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The Impact of Varying Levels of *Laurus nobilis* Leaves as a Sustainable Feed Additive on Ruminal Fermentation: In Vitro Gas Production, Methane and Carbon Dioxide Emissions, and Ruminal Degradability of a Conventional Diet for Ruminants

Ahmed E. Kholif [1,2]

[1] Department of Animal Sciences, North Carolina Agricultural and Technical State University, Greensboro, NC 27411, USA; ae_kholif@live.com or aekholif@ncat.edu
[2] Dairy Science Department, National Research Centre, 33 Bohouth St. Dokki, Giza 12622, Egypt

Abstract: The experiment aimed to evaluate the effects of varying levels of *Laurus nobilis* leaves [0% (control), 0.5%, 1%, 1.5%, and 2%] on the in vitro ruminal fermentation of a ruminant diet consisting of a 50% concentrate mixture, 40% berseem hay (*Trifolium alexandrinum*), and 10% rice straw (*Oryza sativa*). The in vitro incubation lasted 48 h, during which gas production (GP), methane (CH_4), carbon dioxide (CO_2), total and individual short-chain fatty acids (SCFA), and nutrient degradability were measured. The experiment utilized a randomized block design and consisted of two incubation runs. Gas chromatography analysis revealed that 1,8-cineole (81%) was the primary volatile compound in the *L. nobilis* leaves. The 0.5% inclusion level exhibited the highest (linear, $p = 0.006$) asymptotic GP and lowest lag of GP (linear, $p = 0.002$), while the 2% inclusion level had the highest lag of GP. The 2% inclusion level significantly lowered CH_4 (linear, $p = 0.003$) compared to the control, and all levels of the leaves linearly decreased in the proportional CH_4 production ($p = 0.001$), with the lowest value at the 0.5% inclusion level. The highest asymptotic CO_2 production was observed with the 0.5% inclusion level (linear, $p = 0.002$), while the 0.5%, 1%, and 1.5% inclusion levels significantly increased (quadratic, $p = 0.006$) the proportion of CO_2 compared to the control. The 0.5% inclusion level showed the highest ($p < 0.001$) degradable DM and fiber fractions compared to the control, whereas the 2% level decreased them. The 0.5% inclusion level resulted in the highest ($p < 0.01$) production of total SCFA, acetate, and propionate. Additionally, the 0.5% inclusion level demonstrated the highest ($p < 0.05$) metabolizable energy and microbial crude protein, while the 2% level reduced these measures compared to the control. It is concluded that *L. nobilis* leaves can be included at 0.5% of the ruminant diet (e.g., sheep) to improve ruminal fermentation and reduce CH_4 production.

Keywords: degradation; essential oils; greenhouse gases; in vitro fermentation; *Laurus nobilis*

Citation: Kholif, A.E. The Impact of Varying Levels of *Laurus nobilis* Leaves as a Sustainable Feed Additive on Ruminal Fermentation: In Vitro Gas Production, Methane and Carbon Dioxide Emissions, and Ruminal Degradability of a Conventional Diet for Ruminants. *Fermentation* **2024**, *10*, 387. https://doi.org/10.3390/fermentation10080387

Academic Editor: Guijie Chen

Received: 11 July 2024
Revised: 24 July 2024
Accepted: 26 July 2024
Published: 28 July 2024

1. Introduction

Greenhouse gas emissions from livestock contribute to about 14.5% of global anthropogenic gas emissions, posing a significant environmental concern [1]. Methane (CH_4) is particularly problematic, being 28 times more potent than carbon dioxide (CO_2) in terms of its heat-trapping capacity [2]. Moreover, CH_4 production also reduces animal energy efficiency, with 10% of energy being lost through CH_4 production [3]. Therefore, mitigating CH_4 emissions from ruminal fermentation is crucial, as approximately 71% of the CH_4 produced by animals originates from this process [4]. Over the past decade, intensive research has focused on identifying anti-methanogenic feed additives (e.g., plant secondary metabolites) to reduce enteric CH_4 emissions [5–8]. Nonetheless, only a few dietary approaches have been shown to be safe and effective in both in vitro and in vivo settings by the US Food and Drug Administration.

The use of feed additives derived from natural sources such as plant leaves and seeds, essential oils, plant extract, microalgae, etc., has garnered increased interest for organic and sustainable animal production to improve animal performance and reduce CH_4 emissions [7,9–11]. These organic feed additives are favored because they avoid concerns related to antibiotic residues or chemical additives [12,13]. *Laurus nobilis* is one of these additives that may be sustainably used in feed to improve animal performance.

Laurus nobilis L. is a tree native to Mediterranean regions and belongs to the Lauraceae family [14]. *L. nobilis*, known as sweet bay, is an evergreen tree known for its glossy green, spicy leaves and berries. *L. nobilis* leaves have been used for many years in the diet of human beings as a food flavor due to their functional effects on human health [14]. The leaves have strong antibacterial, anti-inflammatory, antioxidant, and other health-promoting qualities because of their abundant bioactive compounds [15]. The additive contains up to 4% methyleugenol [16]. The main components in the leaves are 1,8-cineole (known as eucalyptol), estragole, and α-terpinyl acetate [14].

Recently, *L. nobilis* leaves gained interest from animal nutritionists to evaluate their efficacy as a sensory additive for all animal species [16]. The EU is currently authorized to use *L. nobilis* leaves as a feed additive under European Commission No. 1831/2003 [16]. Khayyal et al. [17] fed Rahmani × Finnish Landrace lambs a diet supplemented with *L. nobilis* leaves at 0.5%, 1.5%, and 2% for 20 weeks, and observed no effects on ruminal pH, ammonia-N (NH_3-N), or short-chain fatty acids (SCFA) concentrations. Sızmaz [18] used the RUSITC technique to investigate the in vitro fermentation of a diet with a 48:52 ratio of hay to concentrate, supplemented with laurel essential oil at concentrations of 50 and 100 mg/L of the fermenter liquid. This study found that the essential oil did not impact rumen fermentation measured by pH, NH_3-N, and SCFA, apparent nutrient degradation, CH_4 emissions, bacterial number, and protozoa, including Holotrichs and Entodiniomorphs. By administering eucalyptus oil with eucalyptol (1,8-cineole) as the primary active component, Sallam et al. [19] evaluated its effect on in vitro fermentation. They observed a significant reduction in gas production (GP) and CH_4 emissions (up to 85.3%) without impacting DM and organic matter (OM) degradation.

The suitability of utilizing *L. nobilis* leaves as a supplement in animal feed has only been the subject of a small number of research to date. Therefore, the aims of this experiment were to assess the effects of varying the amount of dried *L. nobilis* leaves in a total mixed diet on the in vitro fermentation of ruminants, CH_4 and CO_2 productions, and GP. Our hypothesis was that the dried *L. nobilis* leaves' phytochemicals would influence the ruminal fermentation and enhance nutrient degradability.

2. Materials and Methods

2.1. Ingredients and Treatments

To be utilized as substrates, a basal total mixed ration (TMR) containing (per kg DM) 500 g concentrate feed mixture, 400 g berseem hay (*Trifolium alexandrinum*), and 100 g rice straw (*Oryza sativa*) was formulated. The incubated substrate or diet is the same as the control diet previously used by others [6,7,9,20]. Table 1 displays the nutrients' concentrations in *L. nobilis* leaves as well as the ingredients and TMR.

Table 1. Chemical composition of *Laurus nobilis* leaves and incubated diet (g/kg DM).

	Laurus nobilis	**CFM [1]**	**Berseem Hay**	**Rice Straw**	**Diet [2,3]**
Dry matter	940.4	903.2	890.1	940.3	892.8
Organic matter	955.0	922.9	884.4	851.1	819.4
Crude protein	58.7	165.0	128.3	41.8	135.7
Ether extract	101.2	46.8	54.4	18.8	61.8

Table 1. *Cont.*

	Laurus nobilis	CFM [1]	Berseem Hay	Rice Straw	Diet [2,3]
Nonstructural carbohydrates	415.1	414.0	224.2	166.1	359.1
Neutral detergent fiber	380.0	297.1	477.5	624.4	379.0
Acid detergent fiber	229.7	175.1	380.7	394.1	239.8

[1] Concentrate feed mixture (CFM) was made up of 170 g of soybean meal, 395 g of wheat bran, 395 g of corn, 20 g of limestone, 10 g of a combination of vitamins and minerals, and 10 g of salt per kilogram. [2] Diets: 500 g of concentrate mixture, 400 g of berseem hay (*Trifolium alexandrinum*), and 100 g of rice straw (*Oryza sativa*) were contained per kilogram of DM. [3] Previously used by others as a control diet [6,7,9,20].

