UNIVERSIDAD AUTÓNOMA DEL ESTADO DE MÉXICO
FACULTAD DE MEDICINA VETERINARIA Y ZOOTECNIA

"***IMPACT OF Moringa oleifera* LEAF MEAL PROTEIN ON BIOMETHANE AND CARBON DIOXIDE IN STEER DIETS**"

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PRESENTA

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DEDICATORIA

Dedico esta investigación a mis padres que siempre estuvieron apoyándome en cada nuevo logro que ontuve a lo largo de mi carrera.

A todosmis compañeros que siempre me apoyaron y me ayudaron en lo que necesite.

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INDICE

1. INTRODUCCIÓN

El estudio del ecosistema ruminal implica analizar el funcionamiento de una compleja variedad de bacterias anaerobias obligadas, hongos y protozoarios que se rigen a ser seleccionados por la exigencia del rumen (Forsberg y Cheng, 1992).

Debido a la alimentación de los rumiantes el trabajo de las enzimas ruminales debe dirigirse estrictamente a la digestión de compuestos fibrosos que una vez fermentados se transforman en productos como ácidos grasos volátiles que posteriormente serán absorbidos e implementados como fuente de energía (Chesson y Forsberg, 1997).

Así pues, la composición de la pared celular de las plantas está formada por una diversidad de polisacáridos de los que destacan la celulosa, hemicelulosa y la lignina (Jung et al., 1991).

La lignina es particularmente una barrera que dificulta que exista una adecuada hidrolisis de la celulosa y la hemicelulosa (Cheng et al., 1999). Por lo que el ingreso de las enzimas degradantes a la planta se ve limitado.

Aun bajo esta limitación, la alimentación de los rumiantes continúa basándose en una gran cantidad de unidades de producción particularmente en pequeña escala en subproductos del sector agrícola con un elevado contenido lignocelulósico, el cual es de baja calidad nutricional por la dificultad que guarda para ser digerido.

Por lo tanto si la dieta se basa en residuos agrícolas, la degradabilidad de los polisacáridos traspolado al beneficio nutricional debe ser redituable (Hamer, 2003; Angenent *et al.*, 2004, Das y Singh, 2004, Haight, 2005).

2. REVISIÓN BIBLIOGRÁFICA

2.1 FISIOLOGÍA DIGESTIVA DEL RUMIANTE

La particularidad de estos animales se basa en que son capaces de alimentarse de pradera, ensilado y forraje debido a que pueden digerir los componentes de estos forrajes como celulosa y hemicelulosa, condición que los demás animales con un estómago simple no pueden realizar (Relling y Mattioli, 2003). Según (Weimer, 1998), se debe apresurar el trabajo de la microbiota ruminal para la digestión de la fibra.

Cualquier alimento y agua que el animal consume es fermentado dando lugar a las células microbianas, ácidos grasos volátiles y gases como dióxido de carbono y metano (McDonald *et al.,* 1995).

El animal y el rumen trabajan en conjunto ya que el primero suministra el alimento y el medio adecuado como anaerobiosis y pH para el desarrollo de bacterias que le darán a él la energía para su desarrollo y ciclo productivo (Hamada, 1976, citado por Angeles, 2000).

3. ECOSISTEMA RUMINAL

3.1. DESARROLLO PRENATAL DE LOS BOVINOS

El desarrollo fetal del estómago del bovino es relativamente rápido, pudiendo distinguirse los distintos comportamientos a los 56 días (Church, 1974). Los divertículos gástricos se desarrollan a partir de una dilatación fusiforme del intestino primitivo (Michel y Schwarze, 1970; Church, 1975). En los embriones bovinos se presentan a los 28 días un estómago primitivo similar al de otros embriones mamíferos (9,5 mm), mientras que a los 36 días ya se manifiestan algunas

diferencias en el tejido epitelial y a los 56 días se distinguen bolsas definitivas (50 mm), (Warner, 1958). Una vez diferenciados los departamentos gástricos hasta el punto de hacerse evidente los cuatro, se observa que se encuentran alineados uno tras otro, ocupando una posición caudal con respecto al hígado y al diafragma; después se dispone en forma de herradura, desplazándose el esbozo del rumen y el retículo hacia la izquierda y arriba, el omaso hacia la derecha y abajo, y el abomaso más hacia la izquierda (Michel y Schwarze, 1970). El rumen se halla entonces entre el diafragma y el riñón primitivo, aumentando de tamaño y desarrollando sus sacos ciegos; girando en dirección caudal para alcanzar su posición definitiva; el abomaso cambia a la vez de posición hacia el lado derecho, debido a un desplazamiento del hígado; los movimientos descritos permiten a los divertículos gástricos adoptar su forma típica de herradura. El tamaño relativo de los distintos departamentos gástricos varía mucho en el curso del desarrollo prenatal; al principio presentan los cuatro las mismas dimensiones, luego predomina el tamaño del rumen cuando este verifica su giro, observándose más adelante un incremento notable del abomaso hasta el punto de superar el volumen del rumen al nacimiento (Michel y Schwarze, 1970) Conjuntamente con el desarrollo externo, se produce el desarrollo de la mucosa de los compartimentos gástricos, observándose primero el esbozo de las hojas del omaso, después los pliegues del abomaso, a continuación las crestas del retículo y finalmente las vellosidades del rumen (Michel y Schwarze, 1970). La superficie epitelial se desarrolla más lentamente, siendo la superficie interna del rumen lisa durante la etapa fetal, sin papilas visibles (Wardrop, 1961). El desarrollo fetal es relativamente uniforme en cada espacio, ya que el ambiente intrauterino es bastante constante, no disponiéndose de datos suficientes para demostrar cualquier tipo de influencia por parte de la raza, el tamaño, el plano nutricional, etc. (Church, 1974).

