In vitro nutrient degradability and methane production of two dairy diets as affected by nutraceutical plants inclusion levels

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Abstract The present study investigated the effects of two nutraceutical plants – Petiveria alliacea and Waltheria indica on in vitro gas production, dry matter degradability (DMD) and methane production of two dairy cow diets (high forage [HF] and high concentrate [HC]). A factorial arrangement of two diets and two nutraceutical plants with four levels of inclusion (2.5, 5.0, 7.5 and 10.0%) was used. Results demonstrate substrate x inclusion level interactions were significant (P<0.05) for gas volume, DMD and methane production. P. alliacea at 2.5 and 5.0% suppressed gas production in high forage (HF) by 7.5 and 7.9%, respectively and W. indica inclusion levels of 2.5 and 5.0% increased gas production. All inclusion levels of P. alliacea suppressed gas production in high concentrate (HC) diet and the lowest gas volume was noted in the 10.0% treatment. Contrary to this observation, 2.5% inclusion level of W. indica had the lowest gas volume in the HC diet. Compared with the control, DMD of HF diet was improved with the inclusion of both plants except for P. alliacea at 2.5 and 5.0%. There was a significant decrease in methane production across all levels of inclusion for both medicinal plants and substrates; P. alliacea at 10.0% decreased methane by 31.2%. Substrate x inclusion level interactions (P<0.001) were noted for neutral detergent fiber degradability (NDFD) and acid detergent digestibility (ADFD). P. alliacea at 2.5% and 10% inclusion levels reduced NDFD in HF and HC diets, respectively. A similar trend was noted with P. alliacea inclusion. Based on the present results, inclusion of P. alliacea above 5.0% and W. indica at all inclusion levels were able to improve DMD. Both medicinal plants reduced methane concentration irrespective of inclusion level.

Keywords:
Batch culture, dry matter, feed, methane emission, plant nutraceuticals

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Introduction
Ruminants are major contributors to enteric methane production (Bodas et al. 2008). According to the IPCC (2007), ruminant animals produce 10-12% of the world's anthropogenic greenhouse gas emissions in carbon dioxide equivalents, and 2-15% of feed gross energy is lost in the form of methane (Johnson and Johnson 1995). The world population is expected to reach 8.5 billion by 2030, and this will invariably lead to increased demand for ruminant animal products - in the form of milk or meat (United Nations 2022). Recent research efforts have focused on improving dietary feed efficiency through inhibition of methane production.

In the recent past, concerns have been on how to effectively increase animal production with a corresponding reduction in greenhouse gas emissions (Hristov et al. 2013; Brice et al. 2022). The addition of plants rich in secondary metabolites in the feeds of ruminants have been suggested as a strategic nutritional-based approach to methane mitigation (Rira et al. 2015; Meljekal et al. 2017). Some of these plants are referred to as nutraceutical plants because they confer both nutritional and health benefits (Lopreiato et al. 2020; Torres-Fajardo et al., 2021; Uushona et al. 2021) to the animals.

Many of these nutraceutical plants have been known to have antimicrobial properties (Cowan 1999; Egamberdieva et al. 2017; Hossan et al. 2018). Nutraceutical plants have been reported as mitigators of enteric methane (Rira et al. 2015). Nutraceutical plants are known to improve ruminal fermentation efficiency (Khiaosa-ard and Zebeli, 2013) and are rumen modifiers (Khattab et al. 2020). *Petiveria alliacea* and *Waltheria indica* are commonly found in the tropical and subtropical climates of the world (Wagner et al. 1990) and are widely established in Florida USA (Wunderlin et al. 2022). They are widely known for their medicinal values and secondary metabolites such as tannin, saponins and polyphenols (Zongo et al. 2013; San Andres Larrea et al. 2014). There is limited information on the anti-methanogenic properties and feed efficiency potential of these two plants. Hence, the aim of this study was to evaluate the effects of inclusion levels of *P. alliacea* and *W. indica* in two dairy diets on *in vitro* gas production, nutrient degradability and methane production.

Methodology
The two nutraceutical plants used in the present study (*P. alliacea* and *W. indica*) were selected based on their known medicinal properties and location in the tropical climate. Plant materials
(i.e., leaves) were air-dried at room temperature and ground to pass through a 1 mm sieve. Samples were preserved in tightly closed Ziploc bag and stored in a refrigerator maintained at 2°C. The two dietary substrates were high concentrate (HC) made up of grain products, processed grains byproducts, plant protein products, molasses products, multi vitamins and mineral supplements and high forage (HF, corn silage). The samples were collected from North Carolina Agricultural and Technical State University Farm. The dietary substrates were dried in a forced air oven at 55°C to constant weight and milled through a 1 mm sieve and used for the in vitro batch culture.