Dry and clean *L. nobilis* leaves were purchased from a local supplier in Egypt. Before use, the leaves were ground and mixed. At the Central Laboratory of National Research Centre (Dokki, Giza, Egypt), the volatile compounds in the leaves were quantified using a capillary column ZB-5 (60 m × 0.32 mm i.d.; J & W Agilent Technologies Inc., Palo Alto, CA, USA) and a Perkin Elmer Auto System XL GC/MS (J & W Agilent Technologies Inc., Palo Alto, CA, USA). The analysis was carried out in accordance with Qin et al. [21] with some modifications. The temperature was set for three minutes at 100 °C, and then it was raised to 240 °C at a pace of 2.5 °C per minute, where it remained for ten minutes. The injector and flame ionization detector (FID) were calibrated to operate at 250 °C and 285 °C, respectively. With a split vent flow ratio of 1:10, helium was utilized as the carrier gas at a rate of 1 mL/min. Direct introduction of the GC column effluent to the MS source was made. Spectra with an ionization energy of 70 eV were observed in the EI mode. A one-second scan from 40 to 300 amu was programmed into the sector mass analyzer. By comparing the compounds' relative retention time and mass spectra with those of the NIST and WILLY libraries, a preliminary identification of the compounds was carried out.

2.2. In Vitro Fermentation and Biodegradation

Ruminal contents from three fattened Barki male sheep (42 ± 0.6 kg body weight, 25 ± 3 weeks old) were collected from a nearby abattoir in Cairo, Egypt. The sheep were allowed unrestricted access to water and fed a diet consisting of concentrates, berseem hay, and rice straw at a ratio of 500:400:100 (DM basis) for about 15 weeks prior to slaughter. Sheep were fasted for twenty-four hours before being slaughtered. The standardized process for sampling, storing, and using ruminal contents that Fortina et al. [22] advocated was followed while collecting the rumen contents. Less than ten minutes passed at the slaughterhouse between the animal's slaughtering and the collection of rumen fluid. Using a colander, around 150–250 g of the rumen contents were manually sampled and squeezed into a plastic beaker. This technique was repeated until about 1000 mL of rumen fluid was collected. Large feed particles were removed from the collected ruminal contents by filtering it through two layers of cheesecloth. The inoculum's initial pH ranged from 6.8 to 6.9.

Goering and Van Soest's method [23] was followed in the preparation of the in vitro fermentation medium. Just before the rumen fluid was introduced, 2 mL of a sodium sulfide reduction solution was added to the buffer. A mixture of 20 mL ruminal inoculum and 80 mL buffer solution was contained in each 250 mL bottle.

An automatic wireless in vitro GP module (Ankom RF Gas Production System, Ankom Technology, Macedon, NY, USA) with pressure sensors (Ankom Technology, Macedon, NY, USA) was installed in 250 mL ANKOM bottles after a 1 g ± 10 mg sample of TMR was weighed into filter bags (ANKOM F57; Ankom Technology, Macedon, NY, USA). The amount of *L. nobilis* leaves added to the diet was 0 (control), 0.5% (0.005 g), 1% (0.01 g), 1.5% (0.015 g), and 2% (0.02 g). The experiment was repeated twice (2 incubations) in two different weeks. The doses of *L. nobilis* leaves were carefully weighed into the filter bags using a Luna Analytical Balance (LAB 124e, Adam Scales and Balances, Thetford, UK). To establish baseline fermentation GP, two bottles with inoculum but no feed (blanks) were also added to each incubation run (5 treatments × 3 replicates × 2 incubation runs + 2 blank

bottles). The average of the 3 bottles was the experimental unit, which provided a total of three experimental units for testing each of the additive's levels. For 48 h, pressure was measured every 10 min. The total pressure was computed using these readings. At standard pressure and temperature, the gas pressure was translated into volume (mL). To calculate net GP, the gas volume in the blank units was deducted. To quantify the concentration of CH_4 and CO_2, gas samples (5 mL) were obtained from the sampling vent and infused into a Gas-Pro detector (Gas Analyzer CROWCON Model Tetra3, Abingdon, UK) at 2, 4, 6, 8, 10, 12, 24, 36, and 48 h of incubation.

2.3. Sampling and Analysis of Fermentation Variables

At the end of the 48 h incubation period, the bottles were placed on ice for five minutes to stop the fermentation. A pH meter (Thermo Scientific, Orion StarTM A121, Beverly, MA, USA) was used to measure the pH. After that, the ANKOM F57 filter bags were dried for 48 h at 55 °C in a forced air oven. The weights of the dried residue and the initial weight of the dried substrate were subtracted to determine the DM, and neutral detergent fiber (NDF), acid detergent fiber (ADF), and degradation as:

$$\text{Nutrient degradability} = \frac{\text{Initial amount of nutrient} - \text{residual amount of nutrient}}{\text{Initial amount of nutrient}} \times 100.$$

Following a 48 h incubation period, the total gas, CH_4, and CO_2 produced were expressed in relation to the degraded DM (*d*DM), NDF (*d*NDF), and ADF (*d*ADF).

Glass tubes were used to collect samples (5 mL) of the supernatant fermented fluid from each bottle to measure the amounts of NH_3-N, total SCFAs, and individual SCFAs. In accordance with AOAC [24], a subsample of 3 mL was preserved with 3 mL of 0.2 M hydrochloric acid for the determination of NH_3-N concentration. To prepare an aliquot (0.8 mL) for SCFA analysis using steam distillation and the titration method, 0.2 mL of a metaphosphoric acid solution (250 g/L) was combined with it.

2.4. Chemical Analysis

According to AOAC [24] methods, samples of *L. nobilis* leaves, ingredients, and TMR were dried at 55 °C for 48 h to determine DM concentration (method ID 930.15). The samples were burned at 550 °C for 12 h in a muffle furnace to measure ash concentration (method ID 942.05). Crude protein (CP) was measured using the Kjeldahl method (method ID 954.01), and ether extract (EE) was measured using diethyl ether in Soxhlet extractors (method ID 920.39). The samples were then analyzed for ash content. Using sodium sulfite and alpha amylase, the NDF content was ascertained by following Van Soest et al.'s protocol [25]. The ADF content was measured using the AOAC [24] (method ID 973.18), and the results were expressed without accounting for residual ash. The concentrations of OM (100 − ash) and non-structural carbohydrates (1000 − NDF − CP − EE − ash) were determined.

2.5. Calculations and Statistical Analyses

The NLIN procedure of SAS (Version 9.4, SAS Institute Inc., Cary, NC, USA) was used to fit data of total GP, CH_4, and CO_2 (mL/g DM) in accordance with the model of France et al. [26]: $y = A \times [1 - e^{-c(t-Lag)}]$ for the estimation of GP, CH_4, and CO_2 kinetics, where A is the asymptotic GP, CH_4, or CO_2 (mL/g DM); c is the fractional rate of GP, CH_4, or CO_2 (per h); Lag (h) is the discrete lag time before any gas, CH_4, or CO_2 release; and y is the volume of total GP CH_4 or CO_2 production at time t (h).

According to Blümmel et al. [27], the partitioning factor during 24 h of incubation (PF_{24}) was estimated as mg *d*DM/mL gas. The volume of gas produced (mL/200 mg DM) at 24 h incubation (GY_{24}) was calculated. Menke et al.'s formula was used for metabolizable energy (ME) calculation [28]. The production of microbial crude protein (MCP) was estimated in accordance with Blümmel et al. [27].

Data were analyzed using the mixed procedure of SAS in a randomized block design. Each run formed a block, and the experimental unit was specified as the additive level within each block. The model, $Y_{ijk} = \mu + L_i + R_j + (L \times R)_{ij} + \varepsilon_{ijk}$, was employed where Y_{ijk} is the observation, μ is the population mean, L_i is the *L. nobilis* leaves' level effect, R_j is the run (block) effect, $(L \times R)_{ij}$ is the interaction between run and additive level, and ε_{ij} is the residual error. Linear and quadratic contrasts were used to determine the level responses (increasing *L. nobilis* leaves' levels). The effect of run and interaction between run and additive level were nonsignificant (i.e., $p > 0.05$) for most of the measurements; thus, only the main effects of additives are reported.

3. Results

3.1. Laurus nobilis

The essential oil in the laurel leaves was about 3.29% of its weight. The GC analysis showed that the leaves of *L. nobilis* leaves contained 12 volatile compounds (Table 2). Eucalyptol (1,8-cineole) (81.01%), estragole (5.9%), and α-terpinyl acetate (3.91%) were the major compounds.

