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Methane emissions from rams fed kikuyu hay or kikuyu-lotus hay mixture

Vargas, J.J.[1, 2@]; Pabón, M.L.[2] and Carulla, J.E.[2].

[1] Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA). Centro de Investigación Tibaitatá. Mosquera, Cundinamarca, Colombia.
[2] Departamento de Producción Animal, Universidad Nacional de Colombia Bogotá, Colombia.

ADITIONAL KEYWORDS

Feeding systems.
Greenhouse gases.
Methanogenesis.
Contain-tannin legumes.

SUMMARY

Dietary inclusion of contain-tannin legumes may reduce enteric methane emission in ruminants. To evaluate methane emissions from sheep fed a kikuyu grass (Cenchrus clandestinus) diet partially substituted with lotus (Lotus uliginosus), twelve growing rams, with 23±2 kg average liveweight, were assigned randomly to two treatments and with three measurement periods in a switchover design. Treatments consisted of 100% kikuyu hay or 70% kikuyu hay: 30% lotus hay and with 6 rams per treatment. Each of three periods lasted 20 d, where the first 15 d were for acclimatization and the last 5 d for measurements. Rams were placed in metabolic cages and fed once a day (8 AM) at 90% of their voluntary feed intake, with free access to drinking water. Feed intake, fecal production and feed digestibility were determined at each period. Methane production was measured for each treatment group of 6 rams using the polytunnel technique. Legume addition reduced total methane production (27.6 vs. 23.1 g/animal; p<0.01), methane production per dry matter intake (DMI) (18.8 vs. 12.2 g/kg DMI; p<0.01), methane production per digestible OM (DOM) (36.1 vs. 23.4 g/kg DOM; p<0.01) and methane production per digestible NDF (DNDF) (43.5 vs 34.0 g/kg DNDF; p<0.01). In conclusion, lotus inclusion in pasture systems could be a suitable legume to reduce methane emissions in grazing systems.

Producción de metano de corderos alimentados con heno de kikuyo y kikuyo:lotus

PALABRAS CLAVE

Sistemas de alimentación.
Gases efecto invernadero.
Metanogenesis.
Leguminosas taníferas.

RESUMEN

La inclusión de leguminosas taníferas puede reducir las emisiones de metano entérico en rumiantes. Para evaluar la incorporación de lotus (Lotus uliginosus) a una dieta basal de kikuyu (Cenchrus clandestinus) sobre la emisión de metano en ovinos, doce corderos con peso vivo de 23±2 kg fueron asignados aleatoriamente a dos tratamientos en tres periodos de medición en un diseño de sobrecambio. Los tratamientos fueron 100% heno de kikuyo o 70% heno de kikuyo y 30% heno de lotus con seis corderos por tratamiento. Cada uno de los tres periodos tuvo una duración de 20 d, en donde los 15 d primeros días fueron de adaptación y los últimos 5 d de medición. Los corderos fueron ubicados en jaulas metabólicas, alimentados una vez al día (8 AM) al 90% de consumo voluntario y tuvieron agua a voluntad. El consumo, la producción de heces y la digestibilidad fue determinada para cada periodo. La producción de metano fue determinada para cada grupo de 6 corderos a través de la técnica del poli-tunel. La inclusión de leguminosa redujo la emisión de metano total (51.6 vs. 43.1 g/animal; p<0.01), metano respecto a la materia seca consumida (MSC) (18.8 vs. 12.2 g/kg MSC; p<0.01) y metano respecto a la materia orgánica digerida (MOD) (36.1 vs. 23.4 g/kg MOD; p<0.01). En conclusión, el lotus podría ser una leguminosa adecuada para reducir las emisiones de metano en sistemas pastoriles.

INFORMATION

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jvargasm@agrosavia.co

INTRODUCTION

Methane is the main greenhause gas contributed by livestock farming and this gas also represents a energertic loss (Gerber *et al*., 2013). Hence, strategies to reduce methane production could decrease its implications in climate change and the same time increase ruminant energetic efficiency (Makkar, 2016). Nutritional manipulation of the diet characteristics can modify enteric methane emissions and animal efficiency (Johnson and Johnson, 1995, Lovett *et al*., 2005).