3.1.2. DESARROLLO POST-NATAL DE LOS BOVINOS

El desarrollo postnatal del estómago de los rumiantes guarda relación con el tamaño y/o la edad y con la dieta. Una dieta líquida retrasa el desarrollo del rumen-retículo, tanto en el grosor y peso de los tejidos como en el desarrollo papilar. El desarrollo normal determina un crecimiento rápido del rumen-retículo después que el animal comienza a ingerir alimentos sólidos. El consumo de alimentos groseros e inertes estimula el crecimiento; esto se aprecia por el aumento de grosor de los tejidos, aunque la presencia de productos o alimentos capaces de fermentarse originando los ácidos grasos volátiles (A.G.V.) parece un factor necesario para la maduración de las papilas. El tamaño adulto relativo del estómago de los bovinos se alcanza a los 5 ó 6 meses (Church, 1974). Durante el nacimiento y en las tres primeras semanas de vida, el ternero no utiliza los tres primeros compartimentos gástricos (rumen, retículo y omaso); su desarrollo demora algún tiempo y está en dependencia de que el animal ingiera un concentrado seco adecuado; entre tanto es necesario suministrarle leche o un sucedáneo lácteo líquido apropiado. Durante la primera fase de vida el alimento líquido se dirige directamente al cuarto compartimiento gástrico (abomaso), aquí se coagula y la digestión prosigue, como en los monogástricos. Es imponderable la necesidad en la dieta del becerro recién nacido de un concentrado adecuado y especialmente durante las tres primeras semanas de nacido, porque el aparato enzimático del becerro no está bien adaptado a dirigir a no ser una cantidad bastante pequeña de ingredientes alimenticios (Stewart, 1974).

3.1.4. FUNDAMENTOS DEL RUMEN

Se puede definir según (Relling Y Mattioli, 2003), que el rumen y el animal son dos organismos independientes en donde en primer lugar se deben encontrar los ingredientes óptimos para la dieta que alimentará al rumen para que posteriormente se nutra el animal.

Siendo así, la primer característica fundamental es el pH mismo que tiene una varianza entre 5.5 hasta 7 (Krause y Oetzel, 2006 citado por Araujo y Vergara, 2007), considerando el tipo de dieta proporcionada, de él depende que exista una mayor sobrevivencia de la microbiota y por lo tanto una mejor digestión, energía y ganancia de peso en los animales.

La consecuente característica relevante que posee el rumen que es la cámara de fermentación por excelencia que debe tomarse siempre en cuenta es la temperatura que oscila entre los 38 a 42 °C, puesto que es uno de los pilares que permiten que se generen las bacterias ruminales, recordemos que la glucolisis es el medio por el cual dicha microbiota obtiene la energía que necesita por su condición anaeróbica (Relling y Mattioli, 2003).

3.2. PROCESO FERMENTATIVO

Los rumiantes tienen la increíble capacidad de darle trasformación a la celulosa y la hemicelulosa que (Ladisch, et al. 1990), aseguran forman un aproximado del 70 % de la biomasa vegetal.

El tipo y número me microorganismos presentes en el rumen están directamente asociados con los ingredientes de la dieta (Febel y Fekete, 1996).

La finalidad de que los microorganismos lleven a cabo la fermentación es producir Ácidos Grasos Volátiles, acético, butírico, propionico y láctico; mismos que serán la fuente nutricional para la actividad metabólica del rumiante, lo que significa que el rendimiento de producción del animal está directamente relacionado a la actividad y calidad de la microbiota ruminal; además de la formación de otros compuestos como gases en su mayoría metano y dióxido de carbono (Roderick y White, 1990). La composición de los gases según (Calsamiglia y Ferret, 2002); es de 65% de

CO_2, 27% de CH_4, 7% de N_2, 0.6% de O_2, 0.2% de H_2 y 0.01% de H_2S que se expulsan mediante el eructo.

3.2.1 IMPACTO DEL pH EN LA DIGESTIBILIDAD

Los cambios repentinos en el nivel de pH alteran la cantidad y funcionamiento normal del rumen, haciendo que la digestión de la fibra se desplome volviendo al animal susceptible a pérdida del apetito y a una disminución de la motilidad ruminal que lo predispone a consecuentes trastornos metabólicos; siendo los animales más afectados los que están bajo un régimen alimenticio a base de concentrados en su mayoría. Se menciona que lo ideal es que el rumen se mantenga en un punto de equilibrio entre un 6.2 y un 7 (Ash, 1959 citado por Krausen et al., 2002).

La clave de una buena producción de Ácidos Grasos Volátiles es el cuidado del pH, ya que mientras mayor sea la cantidad de bacterias fibroliticas estos estarán siempre disponibles para el desarrollo del rumiante (Dirkensen, 1969 citado por Calsamiglia, 1997).

3.2.2 MICROBIOTA RUMINAL

El proceso de alimentación no sería posible sin la existencia de esos organismos microscópicos que gracias a la labor de investigación ya ha sido posible su cuantificación , de las forma que autores como (McDonald *et al,* 1995), nos dice que de bacterias hay entre 109-1010 por ml y más de 60especies, añadiendo que la población de hongos puede variar entre un 8 a un 10 % del total de la población del rumen y que además el ciclo de vida de estos organismos es en dos tiempos, primero en una zoospora con la capacidad de moverse, seguido de la fase en que ya es un esporangio con la facilidad de adherirse con sus rizoides a las partículas

del alimento mientras que los protozooarios según Shler et al., 1996; se pueden encontrar de 105 a 106 células/gramo de líquido ruminal.

4. PROBIOTICOS EN LA NUTRICION ANIMAL

(Parker., 1974) fue el primero que utilizó la palabra probiòtico dentro del contexto para explicar que eran microorganismos capaces de hacer crecer otros, pero (Fuller.,1989) hace una modificación en el término señalando que es un suplemento alimentante, que mejora el equilibrio intestinal, además de proponer que el modo de acción puede ser a nivel de competir por receptores en el tracto gastrointestinal y de los nutrientes, promotor de sustancias antibacteriana y estimulador de la inmunidad,

Son parte de los aditivos utilizados en la producción animal como una alternativa a la utilización de otros que ocasionan peligro para los consumidores de los productos y subproductos, los probiòticos se definen como organismos vivos que van a tener un efecto positivo en el tracto gastrointestinal del organismo que los recibe sin perjudicar sus funcionamiento normal (Van der Aa Kühle et al., 2005).

