3. Results

Chemical composition and secondary metabolites of the plants and the control diet (g/kg DM) are show in Table 1. The highest CP content ($P<0.05$) was observed in *S. hirsuta*, followed by *T. erecta* among plant species. It can also be observed that the NDF content of *S. hirsuta* and *T. erecta* are the lowest ($P<0.05$) among plant species. The highest concentration of total phenols and tannins ($P<0.05$) was in T. erecta, and the lowest in T. filifolia. On the contrary the second lowest concentration of condensed tannins was observed in T. erecta and the highest in S. hirsuta among plants.

Increasing levels of the plants ($P<0.05$) increase the total phenols, non tannins phenols, total tannins and condensed tannins in the whole diets (Table 2) . Increasing levels of the plants linearly increased ($P<0.05$) GP parameters (i.e., b, c and L, Table 3 and Figure 1).

Plant species decrease ($P<0.05$) GP at GP6, GP12 ,GP24 , GP44 and GP72, but not significant quadratic effect ($P>0.05$) was showed. Increasing levels of plan species decrease ($P<0.05$) GP at GP6 ,GP12 ,GP24 , GP44 and GP72, and also showed quadratic effect ($P<0.05$). In general, accumulated GP was lower ($P<0.05$) for S. hirsuta, intermediate for T.erecta and J. hirta the highest for I. orizabensis (Table 3).

There were lineal and quadratic effects on final ADS, IVOM, NDFD, CH_4, SCFA and MP in all inclusion levels of plants. However, ADS 24, IVOMD24, NDFD72h, ME, GY_{24} and SCFA were increased ($P<0.05$) with plant increase doses compared to control diet. Most of the ruminal fermentation parameters such as ADS, IVOMD, NDFD, and MP

estimated were highest (P<0.05) for *J. hirta* and *S. hirsuta* intermediate for *T. erecta* and lowest for *I. orizabensis and T. filifolia* (Table 3).The inclusion of all plant species resulted in a reduction (P<0.05) of methane produced, the highest reduction was observed production (30 %) was for *T. erecta* compared with the control diet and *S. hirsuta* when it was included at 20%. The inclusion of these two plants resulted in 41.8%, 35.7% and 27.3% less methane produced respectively, in comparison with the control diet. It should be noted that the other three plants evaluated also significantly reduced (P<0.05) methane production approximately 20% when included at 30% inclusion level.

4. Discussion

In vitro gas production profile

In the present study, there was a significant trend (P<0.001) to a lower methane release per unit of apparently fermented OM with increasing supply of total tannin-rich plants like *T. erecta* and plants with high-condensed tannins concentration like *S. hirsuta*. Surprisingly, this methane production pattern was not associated with negative effects on NDF degradation. This result contrasts with Goel *et al*. (2008) who mentioned that there is a positive correlation between NDF degradation and methane production. Similar results to those obtained in the present study were reported by Jayanegara *et al*. (2009) who used *Rhus typhina* and *Salix alba* as a tannin source, the use of both plants significantly decreased (P<0.05) the amount of rumen methane production by 11.2% and 4.3% when added to hay diet, respectively. They also observed that percentage of methane production increased to 15.8% and 6.1%, respectively, when rice straw was added to the diet. Moreover, supplementation of these tannin-containing forages significantly increased

($P<0.05$) organic matter digestibility of hay and straw diets. Concluding that supplementation of tannin-containing forages could strategically be used to decrease methane emission from rumen fermentation *in vitro*, and at the same time increase the quality of the basal diets.

It is well know that tannins are water-soluble polyphenolic compounds with high molecular weights, which have a potentially wide range of effects on rumen fermentation, such as reducing protein degradation in the rumen, decreasing methane production (Puchala *et al*., 2005; Tavendale *et al*., 2005). Moreover, tannins in moderate amounts might improve body weight gain, wool growth, milk yields and reproductive performance (Patra and Saxena, 2011). However, the role of tannins on reducing methane production is not completely elucidated yet, for example Jayanegara *et al.* (2015) found that all tannins decreased methane concentration either linearly or quadratically, as in the present work, but their magnitudes were different. Jayanegara *et al.* (2015) also reported that the magnitude of decrease was greater for the hydrolysable tannins containing plants than for those plants rich in condensed tannins. This may explains why *T. erecta* was not only the plant species with the highest potential in reducing methane production but at the same time did not affect NDFD after 24 h of incubation, its low content of condensed tannins may be a plausible explication for this behavior.

Several authors are agree (Tavendale *et al*., 2005; Patra and Saxena, 2011,) that tanniferous plants reduce methane production due to their antimicrobial properties, for example Francis *et al*. (2002) found that tannins can inhibit directly methanogenic bacteria growth. On the other hand, tannins have an indirect effect by decreasing protozoa number, so affecting on

the associated methanogen bacteria. Jayanegara *et al*. (2015) found that all the purified hydrolysable and condensed tannins significantly decreased, from 22.3 to 36.7% from control, total methanogens population ($P \leq 0.05$) when added at a dose of 1.0 mg/ml. The previous authors concluded that hydrolysable tannins had a greater effect in reducing methane emission with less adverse effect on digestibility than those of condensed tannins. Our result coincide with Jayanegara *et al*. (2015) because it seems that tanniferous plants like *T. erecta* reduced methanogenic bacteria but not fiber digestion bacteria as NDFD remained constant ($P > 0.05$) even at the highest inclusion level. This response is possibly due to the low condensed tannins content in *T. erecta*. In contrast, Goel *et al*. (2008) reported that the extracts of *Sesbania* and Fenugreek *Trigonella foenum-graecum* had antiprotozoal activity, but no methane reduction was observed for these extracts, suggesting that other non-tannin compounds in the plant may play a role in methane reduction, despite the fact that some secondary plant compounds are toxic to rumen protozoa thereby reducing methane production (Moss *et al*., 2000; Patra *et al*., 2006).

Navarro-González *et al*. (2015) found that *T. erecta* is a flavonoid rich plant, the effect of flavonoid rich plants was studied also by Kim *et al*. (2015) who found that the adding these plants increased gas production and the microbial growth at 24 h of incubation, while a significant decrease of methane emission, without adversely effects of ruminal fermentation *in vitro* in 24 h incubation, this results coincides with our results, were *T. erecta* increase GP and reduce CH_4 production.

