



Effect of Fodder Tree Species with Condensed Tannin Contents on *In vitro* Methane Production

Ernestina Gutiérrez Vázquez*, Leonardo Hernández Medina, Liliana Márquez Benavides, Aureliano Juárez Caratachea, Guillermo Salas Razo, Armin Javier Ayala Burgos¹, and Ruy Ortiz Rodríguez²
Agriculture and Forestry Research Institute, Michoacan University of San Nicolas de Hidalgo, Tarímbaro Michoacán, CP 58880, México

ABSTRACT: The objective was to evaluate the effect of fodder tree species (FTS) with condensed tannin contents: *Cordia elaeagnoides*, *Platymiscium lasiocarpum*, *Vitex mollis*, and *Haematoxylon brasiletto*, on *in vitro* methane (CH₄) production at 24 h post incubation. The analysis was performed using the *in vitro* gas production technique, with three levels of inclusion/species: 600, 800, and 1,000 mg and with 4 replicates/species/level of inclusion. The substrate was incubated at 39°C, and the gas and CH₄ production were recorded at 4, 8, 12, and 24 h post incubation. The data collected was analyzed through Pearson correlation, polynomial regression and fixed effects models. There were negative correlations between FTS-total gas volume ($r = -0.40$; $p < 0.001$); FTS-volume of CH₄ produced ($r = -0.40$; $p < 0.001$) and between the inclusion level-volume of CH₄ produced ($r = -0.20$; $p < 0.001$). As well as a positive correlation between hours post incubation-total gas volume ($r = 0.42$; $p < 0.001$) and between hours post incubation-volume of CH₄ produced ($r = 0.48$; $p < 0.001$). The FTS: *C. elaeagnoides*, *V. mollis*, and *H. brasiletto* have potential, in the three inclusion levels analyzed, to reduce CH₄ emission on *in vitro* trials (>32.7%), taking into account the total CH₄ production at 24 h of the forage used as reference (*Avena sativa*). It's suggested that *C. elaeagnoides*-according to its crude protein, neutral detergent fiber, and condensed tannins content- is the best alternative within the FTS analyzed, for feeding ruminants and for the control of CH₄ emissions during the dry season. (**Key Words:** Fodder Tree Species, Condensed Tannins, Methane, *In vitro*)

INTRODUCTION

Currently, global warming is one of the most important crises in the world. Livestock, especially ruminants, are major contributors to this crisis and to the deterioration of the ozone layer by releasing large amounts of greenhouse gases into the atmosphere, including carbon dioxide (CO₂) and methane (Leng, 2010). So CH₄ mitigation strategies in ruminants have focused on obtaining economic and environmental benefits (Thornton, 2010). The nutritional

changes are more likely to be adopted by farmers because they increase the efficiency of feed utilization (Patra, 2012). Also, it has been shown that the use of different plants, such as trees and shrubs, have the potential to reduce CH₄ emissions in ruminants (Patra et al., 2006).

In the region of Tierra Caliente Michoacán, Mexico, a great variety of tree species have been referred by ranchers and identified as fodder tree species (FTS) which livestock (ruminants) consume mainly during the dry season. The FTS with condensed tannin (CT) contents, such as Cueramito (*Cordia elaeagnoides*), Atuto (*Vitex mollis*), Granadillo (*Platymiscium lasiocarpum*), and Brasil (*Haematoxylon brasiletto*), are identified in the region of *Tierra Caliente*, Michoacán, México. These trees are considered promising species in the region, since they have 4 or more of the following attributes: i) produce fruit, with possible energy supply for livestock, ii) provide comfort to the animal since they remain green (fully or partially), iii) contain less than

* Corresponding Author: Ernestina Gutiérrez Vázquez. Tel: +52-443-322-3500, E-mail: ernestinagvazquez@gmail.com

¹ Faculty of Veterinary Medicine and Zootechny, Autonomous University of Yucatan, CP 97315, México.

² Faculty of Veterinary Medicine and Zootechny, Michoacan University of San Nicolas de Hidalgo, CP 58130, México.

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50% of neutral detergent fiber (NDF) and may be highly digestible, iv) have less than 5% of tannins, in order not to affect protein digestibility and v) have a concentration equal or greater than 0.2% of phosphorus. Therefore, the objective of this study was to evaluate the effect of the FTS as a source to inhibit *in vitro* CH₄ production.