5. JUSTIFICACIÓN

En la actualidad es una necesidad constante que los animales tengan una tasa de crecimiento adecuada que les permita llegar a la edad productiva con la mejor condición corporal y con un óptimo estado de salud, es por ello que el pilar para conseguir este resultado es la dieta proporcionada, que además de los ingredientes tradicionales como maíz, pasta de soya, canola, salvado y ensilados, tengan en su composición algunos ingredientes naturales que no repercutan en la salud animal ni humana; pero que si tengan la capacidad de mejorar el trabajo digestivo del rumiante para un mayor aprovechamiento nutricional y por ende elevar la producción haciendo redituables las unidades productivas.

6. HIPÓTESIS

El uso de hojas de *Moringa oleifera* como suplemento en la alimentación de toros puede, aumentar la digestibilidad de la dieta del animal, así aprovechando mejor su valor nutritivo, sin modificar variables de cinética ruminal.

7. OBJETIVO

3.1. D Objetivo general

Evaluar el impacto del uso de hoja de *Moringa oleifera*, sobre la fermentación in vitro de una dieta de concentrado al 100%en bovinos

3.2. E Objetivos específicos

Determinar el efecto de la adición de diferentes niveles de hoja de *Moringa oleifera* en diferentes concentraciones(/100 g DM): 0 g (TMR0, control), 10 g (TMR10), 20 g (TMR20), 30 g (TMR30), 40 g (TMR40), 50 g (TMR50), 60 g (TMR60), 70 g (TMR70), 80 g (TMR80), 90 g (TMR90), and 100 g (TMR100 (0.0, 1.) en la fermentación ruminal in vitro

8. MATERIALES Y MÉTODOS

8.1 UBICACIÓN DEL SITIO EXPERIMENTAL

Colegio de postgraduados de la UAEM

8.2 CRITERIOS DE SELECCIÓN DEL ESTABLO Y MATERIAL BIOLÓGICO

Se aplicará una encuesta al encargado del establo, para conocer las características generales y el manejo de los becerros desde el nacimiento al destete, la sanidad e higiene y el tipo de alimentación de estos.

8.3 TRATAMIENTOS

Se realizó un alimento a base de heno de alfalfa, maíz amarillo, semillas de soja, salvado de trigo y vitaminas y minerales.

- 400 g de heno de alfalfa
- 250 g de maíz amarillo aplastado
- 250 g de semillas de soja
- 100 de salvado.

Al implementar la hoja de moringa en la dieta se fue sustituyendo la soya en diferentes tratamientos empezando con un 10 % hasta llegar a un 100%.

8.4 Manejo de alimentación y aplicación de los tratamientos

El rumen inoculo se preparó a partir de dos novillo Holstein canulados (450±20 kg de peso vivo alojados en corrales individuales y alimentados con una dieta a base de heno de alfalfa y concentrado (Purina) a la proporción de 60:40 con acceso de agua a libre acceso se les alimentaba dos veces al día a las 8 y 16 horas y se estipulaban en las condiciones estipuladas en el documento oficial de especificaciones técnicas para la producción , cuidado, y usos de animales de laboratorio (NOM-062-ZOO-1999).

8.4.2 Alimento sólido

Se ofrecerá un concentrado comercial con un 16% de PC mínimo. Éste alimento será ofrecido a libre acceso desde el nacimiento.

8.5 Parámetros de medida:

Preparación *In Vitro*

La producción de gas se determinó en frascos ámbar de 125 ml en los cuales se incubaron cada tratamiento, utilizando la técnica propuesta por Theodorou *et al,* (1994). Se preparó la solución nutritiva para 900ml; solución micromineral 0.1 ml, solución macromineral 200ml, solución buffer 200ml, resazurina 1ml, agua destilada 500ml mezclada con liquido ruminal, posterior se pesó 1g MS de la ración mixta (50/50) en cada frasco y se adiciono la solución preparada (10 ml), más líquido ruminal (50 ml); para la corrección se utilizaron por triplicado blancos sin sustratos con un total de 576 frascos, los cuales se gasearon con CO_2, se taparon herméticamente y incubaron a 39°C en baño maría, se midió la producción de gas a las 48 horas usando el transductor de presión (Extech, Waltham, USA)

Degradabilidad de la materia seca

Se determinó la degradabilidad de la materia seca por filtración de los contenidos de los frascos al final de la incubación (es decir, después de 72 h). El contenido de cada botella se filtró con un filtro (Porosidad gruesa no. 1, tamaño de poro 100 a 160 m; Pyrex, stone, Reino Unido). Los residuos de fermentación se secaron a 65 °C durante 72 h para estimar desaparición materia seca con la pérdida de peso después del secado siendo la medida de la MS degradada.

8.6 Cálculo y análisis estadísticos

8.6.1 Producción de gas In Vitro

Después del periodo de incubación (72h), se liberó el gas acumulado y se calculó la producción de gas; se estimaron los parámetros kinestésicos (ml/g MS) de acuerdo al modelo propuesto por France *et al.* (2001).

$$A=b\ x\ (1-e^{-c(t-L)})$$

Dónde:

A= volumen de producción de gas al tiempo t

b= producción de gas asintótica (ml/g MS)

c= ritmo de producción de gas (/h) de la fracción de b de alimento fermentable lentamente

L= intervalo discontinuo antes de la producción de gas

La energía metabólica (EM; MJ/kg MS) y la digestibilidad de la materia orgánica *in vitro* (DMO; %) fueron estimados de acuerdo con Menke *et al.* (1979): EM=2.20+0, 136 PG+0.057 PC DMO=14.88+0.889 PG+0.45 PC +0.0651 cenizas.

Donde DMO es degradabilidad de la materia orgánica; PC es proteína cruda (%); cenizas (%); y PG es producción de gas (mL gas / 200 mg de muestra seca después de 24 h).

El factor de reparto (FP) a las 24 h de incubación (FP_{24}; es una medida de la eficiencia de la fermentación) se calculó como la relación de DMS *in vitro* (mg) para el volumen (ml) de PG en 24 h, es decir, DMS/producción total de gas (PG_{24}), según a Blummel *et al.* (1997).