Interaction

The significant interaction observed between inclusion level and plant species for methane and gas production (Table 4), suggest that the behaviors of both variables is the result of the combined effect between the plant chemical composition and the inclusion level of it in the diet. This effect is clearly seen in the case of *T. erecta* where, in addition to its tannin content, its reasonable good nutritional quality had a positive effect on the whole diet. This is clearly seen by the increased ADS, OMD, and NDFD at 24 h post incubation (Table 4). Similar results coincides with Jayanegara *et al.* (2015) who reported a similar interaction between different tanniferous plants and inclusion levels with regard to the diminution of methane production ($P \leq 0.05$).

5. Conclusions

The effectiveness of the plant Species differed among the diets but it can be concluded that *Tagetes erecta, Senna hirsuta* and *Jaegeria hirta* consistently reduced methane production without influencing gas production or digestibility. Increasing the level of plant species had a quadratic effect on both gas and methane production, including fermentation rate and asymptotic gas production. Further investigation is required to elucidate the mechanisms responsible for these finding, while *in vivo* research is needed to confirm the anti-methanogenic properties as well as production capabilities of promising alternative plant species.

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Tables and figures

Table 1.Chemical composition[1] of the control diet and five tanniferous native plants in g/kg DM

ID of samples	DM	OM	CP	NDFom	ADFom	Total phenols	Non-tannins phenols	Total tannins	Condensed tannins
Control diet	909[a]	806[a]	155[a]	440[a]	181[a]	34.4[b]	11.2[b]	22.2[a]	1.19[b]
Ipomoea orizabensis	923[b]	829[b]	86[b]	421[b]	286[c]	96.8[a]	21.0[a]	75.8[b]	1.70[a]
Jaegeria hirta	932[c]	828[b]	86[b]	408[b]	253[d]	96.9[a]	24.7[c]	72.2[c]	0.90[b]
Senna hirsuta	922[b]	775[c]	187[c]	305[c]	198[ab]	70.6[c]	14.3[d]	56.4[d]	2.93[c]
Tagetes erecta	898[d]	815[d]	107[d]	343[d]	214[b]	108.9[d]	21.1[a]	87.9[e]	1.13[b]
Tagetes filifolia	929[bc]	806[a]	89[b]	607[e]	400[e]	43.0[e]	10.9[b]	32.1[f]	0.87[b]
SEM	1.5	2.6	6.4	10.0	8.0	6.9	1.3	5.7	0.18
P value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

[a,b,c,d,e,f] Different superscripts following means among plant species inclusion levels within plant species in the column indicate differences at $P<0.05$.

SEM, Standard Error Mean

Table 2. Chemical composition, total phenols, non tannin phenols, total tannins and condensed tannins (g/ kg DM) in rye grass at different inclusion level of five plant species.

Specie	Level (%)	DM	OM	CP	NDFom	ADFom	Total phenols	Non-tannins phenols	Total tannins	Condensed tannins
Ipomoea orizabensis	0	909[a]	806[c]	155[a]	440[a]	181[a]	34.4[a]	11.2[a]	22.2[a]	1.19
	10	918[b]	818[a]	152[b]	415[b]	190[b]	40.9[b]	12.8[b]	28.3[b]	1.29
	20	907[ac]	813[b]	143[c]	409[b]	198[c]	47.7[c]	14.3[c]	33.8[c]	1.20
	30	913[ab]	819[a]	135[d]	402[c]	204[d]	54.5[d]	15.3[d]	39.5[d]	1.32
Jaegeria hirta	0	909[a]	806	155[a]	440[a]	181[a]	34.4[a]	11.2[a]	22.2[a]	1.19
	10	921[ab]	812	151[b]	413[b]	188[b]	40.9[b]	13.5[b]	27.7[b]	1.12
	20	923[b]	809	143[c]	405[c]	192[bc]	47.8[c]	14.9[c]	33.3[c]	1.23
	30	932[b]	812	135[d]	399[d]	195[c]	54.6[d]	17.3[d]	38.5[d]	1.15
Senna hirsuta	0	909[a]	806	155[a]	440[a]	181	34.4[a]	11.2[a]	22.2[a]	1.19
	10	925[b]	815	162[b]	403[b]	182	38.4[b]	12.3[b]	26.3[b]	1.37
	20	918[ab]	808	163[c]	385[c]	180	42.5[c]	12.6[c]	30.5[c]	1.56
	30	928[b]	812	165[d]	367[d]	178	46.7[d]	13.3[d]	33.6[d]	1.74
Tagetes erecta	0	909[a]	806[a]	155[a]	440[a]	181[a]	34.4[a]	11.2[a]	22.2[a]	1.19
	10	905[b]	812[b]	162[b]	406[b]	183[ab]	42.3[b]	12.6[b]	29.4[b]	1.19
	20	903[b]	808[ab]	163[c]	392[c]	187[b]	50.3[c]	13.8[c]	36.5[c]	1.15
	30	906[b]	816[bc]	165[d]	378[d]	182[a]	58.4[d]	15.3[d]	43.4[d]	1.14
Tagetes filifolia	0	909[a]	806[a]	155[a]	440[a]	181[a]	34.4[a]	11.2[a]	22.2[a]	1.19
	10	922[b]	816[b]	152[b]	433[b]	202[b]	35.5[b]	11.7[b]	23.6[b]	1.24
	20	921[b]	814[b]	144[c]	446[c]	219[c]	37.3[c]	11.7[c]	25.3[c]	1.15
	30	920[b]	814[b]	136[d]	458[d]	239[d]	38.5[d]	12.3[d]	26.5[d]	1.14

SEM	1.5	2.6	6.4	10.0	8.0	0.97	0.23	0.83	0.02
P value									
Specie	0.001	0.054	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Level	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.154
S × Level	0.001	0.700	0.001	0.001	0.001	0.001	0.001	0.001	0.018

[1]OM: organic matter, CP: crude protein, ADFom, acid detergent fibre, NDFom, neutral detergent fibre, SEM, Standard Error Mean.

[a,b,c,d] Different superscripts following means among plant species inclusion levels within plant species in the column indicate differences at $P<0.05$.

Table 3. *In vitro* gas production parameters and cumulative gas volume after 72 h of incubation of five tanniferous plants feeds species and four doses of plant species inclusion (mg/g DM) in cow´s diet.