Worldwide, significant proportion of ruminants use pastures as the main source of feed. In *in vivo* studies, legumes with low content of condensed tannins included in basal diets of grass presented contradictory results. For example, Carulla *et al*. (2005) suggested that methane emissions per unit of dry matter intake by sheep increased when clover was included in a basal diet of ryegrass. On the other hand, Lee *et al*. (2004) reported that methane production per unit of dry matter consumed by cattle decreased when the proportion of white clover in the diet increased. Similar results were reported when tanniferous legumes were included in diets of sheep and cattle (Woodward *et al*., 2004; Tiemann *et al*., 2008a).

There is little information that evaluates methane emissions in tropical highland conditions. Archimède *et al*. (2011) reported reporting trials showing less methane production from C3 compared to C4 grasses and from tropical versus temperate legumes. The objective of this experiment was to determine methane emissions from lambs when lotus (*Lotus uliginosus*) hay, a tanniferus forage legume, was added to a basal diet of kikuyu (*Cenchrus clandestinus*) hay.

MATERIALS AND METHODS

All procedures were approved by the Bioethics Committee of the Facultad de Medicina Veterinaria y de Zootecnia, Universidad Nacional de Colombia, (Act 007 of 2010 with number CBE-FMVZ-026).

Localization

The experiment was conducted at the Tibaitatá Research Center, located in the municipality of Mosquera, Cundinamarca (2560 meters above sea level, 13°C mean temperature, with fluctuations between 0°C and 20°C). The average annual precipitation is 528.9 mm with bimodal distribution of two rainy periods, one between April and May and other from September to November, and 80-85% relative humidity.

Forage Species

Two different paddocks, one of kikuyu grass (*Cenchrus clandestinus*) and another of lotus (*Lotus uliginosus*) were harvested at 50 days of regrowth. The harvested forages were dried at air temperature, packed and stored at room temperature.

Animals and experimental design

Twelve growing rams with 23±2 kg of live weight were placed in individual metabolic cages and randomized between two groups of 6 animals each. The groups were assigned to an experimental switchover design composed of 2 (treatments) x 3 (periods). The experimental treatments were 100% kikuyu hay and 70% kikuyu hay and 30% of lotus hay. Three experimental periods of 20 d were performed. During the first 7 d of each period, feed was supplied *ad libitum* to determine voluntary intake. The following 13 d feed on offer was restricted to 90% voluntary intake to reduce selectivity and ensure total feed consumption. Feed was allocated once a day. Each group of 6 animals placed in cages was located in one of two tunnels of 7 m long, 5 m wide and 2.6 m high (total volume of 83.5 m^3) for methane emissions estimation (Molina et al., 2016).

Dry matter intake, and feces and urine excretions by individual animals were measured during the last 5 d of experimental period. During the last 3 days of each period, methane emissions by the group of 6 animals was estimated. For this gas samples (5 ml) of the tunnels exhaust stream were taken every hour and stored in vacutainers for subsequent methane analysis (Molina *et al*., 2016). The last day of each period, rumen contents (~15 ml) were sampled from each animal using an ororuminal probe, discarding when possible contamination with saliva was suspected. The sample of rumen content was filtered using two layers of cheese cloth. An aliquot was used to measure pH with a potentiometer (Hanna HI 98140) and another was stored at - 20 ° C to posterior volatile fatty acids (VFA) analysis.

Chemical Analysis

Dry matter (DM, AOAC, 2005), crude protein (CP, method of Dumas; AOAC, 2005), ether extract (EE, AOAC, 2005), neutral detergent fiber, acid detergent fiber, and lignin (Van Soest *et al*., 1991), ash (AOAC, 2005) and gross energy (GE, calorimetric bomb Parr® 6510) contents were determined for forage and fecal samples. Additionally, condensed tannin concentration in forages was determined by the butanol-HCL method (Terrill *et al*., 1992). Nitrogen ammonia concentration in rumen fluid was determined by a colorimetric method, according to procedure described by Parra and Avila (2010).

Methane (from tunnel gas sample) and ruminal VFAs were quantified using a gas chromatograph (Shimadzu GC-2014) equipped with a flame ionization (FID) detector as described by Parra and Avila (2010) and Betancour (2001), respectively.