El rendimiento de gas (RG_{24}) se calculó como el volumen de gas (ml gas/g MS) producida después de 24 h de incubación dividido por la cantidad de DMS (g) como:

Rendimiento de gas (RG_{24}) = ml gas/g DMS

Las concentraciones de ácidos grasos de cadena corta (AGCC) se calcularon de acuerdo a Getachew *et al.* (2002) como:

AGCC (mmol / 200 mg MS) = 0,0222 PG -0.00425

dónde: PG neto en 24 h (ml/200 mg MS). La PC microbiana (PCM) o producción de biomasa microbiana, se calculó de acuerdo con Blummel *et al*. (1997) como:

PCM (mg/g MS) = mg DMS-(gas ml x 2,2mg/mL)

Dónde: 2,2 mg/mL es un factor estequiométrico que expresa mg de C, H y O requerido para producción de gas asociado con la producción de AGCC de un ml de gas.

Análisis estadístico

Los datos de las variables (digestibilidad *in vitro* y producción de gas) fueron sujetos a análisis de varianza para un diseño experimental completamente al azar con arreglo factorial 4 x 4 de tratamientos usando el procedimiento GLM de SAS (2002), de acuerdo al siguiente modelo:

$Y_{ijkl} = \mu + SB_j + PM_k + (SB*PM)_{jk} + e_{ijkl\ (\text{dudas})}$

Dónde:

Y_{ij}= Variable de respuesta

μ= Media general

SB_i= es la dosis de extracto de *Salix babilónica* (i= 1-4)

PM_i= Efecto de la dosis mineral (j=1- 4)

SB*PM= Efecto de la interacción extracto y minerales ($SB_i * PM_i$)

e_{ijk}= Error experimental

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ANEXOS

ARTICULO: Impact of Moringa oleifera leaf meal protein on biomethane and carbon dioxide in steer diets

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Short title: *Moringa oleifera as a protein feed*.

Abstract

Ruminal fermentation produces methane (CH_4) and carbon dioxide (CO_2) making the earth warmer. Therefore, the sustainable mitigation of CH_4 and CO_2 emissions as well as ruminal fermentation kinetics when soybean meal was replaced with *Moringa oleifera* leaf meal (MLM) as a protein source at different levels was investigated. A basal total mixed ration (TMR) containing 400 g alfalfa hay and 600 g concentrate feed mixture per kilogram with soybean meal as the sole protein source was formulated. *M. oleifera* leaf meal replaced (/100 g DM): 0 g (TMR0, control), 10 g (TMR10), 20 g (TMR20), 30 g (TMR30), 40 g (TMR40), 50 g (TMR50), 60 g (TMR60), 70 g (TMR70), 80 g (TMR80), 90 g (TMR90), and 100 g (TMR100) of soybean in the rations. Rations were incubated for 48 h using rumen inoculums from goats and steers. Some interactions between inoculum × TMR were observed ($P<0.05$) for GP parameters, CH_4 production, and fermentation kinetics. Moreover, most determined parameters responded in different manners between steers and goats. Rations containing MLM decreased the asymptotic GP ($P<0.01$), while they increased ($P<0.01$) the rate of GP and lag of GP, with both inoculums. Decreased ($P<0.05$) CH_4 production and increased CO_2 production ($P<0.05$) were observed when MLM replaced soybean meal. *M. oleifera* leaf meal containing diets decreased ($P<0.05$) ruminal ammonia-N, and total protozoal number, while they increased ($P<0.05$) total bacterial number with both goat and steer inoculums. Replacing soybean with MLM increased ($P<0.05$) fermentation pH, but decreased ($P<0.05$) organic matter degradability (OMD) with goat inoculum. On the other hand, TMR with MLM had declined ($P<0.05$) SCFA concentrations, and enhanced ($P<0.05$) OMD and DM degradability compared with the control TMR (i.e., TMR0). It is concluded that replacing

soybean meal in the diets of goats and steers negatively affected the nutritive value of the diet; however, decreased CH_4 production. From an environmental point of view, replacing soybean with *M. oleifera* leaf meal can be used as a sustainable strategy to reduce CH_4 production from goats and steers, and thus abate global warming.

Keywords: Greenhouse gases, *in vitro* fermentation, *M. oleifera* leaf meal, protein feeds, sustainable livestock production.

1. Introduction

During ruminal fermentation of feeds, large amounts of greenhouse gases (GHG) are produced making livestock one of most important GHG producers. Food and Agriculture Organization (FAO, 2006) reported that livestock sector accounts for about 18% of methane (CH_4) and 9% of carbon dioxide (CO_2) productions. Many attempts have been made to mitigate CH_4 emission from ruminants including the inclusion of yeast (Elghandour et al., 2017), organic acids salt (Elghandour et al., 2016), exogenous enzymes (Kholif et al., 2017a), and essential oils (Hernandez et al., 2017), with promising results.

A series of problems are facing animal producers; one of them is the availability and price of concentrates, in particular protein sources, which compel nutritionists to seek for less-expensive alternative protein feeds (Kholif et al., 2015). Some concerns should be considered for the alternative protein feeds including the high protein content and balanced amino acids profile, without ignoring suitable cost. *Moringa oleifera* Lam (syns. *Moringa*

pterygosperm family *Moringaceae*) is a tree fodder growing almost worldwide and yields a huge amount of biomass ranging from 43 to 115 tons per hectare (Safwat et al., 2014), with a good protein content. Kholif et al. (2015, 2016) reported an excellent chemical composition as a protein feed containing (/kg DM) 241 to 277 g crude protein (CP), with about 47% of bypass protein (Becker, 1995) and adequate amino acid profile (Sánchez-Machado et al., 2010). The protein content of *M. oleifera* leaf meal (MLM) is less than that of common protein concentrates in ruminant nutrition (e.g., soybean meal and cottonseed meal), but comparable to sesame meal (260 g CP/kg DM). Kholif et al. (2015) reported that the price of branches with leaves and soft twigs used as animal feed can be around 0.25 - 0.5 US$ per kg DM, making it cheaper than traditional protein feeds such as sesame and soybean meal.