MATERIAL AND METHODS

Sampling of fodder tree species

Samples of Cueramo, Atuto, Granadillo, and Brasil were collected in December 2012 from Zacapungamio, municipality of Carácuaro (N 18° 57' 46" W 101° 03' 39") located in the region of *Tierra Caliente, Michoacán*, México. The municipality has elevations ranging from 300 to 1900 MASL. The area receives an annual rainfall of 800 to 1,000 mm, an average annual temperature greater than 22°C and winter temperature ranging around 18°C (INEGI, 2000). The leaves of the FTS were harvested (approximately 4 kg of each tree was collected, consisting of a pool of several trees from the same species) and transported for laboratory analysis to the Institute of Agriculture and Forestry Research, located in the municipality of *Tarimbaro Michoacán*, México. The samples were air dried indoors followed by oven drying for 48 h at 70°C and ground to pass a 1 mm sieve prior to chemical analysis and *in vitro* incubations.

Chemical composition analysis

Ground samples were analyzed by standard procedures. Total ash (AOAC, 1995; method 942.05) and dry matter (DM) (AOAC, 1995; method 930.15), crude protein (CP)(AOAC, 1995; method 984.13) and ether extract (EE)(AOAC, 1995; method 920.29) were determined; neutral detergent fiber (NDF) and acid detergent fiber (ADF) using the procedure of Van Soest et al. (1991); CT and total phenols (TP) using the technique by Price and Butler (1997).

In vitro gas production

Rumen liquor donor: Rumen fluid was obtained from a Creole bull (400 kg BW), which was fed oat hay (*ad libitum*), supplemented with minerals and had access to clear water (to simulate the feeding conditions during the dry season). The ruminal inoculum was obtained before the morning feeding (07:00 am) using a rumen fluid extraction equipment designed by Geishauser (1993). The collected rumen fluid was placed in warm insulated thermos (39°C) under anaerobic conditions and transported immediately to the laboratory. In the laboratory it was strained through four layers of cheesecloth and kept at 39°C under a N₂ atmosphere until utilization.

Culture medium and in vitro incubations: The culture medium and the *in vitro* gas production test were carried out according to Menke and Steingass (1988), modified by using 70 mL serum bottles instead of calibrated glass syringes. The culture media consist of distilled water, macro and micromineral solution, buffer solution, resazurin and a reducing solution. It was prepared and inoculated using aseptic and anaerobic techniques. For the fermentation, 600, 800 and 1,000 mg of each species were incubated (with 4 replicates/species/level of inclusion) along with 10 mL of rumen fluid and 20 mL of the culture media. Additionally, serum bottles containing oat hay (without CT contents) were incubated in quadruplicate, serving as control for the incubation, no blanks were included. The bottles were sealed with rubber stoppers (Suba-Seal rubber septa network # 33, Sigma Aldrich, Milwaukee, WI, USA), the biogas in the headspace was flushed with N₂ and the bottles were incubated without agitation at 39°C for a period of 24 h. After each reading (4, 8, 12, 24 h post incubation) the bottles were agitated and returned to the incubator.

Determination of gas and methane production: To quantify the CH₄ production a gas chromatograph (GC) was used (Varian CP-3800, Walnut Creek, CA, USA), equipped with a flame ionization detector and a packed column Silica Gel 60/80 dimensions 18'×1/8" stainless steel, the operating temperatures of the injector, the detector and the column were 170°C, 170°C, and 90°C respectively. With N₂ as the carrier gas at a flow rate of 30 mL/min. For analysis of CH₄ production, 30 µL were obtained from the headspace of each bottle with a gas-tight syringe (Hamilton Company, Reno, NV, USA; chromatography syringes) and injected in the GC at 4, 8, 12, and 24 h post incubation. Gas production was measured using a biogas measurement system (Beuvink et al., 1992).