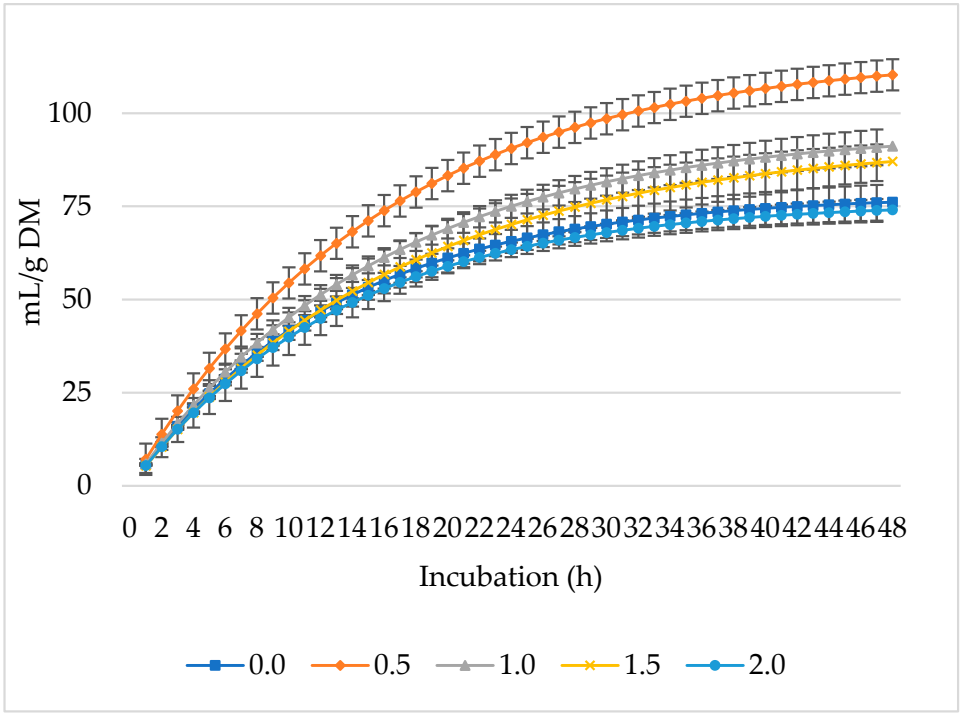
Table 2. Volatile compounds in *Laurus nobilis* leaves identified by GC-MS analysis.

Peak	Compound [1]	Formula	RT [2]	Concentration [3]
1	α-Pinene	$C_{10}H_{16}$	3.67	0.91
2	β-Pinene	$C_{10}H_{16}$	4.75	2.47
3	1,8-cineole	$C_{10}H_{18}O$	6.78	81.01
4	sabinene hydrate isomer	$C_{10}H_{18}O$	8.06	0.5
5	Fenchone	$C_{10}H_{16}O$	8.52	1.18
6	Alloocimene	$C_{10}H_{16}$	8.95	0.33
7	Linalool	$C_{10}H_{18}O$	9.15	0.78
8	Camphor	$C_{10}H_{16}O$	9.93	0.73
9	α-Terpineol	$C_{10}H_{18}O$	11.18	1.06
10	Estragole	$C_{10}H_{12}O$	11.35	5.9
11	α-Terpinyl acetate	$C_{12}H_{20}O_2$	14.17	3.91
12	trans-Caryophyllene	$C_{15}H_{24}$	15.23	0.44

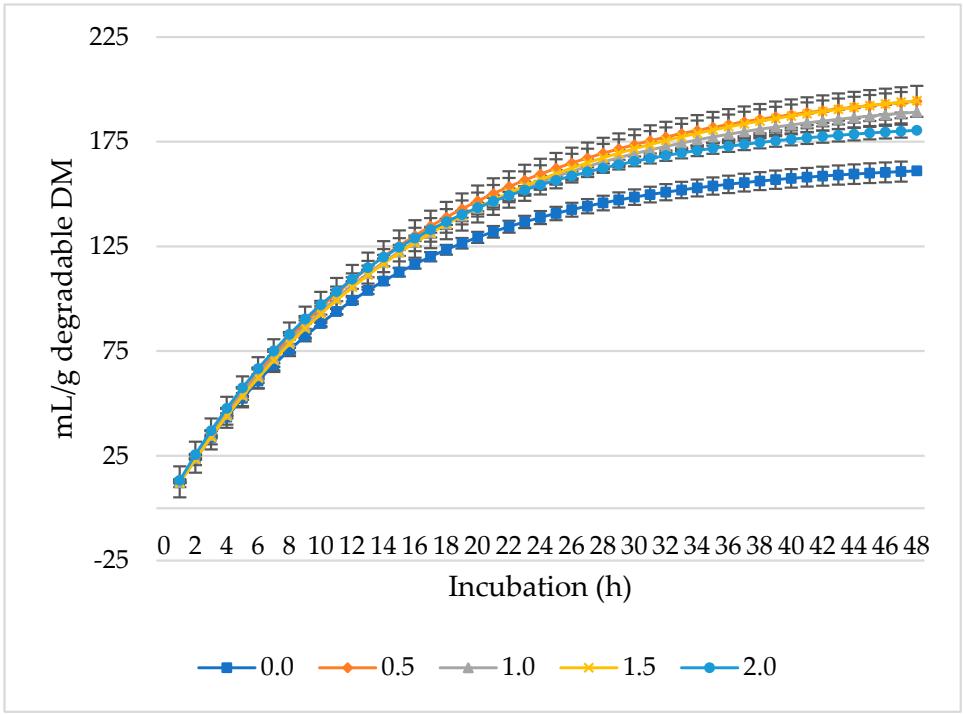
[1] Authentic standards, literature, and library spectra from the National Institute of Standards and Technology (NIST) were used to identify the samples. [2] RT stands for retention time in minutes. [3] Concentration (%) based on the total areas of the identified peaks.

3.2. Biogases Production

Figures 1–3 represent GP, CH_4, and CO_2 as mL/g DM, *d*DM, *d*NDF, and *d*ADF. The maximum GP was noted at 0.5% inclusion of *L. nobilis* leaves, followed by a 1% and 1.5% dietary inclusion. Gas production (mL/g DM) rose linearly with incubation hours (Figure 1A). The lowest GP observed at the 2% *L. nobilis* leaves inclusion. Conversely, 0.5%, 1%, and 1.5% inclusion of *L. nobilis* leaves resulted in optimal GP per gram of *d*DM (Figure 1B), *d*NDF (Figure 1C), and *d*ADF (Figure 1D). The 0.5% inclusion level achieved the highest (linear, $p = 0.006$; quadratic, $p < 0.001$) asymptotic GP, followed by 1% and 1.5%, while the 2% inclusion did not affect it compared to the control level (Table 3). Without affecting the rate of GP, the level 0.5% showed the lowest lag time, while the level 2% showed the highest one (linear, $p = 0.002$, quadratic, $p = 0.039$).

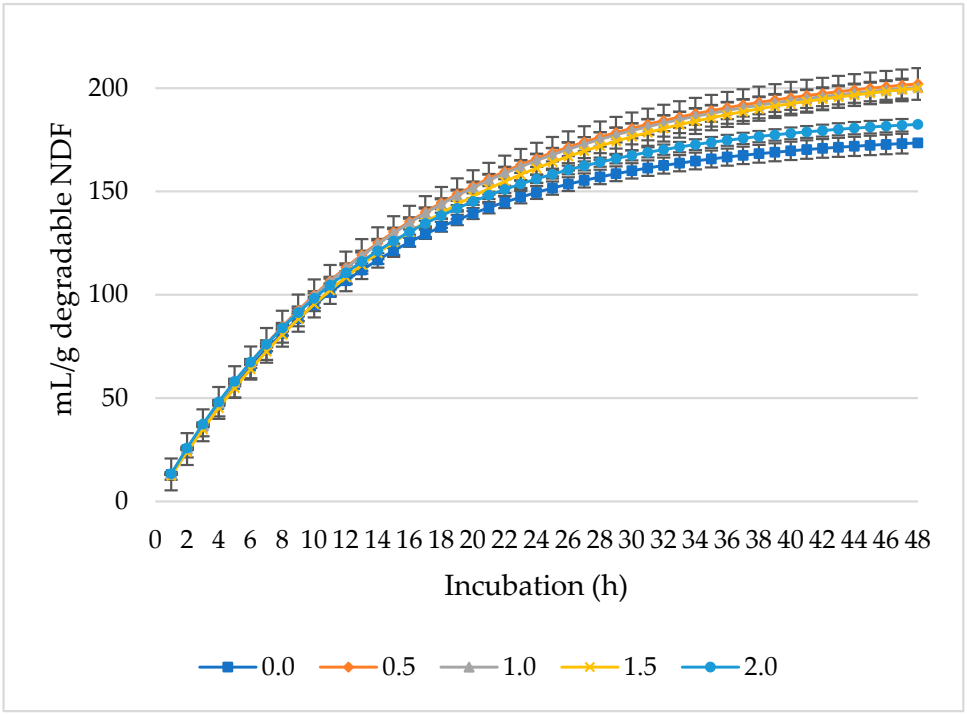


(**A**)



(**B**)