Plant species (S)	Level of inclusion	Gas production parameters[1]			*In vitro* gas production (ml/g DM)				
		b	*c*	*L*	GP_6	GP_{12}	GP_{24}	GP_{44}	GP_{72}
Ipomoea orizabensis	0	272.0^{a}	0.0459^{a}	0.75^{a}	56.1^{a}	114.7^{a}	178.0^{a}	230.3^{a}	265.1^{a}
	10	254.3^{b}	0.0504^{b}	2.26^{b}	38.9^{b}	100.4^{b}	165.9^{b}	222.5^{ab}	245.9^{b}
	20	268.4^{a}	0.0513^{b}	2.02^{c}	43.6^{c}	110.4^{ac}	177.7^{a}	236.3^{ac}	260.1^{a}
	30	263.1^{ab}	0.0512^{b}	2.44^{b}	38.3^{b}	104.1^{bc}	172.6^{ab}	230.6^{a}	255.1^{a}
SEM specie		2.4	0.0007	0.2	2.2	1.8	1.8	1.9	2.5
Pvalue									
Level (lineal)		0.014	0.001	0.001	0.001	0.001	0.018	0.048	0.008
Level (quadratic)		0.224	0.001	0.016	0.040	0.265	0.372	0.814	0.174
Jaegeria hirta	0	272.0^{a}	0.0459^{a}	0.75^{a}	56.1^{a}	114.7^{a}	178.0^{a}	230.3^{a}	265.1^{a}
	10	234.4^{b}	0.0466^{a}	2.85^{b}	30.1^{b}	82.5^{b}	149.6^{b}	198.8^{b}	225.2^{b}
	20	261.1^{a}	0.05420^{b}	2.13^{bc}	44.8^{a}	114.3^{a}	182.3^{a}	232.3^{a}	257.1^{a}
	30	254.0^{a}	0.0537^{b}	1.86^{c}	46.7^{a}	109.6^{a}	179.5^{a}	225.9^{a}	248.6^{a}
SEM species		4.6	0.001	0.2	3.1	4.6	4.6	4.6	5.0
Pvalue									

Level (lineal)		0.003	0.012	0.001	0.003	0.006	0.008	0.006	0.003
Level (quadratic)		0.110	0.772	0.003	0.021	0.165	0.180	0.206	0.139
Senna hirsuta	0	272.0^{a}	0.0459^{a}	0.75^{a}	56.1^{a}	114.7^{a}	178.0^{a}	230.3^{a}	265.1^{a}
	10	256.9^{a}	0.0573^{b}	1.3^{a}	59.9^{a}	123.0^{a}	187.7^{a}	232.9^{a}	255.4^{a}
	20	221.9^{b}	0.0466^{a}	2.8^{b}	26.8^{b}	77.4^{b}	141.3^{b}	188.1^{b}	213.6^{b}
	30	224.3^{b}	0.0550^{b}	2.8^{b}	31.2^{b}	90.8^{b}	157.3^{b}	199.2^{b}	220.6^{b}
SEM specie		6.8	0.002	0.3	4.7	5.7	5.7	6.2	7.0
Pvalue									
Level (lineal)		0.001	0.001	0.003	0.001	0.001	0.001	0.001	0.001
Level (quadratic)		0.214	0.648	0.406	0.961	0.784	0.750	0.645	0.302
Tagetes erecta	0	272.0^{a}	0.0459^{a}	0.75^{a}	56.1^{a}	114.7^{a}	178.0^{a}	230.3	265.1^{a}
	10	235.9^{b}	0.0500^{a}	3.4^{b}	27.3^{b}	83.4^{ab}	154.5^{a}	202.8	229.8^{b}
	20	239.4^{b}	0.0442^{ac}	3.8^{b}	19.9^{c}	72.8^{b}	144.3^{ab}	196.9	229.5^{b}
	30	257.9^{ab}	0.0551^{ab}	1.8^{a}	50.9^{a}	117.1^{a}	184.0^{ac}	230.3	254.9^{ab}
SEM specie		5.2	0.002	0.4	5.3	6.8	6.0	5.7	5.6
Pvalue									
Level (lineal)		0.012	0.034	0.001	0.009	0.012	0.022	0.028	0.015
Level (quadratic)		0.002	0.271	0.001	0.001	0.001	0.003	0.003	0.001

Tagetes filifolia	0	272.0[a]	0.0459[a]	0.75[a]	56.1[a]	114.7[a]	178.0	230.3[a]	265.1[a]
	10	258.4[ab]	0.0478[ab]	1.12[b]	51.6[ab]	106.2[ab]	172.9	221.8[a]	251.8[ab]
	20	254.6[b]	0.0495[bc]	1.23[b]	51.7[ab]	106.0[ab]	172.5	221.0[a]	249.7[ab]
	30	251.1[b]	0.0460[ab]	1.4[b]	44.9[b]	96.6[b]	165.0	212.5[b]	243.7[b]
SEM specie		2.9	0.0005	0.1	1.5	2.3	1.9	2.4	2.9
P value									
Level (lineal)		0.018	0.032	0.003	0.020	0.014	0.10	0.039	0.029
Level (quadratic)		0.189	0.010	0.319	0.572	0.879	0.693	0.990	0.374
SEM (overall)		4.4	0.001	0.2	3.4	4.2	4.0	4.2	4.6
P value									
Species (lineal)		0.001	0.004	0.001	0.001	0.030	0.060	0.001	0.001
Species (quadratic)		0.346	0.598	0.001	0.022	0.076	0.193	0.178	0.323
Level (overall- lineal)		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Level (overall- quadratic)		0.001	0.450	0.001	0.001	0.001	0.003	0.004	0.001
Specie * level (lineal)		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Species * level (quadratic)		0.083	0.296	0.001	0.001	0.002	0.017	0.021	0.063

[1] *b* is the asymptotic gas production (ml/g DM); *c* is the rate of gas production (/h); *L* is the initial delay before gas production begins (h), SEM, Standard Error Mean.

[a,b,c,d] Different superscripts following means among plant species inclusion levels within plant species in the column indicate differences at $P<0.05$.