Statistical analysis

With the gathered data, a database was developed for statistical analysis using Pearson correlation for continuous variables, the omega squared statistics of Hays (ω^2) to determine the degree of association between nominal and continuous variables, polynomial regression, and fixed effects models. The differences between treatments were tested using a least square means test (LSMEANS). The statistical package used was Scientific Applied Statistics (SAS, 2000). The model used to estimate polynomial methane gas production/FTS was:

$$\hat{Y}_i = \beta_0 + \beta_1 \times N + \beta_2 \times t + \beta_3 \times t^2$$

Where, \hat{Y}_i = CH₄ production from: oat hay, Cueramo, Granadillo, Atuto, and Brasil, N = FTS inclusion level, t = hours post-incubation.

While for the fixed effects analysis the following model was used:

$$Y_{ij} = \mu + T_i + E_{ij}$$

Where, Y_{ij} = *in vitro* CH₄ production at 24 h, μ = overall mean, T_i = fixed effect of the *i*th treatment with *i* = oat hay at 600, 800, and 1,000 mg inclusion; Cueramo at 600, 800, and 1,000 mg inclusion; ... Brasil at 600, 800, and 1,000 mg inclusion, E_{ij} = error (NID ~ 0, δ^2).

RESULTS

Chemical composition

In order to evaluate the nutritional quality of the FTS during the dry season, the chemical composition analysis was done. Ash content in two FTS was >100 g/kg DM (Table 1). CP from the four FTS was higher than the reference feed (oat hay), and varied from 88 g/kg DM in Granadillo to 167 g/kg DM in Cueramo. The EE was very similar in all FTS but was much higher (32 to 35 g/kg DM) compared to the reference (6 g/kg DM). NDF in the FTS was very similar, ranging from 409 g/kg DM in Cueramo to 498 g/kg DM in Atuto. ADF varied from 220 g/kg DM in Cueramo to 339 g/kg DM in Atuto. Both NDF and ADF in FTS were lower than the reference oat hay with 633 and 392 g/kg DM, respectively.

CT and TP varied among the FTS (Table 1). Brasil showed the highest concentration of both CT and TP with 119 and 196 g/kg DM, respectively; in comparison with Cueramo, which contained the lowest concentrations of CT and TP with 0.2 and 22 g/kg DM, respectively.

Effect of fodder tree species on *in vitro* CH₄ production

The degree of association observed between FTS and total volume of gas *in vitro* ($\omega^2 = 0.57$; $p < 0.05$) and between FTS and volume of CH₄ produced ($\omega^2 = 0.53$, $p < 0.05$) was moderate (<70%) (Table 2). Implying that 57% and 53% of the variance in the volume of gas and CH₄ produced *in vitro*, respectively, can be attributed to the presence of the FTS analyzed (Figure 1). The total average of CH₄ production at 24 h was 44.7±28 mL/g DM. Average affected by treatment

Table 2. Association degree (ω^2) between nominal and continuous variables and Pearson correlation coefficients for continuous variables: gas volume and methane (CH₄)

	FTS	Inclusion level	Hours post incubation	Gas volume	
	ω^2	ω^2	ω^2	r	R
Gas volume	0.57**	0.04**	0.30**	0.42**	1.0
CH ₄ volume	0.53**	0.12**	0.24**	0.48**	0.87**

FTS, fodder tree species; ω^2 , Omega squared statistics; r, Pearson correlation coefficients.

** $p < 0.001$.

(FTS and inclusion level; $p < 0.001$), hours post-incubation ($p < 0.001$) and the interaction between treatment and hours post incubation ($p < 0.001$).

Regarding the effect of the treatment on total CH₄ production (at 24 h post-incubation), it was found that CH₄ production was lower ($p < 0.05$) with the species Cueramo and Brasil at the three inclusion levels analyzed (Table 3, Figure 2). The percent in CH₄ reduction from the FTS compared to oat hay (reference) showed a trend among the three levels of inclusion (Table 4). Granadillo was the species with the least CH₄ inhibited with a maximum of 18.6%.