Statistical analysis

A switchover model with two treatments (grass only and grass:legume mixture diets) applied in sequence through three periods of evaluation. Each experimental day was independent, and they were considered replicates per treatment. GLM procedure of SAS® version 9.1 was used for variance analysis as described by Martínez et al. (2011). Average values were compared through Tukey's test with a 5% significance. Variables associated with intake, excretion and digestibility considered the animal as experimental unit, while variables associated with methane production considered the group (6 animals) as experimental unit.

RESULTS

Forage chemical composition

In this experiment, addition of legume hay to a hay grass base diet increase protein, lignin and non-structural carbohydrates contents in 42.4, 100.0 and 34.5%, respectively, but decrease structural carbohydrate concentration in 12.5%. Indeed, kikuyu had more total carbohydrate contents than the grass:legume mix diet in 15.2%. However, the mixture diet contained 1.3% of condensed tannins, while kikuyu did not present condensed tannins (**Table I**).

Intake, excretion and digestibility of nutrients

Intake of dry matter (DM), organic matter (OM), lignin (LGN) and acid detergent fiber (ADF) were greater for the mix than for kikuyu only diet ($p<0.05$) but NDF consumption did not differ between treatments. DM, OM, LGN, NDF and ADF excretion was greater in animals that received the forage mix diet ($p<0.05$) (**Table II**). Apparent digestibilities of DM and OM were higher in the animals that received the mixture higher in animals receiving pure grass ($p<0.05$) (**Table III**).

Table I. Chemical composition of the kikuyu and kikuyu:lotus diets (Composición química de las dietas kikuyu y kikuyu:loto).

Composition[1]	Kikuyu	Kikuyu: Lotus
CP, %	11.8	16.8
EE, %	1.3	1.2
NDF,%	69.9	61.2
ADF, %	31.6	31.3
Hemicellulose, %	38.4	29.9
Cellulose, %	27.8	23.7
LGN, %	3.8	7.6
Ash, %	12.1	12.9
Condensed tannins, %	0.0	1.3
Non-structural carbohydrates[2], %	4.9	6.6
Total carbohydrates[3], %	71.0	60.2
OM, %	87.9	87.1
Gross energy (Kcal/g)	3.9	3.9

1. CP: Crude protein, EE: Ether extract, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, LGN: Lignin, OM: Organic matter.
2. NSC: 100-(CP + NDF + Ash + EE + Condensed tannins)
3. NDF+NSC-LGN

Table II. Dry matter intake, feces and urine production from rams feeding kikuyu hay or kikuyu:lotus hay diets (Ingesta de materia seca, heces y producción de orina de carneros alimentados con heno kikuyu o dietas de heno kikuyu:loto).

Variable[1]	Kikuyu	Kikuyu:Lotus	P<
Intake (g)			
DM	475.8[b]	589.3[a]	***
NDF	332.9	360.9	ns
ADF	150.3[b]	184.6[a]	***
LGN	18.2[b]	45.0[a]	***
OM	418.4[b]	513.6[a]	***
Feces (g)			
DM	211.3[b]	242.8[a]	*
NDF	128.9[b]	149.7[a]	*
ADF	70.7[b]	94.9[a]	***
LGN	26.8[b]	46.0[a]	***
OM	174.6[b]	206.6[a]	***
Urine (ml)			
Total	758.8[b]	1211.3[a]	***

1. DM: Dry matter, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, LGN: Lignin, OM: Organic matter.

Ammonia, pH, VFA´s and methane production

Ammoniacal nitrogen in rumen fluid was higher ($p<0.05$) in animals fed kikuyu:lotus than in those fed on kikuyu only (7.95 vs 5.44 vs mmol/L) (**Table III**). Rumen pH and total VFAs concentration in rumen did not differ between treatments. Acetate concentration was lower ($p<0.01$) and propionate ($p<0.1$) and butyrate ($p<0.01$) were greater for kikuyu:lotus than for kikuyu only diet. The ratio acetate:propionate was higher ($p<0.05$) in animals that consumed pure grass (5.1 vs. 4.6) than on the forage mixture diet (**Table III**).

Total methane emissions declined in rams that consumed kikuyu:lotus, regardless of how it was expressed (gross, per unit of feed intake or digested matter; **Table IV**). The animal group that received kikuyu produced less methane if they had previously received kikuyu:lotus, suggesting a residual effect of the diet on methane production (**Figure 1**).