Figure 1. *Cont.*



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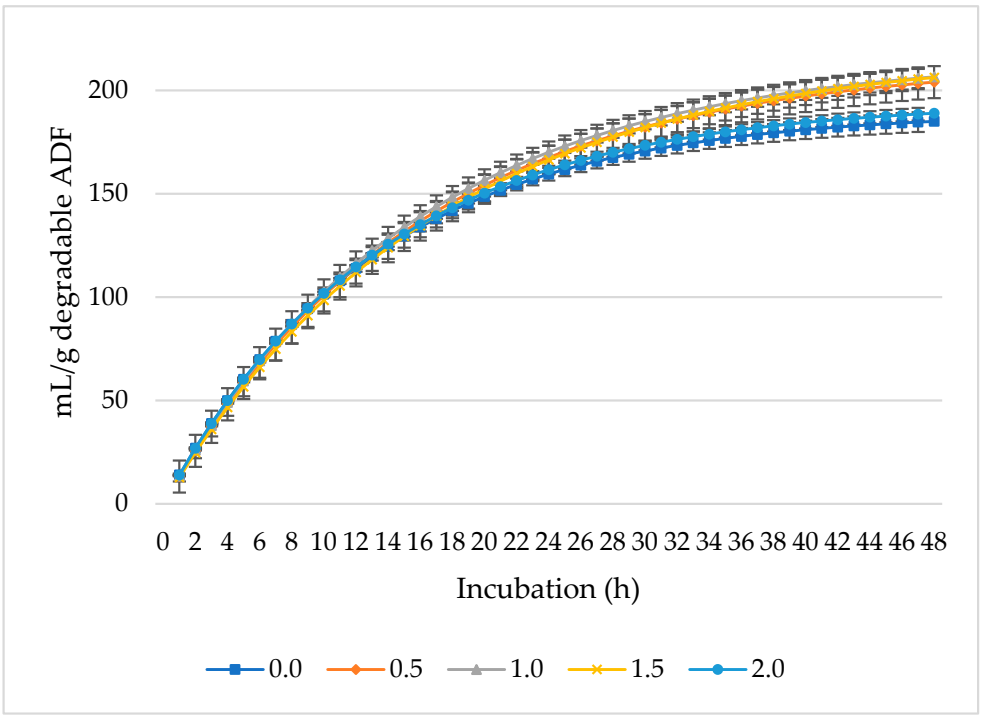
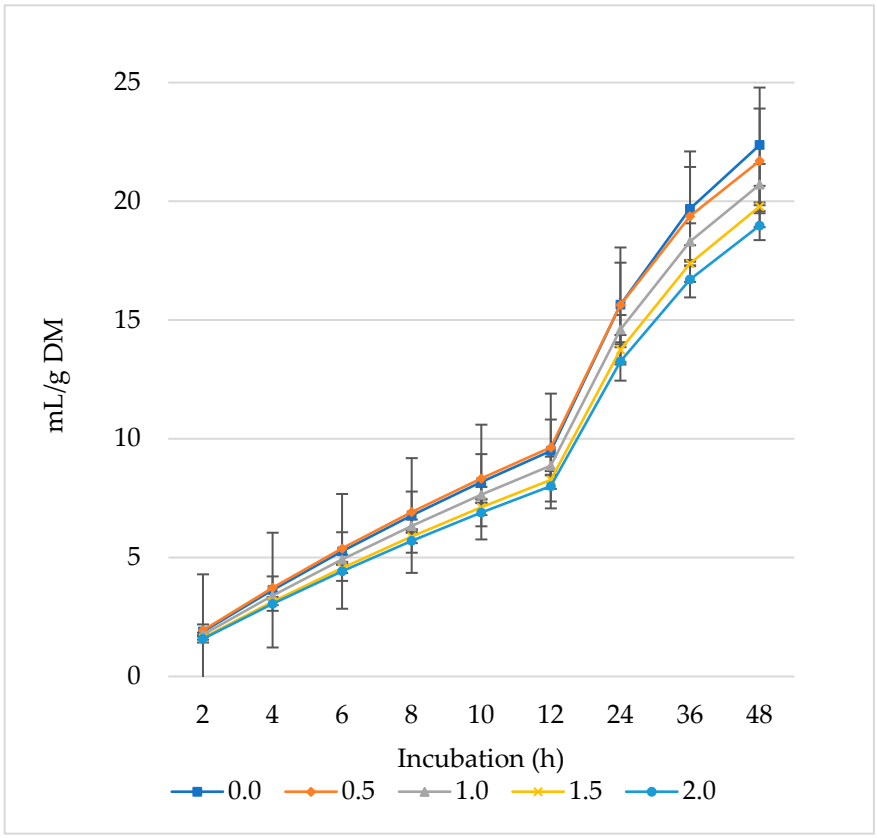


(**D**)

Figure 1. In vitro ruminal gas production: mL/g incubated DM (**A**), mL/g degradable DM (**B**), mL/g degradable NDF (**C**), mL/g degradable ADF (**D**) of a total mixed ration supplemented with different levels of *Laurus nobilis* leaves. ADF refers to Acid detergent fiber, and NDF refers to neutral detergent fiber.



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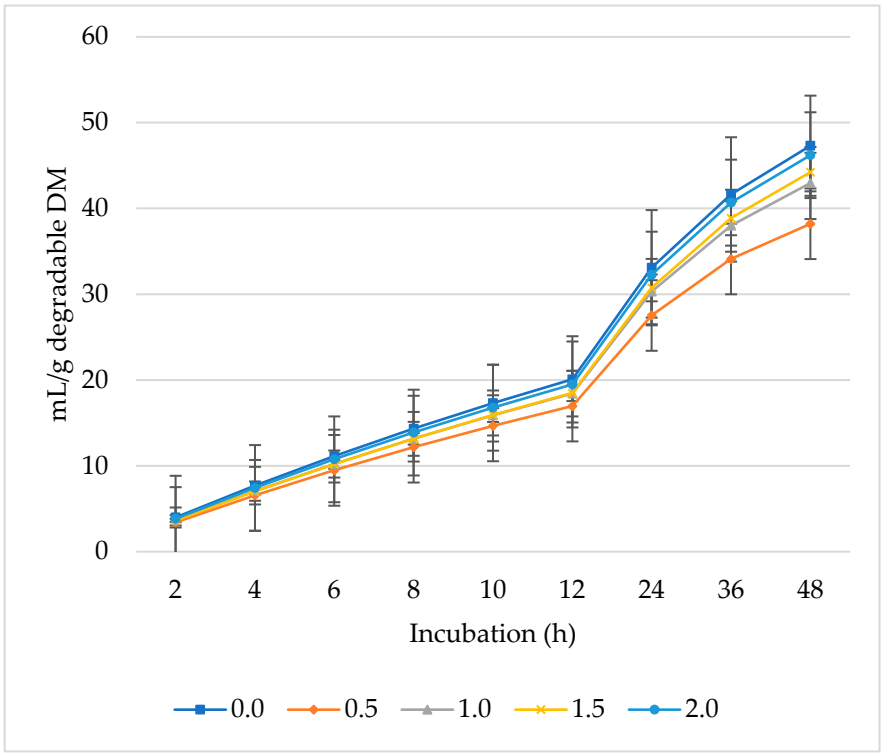
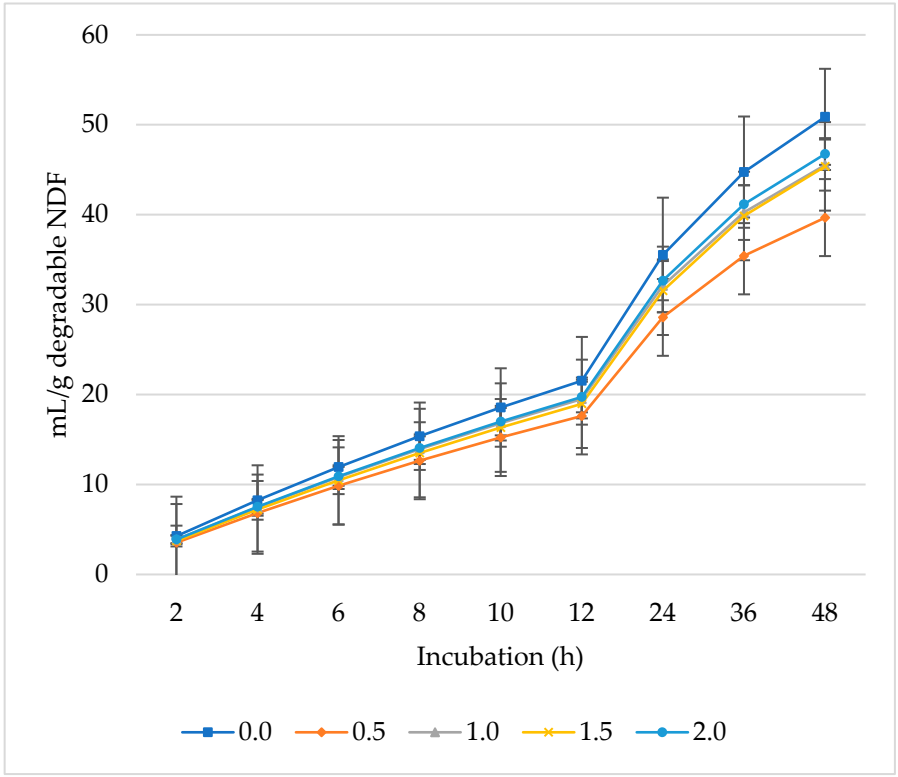


(**B**)