The in vitro batch culture study was done to evaluate the effects of different inclusion levels of the two nutraceutical plants on in vitro gas production, methane, dry matter and fiber digestibility of the two dietary substrates (HC and HF). The study was arranged as a 2 × 9 factorial design to evaluate the effects of the two nutraceutical plants at four inclusion levels (2.5, 5, 7.5 and 10%) as well as the control (i.e., no additive) on the two dietary substrates (HF and HC). The in vitro incubation procedure was based on the method described by Anele et al. (2014). The Ankom bags used for this study were washed in acetone, dried and labeled and weighed. Approximately 0.5 ± 0.05 g of the substrates were measured into their respective bottles. Artificial saliva preparation was based on McDougall’s recipe and maintained in a water bath at 39°C under continuous flushing with CO₂ until dispensed into the serum bottles. Ruminal fluid was collected 3 h after feeding from two ruminally cannulated dairy cows that were fed 18% protein grain, corn silage, and alfalfa hay. The batch culture media was dispensed into the 100 mL glass serum bottles in a 3:1 of artificial saliva-rumen fluid under constant flushing with carbon dioxide at 39°C. The bottles were capped with a rubber stopper and crimped with an aluminum seal cap. The serum bottles were incubated for 24 h at 39°C and agitated at a speed of 125 rpm using a orbital shaker. At 24 h post incubation, gas production was measured using a manometer. Methane concentration was estimated using a portable gas analyzer (Biogas 5000, Landtec, Dexter, MI, USA). Immediately after gas reading and methane determination, the Ankom bags were removed from the serum bottles, rinsed, and dried in a 55°C oven for 48 h and calculated for in vitro dry matter digestibility according to Brice et al. (2022). After incubation, the contents of the bags were digested using Ankom 200 Fiber Analyzer (Ankom, Macedon, NY, USA) to estimate neutral detergent fiber (NDF) and acid detergent fiber (ADF) concentrations. The NDF
content was determined as described by Van Soest et al. (1991) using heat stable α-amylase with sodium sulfite. Acid detergent fiber was determined according to Association of Official Analytical Chemists [AOAC] (1995). Acid detergent lignin (ADL) was determined by soaking in concentrated sulfuric acid based on ANKOM Technologies analytical methods. Data generated were analyzed using the GLM procedure of SAS 9.4 (SAS Inst., Inc., Cary, NC, USA) in a 2 × 9 factorial arrangement. The means were separated using Duncan’s multiple comparisons test at $P < 0.05$.

**Results**

The current study was aimed to determine the effects of *P. alliacea* and *W. indica* leaves on dry matter degradability (DMD), total gas and methane production of two dairy diets using the *in vitro* gas production technique. The effects of inclusion levels of *P. alliacea* and *W. indica* leaves on *in vitro* gas production, digestibility, and methane concentration of the two diets is shown in Table 1. Substrate x inclusion level interactions were significant ($P<0.05$) for total gas production, methane concentration and nutrient degradability except for ADLD. Total gas production of HF was reduced by 7.5% and 7.9% with *P. alliacea* inclusion at 2.5% and 5.0%, respectively. Contrary to this observation, *W. indica* inclusion increased gas production in HF except for 10.0% inclusion level. For the HC, all inclusion levels of *P. alliacea* reduced total gas production and only 2.5% inclusion level reduced total gas production in *W. indica*. The increase in total gas production with the inclusion of *W. indica* in both substrates is consistent with previous studies (Goel et al. 2008; Dey et al. 2014; Sarkar et al. 2018). Higher gas production could be as a result of improved DMD as more gas is released with increasing nutrient digestibility.

Higher DMD with the inclusion of the nutraceutical plants is consistent with previous reports by Goel et al. (2008) and Sarkar et al. (2018) which showed that the inclusion levels of the nutraceuticals encouraged the population and activities of microbes associated with DM degradation.

The inclusion levels of both nutraceutical plants reduced methane in both substrates with *P. alliacea* at 2.5% in HF and *P. alliacea* at 10.0% in HC, reducing methane by 23.8 and 31.2%, respectively. Consistent with results in the present study, Bhatta et al. (2015) reported a reduction in methane production of some tropical tree leaves using in vitro rumen fermentation.
Although there is limited information on the methane reduction potential of the nutraceutical plants in the current study, there are documented reports that they are rich in secondary metabolites such as tannins (Zongo et al. 2013; San Andres Larrea et al. 2014). Tannins are known to reduce total gas and methane production (Bhatta et al. 2009). The reduction in methane production with the inclusion of both nutraceuticals could help reduce energy loss in form of methane in ruminants.