Table 4. *In vitro* rumen fermentation profile[1] of five tanniferous plants feeds species and four levels of plant species inclusion levels (mg/g DM) in cow´s diet

Plant species	Level of inclusion	ADS 24h	ADS 72h	IVOMD 24h	IVOMD 72h	NDFD 24h	NDFD 72h	CH_4 24h	ME	GY_{24}	SCFA	MP
Ipomoea orizabensis	0	739.3^{a}	813.6	742.4^{a}	820.5	657.1^{a}	754.7^{a}	65.6^{a}	11.7^{a}	178.0^{a}	0.79^{a}	156.2^{a}
	10	683.0^{b}	826.0	688.8^{b}	847.2	694.1^{b}	727.1^{b}	52.3^{b}	10.8^{b}	165.9^{b}	0.73^{b}	142.1^{a}
	20	684.7^{b}	811.5	690.4^{b}	820.2	674.1^{a}	729.2^{b}	56.2^{c}	10.8^{b}	177.7^{a}	0.78^{a}	112.39^{b}
	30	710.6^{c}	808.5	719.0^{c}	818.8	680.1^{b}	684.6^{c}	53.3^{bc}	11.3^{c}	172.6^{ab}	0.76^{ab}	149.4^{a}
SEM species		7.0	2.8	6.8	4.7	4.5	7.9	1.6	0.1	1.8	0.008	5.9
P value												
Level (lineal)		0.001	0.097	0.001	0.063	0.005	0.001	0.001	0.001	0.018	0.018	0.013
Level (quadratic)		0.001	0.166	0.001	0.152	0.077	0.286	0.024	0.001	0.372	0.372	0.022
Jaegeria hirta	0	739.3^{a}	813.6^{a}	742.4^{a}	820.5^{a}	657.1^{a}	754.7^{a}	65.6^{a}	11.7^{a}	178.0^{a}	0.79^{a}	156.2
	10	697.8^{b}	836.7^{b}	715.4^{b}	848.7^{b}	722.4^{b}	766.7^{a}	49.3^{b}	11.2^{b}	149.6^{b}	0.66^{b}	202.3
	20	750.3^{c}	841.1^{b}	757.7^{c}	856.8^{b}	728.8^{b}	747.5^{a}	63.0^{a}	11.9^{c}	182.3^{a}	0.81^{a}	184.7
	30	742.1^{a}	805.3^{a}	750.0^{ac}	819.6^{a}	658.0^{a}	703.3^{b}	63.8^{a}	11.8^{ac}	179.5^{a}	0.79^{a}	195.1

SEM species		6.2	4.9	5.0	5.5	10.9	7.5	2.1	0.08	4.6	0.02	7.0
P value												
Level (lineal)		0.001	0.001	0.001	0.002	0.001	0.001	0.001	0.001	0.008	0.008	0.064
Level (quadratic)		0.186	0.001	0.334	0.001	0.001	0.001	0.043	0.334	0.180	0.180	0.174
Senna hirsuta	0	739.3^{a}	813.6^{a}	742.4^{a}	820.5^{a}	657.1^{a}	754.7^{a}	65.6^{a}	11.7^{a}	178.0^{a}	0.79^{a}	156.2^{a}
	10	741.2^{a}	838.9^{b}	748.5^{a}	847.5^{b}	738.7^{b}	748.4^{a}	64.6^{a}	11.8^{a}	187.7^{a}	0.83^{a}	179.4^{a}
	20	733.5^{a}	805.2^{a}	743.4^{a}	825.9^{ab}	697.9^{c}	749.3^{a}	47.8^{b}	11.7^{a}	141.3^{b}	0.62^{b}	263.6^{b}
	30	723.5^{b}	843.3^{b}	730.7^{b}	848.1^{b}	703.7^{c}	702.9^{b}	53.8^{c}	11.5^{b}	157.3^{b}	0.69^{b}	238.2^{b}
SEM species		2.3	5.2	2.1	4.4	9.4	6.7	2.3	0.03	5.7	0.03	14.0
P value												
Level (lineal)		0.002	0.001	0.001	0.011	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Level (quadratic)		0.020	0.556	0.001	0.784	0.029	0.015	0.261	0.001	0.750	0.750	0.188
Tagetes erecta	0	739.3^{a}	813.6^{a}	742.4^{a}	820.5^{a}	657.1^{a}	754.7^{a}	65.6^{a}	11.7^{a}	178.0^{a}	0.79^{a}	156.2

	10	667.8^{b}	850.4^{b}	675.2^{b}	867.3^{b}	706.1^{b}	775.58^{a}	42.2^{b}	10.6^{b}	154.5^{a}	0.68^{a}	162.2
	20	714.8^{c}	841.7^{b}	725.1^{c}	857.5^{b}	657.1^{a}	751.6^{ab}	38.3^{b}	11.4^{c}	144.3^{ac}	0.64^{ab}	209.9
	30	774.9^{d}	832.9^{ab}	798.2^{d}	842.9^{ab}	658.3^{a}	725.1^{b}	58.1^{c}	12.5^{d}	184.0^{ab}	0.81^{ac}	214.1
SEM species		11.8	4.7	13.2	5.8	7.1	6.1	3.4	0.2	6.0	0.03	10.1
P value												
Level (lineal)		0.001	0.007	0.001	0.001	0.004	0.004	0.001	0.001	0.022	0.022	0.041
Level (quadratic)		0.001	0.005	0.001	0.001	0.104	0.007	0.001	0.001	0.003	0.003	0.953
Tagetes filifolia	0	739.3^{a}	813.6^{a}	742.4^{a}	820.5^{a}	657.1^{a}	754.7^{a}	65.6^{a}	11.7^{a}	178.0	0.79	156.2^{a}
	10	674.6^{b}	775.6^{b}	684.1^{b}	786.2^{b}	649.8^{a}	675.7^{b}	57.5^{b}	10.7^{b}	172.9	0.76	120.6^{ac}
	20	687.6^{c}	773.0^{b}	697.5^{b}	775.0^{b}	561.4^{b}	658.5^{b}	57.9^{b}	11.0^{b}	172.5	0.76	138.4^{ac}
	30	721.0^{d}	726.8^{cb}	729.6^{a}	728.1^{c}	594.5^{b}	569.8^{c}	54.0^{b}	11.5^{a}	165.0	0.72	184.8^{ab}
SEM species		7.8	9.5	7.2	10.2	12.5	20.4	1.4	0.1	1.9	0.008	8.2
P value												
Level (lineal)		0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.100	0.100	0.007

Level (quadratic)	0.001	0.572	0.001	0.362	0.265	0.746	0.153	0.001	0.693	0.693	0.001
SEM (overall)	7.0	5.4	6.9	6.1	8.9	9.7	2.2	0.1	4.0	0.02	9.0
P value											
Species (lineal)	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.060	0.060	0.001
Species (quadratic)	0.001	0.024	0.001	0.024	0.002	0.133	0.001	0.001	0.193	0.193	0.011
Level (overall-lineal)	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Level (overall-quadratic)	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.003	0.003	0.419
Specie * level (lineal)	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Species * level (quadratic)	0.001	0.004	0.001	0.012	0.001	0.278	0.001	0.001	0.017	0.17	0.003

[1] ADS is Apparently degraded substrate (mg/g DM); IVOMD is *in vitro* organic matter digestibility (g/kg MS); CH_4, Methane production (ml CH_4/ g DM); ME is metabolizable energy (MJ/kg DM); GY_{24} is gas yield at 24h (ml gas/g ADS); SCFA is short chain fatty acids (mmol/g DM); MP is microbial protein production (mg/g DM), SEM, Standard Error Mean.