Figure 2. *Cont.*



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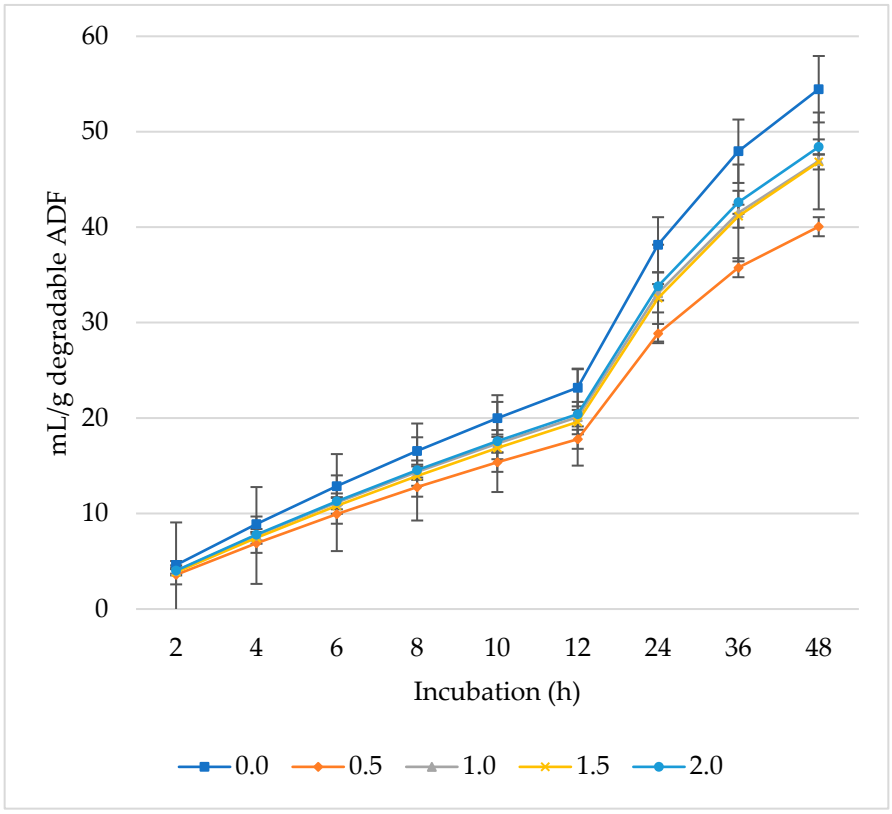
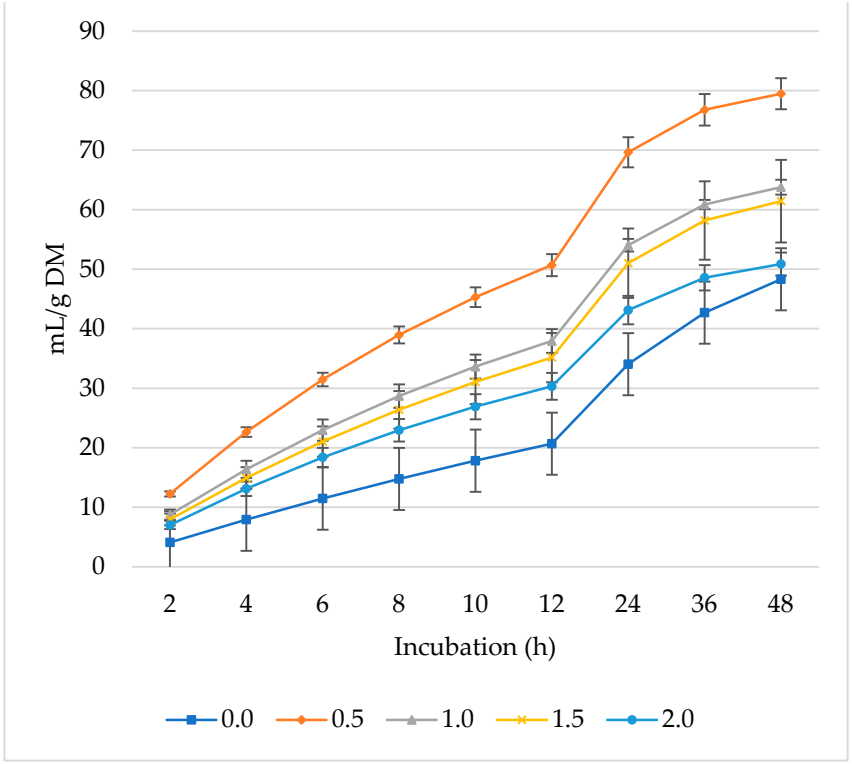


(**D**)

Figure 2. In vitro ruminal methane (CH_4): mL/g incubated DM (**A**), mL/g degradable DM (**B**), mL/g degradable NDF (**C**), mL/g degradable ADF (**D**) of a total mixed ration supplemented with different levels of *Laurus nobilis* leaves. ADF refers to Acid detergent fiber, and NDF refers to neutral detergent fiber.



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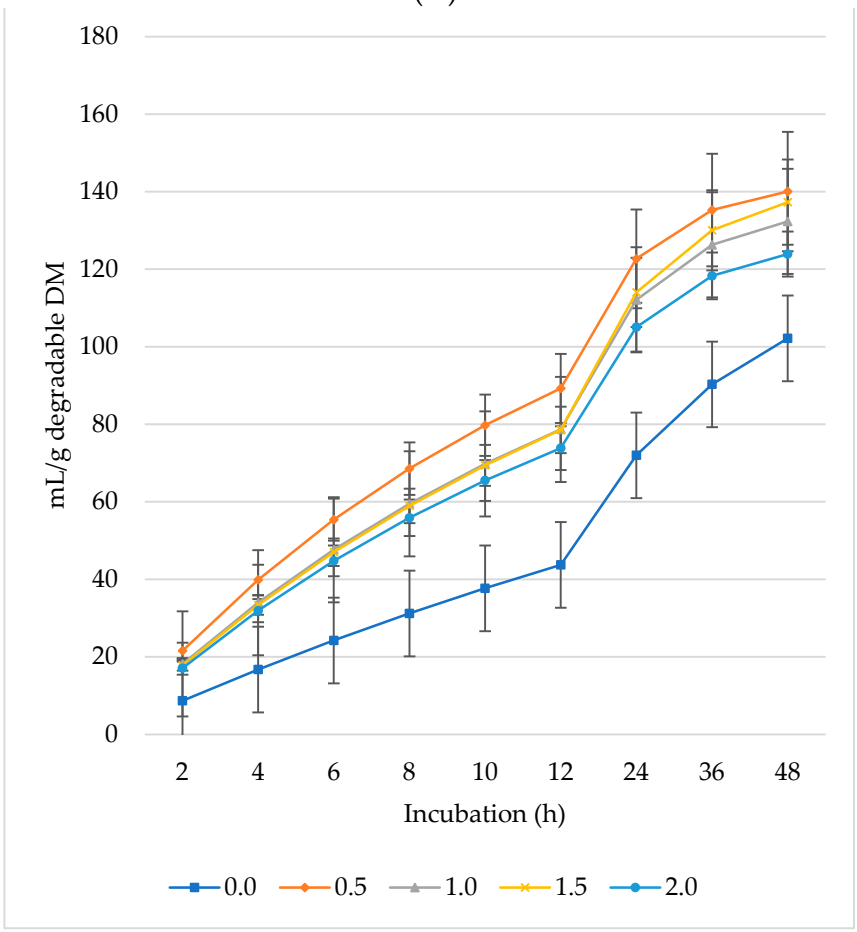

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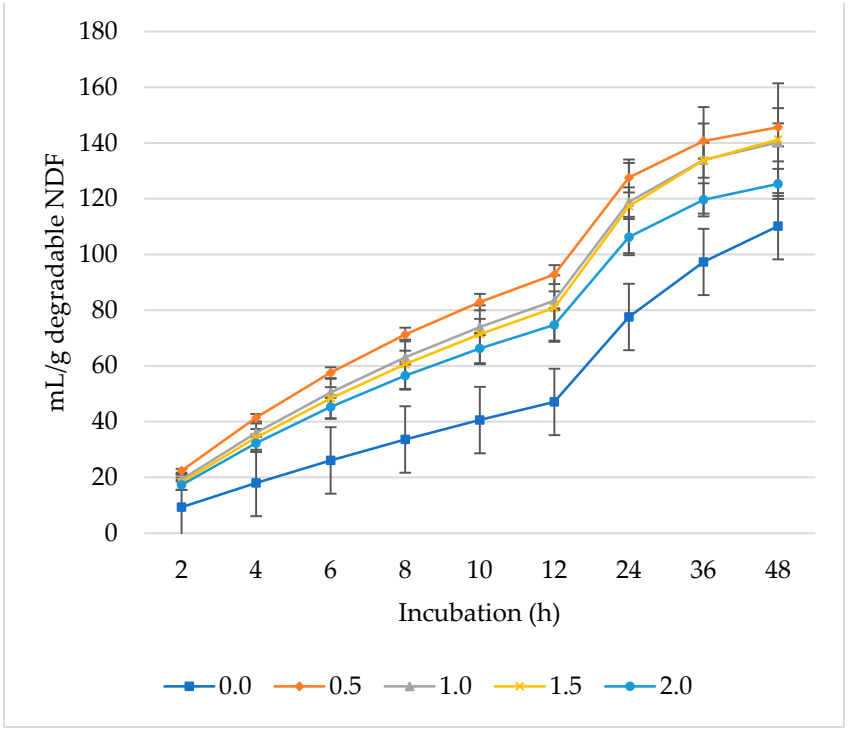
Figure 3. *Cont.*