Little information about MLM as a protein source in the diet of ruminants is available; however, recent experiments that included MLM as a protein feed are gaining increasing interests, with promising results such as enhanced feed utilization and milk production from goats (Kholif et al., 2015, 2016). Unfortunately, they did not study the effect of replacing soybean meal with MLM as a protein source on GHG production. Therefore, the present study aimed to evaluate the effects of replacing soybean meal at different levels with MLM in the diet for ruminant animals, as a clean feed for the environment on the sustainable mitigation of CH_4 and CO_2 production, ruminal fermentation, and CH_4 producing protozoa and bacteria using rumen inoculums from goats and steers.

2. Materials and methods

2.1. Substrate and treatments

M. oleifera leaf meal was prepared as previously described in Kholif et al. (2016). Briefly, *M. oleifera* biomass composing of leaves and small twigs was harvested at 40 d age. *M. oleifera* was air-dried at 60 °C for 48 h, and then kept for further use. The concentration of total tannins in *M. oleifera* leaves was determined according to Makkar (2003), and total phenolic content was determined chromatographically as described by Meier et al. (1988). *M. oleifera* contained 22 g/kg DM total tannins and 48 g/kg DM total phenolics. A total mixed ration (TMR) was prepared, as a substrate containing (/kg DM) 400 g alfalfa hay (*Medicago sativa*), 250 g crushed yellow corn, 250 g soybean meal, and 100 g wheat bran, and considered as a control. Rations were balanced for minerals and vitamins contents. In the basal TMR, dried MLM replaced soybean meal at (/100 g DM): 0 g (TMR0, control), 10 g (TMR10), 20 g (TMR20), 30 g (TMR30), 40 g (TMR40), 50 g (TMR50), 60 g (TMR60), 70 g (TMR70), 80 g (TMR80), 90 g (TMR90), and 100 g (TMR100). The chemical composition of ingredients and TMRs used as substrates is shown in Table 1.

2.2. In vitro fermentation and biodegradation

Rumen inoculum was collected from two cannulated Holstein steers (450 ± 20 kg LW), and two cannulated Creole goats (50 ± 2 kg LW), housed in individual pens and fed *ad libitum* on a diet consisting of oat hay and concentrate (PURINA®, Toluca, Mexico) at 60:40 ratio,

with free access to water. Animals were fed twice daily at 08:00 and 16:00 h, and managed under the conditions stipulated in the Official Mexican Standard of technical specifications for the production, care and use of laboratory animals (NOM-062-ZOO-1999). Rumen contents were placed in a plastic thermo preheated at 39 °C, and transported to the laboratory where they were flushed with CO_2, mixed and strained through four layers of cheesecloth into a flask with O_2-free headspace, and maintained at a constant temperature of 39 °C and continuous CO_2 flow.

Before the incubation process, incubation medium containing buffer, macromineral, micromineral and resarzurin solutions, and distilled water were prepared according to Goering and Van Soest (1970), mixed in a volumetric flask using a platen and magnetic stirrer at 39 C to maintain the temperature and homogenize the solution. After, the ruminal inoculum and the reducing solution were added at 1:4 vol/vol, respectively.

Samples (0.5 g) of the substrate were weighed into 120 mL serum bottles. Consequently, 50 mL of previously prepared rumen liquor and the buffer were added. Bottles were maintained at constant CO_2 flow for 30 sec, and then capped with neoprene plugs and sealed with aluminum rings. The vials were placed in an incubator (Riossa®, F-51 D, Mexico State, Mexico) at 39 °C for 48 h. Moreover, three bottles as blanks (rumen fluid only) were incubated for 48 h. Three incubation runs were performed in three weeks.

The gas production (GP) readings were performed at 2, 4, 6, 8, 10, 12, 24 and 48 h of incubation. A water displacement apparatus was used according to Fedorak and Hrudey (1983). The apparatus was designed with a universal support, with a conical funnel, a 100

mL burette and two latex hoses of 0.5 and 1 m in length and 3/8-inch diameter. The vials were punctured with a 16-gauge needle placed at the end of the hose. Gas production (mL) was measured by the displacement of water in the burette.

After 48 h of incubation, 5 ml of gas were taken and stored in the vials with saturated saline solution prepared with 400 g of NaCl in 1 L of distilled water, and the pH adjusted at 2 and 5 mL of 20% methyl orange added as an indicator for CH_4 and CO_2 concentrations determination. The saturated saline solution was previously prepared and stored in 60 mL serological vials, leaving no space; and neoprene plugs were placed and sealed with aluminum rings, and stored away from light. For the determination of CH_4 and CO_2 from the vials with saturated saline, a sample of 10 μL of the gas phase was taken and injected into a PerkinElmer, Claurus 500 gas chromatograph (Mexico City, Mexico) with a flame ionization detection, and helium as the carrier gas. A thermal conductivity detector was used, the oven, column and TCD temperatures were 80°C, 170°C and 130°C respectively. Retention times were 0.73 min and 1.05 min for CH_4 and CO_2 respectively.

At the end of incubation at 48 h, the fermentation process was stopped by swirling the bottles in ice for 5 minutes, then the bottles were uncapped and the pH was measured using a pH meter (Thermo Scientific, Orion Star™ A121, Beverly, MA, USA). The contents of the bottles were filtered in Ankom® Technologies F57 bags (at constant weight), with the aid of a filtration system connected to a vacuum pump. The bottles were rinsed with a hot water 3 times to ensure recovery of all the residue of the fermentation. The bags were placed in a

forced-air oven at 55 °C for 48 h. Dry matter degradation was calculated by considering the initial weight of the substrate and the weight of the residue.

After pH measure and filtration, 4 mL of the medium were obtained with a syringe and mixed with 1 mL of 25% metaphosphoric acid, shaken slightly and placed under freezing until analysis of ammonia-N (NH_3-N) concentration. Other 4 mL of the medium were mixed with 1 mL 10% formaldehyde, shaken slightly and placed in refrigeration until analysis of bacterial and protozoal counting.