[a,b,c,d] Different superscripts following means among plant species inclusion levels within plant species in the column indicate differences at $P<0.05$

Figure 1 : Cumulative gas production profiles (ml gas/g DM)

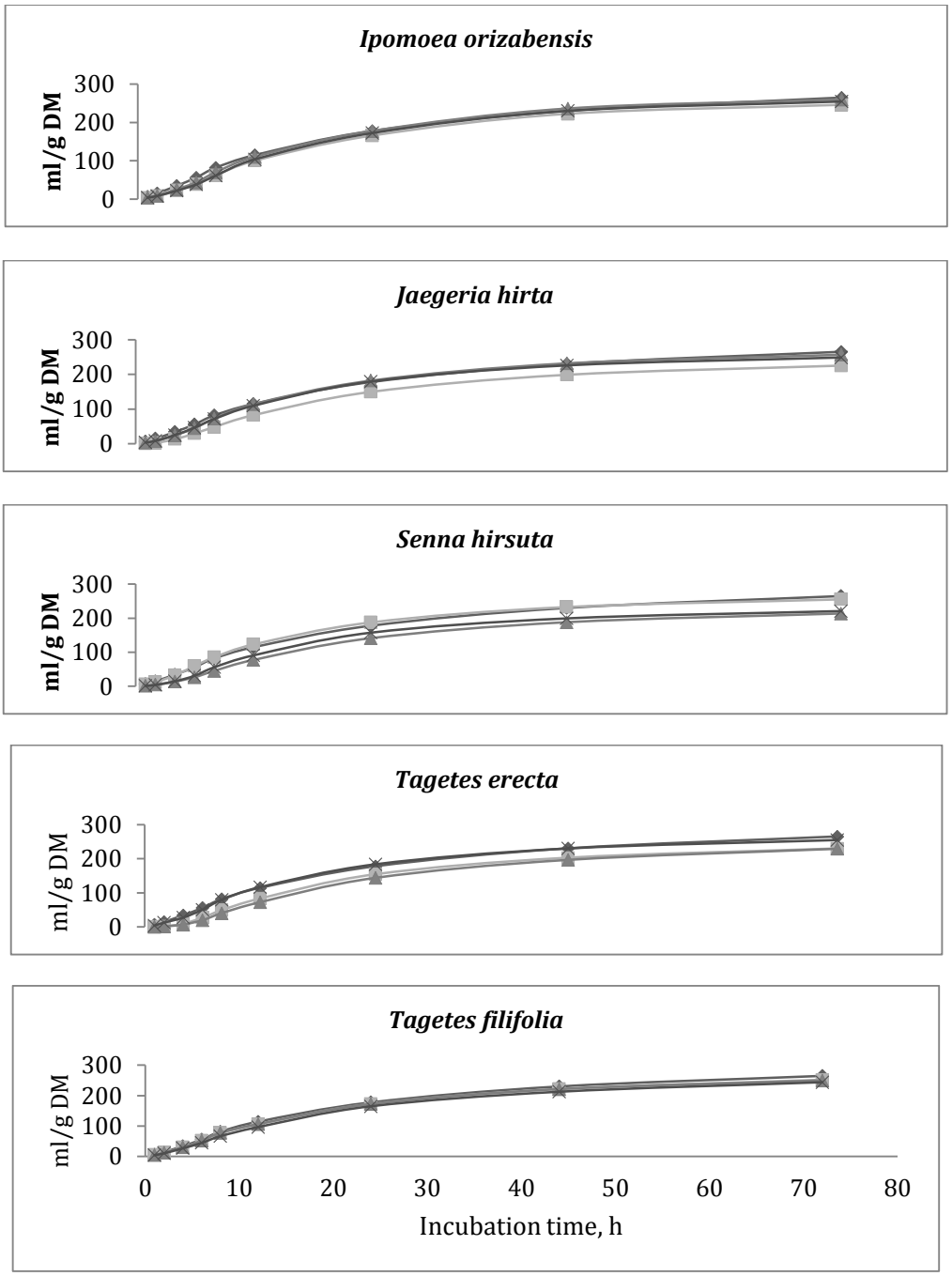


Fig. 1. Cumulative gas production profiles (ml gas/g DM) from *in vitro* fermentation of five plant species at four levels of inclusion in cows diets. (-♦-, 0% ; -■-, 10% ; -▲-, 20% ; -×-, 30% of plant species; SEM is for the overall fit).

VIII. DISCUSIÓN GENERAL

Efecto del contenido de taninos en la producción de metano

En el presente estudio, se observó una tendencia significativa a una menor liberación de metano por unidad de materia orgánica fermentada al parecer con el aumento de suministro de plantas ricos en taninos totales como *T. erecta* y plantas con la concentración de taninos de alto condensó como S. hirsuta. Sorprendentemente, este patrón de producción de metano no se asoció con efectos negativos sobre la degradación de la FDN. Este resultado contrasta con Goel *et al*. (2008) quien mencionó que existe una correlación positiva entre la degradación de la FND y la producción de metano. Resultados similares a los obtenidos en el presente trabajo fueron reportados por Jayanegara *et al*. (2009), que utiliza *Rhus typhina* y *Salix alba* como fuente de taninos, el uso de ambas plantas se redujo significativamente ($P < 0.05$) la cantidad de la producción de metano ruminal en un 11.2% y un 4.3% cuando se añade a la dieta de heno, respectivamente. También observaron que el porcentaje de la producción de metano se incrementó a 15.8% y 6.1%, respectivamente, cuando se añadió la paja de arroz a la dieta. Por otra parte, la suplementación de estos forrajes que contienen taninos aumentó significativamente ($P < 0.05$) digestibilidad de la materia orgánica del heno y paja dietas. Los antiguos autores concluyeron que la suplementación de los forrajes que contienen taninos estratégicamente podría utilizarse para disminuir la emisión de metano de la fermentación del rumen *in vitro*, y al mismo tiempo aumentar la calidad de las dietas basales.