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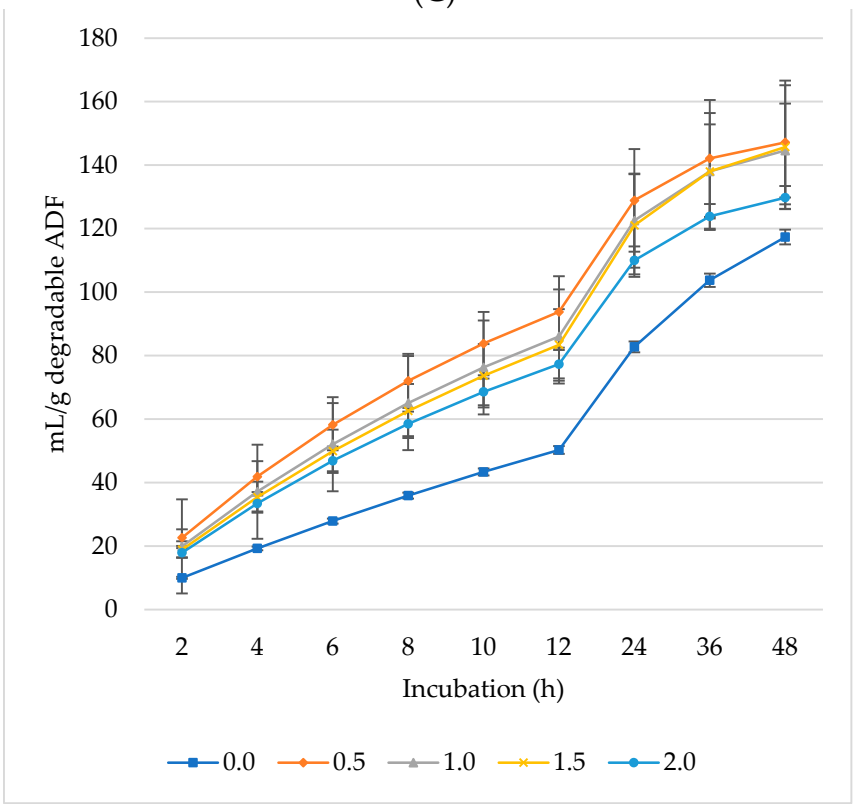

(**D**)

Figure 3. In vitro ruminal carbon dioxide (CO_2): mL/g incubated DM (**A**), mL/g degradable DM (**B**), mL/g degradable NDF (**C**), mL/g degradable ADF (**D**) of a total mixed ration supplemented with different levels of *Laurus nobilis* leaves. ADF refers to Acid detergent fiber, and NDF refers to neutral detergent fiber.

Table 3. In vitro rumen gas production (GP), methane (CH_4), and carbon dioxide (CO_2) kinetics as affected by increasing levels of *Laurus nobilis* leaves (%, DM).

	GP Parameters [1]			CH_4 Parameters [2]				CO_2 Parameters [3]			
Level	***A***	***c***	**Lag**	***A***	***c***	**Lag**	**% [4]**	***A***	***c***	**Lag**	**% [4]**
0	78.1 ^{c}	0.076	1.55 ^{bc}	28.6 ^{a}	0.035	1.42 ^{b}	29.3 ^{a}	58.8 ^{bc}	0.036 ^{c}	2.47	63.5 ^{b}
0.5	115.9 ^{a}	0.064	1.47 ^{c}	25.6 ^{ab}	0.039	1.65 ^{ab}	19.7 ^{c}	81.2 ^{a}	0.082 ^{a}	2.27	72.1 ^{a}
1	95.6 ^{b}	0.064	1.58 ^{bc}	25.2 ^{ab}	0.036	1.74 ^{ab}	22.7 ^{bc}	65.9 ^{b}	0.071 ^{ab}	2.27	70.1 ^{a}
1.5	92.7 ^{b}	0.060	1.82 ^{ab}	24.5 ^{ab}	0.034	1.97 ^{a}	22.8 ^{bc}	64.1 ^{b}	0.066 ^{b}	2.05	70.5 ^{a}
2	76.3 ^{c}	0.074	1.96 ^{a}	23.4 ^{b}	0.035	1.87 ^{a}	25.7 ^{ab}	52.6 ^{c}	0.072 ^{ab}	2.13	68.7 ^{ab}
SEM	2.42	0.0072	0.064	0.90	0.0035	0.088	0.99	2.180	0.0031	0.197	1.38
p value											
Treatment	<0.001	0.117	0.001	0.023	0.841	0.010	0.005	<0.001	<0.001	0.626	0.012
Linear	0.006	0.486	0.002	0.003	0.701	0.001	0.001	0.002	0.002	0.176	0.072
Quadratic	<0.001	0.102	0.039	0.321	0.636	0.126	0.214	<0.001	<0.001	0.658	0.006

^{a,b,c} Different superscripts indicate a difference in means ($p < 0.05$) within the same column. The standard error of the mean is denoted by SEM, and the observed significance level of the *F*-test for the treatment effect is represented by the *p*-value. [1] GP parameters: *A* is the asymptotic GP (mL/g DM), *c* is the rate of GP (per h), and Lag is the first delay before GP starts (h). [2] Methane (CH_4) production characteristics are as follows: *A* is the asymptotic CH_4 (mL/g DM), *c* is the rate of CH_4 (per h), and Lag is the first delay before CH_4 starts (h). [3] Carbon dioxide (CO_2) production characteristics are as follows: *A* is the asymptotic CO_2 (mL/g DM), *c* is the rate of CO_2 (per h), and Lag is the first delay before CO_2 starts (h). [4] The proportion at the end of incubation (48 h).

Ruminal CH_4 production decreased per gram DM (Figure 2A), *d*DM (Figure 2B), *d*NDF (Figure 2C), and *d*ADF (Figure 2D) with increasing levels of *L. nobilis* leaves in the diet. Without significant differences compared to the control, the 0.5%, 1%, and 1.5% inclusion levels gradually decreased asymptotic CH_4 production, while the 2% inclusion level significantly showed the lowest production ($p = 0.0032$) compared to the control (Table 3). All levels of *L. nobilis* leaves linearly decreased proportional CH_4 production ($p = 0.001$), with the lowest value observed at the 0.5% inclusion level. None of the *L. nobilis* leaves' inclusion levels affected the rate; however, the levels 1.5% and 2% linearly increased ($p = 0.001$) the lag phase of CH_4 production.

The production of CO_2 increased per gram DM (Figure 3A), *d*DM (Figure 3B), *d*NDF (Figure 3C), and *d*ADF (Figure 3D) with all levels of *L. nobilis* leaves in the diet. The 0.5% inclusion level significantly (linear, $p = 0.002$; quadratic, $p < 0.001$) increased asymptotic CO_2 production compared to the control, with no significant differences observed between the other inclusion levels compared to the control (Table 3). All inclusion levels increased the rate of CO_2 production (linear, $p = 0.002$; quadratic, $p < 0.001$) compared to the control. The 0.5%, 1%, and 1.5% inclusion levels significantly increased ($p = 0.006$) the proportion of CO_2 compared to the control. None of the inclusion levels affected the rate or the lag phase of CO_2 production.

3.3. Degradability and Fermentation

The 0.5% level of *L. nobilis* leaves exhibited the highest *d*DM (linear and quadratic $p < 0.001$), *d*NDF (linear $p = 0.045$, quadratic $p = 0.043$), and *d*ADF (linear $p = 0.041$, quadratic $p = 0.42$) compared to the control, whereas the 2% level decreased all these parameters (Table 4).

Compared to the control, the additive did not affect the proportions of individual SCFA; however, both the 0.5% and 1% levels increased the production of total SCFA (linear $p = 0.001$, quadratic $p < 0.001$) (Table 4). The highest acetate concentration (linear $p = 0.006$, quadratic $p = 0.009$) was observed at the 0.5% level, with no significant differences between other levels and the control. Additionally, the 0.5%, 1%, and 1.5% levels increased the propionate concentration (linear $p = 0.039$, quadratic $p = 0.004$). However, the treatments did not affect the butyrate concentration.

Table 4. In vitro rumen fermentation profile of diet with increasing levels of *Laurus nobilis* leaves (%, DM).