2.3. Total bacteria and protozoa counting

The population of total bacteria was determined at 48 h of incubation using a count chamber bacterium Petroff-Hausser (Hausser Scientific®, 3900, Horsham, PA) and a phase contrast microscope (Olympus®, BX51, Mexico City, Mexico) at a magnification of 100x. Exactly, 0.5 mL of the 10% formaldehyde fixed medium sample was taken and diluted in 4.5 mL of distilled water. The concentration of bacteria per mL was determined as the average of bacteria observed in each grid, multiplied by the dilution factor and the chamber factor (2×10^7), according to the following formula: $\text{Bacterial number/mL} = \mu \times \text{FD1} \times \text{FD2} \times 2^7$

Where: μ is the average of bacteria in each grid per treatment, FD1 is the first dilution factor (1.25), and FD2 is the second dilution factor (10)

For the protozoal number determination, 1 mL of the 10% formaldehyde fixed sample was obtained and diluted in 1 mL of distilled water; then 0.5 mL of the mixture was taken with a Pasteur pipette (BRAND, 7712, Wertheim, Germany) which were deposited in a Neubauer chamber (BRAND, 7178-10, Wertheim, Germany) and subsequently observed on a contrast microscope (Carl Zeiss®, Axiostar, Mexico City, Mexico) at 400× magnifications. The count of protozoa was made in eight quadrants (4 of each grid), taking as viable protozoans those that maintained their morphological integrity. The concentration of protozoa per mL of culture medium was estimated as the average of protozoa observed in each grid, multiplied by the dilution factor and the chamber factor (1×10^4), according to the following formula: $\text{Protozoal number} = \mu \times \text{FD1} \times \text{FD2} \times 10^4$

Where: = μ is the average of protozoa in each grid per treatment, FD1 is the first dilution factor (5), and FD2 is the second dilution factor (3).

2.4. Chemical analyses

Samples of the diets were analyzed for DM (#934.01), ash (#942.05), N (#954.01) and EE (#920.39) according to AOAC (1997), while the neutral detergent fiber (NDF) (Van Soest et al., 1991), and acid detergent fiber (ADF) and lignin (AOAC, 1997; #973.18) analyses were carried out using an ANKOM^{200} Fiber Analyzer Unit (ANKOM Technology Corp., Macedon, NY, USA) with the use of an alpha amylase and sodium sulfite.

The concentration of ruminal NH_3-N was determined according to Broderick and Kang (1980) methods. Samples of the incubation medium were centrifuged at 3000×g for 10 min, and 20 μL of the supernatant was mixed with 1 mL of phenol and 1 mL of hypochlorite, and the mixture was incubated at 39 °C for 30 min, after they were diluted with 5 mL of distilled water. Samples were read on a visible ultraviolet light spectrophotometer (Varian, model Cary 1E, California, USA) at 630 nm. The resulting g/L concentration was divided by 0.8 which was the 25% metaphosphoric acid dilution factor.

2.4. Calculations and statistical analyses

For estimation of gas production (GP) kinetic, recorded gas volumes (mL/g DM) were fitted using the NLIN procedure of SAS (2002) according to France et al. (2000) model as:

(1) $y = b \times [1 - e^{-c\,(t-L)}]$

where y is the volume of GP at time t (h); b is the asymptotic GP (mL/g DM); c is the fractional rate of fermentation (/h), and L (h) is the discrete lag time prior to the time any gas was released.

Metabolizable energy (ME, MJ/kg DM) and *in vitro* organic matter digestibility (OMD, g/kg DM) were estimated according to Menke et al. (1979) as:

(2) ME = 2.20 + 0.136 GP (mL/0.5 g DM) + 0.057 CP (g/kg DM)

(3) OMD = 148.8 + 8.89 GP + 4.5 CP (g/kg DM) + 0.651 ash (g/kg DM)

where GP is net GP in mL from 200 mg of dry sample after 24 h of incubation.

The partitioning factor at 24 h of incubation (PF_{24}, a measure of fermentation efficiency) was calculated as the ratio of DM degradability in vitro (mg) to the volume (mL) of GP at 24 h (i.e., DMD/total GP (GP_{24})) according to Blümmel et al. (1997). Gas yield (GY_{24}) was calculated as the volume of gas (mL gas/g DM) produced after 24 h of incubation divided by the amount of DMD (g) as:

(4) GY_{24} = mL gas/g DM/g DMD

Short chain fatty acid concentrations (SCFA) were calculated according to Getachew et al. (2002) as:

(5) SCFA (mmol/200 mg DM) = 0.0222 GP − 0.00425

where: GP is the 24 h net GP (mL/200 mg DM).

Microbial biomass production (MCP) was calculated (Blümmel et al., 1997) as:

(6) MCP (mg/g DM) = Milligrams DMD − (Milliliter gas × 2.2 mg/mL)

where the 2.2 mg/mL is a stoichiometric factor that expresses mg of C, H and O required for the SCFA gas associated with production of 1 mL of gas (Blümmel et al., 1997).

Data of each of the three runs within the same sample of each of the three individual samples of rations were averaged prior to statistical analysis and the mean values of each individual sample were used as the experimental unit. The experimental design was a

factorial arrangement with 3 replicates in a randomized complete block design. Data were analyzed using the GLM procedure (SAS, 2002) with the model: $Y_{ijk} = \mu + R_i + M_j + (R \times M)_{ij} + \varepsilon_{ijk}$ where: Y_{ijk} is the observation, μ is the population mean, R_i is the inoculum source effect, M_j is the level of MLM in the ration, $(R \times M)_{ij}$ is the interaction between MLM level and inoculum type, and ε_{ijk} is the residual error. Tukey test was used to compare means.

3. Results

3.1. Chemical composition

Replacing soybean meal with MLM gradually decreased DM, OM, CP, NSC, and hemicellulose, while it gradually increased EE, NDF, ADF, and cellulose contents of the rations (Table 1).

3.2. Gases production

Inoculum type × TMR interactions were observed ($P<0.05$) for GP parameters and CH_4 production (Table 2). Gas production parameters, CH_4 production and CO_2 production differed ($P<0.001$) between goat and steer rumen liquors. Moreover, replacing soybean meal with MLM affected GP parameters and CH_4 production.