Es bien conocido que los taninos son compuestos polifenólicos solubles en agua con pesos moleculares elevados, que tienen una potencialmente amplia gama de efectos sobre la fermentación del rumen, tales como la reducción de la degradación de proteínas en el rumen, la disminución de la producción de metano (Puchala *et al*, 2005; Tavendale *et al*., 2005). Por otra parte, los taninos en cantidades moderadas pueden mejorar la ganancia de peso corporal, crecimiento de la lana, la producción de leche y el rendimiento reproductivo (Patra y Saxena, 2011). Sin embargo, el papel de taninos en la reducción de la producción de metano no se aclara completamente todavía, por ejemplo Jayanegara *et al*. (2015) encontraron que los taninos se redujo la concentración de metano, ya sea de forma lineal o cuadrática, como en el presente trabajo, pero sus magnitudes eran diferentes. Jayanegara *et al*. (2015) también informó que la magnitud de la disminución fue mayor para los taninos hidrolizables que contienen las plantas que para esas plantas ricas en taninos condensados. Esto explica por qué puede *T. erecta* era no sólo las especies de plantas con el mayor potencial en la reducción de la producción de metano, pero al mismo tiempo no afectó dNDF después de 24 h de incubación, su bajo contenido de taninos condensados puede ser una explicación plausible para este comportamiento .

Varios autores coinciden (Tavendale *et al*, 2005; Patra y Saxena, 2011) que las plantas tanniferous reducir la producción de metano debido a sus propiedades antimicrobianas, por ejemplo Francis *et al*. (2002) encontraron que los taninos pueden inhibir el crecimiento de bacterias metanogénicas directamente. Por otro lado, taninos tienen un efecto indirecto por la disminución de número de protozoos, por lo que afecta a las bacterias metanógenas asociados. Jayanegara *et al*. (2015) encontraron que todos los taninos hidrolizables y condensados purificados disminuyeron significativamente, de 22.3 a la 36.7% del control, la población total de los metanógenos ($P > 0.05$) cuando se añade a una dosis de 1.0 mg / ml. Los autores anteriores la conclusión de que los taninos hidrolizables tenían un mayor efecto en la reducción de las emisiones de metano con menos efectos adversos sobre la digestibilidad que las de los taninos condensados. Nuestro resultado están en línea con Jayanegara *et al*. estudio (2015), ya que parece que las plantas tanniferous como *T. erecta* reducen las bacterias metanogénicas, pero no las bacterias digestión de la fibra como dNDF se mantuvo constante ($P> 0.05$), incluso en el nivel más alto de inclusión. Esta respuesta es posiblemente debido al bajo contenido en taninos condensados *T. erecta*. Por el contrario, Goel *et al*. (2008) informaron de que los extractos de Sesbania y alholva tenían actividad antiprotozoaria, pero no se observó reducción de metano para estos extractos, lo que sugiere que otros compuestos no tanino en la planta pueden jugar un papel en la reducción de metano, a pesar del hecho de que algunos planta secundaria compuestos son tóxicos para rumen protozoos lo que se reduce la producción de metano (Moss *et al*., 2000) y Patra *et al*. (2006). Por lo tanto, se necesita más investigación con el fin de aclarar el papel de las plantas tanniferous, las fracciones de taninos y sus diversos metabolitos secundarios en la producción de metano a la vista de los resultados contrastantes.

En el presente estudio, hemos encontrado niveles significativamente más bajos de metano liberado (por unidad de materia orgánica fermentada aparentemente) con el aumento de los niveles de la mayoría de las plantas ricas en tanino añadido a la dieta experimental. Esta tendencia no se asoció con efectos negativos sobre la degradación de la fibra, de hecho, algunos casos como *A. spinosus*, *C. bipnnatus* la dNDF aumentaron. Los taninos se observaron con frecuencia para reducir la degradación de los hidratos de carbono estructural al reducir el número de microbios celulolíticas en fluido ruminal (Singleton, 1981), inhibición de la enzima celulasa (Leinmüller *et al* 1991;. Makkar, 1993a), la prevención de la adhesión de microbios sobre partículas de alimentos (Leinmüller y Menke, 1990) y / o la supresión de la degradación por la formación de complejos con celulosa (McSweeney *et al*. 2001). Sin embargo, no se da información por estos autores en cuanto a si existe una diferencia entre los efectos de taninos hidrolizables y condensados, pero Leinmüller *et al*. (1991) consideró taninos hidrolizables como menos adverso a este respecto.

La producción de gas

La producción de gas es generalmente reconocida como un método más sensible para la detección de diferencias en la fermentación del sustrato utilizando sistemas in vitro que la desaparición de DM (Xu *et al*., 2010). Nuestros resultados muestran que GP se vio afectada negativamente por algunos niveles de inclusión de las plantas tanniferous en la dieta experimental. Este efecto podría atribuirse a las propiedades antimicrobianas de taninos y compuestos fenólicos en las plantas experimentales, que a su vez causan una reducción en la producción de gas (Francis *et al*., 2002). Alternativamente, el estudio de Patra *et al*. (2010) demostraron que la producción de gas aumentó con extractos de plantas que contienen fenoles o saponinas y que esto probablemente fue causado por el aumento de azúcares solubles en la dieta (Santoso *et al*., 2013). Es posible plantear la hipótesis de que esto puede haber ocurrido en nuestro trabajo para el caso de *A. spinosus*, que es una especie de baja en taninos y con alto contenido de azúcares solubles y CP. Esta combinación probablemente resultó en un efecto modulador, en donde por un lado el medio ambiente rumen se mejoró por la proteína adicional, y por otro lado, *A. spinosus* mantiene baja producción de metano por los efectos de taninos sobre las bacterias metanogénicas. Esta hipótesis podría ser sostenida por el hecho de que *A. spinosus* no afectó la digestibilidad a las 24 h de incubación y el aumento de la digestibilidad a las 72 h, mientras que la producción de gas se mantuvo bastante constante en todos los niveles, pero el nivel de 20%. Por otra parte, una correlación positiva (r = 0.54) entre la producción de gas y el contenido de CP en hojas que contienen taninos ha sido reportado por Kamalak *et al*. (2004). Por lo tanto, parece que las plantas tanniferous como *A. spinosus* podrían reducir las bacterias metanogénicas pero no bacterias digestión de la fibra lo que sugiere que menos energía se desvía a metano formación. El efecto moderado de *A. spinosus* en el GP puede ser relevante debido a que la cantidad de gas producido está muy relacionada con la digestibilidad y por lo tanto al valor de alimentación energética de los piensos para rumiantes (Menke y Staingass, 1988). Nuestros resultados también están en línea con Jayanegara *et al*. (2015) que evaluó el efecto de diferentes fracciones de tanino en la producción de metano y concluyó que estas fracciones pueden actuar diferencialmente.