	Degradability [1]			SCFA [2]			SCFA [3]				Fermentation [4]					
Level	***d*DM**	***d*NDF**	***d*ADF**	**Total**	**C_2**	**C_3**	**C_4**	**C_2**	**C_3**	**C_4**	**pH**	**NH_3-N**	**ME**	**PF_{24}**	**MCP**	**GY_{24}**
0	473 ^{b}	439 ^{bc}	412 ^{b}	23.4 ^{b}	11.4 ^{b}	7.90 ^{c}	4.08	48.8	33.8	17.4	6.27	10.4	4.68 ^{b}	7.22	328.3 ^{ab}	138.8
0.5	568 ^{a}	547 ^{a}	542 ^{a}	27.5 ^{a}	13.5 ^{a}	9.29 ^{ab}	4.74	49.0	33.8	17.2	6.13	11.7	5.00 ^{a}	6.27	368.8 ^{a}	159.5
1	483 ^{b}	455 ^{b}	441 ^{b}	25.9 ^{a}	12.4 ^{ab}	9.39 ^{a}	4.17	47.7	36.2	16.1	6.20	11.8	4.58 ^{b}	6.46	318.3 ^{bc}	155.7
1.5	447 ^{bc}	436 ^{bc}	422 ^{b}	23.3 ^{b}	10.7 ^{b}	8.10 ^{bc}	4.50	45.9	34.7	19.3	6.17	11.5	4.44 ^{bc}	6.42	293.1 ^{bc}	156.8
2	411 ^{c}	406 ^{c}	392 ^{b}	21.7 ^{b}	10.6 ^{b}	7.46 ^{c}	3.60	49.0	34.4	16.6	6.13	10.8	4.26 ^{c}	6.49	271.3 ^{c}	154.3
SEM	8.9	10.5	10.7	0.407	0.40	0.276	0.195	1.51	1.08	0.71	0.039	0.38	0.063	0.258	10.79	6.15
p value																
Treatment	<0.001	0.047	0.042	<0.001	0.002	0.002	0.108	0.571	0.517	0.171	0.171	0.123	0.001	0.161	0.009	0.212
Linear	<0.001	0.045	0.041	0.001	0.006	0.039	0.082	0.583	0.535	0.091	0.091	0.631	<0.001	0.139	0.002	0.175
Quadratic	<0.001	0.043	0.042	<0.001	0.009	0.004	0.103	0.363	0.276	0.513	0.513	0.014	0.012	0.091	0.034	0.102

[a,b,c] The means within the same column that have distinct superscripts exhibit a significant difference ($p < 0.05$). The standard error of the mean is denoted by SEM, and the *p*-value represents the observed significance level of the F-test for the treatment effect. [1] *d*DM represents DM degradability (g/g incubated), *d*NDF stands for NDF degradability (g/kg incubated), and *d*ADF signifies ADF degradability (g/kg incubated). [2] SCFA refers to short-chain fatty acids (mmol/L), C_2 indicates acetate (mmol/L), C_3 represents propionate (mmol/L), and C_4 stands for butyrate (mmol/L). [3] C_2 indicates acetate (%), C_3 represents propionate (%), and C_4 stands for butyrate (%). [4] NH_3-N stands for ammonia-N (mg/g DM), GY_{24} is gas yield at 24 h (mL gas/g dDM), ME represents metabolizable energy (MJ/kg DM), PF_{24} is the partitioning factor at 24 h of incubation (mg *d*DM: mL gas), and MCP stands for microbial crude protein production (mg/g DM).

Without affecting pH, NH_3-N, PF_{24}, and GY_{24}, the 0.5% level demonstrated the highest ME (linear $p < 0.001$, quadratic $p = 0.012$) and MCP (linear $p = 0.002$, quadratic $p = 0.034$), while the 2% level reduced them compared to the control (Table 4).

4. Discussion

4.1. Laurus nobilis

Many pharmacological characteristics, such as antibacterial, anti-inflammatory, and antioxidant properties, are possessed by plant volatile molecules [29]. The extraction of essential oil from the leaves revealed a yield of 3.29% by weight, which is aligned with the findings of Fidan et al. [30], who reported that the essential oil content in laurel leaves could reach up to 3.25% by weight, and with Khayyal et al. [17], who observed a 3% essential oil content in the leaves. In the present experiment, 1,8-cineole at about 81% and estragole at about 6% were the major compounds in the *L. nobilis* leaves. However, Fidan et al. [30] stated that 1,8-cineole (41.0%), α-terpinyl acetate (14.4%), sabinene (8.8%), methyl eugenole (6.0%), β-linalool (4.9%), and α-terpineol (3.1%) were the essential oils found in *L. nobilis* leaves. Others [31] showed that 1,8-cineole (18.2%), α-phellandrene (15%), β-pinene (9.4%), α-pinene (9.1%), α-terpinyl acetate (7.9%), sabinene (6.3%), camphene (4.2%), germacrene D (3.7%), and β-caryophyllene (3%) were the major constituents of *L. nobilis* essential oils. Sızmaz [18] reported that limonene was the major constituent of the laurel oil (64.6%). Choudhary et al. [32] reported that *L. nobilis* leaves from India and Nepal were found to have linalool as a significant component, followed by 1,8-cineole and α-pinene. Under the Egyptian conditions, Khayyal et al. [17] reported that 1,8-cineole (38.9%), α- pinene (17.0%), and terpinene-4-ol (15.01%) are the major volatile compounds in *L. nobilis* leaves. The variations across research could be attributed to environmental factors such as the kind of soil, temperature, season, location, and timing of plant harvesting, as well as strategies for drying, extracting, and analyzing data [15,33].

Due to their effect on ruminal bacteria, plant volatile compounds that modify the rumen microbiome offer a variety of dietary interventions that may affect and modulate rumen fermentation [34]. For example, rumen microbes' growth and activity have been shown to be strongly inhibited by terpenoids, such as 1,8-cineole [35]. According to de Sousa et al. [29], these compounds also have antibacterial properties against bacteria, protozoa, and fungus. They influence enzyme activity, signal transduction pathways, bacterial colonization, and cell membrane integrity. The ruminal fermentation profile,

however, varies depending on the source and concentration of volatile chemicals, which affect ruminal bacteria through different ways.

4.2. Gas Production

In order to evaluate the effect of any feed addition on ruminal microbes, GP kinetics offer important insights into the digestibility of feedstuffs, ruminal fermentation processes, and the activity of ruminal microorganisms [36,37]. The additives did not affect the lag time of GP, indicating that the additives did not affect the microbial activity on the incubated substrate [38]. *L. nobilis* leaves at 0.5% increased asymptotic GP by about 48%, indicating that *L. nobilis* leaves improved the ruminal fermentation. The presence of volatile compounds in the leaves may be the main reason for the increased GP [39,40]. Essential oils have multiple mechanisms through which they affect GP; however, their primary mechanism is their antimicrobial properties. Due to the nature of the plants, the amount and concentration of bioactive components in essential oils might vary, which affects how efficient their antibacterial qualities are [41,42]. In light of the specific chemical composition of certain feed additives, higher doses may inhibit a broad spectrum of microorganisms in the rumen, consequently altering GP characteristics. In the present study, it was anticipated that the highest level of *L. nobilis* leaves would reduce GP due to the antimicrobial impact of essential oils present in *L. nobilis* leaves on various ruminal microorganisms. However, contrary to expectations, minimal differences were observed between the highest level of *L. nobilis* leaves and the control treatment. The increased *d*DM, *d*NDF, and *d*ADF at 0.5% inclusion also explains the highest GP compared to other levels. Furthermore, there is evidence that certain components in essential oils from plant extracts, especially those with lower antimicrobial potential like monoterpenoids (e.g., 1,8-cineole, estragole, and α-terpinyl acetate) with hydrocarbon and alcohol structures, could act as a carbon source for specific rumen microorganisms. This means that the high concentration of volatile compounds in *L. nobilis* leaves, which are sources of monoterpene hydrocarbons, may potentially alter rumen fermentation and GP [43].

Kumar et al. [44] reported that administering eucalyptus fresh leaf extracts rich in 1,8-cineole at 0.5 mL per 30 mL of buffered rumen fluid containing oats hay increased GP per gram DM or *d*DM. However, increasing the dose to 2 mL resulted in decreased GP. However, Sallam et al. [19] conducted an incubation study of a total mixed ration (1:1 roughage to concentrate) with eucalyptus oil containing mainly 1,8-cineole added at concentrations of 25, 50, 100, and 150 µL per 75 mL of buffered rumen fluid. They observed a reduction in GP by 5.3%, 24.2%, 44.6%, and 56.7%, respectively, with increasing levels of eucalyptus oil.

All levels of *L. nobilis* leaves' administration did not affect the rate of GP; however, numerical differences were observed between different treatments. Moreover, the level 2% NLM increased the lag time of GP by 26.5% compared to the control level, confirming the inverse relationship between the lag phase and asymptotic GP [6]. A period of adjustment is required for the bacteria to acclimate to the addition of *L. nobilis* leaves, which may be the cause of the extended lag time seen at 2% level inclusion. This leads to a delayed beginning of fermentation and GP.