The NDFD of HF was suppressed by the inclusion levels of the nutraceutical plants except for \textit{W. indica} at 2.5 which improved NDFD by 5.1%. For the HC diet, only \textit{P. alliacea} at 10.0% reduced NDFD. The inclusion of \textit{W. indica} at 5.0% improved NDFD of HC the most by 11%. The inclusion of \textit{P. alliacea} at 7.5 and 10.0% and \textit{W. indica} at 5.0 and 7.5% improved ADFD of HF. For HC, the inclusion of \textit{P. alliacea} at 10.0% and \textit{W. indica} at 5.0% improved ADFD by 2 and 10.5%, respectively. The improvement of NDFD of HF by the inclusion of \textit{W. indica} at 2.5 and 10.0% is consistent with a previous report by Tekipp et al. (2012) where different plants of North American origin (\textit{Artemisia absinthium}, \textit{A. atra} and \textit{A. annua}) were evaluated as additives in a batch culture. Consistent with results noted for HC in the present study, Bhat et al. (2018) and Ishtiyak et al. (2010) reported an increase in NDFD when \textit{A. absinthium} and \textit{Trigonella foenumgraecum} were used as feed additives. Current results showed that the nutraceutical plants improved the activities and/or population of fiber degrading bacteria and that they might be dose or inclusion level dependent. For HC, NDFD was reduced at 10.0% inclusion of \textit{P. alliacea} which could suggest a suppression of the activities of the fiber degrading bacteria and that inclusion level above 7.5% might not be useful.

**Conclusions**

From this study, it can be concluded that the inclusion levels of \textit{P. alliacea} above 5.0% and \textit{W. indica} at all levels of inclusion were able to improve DMD and reduce methane production.
Table 1. Effect of nutraceutical plant inclusion levels on total gas, in vitro nutrient degradability and methane production of two dairy dietary substrates

<table>
<thead>
<tr>
<th>NPIL (%)</th>
<th>Gas</th>
<th>DMD</th>
<th>Methane</th>
<th>NDFD</th>
<th>ADFD</th>
<th>ADLD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HF</td>
<td>HC</td>
<td>HF</td>
<td>HC</td>
<td>HF</td>
<td>HC</td>
</tr>
<tr>
<td>Control</td>
<td>92.8&lt;sup&gt;g&lt;/sup&gt;</td>
<td>108&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38.7&lt;sup&gt;e&lt;/sup&gt;</td>
<td>47.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PA2.5</td>
<td>85.8&lt;sup&gt;i&lt;/sup&gt;</td>
<td>103&lt;sup&gt;d&lt;/sup&gt;</td>
<td>38.8&lt;sup&gt;e&lt;/sup&gt;</td>
<td>47.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.58&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.57&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PA5.0</td>
<td>85.5&lt;sup&gt;i&lt;/sup&gt;</td>
<td>102&lt;sup&gt;d&lt;/sup&gt;</td>
<td>39.0&lt;sup&gt;e&lt;/sup&gt;</td>
<td>50.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.61&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.47&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>PA7.5</td>
<td>95.3&lt;sup&gt;f&lt;/sup&gt;</td>
<td>101&lt;sup&gt;d&lt;/sup&gt;</td>
<td>42.1&lt;sup&gt;ed&lt;/sup&gt;</td>
<td>50.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.09&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>3.85&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>PA10.0</td>
<td>94.5&lt;sup&gt;f&lt;/sup&gt;</td>
<td>87.0&lt;sup&gt;h&lt;/sup&gt;</td>
<td>43.8&lt;sup&gt;ed&lt;/sup&gt;</td>
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<td>4.07&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>3.64&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>WI2.5</td>
<td>102&lt;sup&gt;d&lt;/sup&gt;</td>
<td>102&lt;sup&gt;d&lt;/sup&gt;</td>
<td>43.3&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>50.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.27&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.93&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>WI5.0</td>
<td>98.6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>115&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.9&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>4.65&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>WI7.5</td>
<td>94.0&lt;sup&gt;f&lt;/sup&gt;</td>
<td>116&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.8&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>4.73&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>WI10.0</td>
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<td>112&lt;sup&gt;b&lt;/sup&gt;</td>
<td>40.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>53.8&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>1.8023</td>
<td>0.7799</td>
<td>0.7799</td>
<td>0.1011</td>
<td>0.1011</td>
</tr>
</tbody>
</table>

P-value

| Substrate | 0.0012 | 0.0012 | <.0001 | <.0001 | 0.0254 | 0.0254 | <.0001 | <.0001 | <.0001 | <.0001 | 0.1215 | 0.1215 |
| NPIIL     | 0.3742 | 0.3742 | 0.0748 | 0.0748 | 0.0373 | 0.0373 | 0.0502 | 0.0502 | 0.0117 | 0.0117 | 0.5228 | 0.5228 |
| Substrate*NPIL | 0.0017 | 0.0017 | <.0001 | <.0001 | 0.0392 | 0.0392 | 0.0001 | 0.0001 | 0.4381 | 0.4381 |

NPIL: Nutraceutical plant inclusion level; PA: P. alliacea; WI: W. indica; SEM: Standard error of means; HF: High forage; HC: High concentrate

<sup>a</sup>-<sup>1</sup>:Means with different superscripts within the same column differ, p < 0.05
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