La producción de metano

Los resultados del presente estudio muestran que el metano se redujo constantemente por todas las cinco plantas tanniferous, y que la mayor reducción se observó por la inclusión de *C. coelestis* y *A. spinosus* en 30 y 20% de nivel de inclusión respectivamente. El mismo efecto de plantas ricos en taninos fue observado por Rodríguez *et al*. (2011) informó que la producción de metano reducida con la adición de extracto vegetal que contiene taninos. Por

el contrario, Beauchemin *et al*. (2007) describieron que la suplementación de una dieta basada en forraje con extracto de quebracho tanino no logró reducir la producción de metano en el ganado, lo que sugiere que se necesita más investigación para aclarar el efecto de los taninos en los extractos de las plantas y en toda la planta como en el presente trabajo.

Esto es importante porque en el presente estudio no se encontró la mayor reducción en la producción de metano para coincidir con el más alto contenido de fenol o tanino en las plantas de aditivos. Goel *et al*. (2008) informaron de que los extractos de Sesbania y alholva tenían actividad antiprotozoaria, pero no se observó reducción de metano para estos extractos, lo que sugiere que otros compuestos no tanino en la planta pueden jugar un papel en la reducción de metano, a pesar del hecho de que algunos planta secundaria compuestos son tóxicos para rumen protozoos lo que se reduce la producción de metano (Patra *et al*., 2006). Que es posible sugerir que *A. spinosus* y *C. coelestis* pueden haber tenido un efecto importante en la producción de metano porque tenían más fenoles no tanino que las otras especies, que pueden ser más activo en la reducción de la producción de metano.

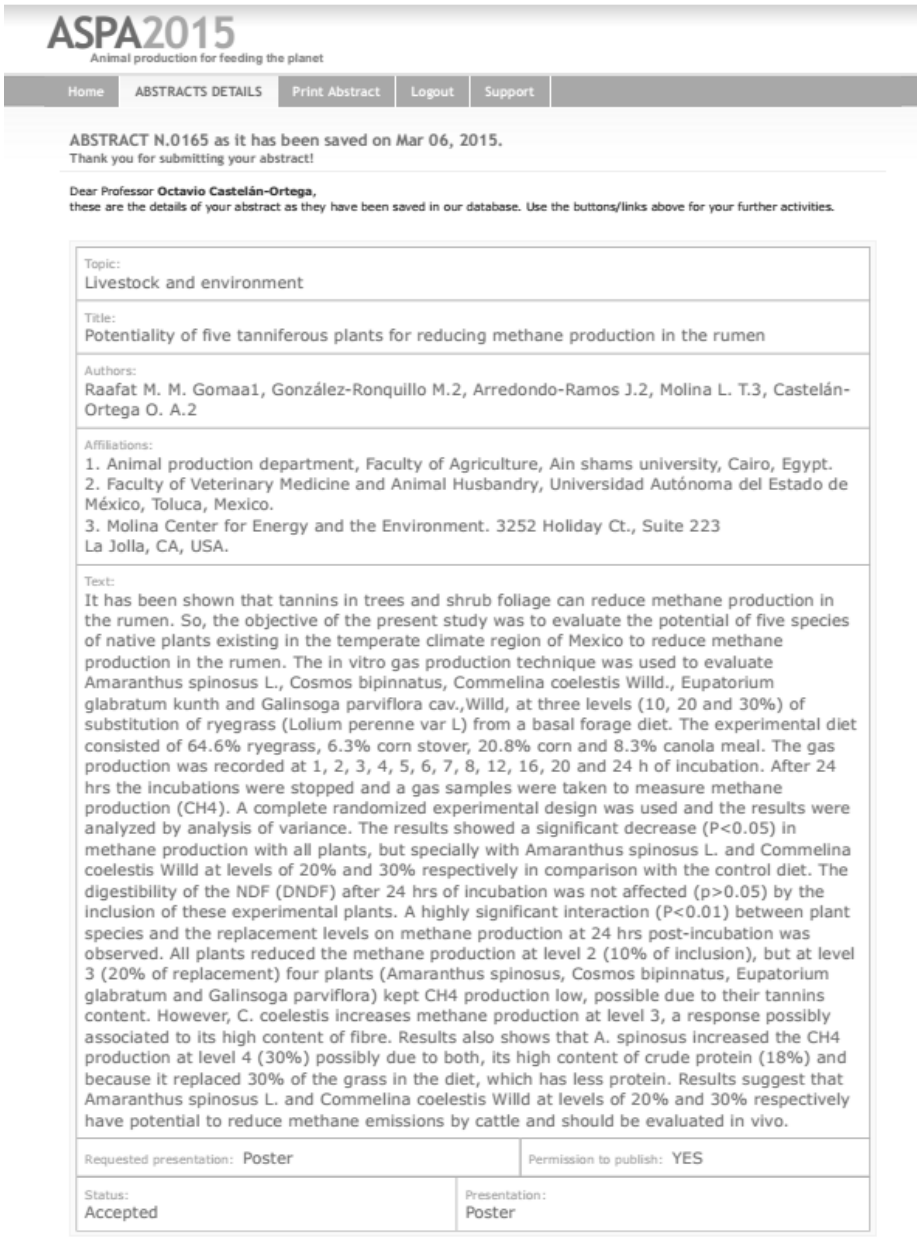
La interacción significativa entre las especies a nivel de inclusión y de plantas para la producción de metano y gas, indican que el comportamiento de ambas variables son el resultado del efecto combinado entre la planta y el nivel de inclusión de la misma en la dieta. Se concluye que *Amaranthus spinosus* y *coelestis Commelina* reducen consistentemente la producción de metano sin influir en la producción de gas in vitro o digestibilidad. El impacto de esta inhibición sobre todo rumiantes animales energética debe ser evaluado in vivo con el fin de utilizar estas plantas en las estrategias de mitigación de metano sostenibles siempre que muestren mismos efectos que en vitro.