DISCUSSION

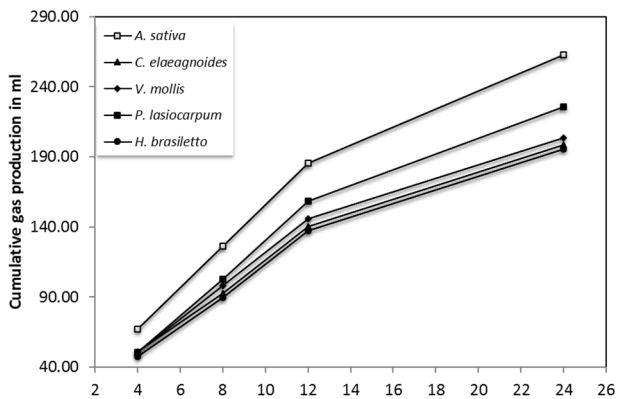
The use of plant secondary metabolites has been previously studied for the inhibition of CH₄ production in ruminants. Hess et al. (2006) established that the strategic use of tannins is a means to limit CH₄ emission 13%. This is consistent with other researchers (Min et al., 2006), who determined that CT decreases (in a dose-dependent response) the rate of *in vitro* gas production. Dietary CP and NDF concentrations influence the amount of substrate organic matter fermented and the short chain fatty acids that are produced (Njidda and Nasiru, 2010). Crude protein concentrations below the threshold of 70 g CP/kg DM, restrict microbial activity due to a lack of nitrogen (Hariadi and Santoso, 2010). However, protein levels above this threshold, as seen in this study, are considered to enhance microbial multiplication in the rumen, thus improving fermentation (Njidda and Nasiru, 2010).

Table 1. Chemical composition and secondary metabolites of the experimental plants (g/kg DM)¹

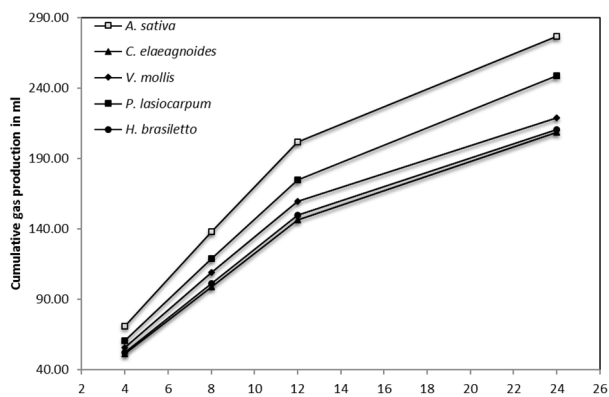
Species	Ash	Crude protein	Ether extract	NDF	ADF	CT	TP
Oat hay	97	78	6	633	392	ND	ND
Atuto	99	121	33	498	339	10.1	92.2
Cueramo	149	167	35	409	220	0.2	22.1
Granadillo	105	88	35	476	270	0.9	44.3
Brasil	93	112	32	488	303	119.3	196.2

DM, dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; CT, condensed tannins, equivalent to catequin; TP, total phenols, equivalent to gallic acid; ND, not detected.

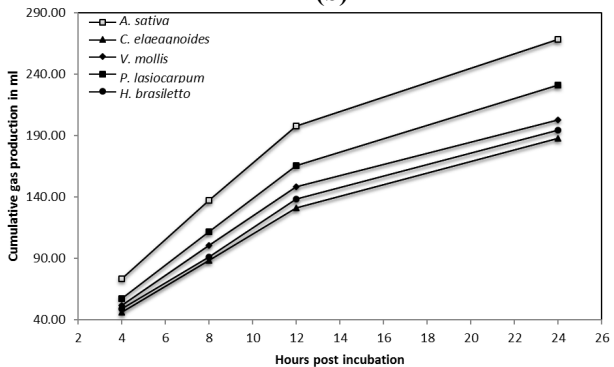
¹ Samples pooled (n = 4).



(a)

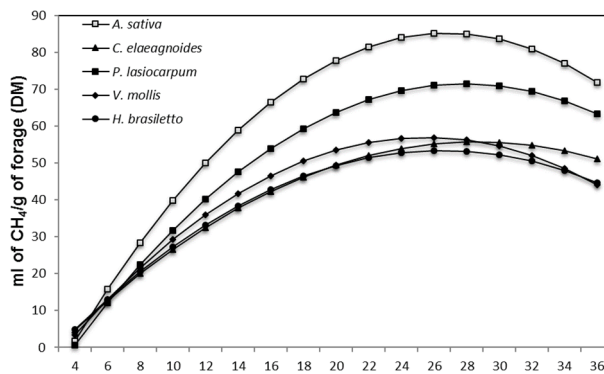


(b)