Fig. 1 and 2 show GP of TMR with different levels of MLM incubated with rumen inoculums from goats and steers at different incubation hours. With goat rumen inoculum,

replacing soybean with MLM decreased the asymptotic GP (linear and quadratic effects; $P<0.01$), but increased the rate of GP (linear and quadratic effects; $P<0.01$) and lag of GP (linear effect; $P<0.001$). On the other hand, excluding TMR10 and TMR40 treatments, MLM containing rations quadratically decreased ($P=0.011$) the asymptotic GP, and linearly increased ($P\leq0.003$) the rate of GP and the lag time of GP.

With rumen inoculum from goats, the inclusion of MLM decreased (linear and quadratic effects, $P<0.05$) CH_4 production and also the proportions of CH_4 production (linear effect, $P<0.05$), while it increased (linear effect, $P=0.034$) CO_2 production (Table 2). With steers inoculum, rations containing MLM quadratically decreased ($P=0.012$) CH_4 production without affecting its proportion, but linearly increased ($P=0.032$) CO_2 production (ml/g degraded DM).

3.3. Fermentation kinetics

Inoculum × TMR interactions were observed ($P<0.05$) for SCFA, NH_3-N, OMD, ME, PF24, GY24, and MCP (Table 3). All determined fermentation parameters differed between steers and goats. The ration effect on these parameters was quadratic ($P<0.01$).

With goats' rumen liquor, MLM increased fermentation pH (linear effect, $P=0.037$) and total ruminal bacteria (quadratic effect, $P=0.045$), but decreased ruminal NH_3-N (linear and quadratic effects, $P<0.05$), OMD (quadratic effect, $P=0.033$), and total protozoa number ($P=0.015$). With the steer inoculum, decreased concentrations of SCFA (quadratic effect,

P=0.005), NH_3-N (linear and quadratic effects, P<0.001), and total protozoa number (quadratic effect, P=0.027) were observed when soybean was replaced with MLM. However, MLM containing rations increased DMD (linear effect, P=0.002), OMD (quadratic effect, P=0.002), ME (quadratic effect, P=0.002), MCP (quadratic effect, P=0.005), and total bacterial number (linear effect, P=0.021).

4. Discussion

4.1. Chemical composition

The observed changes in the chemical composition when MLM replaced soybean meal were expected. *M. oleifera* leaf meal contains a high fiber fractions and less protein compared with soybean meal. In the present experiment, MLM contained 281 g CP which represents about 69% compared with soybean meal which contained 408 g CP. On the other hand, MLM contained about 345 g NDF versus 143 g for soybean meal. The chemical composition is expected to affect the fermentation of each ration, as explained later. The protein content of MLM is comparable to sesame meal (26%), however, less than soybean meal (approximately 40-44% CP), cottonseed meal (approximately 40% CP) and sunflower seed cake (approximately 35% CP), which are mostly used as protein concentrates in ruminant nutrition. Kholif et al. (2015) replaced sesame meal in the diets of goats with fresh MLM at 0, 50, 75, and 100%, and found that the CP content of the diets was not changed significantly; however, the NDF content of the diets increased. This is a result of almost equally CP content and high NDF content of MLM and sesame meal.

4.2. Gas production

The observed interactions between inoculum type and MLM level for most measured parameters of GP and fermentation reveal that the response to replacing soybean meal with MLM differed between goats and steers. These differences may be supported by the significant different response between steers and goat inoculums, and explain possible differences in the ruminal microbial population and the digesting capacity of the two ruminant species. This is an important indicator of the importance of using rumen fluid from different ruminant species to inoculate the substrates *in vitro* incubation cultures to evaluate feed nutritive value. Aderinboye et al. (2016) reported different fermentation parameters among cows, sheep and goat inoculums.

Replacing soybean with MLM affected negatively GP. Soliva et al. (2005) observed that complete replacing of soybean meal and rapeseed meal with MLM decreased *in vitro* total GP. The decreased GP with increasing lag of GP is a direct result of increased fiber concentration in MLM-containing rations. Kholif et al. (2017b) stated that the increasing fiber portion in TMR decreased the asymptotic GP. In another experiment, Elghandour et al. (2015) observed that increasing ration content of corn silage instead of concentrate feed mixture decreased GP and increased the lag time of GP. Moreover, the decreasing CP concentration in rations of MLM can partially explain the decreased GP (Elghandour et al., 2017). They showed that increasing CP content of a ration increases GP. The crude protein content is a secondary reason for the decreased GP compared with fiber content because fermentability of protein produces relatively small GP compared to carbohydrate

fermentation (Makkar et al. 1995). It was expected that the observed increased bacterial number with MLM would increase GP, but this expectation did not exist. The reasons for these observations are unknown.

Plant secondary metabolites (PSM) presented in MLM can be another reason for the negatively affected ruminal fermentation. Generally, plant secondary compounds at high doses have a great antimicrobial activity against ruminal bacteria, protozoa and fungi (Bodas et al., 2012). The antimicrobial effect depends on plant species, the chemical composition of the plant, and the dose fed to animal (Bodas et al., 2012). Therefore, PSM sometimes can stimulate rumen microbial activity (Benchaar et al., 2008), and this may explain the greater GP and shorter lag time in the case of TMR10 and TMR40 rations for steers inoculum. Ruminal microflora can tolerate and degrade low and moderate concentrations of PSM such as phenolic compounds (Varel et al., 1991) and tannins (Frutos et al., 2004), and utilize them as energy sources.

Many reports showed that goats have a high ability to tolerate high levels of tannins compared with other ruminant species (Frutos et al., 2004; Yisehak et al., 2016). In the present experiment, ruminal microflora from steers showed better response compared with that of goats, which is not in line with Frutos et al. (2004) and Yisehak et al. (2016). This may be due to the previous feed fed to the goats and steers before starting the experiment. Ruminal microbial population depends mainly on the type of diet fed; therefore, based on the fact that both the steers and goats in the present experiment were maintained on the same diet, microbial species were not expected to vary (Mould et al., 2005).