IX. CONCLUSIONES GENERALES

Se concluye que las plantas *Amaranthus spinosus, Commelina coelestis, Tagetes erecta, Senna hirsuta* y *Jaegeria hirta* redujeron constantemente la producción de metano sin influir en la producción o digestibilidad del gas medidas después de una fermentación in vitro de 24 h y 72 h. El aumento del nivel de especies de plantas tuvo un efecto cuadrático en la producción de gas y metano, incluyendo la tasa de fermentación y la producción de gas. Se requiere una investigación adicional para dilucidar los mecanismos responsables de estos hallazgos, mientras que la investigación *in vivo* es necesaria para confirmar las propiedades antimetanógenas, así como las capacidades de producción de las especies vegetales alternativas.

X. ANEXOS

11.1 Trabajo presentado en formato de cartel en el "Animal Science and Production Association Conference 2015 : "Animal production for feeding the planet 9 - 12 June 2015. Milano, Italy



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La Jolla, CA, USA.

Text:
It has been shown that tannins in trees and shrub foliage can reduce methane production in the rumen. So, the objective of the present study was to evaluate the potential of five species of native plants existing in the temperate climate region of Mexico to reduce methane production in the rumen. The in vitro gas production technique was used to evaluate Amaranthus spinosus L., Cosmos bipinnatus, Commelina coelestis Willd., Eupatorium glabratum kunth and Galinsoga parviflora cav.,Willd, at three levels (10, 20 and 30%) of substitution of ryegrass (Lolium perenne var L) from a basal forage diet. The experimental diet consisted of 64.6% ryegrass, 6.3% corn stover, 20.8% corn and 8.3% canola meal. The gas production was recorded at 1, 2, 3, 4, 5, 6, 7, 8, 12, 16, 20 and 24 h of incubation. After 24 hrs the incubations were stopped and a gas samples were taken to measure methane production (CH4). A complete randomized experimental design was used and the results were analyzed by analysis of variance. The results showed a significant decrease ($P<0.05$) in methane production with all plants, but specially with Amaranthus spinosus L. and Commelina coelestis Willd at levels of 20% and 30% respectively in comparison with the control diet. The digestibility of the NDF (DNDF) after 24 hrs of incubation was not affected ($p>0.05$) by the inclusion of these experimental plants. A highly significant interaction ($P<0.01$) between plant species and the replacement levels on methane production at 24 hrs post-incubation was observed. All plants reduced the methane production at level 2 (10% of inclusion), but at level 3 (20% of replacement) four plants (Amaranthus spinosus, Cosmos bipinnatus, Eupatorium glabratum and Galinsoga parviflora) kept CH4 production low, possible due to their tannins content. However, C. coelestis increases methane production at level 3, a response possibly associated to its high content of fibre. Results also shows that A. spinosus increased the CH4 production at level 4 (30%) possibly due to both, its high content of crude protein (18%) and because it replaced 30% of the grass in the diet, which has less protein. Results suggest that Amaranthus spinosus L. and Commelina coelestis Willd at levels of 20% and 30% respectively have potential to reduce methane emissions by cattle and should be evaluated in vivo.

Requested presentation: Poster | Permission to publish: YES

Status: Accepted | Presentation: Poster

11.2 Resumen Aceptado en la reunión de GGAA 2016 in Australia: "Reduction of methane production from ruminant livestock using tropical tanniferous plants: a sustainable option for mitigation"

GGAA2016

Melbourne Australia

Wednesday, 30 September 2015

Dr OCTAVIO CASTELAN-ORTEGA
UNIVERSIDAD AUTONOMA DEL ESTADO DE MEXICO, FACULTY OF VETERINARY MEDICINE
Instituto Literario No.100, Colonia Centro
TOLUCA ESTADO DE MEXICO 50000
MEXICO

Reference Number: 735

Dear Dr CASTELAN-ORTEGA,

Thank you for your abstract submission for presentation at the 6th Greenhouse Gas and Animal Agriculture Conference (GGAA2016), from 14 to 18 February 2016, to be held in Melbourne, Australia.
We were delighted to receive a large number of abstract submissions for the conference. Following an examination of the feedback provided by the conference reviewers, the committee has decided that your submission has been accepted as a **Poster** presentation and have provided feedback below (under 'Review Feedback') for your consideration.

Abstract Details	
Title:	Reduction of methane production from ruminant livestock using tropical tanniferous plants: a sustainable option for mitigation
Reviewer Feedback:	The written English in this paper needs to be revised (eg raygrass for ryegrass).It is of merit but not definitive in its scope. For example, the plants contain tannins but are the tannins what are causing the difference in emissions ? You are assuming so but it may not be true. Please revise the English and have it proof-read by native English speaker
Title:	Construction and operation of the first low cost ventilated-hood system for methane

We believe your submission has great potential and sincerely hope you will take advantage of this opportunity to consider the reviewers' commentary and amend your proposal accordingly. Please email your revised abstract to us as a Microsoft Word document, to abstracts@ggaa2016.org. No later than **Friday 23 October 2015.**

Further information on the scheduled poster sessions will be sent out at a later date. Posters should be no larger than portrait A0 (841mmx1189mm). All posters must be produced in English. All posters must be a single presentation; multiple pages, (eg A4, A3 stuck together) are not permitted.

11.3. Trabajo presentado en la "XVII Congreso BIENAL AMENA" 20 - 23 Octobre 2015. Puerto Vallarta, Jalisco. México

Santiago de Querétaro, a 26 de julio del 2015.

Gomaa RMM, González RM, Arredondo RJ, Pedraza BP, Molina TL, Castelán OO*

Para el Comité Científico es un placer informarle que su ponencia titulada **"Potencial de cinco plantas taníferas para reducir la producción de metano en el rumen de bovinos"** ha sido aceptada para presentarse en el XVII Congreso Bienal AMENA.

Debido a la gran cantidad de trabajos libres recibidos, el Comité Científico definirá de acuerdo al tema y disponibilidad de tiempo en el programa, si su trabajo será presentado en forma oral o cartel. Esta información será publicada en la página Web del Congreso www.congresoamena.mx a partir del lunes 3 de agosto del presente.

Le recordamos que el proceso de aceptación, incluyendo la publicación del resumen en las memorias del evento, no termina hasta recibir el pago de su inscripción, mismo que deberá completarse antes del 31 de Agosto del 2015. Para cualquier duda o información adicional agradeceremos se ponga en contacto con la administración de AMENA (administracion@amena.org.mx).

Sin más por el momento reciban un cordial saludo.

Atentamente

Dr. Gerardo Mariscal Landín
Presidente del Comité Científico de AMENA

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