Table III. Apparent digestibilities, rumen pH and fermentation products by rams fed kikuyu hay or kikuyu:lotus hay diets (Digestibilidades aparentes, pH ruminal y productos de fermentación de carneros alimentados con heno kikuyu o dietas de heno kikuyu:loto)

Variable[1]	Kikuyu	Kikuyu:lotus	P<
Apparent digestibility (%)			
DM	56.0[b]	58.9[a]	***
NDF	61.5[a]	58.7[b]	*
ADF	53.4[a]	48.8[b]	*
OM	58.7[b]	59.9[a]	+
pH	7.4	7.6	ns
Ammonia (mmol/L)	5.4[b]	7.9[a]	***
Volatile fatty acids			
Total (mmol/L)	42.9	37.2	ns
Acetate (%)	79.4[a]	76.0[b]	***
Propionate (%)	15.7[b]	16.7[a]	+
Butyrate (%)	4.0[b]	6.0[a]	***
Acetate:propionate ratio	5.1[a]	4.6[b]	*

1. DM: Dry matter, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, OM: Organic matter.
ab. Within rows, values followed by the same letter are not significantly different. + p<0,1. * p<0,05. *** p<0,01. ns: non-significant.

DISCUSSION

Kikuyu is the main grass species used in cattle production systems in Colombian highlands (Carulla and Ortega, 2016). Recently, the legume *Lotus uliginosus* has been introduced to this region with excellent animal production results (Castro *et al*, 2008; Morales *et al.*,

Table IV. Methane emissions from rams feeding kikuyu hay or kikuyu:lotus hay diets (Emisiones de metano de carneros alimentados con heno kikuyu o dietas de heno kikuyu:loto).

Variable[1]	Kikuyu	Kikuyu:Lotus	P<
g/animal	27.6[a]	23.1[b]	***
g/kg DM intake	18.8[a]	12.2[b]	***
g/kg FDN intake	26.9[a]	19.9[b]	***
g/kg OM intake	21.4[a]	14.0[b]	***
g/kg DM digested	33.2[a]	20.7[b]	***
g/kg FDN digested	43.5[a]	33.9[b]	***
g/kg OM digested	36.1[a]	23.4[b]	***

[1.] DM: Dry matter, NDF: Neutral detergent fiber, OM: Organic matter.
[ab.] Within rows, values followed by the same letter are not significantly different. *** p<0,01.