4.3. Greenhouse gases production

Reducing CH_4 production from livestock is always desirable from environmental point of view. The decreased CH_4 production with MLM-containing rations may be related to the PSM such as tannins and saponins in MLM, or the high proportion of α-linolenic acid (Machmüller et al., 2000) in MLM (Soliva et al., 2005). A decreased CH_4 production was observed by Soliva et al. (2005) from MLM compared with soybean meal. The antimicrobial and protozoal effects of PSM such as tannins can be a direct reason for the declined CH_4 production (Bodas et al., 2012). Moreover, the adverse effect of PSM on cellulolytic bacteria (Patra and Saxena, 2009) can cause a reduction in CO_2 and H_2 formation, which are required for methanogenesis, as a result of decreased SCFA production, in particular acetate (Goel and Makkar, 2012) causing a reduction in CH_4 production. Moreover, Jayanegara et al. (2011) reported mitigating effects of phenolic compounds on CH_4 production. Goel and Makkar (2012) reported a decreased CH_4 production up to 50% in response to tannins and phenolic compounds.

4.4. Ruminal microflora

Increasing ruminal bacterial number with MLM was not expected based on the antimicrobial properties of PSM in MLM (Bodas et al., 2012). However, the reports of Varel et al. (1991) and Frutos et al. (2004) about the ability of ruminal microflora to degrade and utilize PSM as energy sources can explain the increased ruminal bacterial number. The increased bacterial populations seem to be a consequence of the observed inhibition of

ruminal protozoa (Newbold et al., 1997; Goel et al., 2008), as ruminal protozoa is the main predators of bacteria in the rumen (Mathieu et al., 1996).

However, the increased bacterial number with MLM rations did not result in a greater GP or ruminal nutrient degradability or SCFA production. This may be due to the fact that not all bacteria species are affected in the same way. For example, tannins and saponins are particularly able to inhibit Gram-positive bacteria more than Gram negative bacteria (Bodas et al., 2012). Thus, the increased bacterial number might be due to increase in other species and not cellulolytic bacteria.

The decreased protozoal number when soybean was replaced with MLM is a result of the marked anti-protozoal activity of PSM such as saponins, tannins and phenolic compounds (Makkar et al., 1995; Bhatta et al., 2009; Bodas et al., 2012). Bhatta et al. (2009) reported that tannins have a clear defaunating effect, without a clear mode of action. Decreasing ruminal protozoa population is desirable because this will allow for a lower CH_4 production and higher bacterial numbers.

4.5. Fermentation kinetics

Increasing ruminal pH in goat nutrition is a good subject to allow better ruminal condition for cellulolytic bacteria activity. In the present experiment, ruminal pH values ranged between 6.42 and 6.48, and fell within the range considered acceptable for fiber digestion (Ørskov and Ryle, 1990).

In the present experiment, ruminal NH_3-N concentrations ranged between 49.0 and 69.6 g/L, and were above the range required for sufficient microbial protein synthesis (Satter and Slyter, 1974). The decreased ruminal NH_3-N with MLM is a result of the reported low degradability of MLM protein in the rumen (Kholif et al., 2015, 2016) due to tannins and phenolic compounds in MLM (Bodas et al., 2012). Tannins in feeds may reduce ruminal protein degradation because tannins have an ability to bind to dietary protein and protect it from ruminal degradation (Frutos et al., 2004). Besides, PSM such as saponins and tannins have the ability to decrease ruminal protozoa (Newbold et al., 1997) as we previously showed. Protozoa play a major role in ruminal feed protein degradation (Jouany, 1996). Another probable reason for the decreased NH_3-N is the inhibition of hyper NH_3-producing bacteria activity and their deaminase activity (Newbold et al., 2004).

The increased bacterial numbers with MLM-containing diets did not result in increased OMD or SCFA production in goat inoculum. The negatively affected nutrient degradability in MLM-containing rations may be due to the negative effects of increasing fiber concentration and the declining CP concentration on ruminal fermentation. Frutos et al. (2004) reported that less than 50 mg/g DM is the acceptable level of tannins in feeds without negative effects on digestibility. In the present experiment, tannins concentration was 22 mg/g DM, which is less the critical level that suppresses ruminal fermentation. Therefore, tannins cannot be the main reason for the decreased degradation but the increasing fiber concentration in MLM rations, as earlier speculated. Elghandour et al. (2015) observed that increasing fiber concentration in a ration reduced nutrient degradability. In steer nutrition,

the result of DMD was in contrary to the observed result in goat nutrition. Differences in ruminal microflora response to PSM might be the reason.

Decreased SCFA concentration can be interpreted as a result of declined digestion of MLM containing ration. Flatt et al. (1956) reported that the concentrations of ruminal SCFA production depend on nutrient digestibility and the activity of microbial population in the rumen. Results of decreasing NH_3-N concentration with decreasing total SCFA concentration is an evidence of improved synchronization between dietary energy and protein, which is expected to increase microbial-N production within the rumen (Seo et al., 2014). Soliva et al. (2005) compared the ruminal fermentation of MLM with soybean meal and rapeseed meal, and observed unaffected ruminal pH values and SCFA concentration, and a decreased ruminal NH_3-N concentration with MLM.

5. Conclusion

From the nutritional perspective, *M. oleifera* cannot replace soybean meal as a protein feed in diets for goats and steers because of the negative effect on ruminal fermentation. However, from an environmental point of view, replacing soybean meal with *M. oleifera* leaf meal reduced CH_4 production, which can be used as a good cleaner product for the environment and feedstuff for ruminant livestock to control the environmental contamination by biogases pollution from ruminants. More research will be desirable to determine the best levels of replacement on feed utilization and methane production in dairy and beef cattle, wool and meat sheep and dairy goats. Besides, more experiment should be carried out to

replace other protein feeds with low protein concentrates such as sesame meal and rapeseed meal with *M. oleifera* at different levels in both *in vitro* and *in vivo* trials.

Conflict of interest

All authors declare that there are no present or potential conflicts of interest between them and other people or organizations that could inappropriately bias their work.

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