4.3. Methane and Carbon Dioxide Production

It was expected that varying concentrations of *L. nobilis* leaves would demonstrate anti-methanogenic activity by improving the ruminal fermentation profile and substrate degradability while suppressing methanogenic archaea and lowering the generation of CH_4. Without affecting the rate of CH_4, all levels of *L. nobilis* leaves lowered the asymptotic CH_4 production and proportion with the lowest CH_4 production at 2% (reduced CH_4 by about 18%) and lowest proportion at 0.5% (reduced CH_4 proportion by about 33%). Moreover, the 1.5 and 2% levels increased the lag of CH_4 production by 38.7% and 31.7%, respectively, indicating that methanogens needed more time to start producing CH_4. The impact of essential oils (e.g., 1,8-cineole) on reducing the methanogenic archaeal popula-

tion and methanogenesis was previously approved [45,46]. The molar proportion of each individual SCFA and the total SCFA concentration may also contribute to the decreasing CH_4 generation with *L. nobilis* leaves. For instance, the levels 0.5% and 1% increased the propionate concentration which can act as a hydrogen sink, limiting the availability of CH_4 production, thereby decreasing methanogenesis [47,48]. Previous research has demonstrated that secondary metabolites from plants can alter the metabolic processes of hydrogen-consuming bacteria and methanogens, as well as affect protozoa populations [49,50]. Similar results were observed by Sallam et al. [19] when they administered eucalyptus oil rich in 1,8-cineole at concentrations of 25, 50, 100, and 150 µL per 75 mL of buffered rumen fluid containing a diet with equal portions of concentrates and roughages. The production of CH_4 was shown to have dropped by 26.0%, 46.8%, 77.3%, and 85.3%, respectively. Moreover, Kumar et al. [44] reported that administering eucalyptus fresh leaf extracts rich in 1,8-cineole at 0.5 or 2 mL per 30 mL of buffered rumen fluid containing oats hay decreased CH_4 production at both doses. However, as previously mentioned, the higher dose reduced GP.

The inclusion of 0.5% of *L. nobilis* leaves showed the highest asymptotic CO_2 production by about 38%. Kholif et al. [6] found comparable outcomes when applying *Salvia officinalis*, which is abundant in volatile compounds, to the substrate employed in our study. Moreover, the administration of *L. nobilis* leaves increased the rate of CO_2 production by about 83% to 128% compared to the control. Increasing the propionate concentration at the expense of acetate could increase CO_2 levels [51]. Although the highest propionate level was found at 1% *L. nobilis* leaves' inclusion without a corresponding decrease in acetate or butyrate (both acetate and butyrate increased alongside propionate), the mechanism underlying the increased CO_2 with rising levels of *L. nobilis* leaves in this study is difficult to reconcile. Therefore, the inhibition of CH_4 generation by the plant secondary compounds in *L. nobilis* leaves may be responsible for the decreased utilization of CO_2.

4.4. Degradability and Fermentation

The ruminal pH, which normally ranges from 5.0 to 7.5, should be measured in order to evaluate the stability and balance of the rumen environment in ruminant animals [52,53]. The administration of *L. nobilis* leaves at different levels did not affect pH and NH_3-N. The values of pH [54] and concentration of NH_3-N [55] were within the reference ranges required for optimal microflora growth and activity for nutrient digestion. Similar results were observed by Khayyal et al. [17] fed growing lambs were fed diets supplemented with *L. nobilis* leaves. It was expected that the high levels of *L. nobilis* leaves and its active component 1,8-cineole will decrease the concentration of ruminal NH_3-N [19,44] because essential oils at a high concentration inhibit the hyper-NH_3 producing bacteria [45]. But this was not observed, which may be related to the concentration of 1,8-cineole in their treatments or the incubated substrates.

All of *d*DM, *d*NDF, and *d*ADF exhibited a dose-dependent response, with higher values observed at 0.5% inclusion of *L. nobilis* leaves. This level improved the degradability of DM by 20%, NDF by 25%, and ADF by 32%, whereas the 2% level decreased them by about 13%, 8%, and 5%, respectively, indicating the importance of defining the optimal doses of this feed additive. Essential oils at appropriate doses, typically low, are beneficial for ruminal microbial activity and growth, especially fibrolytic bacterial activity [45], which enhance the degradation and fermentation of substrates. Higher concentrations of essential oils derived from plant extracts have been demonstrated in earlier studies to potentially inhibit the growth of cellulolytic bacteria in the rumen and decrease the feedstuffs' capacity to degrade feeds [44,56]. Lee et al. [57] reported that administering San wormwood essential oil, containing 56.7% 1,8-cineole, to Bermuda grass hay increased the populations of *Ruminococcus albus* and *Streptococcus bovis*, which are directly linked to cellulose/hemicellulose digestion [58]. The negative effects of increasing the level of *L. nobilis* leaves, and subsequently the level of 1,8-cineole, were previously reported by Kumar et al. [44]. They observed that a low level of Eucalyptus fresh leaf extracts rich in 1,8-cineole, at 0.5 mL per 30 mL

of buffered rumen fluid containing oats hay, enhanced *d*DM, while increasing the dose to 2 mL resulted in decreased *d*DM, *d*NDF, and *d*ADF. Khayyal et al. [17] reported that the inclusion of *L. nobilis* leaves in diets of growing lambs did not affect nutrient digestibility. The presence of active compounds like 1,8-cineole and other components in *L. nobilis* leaves may act as stimulants for rumen microflora, enhancing their efficiency in producing essential vitamins and enzymes needed to optimize digestibility [45]. Moreover, administering San wormwood essential oil, containing 56.7% 1,8-cineole, at 5 mg/kg improved *d*DM of Bermuda grass during fermentation [57]. Sızmaz [18] reported that laurel essential oil at concentrations of 50 and 100 mg/L of fermenter liquid did not affect apparent nutrient degradation. The low doses used in Sızmaz's experiment (equal to 2.92 and 5.84 mg/g DM) and the continuous dilution of the fermenter liquid with buffer may be the main reason for the weak effects on nutrient degradability [18].

The total SCFA (by about 18% and 11%, respectively), acetate (by about 18% and 9%, respectively), and propionate (by about 18% and 19%, respectively) increased with the administration of *L. nobilis* leaves at 0.5% and 1% levels. The enhancements in the production of the total SCFA may be due to the positive effects of the essential oils in *L. nobilis* leaves on nutrient digestion [7,45]. Administering Eucalyptus fresh leaf extracts rich in 1,8-cineole at 0.5 mL per 30 mL of buffered rumen fluid containing oats hay increased the production of total SCFA, acetate, propionate, and butyrate; however, increasing the dose to 2 mL reduced their production [44].

Without affecting PF_{24} or GY_{24}, increased estimated ME, by about 7%, and MCP, by about 12%, were observed when *L. nobilis* leaves were administered at 0.5%, while 2% administration lowered them by about 13% and 17%, respectively. These findings point to the ideal ratio of protein to energy that encourages higher microbial protein production [6,7,20]. Since *L. nobilis* leaves include phytochemicals that regulate both pathways, phenolic compounds in *L. nobilis* leaves at a concentration of 0.5% may interact with the biosynthesis of aromatic amino acids [59]. The increase in MCP indicates that a significant portion of NH_3-N and SCFA were utilized for microbial protein synthesis [60].

5. Conclusions

By adding 0.5% *L. nobilis* leaves (DM basis) to a diet that included concentrate and roughages at a 1:1 ratio, it was possible to minimize ruminal CH_4 production and boost GP in vitro, which could help reduce the environmental impact of ruminants (e.g., sheep) and promote sustainability. Additionally, this study showed that supplementing the diet with 0.5% *L. nobilis* leaves improved the total and individual SCFA, primarily acetate and propionate, as well as the nutrient degradability (*d*DM, *d*NDF, and *d*ADF). Higher quantities of *L. nobilis* leaf supplementation in vivo should be investigated further to evaluate their effects on ruminant animal production performance and rumen microbiota alterations. These studies will yield important information about how to best utilize *L. nobilis* leaves in livestock farming practices to enhance animal health and environmental sustainability. In addition, methods for gathering leaves of *L. nobilis* should be assessed to create a more uniform product that can handle possible issues with broad distribution.

Funding: This research received no external funding.

Institutional Review Board Statement: This was an in vitro experiment using ruminal fluid obtained from nearby butcher facilities; therefore, ethical reviews and permission were not needed. The Assistant Editor received a formal letter of exemption signed by the Dean of the Faculty of Agriculture at New Valley University in Egypt as well as the Head of Department. There was no further use of live animals during any of the in vitro experimental processes. Following the guidelines set forth by the animal welfare laws, the animals were killed without experiencing any kind of pain, suffering, or mistreatment.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article, and further inquiries can be directed to the corresponding author.

Acknowledgments: The author wishes to thank Tarek A. Morsy, Gouda A. Gouda, and Mahmoud Fahmy from the Dairy Science Department at the National Research Centre (Egypt) for their assistance during the experimental work.

Conflicts of Interest: The author declares no conflicts of interest.

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