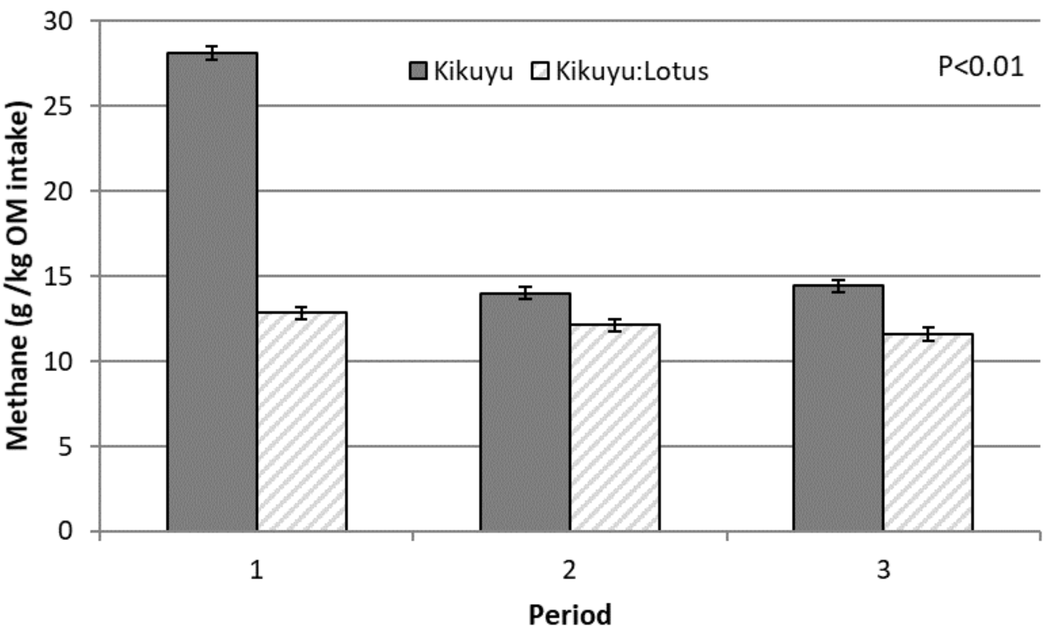

Figure 1. Methane emission (g/kg OM intake) from rams feeding kikuyu hay or kikuyu:lotus hay diets during the experimental period (Emisión de metano (g/kg de ingesta de OM) de carneros alimentados con heno kikuyu o dietas de heno kikuyu:loto durante el período experimental).

2013). Some studies suggest that the use of legumes and particularly tanniferous legumes, could reduce methane production by grazing ruminants (Molina *et al.*, 2016; Waghorn, 2008). The aim of the present experiment was to evaluate the effect of lotus hay inclusion to a basal kikuyu hay diet on methane emissions in sheep.

In our work 30% lotus hay inclusion decreased methane emission, both total emissions and emissions per unit of feed intake. Additionally, feed allowance and the level of legume inclusion may modify methane emissions.

Methane emission by ruminants fed legumes has presented contradictory responses. Clover hay inclusion to a basal diet of ryegrass hay increased methane emissions per unit of dry matter consumed in sheep (Carulla *et al.*, 2005). However, other work found that the increase in the proportion of clover to ryegrass basal diet decreases methane emission per unit of dry matter consumed in cattle (Lee *et al.*, 2004). These experiments have some differences that may explain the contradictory results between these works. In the first experiment, feed supply was restricted to 75 g/kg metabolic weight, while in the second experiment the intake was not restricted.

Some studies show that a greater forage allowance and legume inclusion to grass-based diets increases dry matter intake (Ribehiro-Filho *et al.*, 2005; McCaugey *et al.*, 1999) and when dry matter consumption increases there is a reduction in methane emissions per unit of dry matter intake due to a reduction in forage digestibility (Blaxter and Clapperton, 1965) and increase in passage rate (Pinares-Patiño *et al.*, 2007). In our work, diets were offered and voluntary intake of animals was determined during the first part of each experimental period. Later, offer was restricted to 90% of voluntary intake. Despite this restriction, dry matter intake increased as a consequence of legume addition.

Higher consumption in diets with legumes has been linked to increasing passage rate, due to decrease in structural carbohydrates concentration (Pinares-Patiño *et al.*, 2007). Pinares-Patiño *et al.* (2003) reported an inverse relationship between passage rate and enteric methane production. The increase in passage rate decreases feed permanence time in rumen, which limits nutrients degradation and H_2 and CO_2 production. However, in our work DM and OM apparent digestibility were higher in kikuyu and lotus diet but NDF degradability was lower. A higher DM intake and digestibility would imply higher methane emissions, such as that observed in this work, unless it would be mainly related with FDN degradation (Tiemann *et al.* 2008b).

Other possible explanation for lower methane emissions in diet with legumes could be related to changes in fermentation patterns. In our work, although VFA production did not presented significant differences among treatments, we observed 15.3% higher VFA concentration in rams that received pure grass. Similar response was observed in vitro, when increased lotus proportion in grass:legume mixture decreased VFA production, that was attributed to a higher protein and condensed tannins concentration in the mixture (Vargas et al. 2014). In addition, we observed that including lotus hay increased propionate concentration and decreased acetate and acetate:propionate ratio in rumen fluid. Methane formation, as well as propionate synthesis require H2, whereas during acetate synthesis H2 is released. For this reason, a decrease in acetate:propionate ratio or acetate concentration have been associated with methane reduction (Moss et al., 2000). Acetate:propionate ratio decreases, as a consequence of legume addition to a grass diet has been reported by other authors in in vitro studies (Stürm et al., 2007). However, in vitro (Hess et al., 2003) or in vivo (Carulla et al., 2005) did not report differences in acetate:propionate ratio.