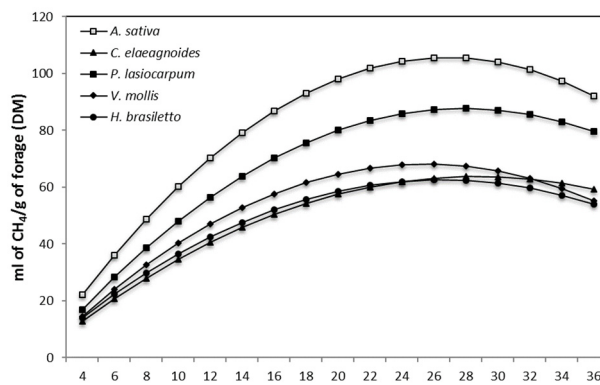


(c)

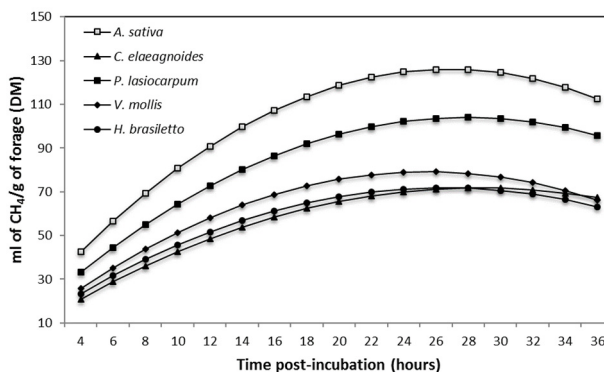
Figure 1. Cumulative gas production at 24 h post incubation with (a) 1,000 mg, (b) 800 mg and (c) 600 mg of FTS and with oat hay as reference forage. FTS, fodder tree species.



(a)



(b)



(c)

Figure 2. CH₄ production at 24 h post-incubation with (a) 1,000 mg, (b) 800 mg and (c) 600 mg of FTS and with oat hay as reference forage. FTS, fodder tree species.

Table 3. Polynomial regression coefficients to determine CH₄ volume produced at 24 h post incubation according to the species, inclusion level and h post incubation

Species	Intercept β_0	Inclusion level β_1	Hours β_2	Hours ² β_3
Oat hay	71.9596639**	-102.0765625**	8.5966661**	-0.1601629**
Cueramo	27.21563076**	-40.3234375**	4.80322169**	-0.08381**
Atuto	37.16741022**	-55.55**	5.92138755**	-0.11633489**
Granadillo	56.30807551**	-81.303125**	6.91893934**	-0.12409861**
Brasil	32.21148941**	-46.175**	5.08404293**	-0.09595419**

** p<0.001.

Table 4. CH₄ reduction according to the FTS, inclusion level and hour of maximum production with respect to oat hay

Species	Inclusion level (mg DM)	MPCH ₄ * (mL)	CH ₄ reduction (%)
Oat hay	1,000	66.6	-
	800	109.9	-
	600	136.1	-
Atuto	1000	44.8	32.7
Granadillo	1000	54.2	18.6
Cueramo	1000	43.1	35.3
Brasil	1000	41.9	37.1
Atuto	800	72.6	33.9
Granadillo	800	90.6	17.6
Cueramo	800	66.2	39.8
Brasil	800	63.9	41.9
Atuto	600	85.8	37.0
Granadillo	600	112.4	17.4
Cueramo	600	76.2	44.0
Brasil	600	76.9	43.5

FTS, fodder tree species.

* Average CH₄ production from the start of incubation to 24 h post incubation.

Nevertheless, Jayanegara et al. (2009) suggest that CT, in synergy with fiber digestion, decrease CH₄ production. The FTS in the region of *Tierra Caliente, Michoacán*, contain concentrations of CT, CP concentrations above the threshold and lower NDF concentrations than the rough forages used as feed during the dry season; thus, the four species used in our study were selected in order to test their capacity to decrease CH₄ production *in vitro*.

Effect of fodder tree species on *in vitro* CH₄ production

Total CH₄ production decreased (mL/g DM) as the CT content increased, which occurred with the species Granadillo, Atuto, and Brasil. This was consistent with Bhatta et al. (2009) and Jayanegara et al. (2011); they reported a negative correlation between tannin content and CH₄ production. Similarly, other researches (Meagher et al., 2005; O'kiely et al., 2011) concluded that a greater quantity of secondary metabolites present in FTS will lead to a lower *in vitro* gas and CH₄ production.