Fermentation patterns are related to diet composition (Lovett *et al*., 2004). Diets rich in structural carbohydrates results in higher acetate proportion (Annison and Anrmstrong 1970). In our study, kikuyu diet had greater structural carbohydrate proportion, but NDF intake was similar between treatments. Likewise, structural carbohydrates in kikuyu had higher digestibility than in the mixed diet (**Table II**). Murphy *et al*. (1982) reported that fermentation products of the same carbohydrates in rumen may be different when amending microbial populations according to diet characteristics and ruminal environment. In our experiment, ruminal environment characteristics were not very different, except by higher ammonia concentrations in the rumen fluid of those animals that included lotus hay due to higher concentration and protein degradation. However, it is also probably that carbohydrate concentration could modify VFA fermentation pattern. Other factors such as the presence of tannins in lotus could help to explain these changes, as some studies have reported changes in VFA profile due to tannins inclusion (Bhatta *et al*, 2009).

L. uliginosus is a legume with moderate condensed tannins content and in this study the mixture contained low concentrations of condensed tannins. Tanniferous legumes inclusion in ruminant diet has been linked to methane emissions reduction (Waghorn 2008; Woodward *et al*., 2004). In studies with animals feeding tanniferous plants like Lotus corniculatus or Hedysarum coronarium reductions between 18 and 23% in methane emissions per unit of dry matter in sheep and cattle (Woodward *et al*., 2004; Woodward *et al*., 2001). In the present experiment, inclusion of 30% lotus hay decreased methane emission per unit of dry matter intake by 35%. Waghorn (2008) suggests that condensed tannins presence limits degradation affecting nutrient availability, ruminal enzyme activity or ruminal microorganisms and could decrease methane production. We observed lower lotus digestibility respect to other species evaluated in *in vitro* assays (Vargas *et al*., 2018), as well as lower digestibility of fodder mixture when lotus inclusion was increased (Vargas *et al*., 2014). However, in the present experiment, DM and OM digestibility were higher in animals that received kikuyu and lotus mixture therefore lower digestibility as a consequence of tannins addition cannot be the explanation for the observed lower methane production.

Tavendale *et al*. (2005) suggest that condensed tannins in lotus could affect specific methanogenic populations and we found that *in vitro,* incubation of lotus decreased methane production (Vargas *et al*., 2018). The inhibitory effect of condensed tannins on methanogenic populations may in alternative routes of use of H_2, such as propionate synthesis, nitrate reduction and lipid bio-hydrogenation (Janssen, 2010; Moss *et al*., 2000). H_2 concentration increases in animal gas exhaled soon after feeding forage:concentrate diets (Pinares-Patiño *et al*., 2011, Lopes *et al*., 2016). It has also been suggested that condensed tannins presence could affect cellulolytic bacteria (Patra and Saxena, 2010) reducing fiber degradation, then decreasing acetate production and consequently methane emission. In this study, the fiber fraction degradation and acetate proportion decreased, and the propionate proportion increased as a consequence of lotus addition, which could partially explain the lower methane emissions.

In our work we found a residual effect due to lotus inclusion for the majority of the variables evaluated despite having made an adjustment period of 15 days between experimental periods. For example, despite that methane production per unit of dry matter intake was lower in animals that received 30% lotus hay in diet, while animals fed with kikuyu diet showed a residual effect on methane production after receiving lotus hay (**Figure 1**). This observation indicates that methane production is lower in animals that have previously consumed the mixed diet. The decrease in methane production is possibly due to the inhibitory effect of tannins on some rumen microbial populations, maintained over time, at least for this experiment, for 15 days.

Results of this study suggest that *L. uliginosus* addition could be a valid strategy to reduce methane emissions in animals grazing kikuyu grass. However, other proposals should be subsequently analyzed using carbon footprint methodology in order to determine in a comprehensive manner CO_{2eq} emissions when replacing this legume in a basal diet of grasses.

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CONFLICTS OF INTEREST

The authors declare they have no conflicts of interest with regard to the work presented in this report.

AUTHOR CONTRIBUTIONS

Conceptualization and experimental design: Juan J. Vargas; Juan E. Carulla. Funding acquisition: Juan E. Carulla. Collect and analysis data: Juan J. Vargas. Writing and reviewing: Juan de J. Vargas; Martha L. Pabón, Juan E. Carulla.

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