The degree of association between level of inclusion and volume of CH₄ produced with the FTS was weak ($\omega^2 = 0.12$; $p < 0.001$), thus 12% of the variance in CH₄ production can be explained by the level of inclusion of the FTS analyzed. However, in other studies (Bhatta et al., 2009; Huang et al., 2010) it was reported that the greater the inclusion level of CT, the lower the CH₄ produced. This is consistent with the results found in this experiment (Table 4). As for the association between hours post-incubation and volume of gas produced ($\omega^2 = 0.30$; $p < 0.001$ and $r = 0.42$; $p < 0.001$) and between hours post-incubation and volume of CH₄ produced ($\omega^2 = 0.24$; $p < 0.001$ and $r = 0.48$,

$p < 0.001$), these determined that as the hours of incubation elapse, gas volume and CH₄ production increase. Similarly, Abdalla et al. (2012) determined that the profile of cumulative gas production at 24 h is in function of the hours post-incubation, and depends on the CT concentration.

The reduction in CH₄ production by the species Brasil is attributed to its high CT content. Tannins have been shown to reduce CH₄ production by reducing fiber digestion (Tiemann et al., 2008); binding with proteins thus reducing degradation of the plant protein in the rumen and through the direct inhibition of the growth of methanogens (Tavendale et al., 2005). It has been established that a greater concentration of CT will lead to a lower total CH₄ production (Huang et al., 2010). Carulla et al. (2005) attributed the inhibition of CH₄ production from the species *A. mearnsii* to the direct effect of the CT.

The FTS Cueramo showed the least concentration of CT in comparison to the other species analyzed, but had a greater concentration of CP (167 g/kg DM) and a lower concentration of NDF (409 g/kg DM) compared to the rest of the FTS and oat hay (Table 1). The CP and the NDF content in Cueramo may be the responsible for the inhibition of CH₄ production. Kume (2002) suggest that CH₄ reduction is attributed to a low content of NDF and a high content of CP. This is consistent with Meagher et al. (2005), they analyzed the species *M. sativa*, without CT, with 348 g of NDF/kg DM and 301 g of CP/kg DM, which led to a reduction in the volume of CH₄ produced. Jayanegara et al. (2009) determined that high concentrations of NDF in forages causes an increase of CH₄ production, due to a shift in the proportion of short chain volatile fatty acids towards acetate, which produces more H₂.

It is generally accepted that feedstuffs which have higher gas production tend to have higher CH₄ production per gram DM incubated (Njidda and Nasiru, 2010; Jayanegara et al., 2011). This tendency was evident in the forages used in this study. This relationship is suggested to be a reflection of feed digestibility (Holtshausen et al., 2009). Increasing cell wall content (i.e. NDF and ADF) is considered to suppress microbial activity through a reduction in the availability of rapidly fermented carbohydrates (Wilson and Hatfield, 1997). This in turn, is negatively correlated with gas production (Njidda and Nasiru, 2010). This is consistent with the results obtained from the forage used as reference which had high concentrations of NDF (632 g/kg DM).

However, Getachew et al. (1998) indicate that the concentration level of the substrate reduces ruminal gas production per gram of DM, due to the low proportion of microorganisms in relation to the substrate or due to the buffer depletion. This could be associated with the decrease in CH₄ production by Cueramo and Brasil analyzed at a concentration of 1,000 mg.

CONCLUSIONS

The species with the greatest potential in CH₄ reduction were Brasil and Cueramo. The FTS with the most CH₄ produced at 24 h of incubation was Granadillo in the three inclusion levels (600, 800, and 1,000 mg). According to its CP, NDF, and CT content, Cueramo is the best alternative for ruminant nutrition during the dry season in the region of Tierra Caliente Michoacán and showed the best CH₄ reduction potential during the *in vitro* trials. However, there is a great variety of FTS which are used as a source of feed during the dry season in the region and with a CT content that will have no negative effects in ruminant nutrition. Further research is needed toward finding better alternatives in ruminant feeding and to determine how the FTS in the region of Tierra Caliente Michoacán can inhibit CH₄ production.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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